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# New monoclonal anti-human Fc gamma receptor II antibodies induce platelet aggregation

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## SUMMARY

We developed two new monoclonal antibodies, designated NNKY3-2 and NNKY4-7, that recognized a 40-kD platelet protein. They appeared to be monoclonal anti-Fc gamma receptor II (FcγRII) antibodies from the results of flow cytometric binding inhibition studies using another monoclonal anti-FcγRII antibody (2E1). The addition of NNKY3-2 or NNKY4-7 to platelet-rich plasma (PRP) led to a typical aggregation pattern preceded by a lag phase, but their addition to washed platelets did not induce aggregation. The aggregation of PRP by these antibodies was inhibited by prostaglandin $E_1$ ($PGE_1$) or staurosporine (protein kinase C inhibitor), whereas it was only slightly affected by a monoclonal anti-GPIIb/IIIa antibody or Arg–Gly–Asp–Ser. Furthermore, these antibodies induced the aggregation of washed platelets plus normal serum, but not that of washed platelets plus heat-treated serum (destruction of complement activity). These results suggest that NNKY3-2 or NNKY4-7-induced aggregation involves an unusual pathway independent of fibrinogen, and that the important factor is the participation of complement. NNKY3-2 and NNKY4-7 may be useful to study the relationship between autoantibodies, the Fc receptor, and complement in idiopathic thrombocytopenic purpura.

**Keywords** IgG Fcγ receptor II monoclonal antibodies platelet aggregation flow cytometry 40-kD molecule

## INTRODUCTION

Receptors for the Fc portion of IgG (FcγR) play an important role in the regulation of immune processes, such as the clearance of immune complexes, phagocytosis, and antibody-dependent cellular cytotoxicity. Three classes of human FcγR have been defined and can be distinguished by several criteria. The high-affinity receptor (FcγRI) has an average molecular mass of 72 kD (Anderson & Looney, 1986), and the two low-affinity receptors (FcγRII and FcγRIII) have molecular masses of 40 kD and 50–70 kD, respectively (Looney, Abraham & Anderson, 1986). FcγRII is expressed by monocytes, neutrophils, eosinophils, B cells, and platelets (Anderson & Looney, 1986; Unkeless, 1989). It plays a significant role in transmembrane signalling and cellular activation (Unkeless, 1989), and recently it was reported to mediate platelet activation by CD9 monoclonal antibodies (Worthingon, Carroll & Boucheix, 1990).

Idiopathic thrombocytopenic purpura (ITP) is a syndrome caused by circulating antibodies that react with the platelet membrane (Karpatkin, 1980; McMillan, 1981), and it has been found that platelet-associated IgG is particularly important in the mechanism of ITP. Platelet-associated IgG includes immune complexes that bind to platelets via Fc receptors on the platelet membrane, but the significance of immune complexes and the role of the platelet-related Fc receptor in the mechanism of ITP remain obscure. Recently, McCrae, Shattil & Cines (1990) reported that platelet activation induced increased FcγR expression on the platelet membrane and that this change may promote the clearance of IgG-containing immune complexes from the circulation and also contribute to the development of immune complex-mediated thrombocytopenia.

Here we report the characteristics of two monoclonal antibodies, designated NNKY3-2 and NNKY4-7, that recognize the platelet FcγRII and induce a unique form of platelet aggregation.

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## MATERIALS AND METHODS

*Production of monoclonal antibodies*

Monoclonal antibodies were produced as described previously (Nomura *et al.*, 1987; Nomura & Kokawa, 1990). The molecular weights of the antigens corresponding to each monoclonal

antibody were determined by SDS–PAGE and Western blotting. Western blotting was performed using a modification of the method of Beardsley *et al.* (1984). The proteins were electrophoretically transferred to nitrocellulose paper in 25 mM Tris (pH 8·3). Nitrocellulose strips containing a single lane of platelet proteins were incubated first with 3% bovine serum albumin (BSA) containing 0·05% Tween 20/phosphate-buffered saline (PBS) for 1 h at room temperature, and then with one of the monoclonal antibodies. The strips were then washed three times with 0·05% Tween 20/PBS and anti-platelet antibodies bound to the nitrocellulose were detected by the avidin–biotin–peroxidase complex (ABC) method using biotinylated anti-human IgM (Vector Laboratories). Molecular weight standards (BioRad) were subjected to simultaneous electrophoresis with the samples. The following antibodies were used for comparison: 2E1 (anti-Fc$\gamma$II receptor; Immunotech) (Farace *et al.*, 1988); ALB6 (anti-CD9; Cosmo Bio) (Baucheix *et al.*, 1983); NNKY1-32 (anti-GPIIb/IIa) (Nomura *et al.*, 1987; Nomura & Kokawa, 1990); SZ22 (anti-GPIIb; Cosmo Bio) (Ruan *et al.*, 1987a); SZ21 (anti-GPIIIa; Cosmo Bio) (Ruan *et al.*, 1987a); SZ1 (anti-GPIb-IX; Cosmo Bio) (Ruan *et al.*, 1987b); NNKY5-5 (anti-GPIb) (Nomura *et al.*, 1988; Nomura & Kokawa, 1990); and OKM5 (anti-GPIV; Ortho Diagnostics) (Talle *et al.*, 1983).

