行政院國家科學委員會專題研究計畫結案報告

計畫編號：NSC 89-2318-B-006-011-M51
計畫名稱：登革病毒感染的登革出血熱/登革休克症候群：動物模式 (Dengue hemorrhage fever/dengue shock syndrome in dengue infection: the animal model)
執行期限：90年 8月 1日至 90年 7月 31日
主持人：黎煥耀 執行機構及單位名稱：成大醫學院微生物及免疫研究所

中文摘要

登革熱的感染，不同於其他病毒的感染，是在於登革病毒感染有時會引起登革出血熱 (DHF) 及登革休克症候群 (DSS)，在 DHF/DSS 的過程中，最重要的一個變化是血小板減少，它的機制一直不清楚。我們利用所設立的登革病毒感染老鼠模式，在十天左右會產生血小板減少的現象，伴隨著抗血小板的抗体產生。利用融合瘤的技術產生很多單株抗体，分析這些抗血小板自體抗体，有 IgM 及 IgG1，會和病毒的 NS-1 結合，同時會增強活化血小板的凝集，在補体存在下也會溶解血小板，這種抗血小板自體抗体的產生及作用可能是登革病毒感染造成血小板低下的主因。對於病毒感染和自體免疫力間的分子模擬也提供了一個模式。

關鍵詞：登革病毒，登革出血熱/登革休克症候群，自體抗體，血小板。

Abstract

Dengue virus infection causes dengue fever, dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS). Thrombocytopenia is common in dengue fever, and is always found in DHF/DSS. The pathogenesis of thrombocytopenia is poorly understood. IgM anti-platelet auto-antibody was found in dengue patients. To further understand the relationship between anti-dengue virus antibody and anti-platelet antibody, we generated monoclonal anti-dengue virus antibodies from the dengue virus infected mice that developed transient thrombocytopenia post dengue infection. The analysis of a panel of monoclonal anti-NS-1 antibodies reveals three different patterns of platelet binding: strong, intermediate, or dull. Their isotypes are different, some are IgM while others are IgG1. Most of anti-platelet antibodies are cross-reactive with NS-1 of dengue virus, and can be competitively inhibited by recombinant NS-1 protein, suggesting a molecular mimicry between dengue virus NS-1 protein and platelet. A clone, I3-F4-G5, preferentially bound activated platelets, can recognize two or three proteins around 150 kD on platelets. The binding to platelet would lyse the platelet in the presence of complement or enhance the ADP-induced platelet aggregation. Furthermore, some of these monoclonal antibodies would also react with the
cellular antigens of BHK. Based on the data, we conclude that dengue virus infection induces auto anti-platelet antibodies which thereafter may involve in the manifestation of thrombocytopenia. A molecular mimicry between NS-1 and platelet is demonstrated.

Keywords: Dengue virus, DHF/DSS, anti-platelet autoantibody, thrombocytopenia

Introduction
Dengue fever (DF) is an acute infectious disease caused by dengue virus which has four serotypes. It is characterized by biphasic fever, headache, pain in various parts of the body, rash, lymphadenopathy, and leukopenia. In most cases, the disease of dengue fever is self-limited. However, there is risk to progress into dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) especially when cross infection of different serotypes occurs. DHF is a severe febrile disease characterized by abnormalities of hemostasis and increased vascular permeability, which in some instances results in DSS. DSS is a form of hypovolemic shock that is associated clinically with hemoconcentration and frequently leads to death if appropriate care is not given (2,3,9). Thrombocytopenia is common in dengue fever, and is always found in DHF/DSS. Its pathogenesis is poorly understood. La Russa and Innis reported dengue-virus-induced bone marrow suppression that depressed platelet synthesis (15). Wang et al. found that dengue-2 virus can bind to human platelets in the presence of virus-specific antibody (28). We also reported the presence of IgM anti-platelet auto-antibody in the sera of dengue patients, and its titer is higher in DHF/DSS patients than in DF patients (18). To further understand the relationship between anti-dengue virus antibody and anti-platelet antibody, a murine model of dengue virus infection was setup. Transient thrombocytopenia developed at 10-13 days after primary or secondary infection and was associated with the generation of anti-platelet antibody (12). A panel of monoclonal antibodies was generated from these dengue virus-infected mice. In this study, it was reported that anti-dengue virus antibodies, especially anti-NS-1 ones, could cross-react with platelet. The molecular mimicry between dengue virus and self-antigens was discussed.