*Preparation of washed human platelets*

Platelet-rich plasma (PRP) and washed platelets were obtained as described previously (Nomura *et al.*, 1987; Nomura & Kokawa, 1990). After washing, the platelets were resuspended in Tyrode's buffer (137 mM NaCl, 12 mM $NaHCO_3$, 5·5 mM glucose, 2 mM KCl, 1 mM $MgCl_2$, and 0·3 mM $Na_2HPO_4$, pH 7·4).

*Platelet aggregation and ATP secretion*

Platelet aggregation studies were performed on citrated PRP or washed platelets using an NKK Hematracer 1 (Niko Bioscience), and the results were expressed as the maximal percentage change in light transmission relative to platelet-poor plasma. ATP secretion was measured with a Lumi-aggregometer (Payton Association) using PRP or washed platelets. Both aggregation curves and ATP secretion were measured after the addition of the monoclonal antibodies. The inducers ADP or A23187 were used for comparison. The sources of the agents used in the study of monoclonal antibody-induced aggregation were as follows: prostaglandin $E_1$ ($PGE_1$) and apyrase (Sigma); EDTA and acetyl-salicylic acid (aspirin) (Wako Pure Chemical Industries); the protein kinase C inhibitor staurosporin (Kyowa Medes). The peptides Arg–Gly–Asp–Ser (RGDS), Arg–Gly–Glu–Ser (RGES), and His–His–Leu–Gly–Gly–Ala–Lys–Gln–Ala–Gly–Asp–Val (HHLGGAKQAGDV; $\gamma$ 400–411) were purchased from Peninsula Laboratories.

*Preparation of paraformaldehyde-treated platelets*

Platelets were resuspended in 1% paraformaldehyde (PFA), dispersed by gentle pipetting, and incubated at room temperature for 15 min. They were then pelleted and resuspended in washing buffer twice more under the same conditions. After the final centrifugation, the platelets were resuspended in stock solution (9 mM $Na_2EDTA$, 26·4 mM $Na_2HPO_4$ $2H_2O$, 140 mM NaCl, 0·1% $NaN_3$, and 2% fetal bovine serum, pH 7·2), and stored at 4°C until analysis.

*Flow cytometric analysis*

*Binding inhibition test of monoclonal antibodies.* To determine whether the two antibodies stained additively or competitively, immunofluorescence intensities were compared according to the method of Zola *et al.* (1984). A mixture of equal volumes of the two antibodies was reacted with PFA-fixed platelets and the fluorescence intensity histogram of this mixture was compared with those for the individual antibodies. To obtain a numerical value representing fluorescence intensity, the peak channel number was recorded after ensuring that the shapes of the histograms were comparable. Staining was considered additive if the model intensity of the mixture was greater than the value for the brighter of the two antibodies.

*Platelet activation.* Stimulation with agonists was performed by incubating washed platelets with normal serum or heat-treated serum (56°C for 30 min) for 10 min at 22°C without stirring in the presence of 20 $\mu$M ADP, 2 $\mu$M A23187, 5 $\mu$g/ml NNKY3-2, or 5 $\mu$g/ml NNKY4-7. Following incubation, samples were immediately prepared without centrifugation using PFA with EDTA–PBS (the final concentration of PFA was 1%, pH 7·2). Activated or control platelets were incubated with saturated concentrations of fluorescein-conjugated anti-C1q (5 $\mu$g/ml; Binding Site) or anti-C3 (5 $\mu$g/ml; Binding Site).

*Operating conditions for the FACS analyser.* Samples were assayed on a Becton Dickinson FACS Research analyser, the operating conditions for which have been published previously (Nomura *et al.*, 1987; Nomura & Kokawa, 1990). To quantify fluorescence, the following formula was used: $\Sigma$ = [Mean channel of the positive region × total platelet count of the positive region]/$10^3$. Ten-thousand events were analysed in the one-colour analysis.

## RESULTS

The monoclonal antibodies NNKY3-2 and NNKY4-7 were shown to be of the IgM class by Ouchterlony immunodiffusion analysis. The antigen defined by both antibodies had a molecular weight of 40 kD (Fig. 1).

Table 1 shows results of binding inhibition tests using the monoclonal antibodies. A mixture of NNKY3-2 plus NNKY4-7 did not produce an increase in the peak channel, indicating that the two antibodies do not stain additively. However, when either antibody was mixed with 2E1, there was slight additive staining. In contrast, when NNKY3-2 or NNKY4-7 was combined with any of the other monoclonal antibodies tested (anti-CD9, anti-GPIIb and/or IIIa, anti-GPIb-IX, anti-GPIb, and anti-GPIV), there was a marked increase in staining.

As shown in Fig. 2a, addition of NNKY3-2 or NNKY4-7 to PRP led to a typical aggregation pattern preceded by a lag phase. Addition of 2E1 itself did not induce the aggregation of PRP, but did prolong the lag phase of NNKY4-7-induced aggregation and 2E1 plus goat anti-mouse antibody induced aggregation (Fig. 2a). Furthermore, the aggregation produced by NNKY3-2 and NNKY4-7 involved ATP secretion (data not shown). Although 2E1 induced the aggregation of washed platelets without the need for goat anti-mouse antibody, neither NNKY3-2 nor NNKY4-7 induced the aggregation of washed platelets (Fig. 2b). NNKY4-7 prolonged the lag phase of the 2E1-induced aggregation of washed platelets and NNKY3-2 completely inhibited such aggregation (Fig. 2b).

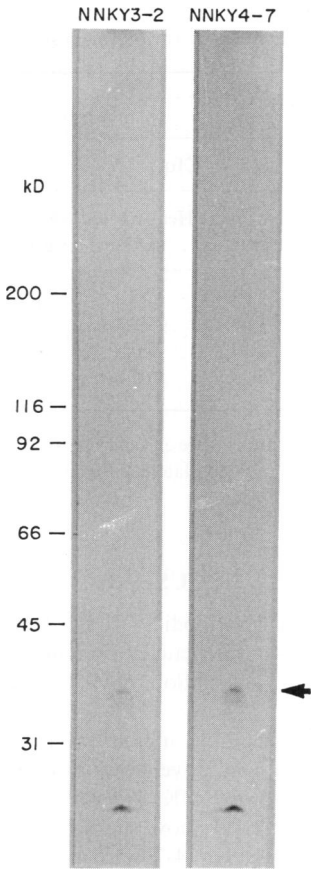


**Fig. 1.** Immunoblots of the monoclonal antibodies NNKY3-2 and NNKY4-7 with human platelets. The arrow indicates 40 kD.

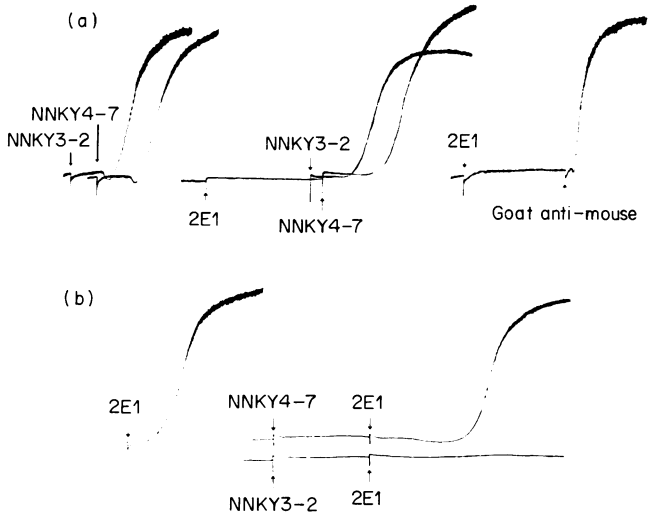


**Fig. 2.** Aggregation studies performed using NNKY3-2 and NNKY4-7 [I]. (a) 2E1 at a final concentration of 10 μg/ml did not induce aggregation in platelet-rich plasma, but did not affect the prolongation of the lag phase by NNKY4-7-induced aggregation, and 2E1 plus goat anti-mouse antibody induced aggregation; (b) 2E1 (10 μg/ml) induced platelet aggregation in washed platelets without goat anti-mouse antibody. NNKY4-7 prolonged the lag phase of 2E1-induced washed platelet aggregation and NNKY3-2 completely inhibited the same aggregation. (Final concentration of NNKY3-2 or NNKY4-7 was 5 μg/ml).

**Table 1.** Additive binding studies

| Antibody | Peak channel number | | | |
|---|---|---|---|---|
| | Undiluted antibody | Antibody +PBS | Antibody +NNKY3-2 | Antibody +NNKY4-7 |
| NNKY3-2 | 84 | 84 | 83 | 88 |
| NNKY4-7 | 80 | 78 | 82 | 80 |
| 2E1 (FcγRII) | 72 | 74 | 90 | 98 |
| ALB6 (CD9) | 90 | 92 | 129 | 126 |
| NNKY1-32 (GPIIb/IIIa) | 108 | 110 | 154 | 150 |
| NNKY5-5 (GPIb) | 126 | 124 | 150 | 159 |
| SZ1 (GPIb-IX) | 116 | 118 | 145 | 142 |
| SZ21 (GPIIIa) | 120 | 121 | 155 | 160 |
| SZ22 (GPIIb) | 100 | 100 | 139 | 139 |
| OKM5 (GPIV) | 66 | 68 | 120 | 122 |

Results are the mean of three experiments.

**Table 2.** Inhibition of aggregation induced by 2E1, NNKY3-2, or NNKY4-7

| Treatment | Washed platelets 2E1 (10 μg/ml) | Platelet-rich plasma NNKY3-2 (5 μg/ml) | Platelet-rich plasma NNKY4-7 (5 μg/ml) |
|---|---|---|---|
| None | 83±5 | 78±4 | 80±5 |
| NNKY3-2 (5 μg/ml) | 0 | NT | NT |
| 2E1 (10 μg/ml) | NT | 76±3 | 80±4 |
| NNKY1-32 (10 μg/ml) | 0 | 56±5 | 58±4 |
| NNKY5-5 (10 μg/ml) | 38±4 | 80±3 | 78±5 |
| RGDS (500 μM) | 0 | 60±4 | 61±5 |
| RGES (500 μM) | 79±5 | 80±5 | 82±4 |
| γ400-411 (500 μM) | 66±4 | 80±4 | 79±4 |
| EDTA (10 mM) | 0 | 0 | 0 |
| $PGE_1$ (5 μg/ml) | 0 | 28±3 | 25±3 |
| Aspirin (500 μM) | 0 | 42±3 | 44±4 |
| Apyrase (5 mg/ml) | 74±5 | 73±3 | 75±4 |
| Staurosporine (10 μM) | 0 | 14±3 | 10±2 |

Percentage of aggregation is expressed as the ratio of the maximum aggregation after 5 min.

NT, not tested.

Results are expressed as the mean ± s.d. of three experiments.

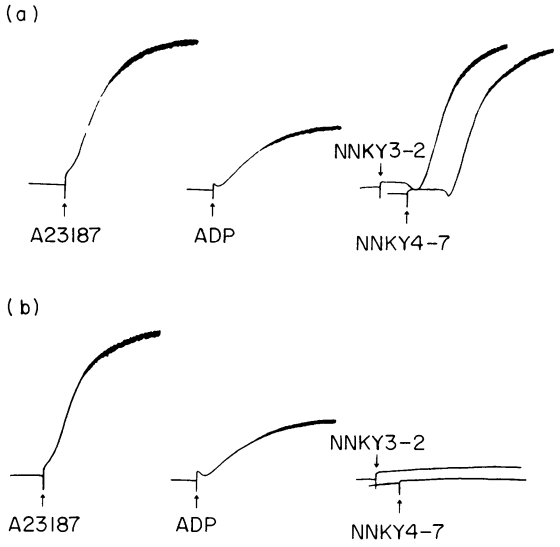


**Fig. 3.** Aggregation studies performed using NNKY3-2 and NNKY4-7 [II]. A23187- or ADP-induced aggregation of washed platelets plus heat-treated serum (b) was same as that of washed platelets plus normal serum (a). NNKY3-2 and NNKY4-7 did not induce the aggregation of washed platelets plus heat-treated serum. Serum was incubated at 56°C for 30 min to perform heat treatment. The stimulators of platelet aggregation were used at the following final concentrations: NNKY3-2 and NNKY4-7, 5 μg/ml; A23187, 2 μM; and ADP, 20 μM.

As seen in Table 2, the monoclonal anti-GPIIb/IIIa antibody (NNKY1-32) and the fibrinogen-related synthetic peptide, RGDS, completely inhibited 2E1-induced aggregation and partially inhibited NNKY3-2- or NNKY4-7-induced aggregation. Another fibrinogen synthetic peptide, γ 400–411, partially inhibited 2E1-induced aggregation, but had little effect on NNKY3-2- or NNKY4-7-induced aggregation. EDTA completely inhibited the aggregation induced by all three antibodies. $PGE_1$, aspirin, and staurosporine completely inhibited 2E1-induced aggregation, and the aggregation induced by NNKY3-2 or NNKY4-7 was also markedly inhibited by $PGE_1$ or staurosporine. However, aspirin only slightly inhibited NNKY3-2- or NNKY4-7-induced aggregation. On the other hand, apyrase scarcely affected the aggregation.

As seen in Fig. 3a, NNKY3-2 and NNKY4-7 induced the aggregation of washed platelets plus normal serum. However, both antibodies did not induce the aggregation of washed platelets plus heat-treated serum, although the A23187- or ADP-induced aggregation of washed platelets plus heat-treated serum was the same as that of washed platelets plus normal serum (Fig. 3b).

Table 3 shows certain surface events occurring on platelets following activation with specific agonists. Platelets stimulated by NNKY3-2 and NNKY4-7 showed the marked expression of C1q and C3 on their surface membranes in washed platelets plus normal serum. The binding of C1q and C3 to washed platelets in the presence of NNKY3-2 or NNKY4-7 increased significantly after normal serum was added, but heat-treated serum did not have such an effect. C1q and C3 binding to platelets was little changed by stimulation with ADP or A23187 when washed platelets plus either normal serum or heat-treated serum were tested.

**Table 3.** Analysis of events occurring at the platelet surface after activation by various agonists

| | Ratio | | | |
|---|---|---|---|---|
| | C1q | | C3 | |
| Agonist | Normal serum | Heat-treated serum | Normal serum | Heat-treated serum |
| ADP (10 μM) | 1·18 | 1·21 | 1·16 | 1·25 |
| A23187 (2 μM) | 1·67 | 1·29 | 1·65 | 1·23 |
| NNKY3-2 (5 μg/ml) | 5·17 | 1·36 | 5·37 | 1·29 |
| NNKY4-7 (5 μg/ml) | 5·03 | 1·34 | 5·06 | 1·27 |

The changes of C1q and C3 are expressed as the ratio in comparison to the Σ value for unstimulated platelets. Results are the mean of three experiments.

## DISCUSSION

Our new monoclonal antibodies NNKY3-2 and NNKY4-7 both recognized a 40-kD platelet protein, and induced the aggregation of PRP in association with ATP secretion after a lag phase.

Flow cytometric binding inhibition studies showed that NNKY3-2 did not stain additively with NNKY4-7. However, a mixture of NNKY3-2 or NNKY4-7 with 2E1 (a monoclonal anti-FcγRII antibody) did show slight additive staining and when NNKY3-2 or NNKY4-7 were mixed with the other monoclonal antibodies tested, there was a marked additive effect. These results suggest that the epitope recognized by NNKY3-2 is located near that for NNKY4-7, while the epitope recognized by 2E1 is slightly further away from both these binding sites.

In the current study, the addition of NNKY3-2 or NNKY4-7 to PRP led to a typical pattern of aggregation preceded by a lag phase. However, 2E1 did not induce the aggregation of PRP, while 2E1 plus goat anti-mouse antibody induced its aggregation (Fig. 2a). This latter result is in accordance with the report of Rosenfeld & Anderson (1989). Furthermore, although 2E1 induced the aggregation of washed platelets without goat anti-mouse antibody, neither NNKY3-2 nor NNKY4-7 induced aggregation of washed platelets (Fig. 2b). However, NNKY4-7 prolonged the lag phase of the 2E1-induced aggregation of washed platelets and NNKY3-2 completely inhibited the same aggregation process. These results suggest that NNKY3-2 and NNKY4-7 can act mutually with 2E1 in platelet activation, but that their platelet activating mechanisms differ. One of the causes may be the difference of subclass in monoclonal antibodies. Our new monoclonal antibodies are IgM, and the 2E1 is IgG2a. The interaction of IgM and IgG antibodies with FcγRII will be different since IgG can bind both through its $F(ab)_2$ and Fc regions.

The 2E1-induced aggregation of washed platelets appeared to depend on the binding of fibrinogen to GPIIb/IIIa on the platelet surface, because it was inhibited by NNKY1-32 (an anti-GPIIb/IIIa antibody), RGDS, and EDTA. Furthermore, this aggregation was also completely inhibited by treatment with $PGE_1$, aspirin, and staurosporine, showing that it was dependent on both thromboxane $A_2$ formation and protein

kinase C. Previous reports have suggested that signal transduction by the human platelet FcγRII occurs via the G-protein-mediated activation of a phospholipase C (Rosenfeld & Anderson, 1989). Our results are partially in accordance with such a concept.

Aggregation of PRP induced by NNKY3-2 or NNKY4-7 was reduced by $PGE_1$, aspirin, and staurosporine, but was only slightly decreased by NNKY1-32 (anti-GPIIb/IIIa) or RGDS. Thus, this aggregation appeared to involve an unusual mechanism independent of fibrinogen, and the important factor appeared to be the participation of complement. The findings suggesting this were that NNKY3-2 induced the aggregation of washed platelets plus normal serum, but not that of washed platelets plus heat-treated serum (destruction of complement activity), despite the fact that A23187- or ADP-induced aggregation of washed platelets plus heat-treated serum was the same as that for washed platelets plus normal serum (Fig. 3). Furthermore, flow cytometric analysis showed that the binding of C1q and C3 to washed platelets in the presence of NNKY3-2 or NNKY4-7 increased significantly after normal serum was added, while heat-treated serum did not have such an effect, suggesting that the aggregation induced by NNKY3-2 or NNKY4-7 involved the complement system. The complement system includes the classical pathway and the alternative pathway, both of which finally initiate the assembly of the membrane attack complex (C5b-9), which has the potential to insert itself into lipid-containing structures and cause plasma membrane damage (Sims, 1989). It has been reported that platelet activation by the C5b-9 protein can occur without the need for functional expression of the fibrinogen receptor on the platelet membrane GPIIb/IIIa complex (Sims, 1989). The NNKY3-2- or NNKY4-7-induced aggregation that was observed after treatment with the monoclonal anti-GPIIb/IIIa antibody or RGDS appeared to be independent of fibrinogen, and may occur in a manner similar to aggregation provoked by C5b-9. Sims (1989) reported that the C5b-9-mediated release response was blocked by sphingosine, a potent inhibitor of protein kinase C, but was unaffected by the cyclooxygenase inhibitor indomethacin. This indicates that C5b-9 can produce the direct activation of protein kinase C. NNKY3-2- or NNKY4-7-induced aggregation was also inhibited by staurosporine (a protein kinase C inhibitor), so it also fits the pattern reported by Sims (1989).

Since the aggregation induced by both antibodies was partially inhibited by aspirin, it apparently also involved a mechanism other than C5b-9 that was dependent on thromboxane $A_2$. The first component of the classical complement pathway, C1q, is liable to activation by immune complexes, immune aggregates, or immunoglobulins. In addition, it has been reported that the C1q receptor is present on the platelet surface as part of the collagen receptor (Peerschke & Ghebrehiwet, 1987, 1990). Although the *in vivo* interaction between C1q and platelets remains unclear, it may promote platelet activation in association with clusters of multivalent C1 activators such as immune complexes (Peerschke & Ghebrehiwet, 1987). It is also possible that NNKY3-2 or NNKY4-7 activated C1q and generated a signal transduction response in the same manner as collagen. Thus, NNKY3-2 and NNKY4-7 appear to stimulate two platelet aggregation mechanisms, one that is dependent on GPIIb/IIIa via C1q and another that is independent of GPIIb/IIIa and acts via C5b-9.

The participation of platelet-associated IgM and platelet-associated C3 has been reported in addition to platelet-associated IgG in the mechanism of thrombocytopenia in ITP (Panzer *et al.*, 1986). NNKY3-2 and NNKY4-7 are of the IgM subclass, bind to the FcγRII to which immune complexes also bind, and appear to activate platelets in relation to complement. These two antibodies may therefore be useful to study the relationship between autoantibodies, the Fc receptor, and complement in ITP.

## ACKNOWLEDGMENTS

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