Results
Different patterns of platelet binding by monoclonal antibodies derived from dengue-2-virus-infected mice. Dengue virus infection can induce anti-platelet antibodies in human or mice (12,18). The relationship between anti-dengue virus antibody and autoantibody was elucidated with monoclonal antibodies generated from dengue virus-infected mice. ELISA binding on either dengue antigen or dengue virus infected cell was used to screen the anti-dengue virus antibodies. We are particularly interested in the cross-reactive autoantibodies, therefore, the anti-platelet binding by FACScan analysis was also used to screen the monoclonal antibodies. More than 20 different clones were generated from several
fusions. Among the anti-platelet antibodies, Figure 1 showed several monoclonal antibodies that react with human platelet. Three patterns of platelet binding were observed based on the degree of fluorescent intensity. Each category of platelet binding had several clones, only representative ones were shown. The strong binding clones are 11-F6-C3, 3-D7-D3, 13-F4-G5, 15-B11-D10, and 16-G3-C3; the intermediate binding, 15-G10-B9; the dull binding, 8-F1-B6. Their immunoglobulin classes are different; 3-D7-D3 and 8-F1-B6 are IgM while the rest are IgG1.

**Cross-reactivity between dengue virus NS-1 protein and platelet or other self-antigens.** Among the anti-platelet antibodies, the dengue antigen specificity was determined. It was found that 13-F4-G5 can specifically recognize the dengue virus, but not herpes simplex virus antigen as shown by immunohistochemical staining and FACScan analysis on dengue virus infected BHK cells (Fig. 2B & 2C). To determine which protein is recognized, dengue virus-infected C6/36 cell lysate was run on PVP membrane and stained with 13-F4-G5 antibody. As shown in Fig. 2A, it is the NS-1 from dengue virus infected C6/36 cell lysate that was bound by 13-F4-G5 on Western blot. Clones such as 8-F1-B6, 15-G10-B9, 11-F6-C3, 3-D7-D3, and 13-F4-G5 can bind recombinant NS-1 protein by ELISA, but 15-B11-D10 and 16-G3-C3 are not NS-1-reactive (data not shown). Using NS-1 to competitively block the binding, the platelet binding of 13-F4-G5 could be dose-dependently inhibited (Fig. 3). This suggests that a cross-reactive epitope between NS-1 and platelet antigen was recognized by 13-F4-G5 monoclonal antibody. The platelet antigens recognized by 13-F4-G5 were then further determined by Western blot analysis on platelet lysate. Several bands with high molecular weight around 150 kDa were recognized by 13-F4-G5 (Fig. 4A), the binding were more intensive on thrombin (1 U/ml)-treated platelet than untreated platelet. This indicates that 13-F4-G5 preferentially recognize activated platelets. This preferential binding by 13-F4-G5 on thrombin-activated platelet was also demonstrated with FACScan analysis (Fig. 4B). The fluorescent intensity of 13-F4-G5 binding was higher in thrombin-treated platelet than non-treated platelet. Furthermore, P1 (amino acids 1-15) is the immunodominant linear epitope of NS-1 recognized by dengue patient sera (11). We tested whether P1 is also dominant in these anti-NS-1 antibodies. Only 8-F1-B6 recognized the P1-peptide, the rest of anti-NS-1 antibodies are P1-peptide binding negative (data not shown).

The cross-reactivity with other cellular antigens was further demonstrated by FACScan analysis on dengue virus infected cells. Surface or intracellular staining was used to localize the antigen on membrane or in cytoplasm, respectively. Both 13-F4-G5 and 15-B11-D10 recognized dengue virus infection-induced antigen in cytoplasm (Fig. 5). But 3-D7-D3 would stain BHK intracellularly irrespective of the dengue virus infection, which suggests that 3-D7-D3 recognized cellular antigen of BHK. Similarly, 11-F6-C3, 16-G3-C3, and
15-G10-B9 could stain the BHK cells on the surface and intracellularly, which indicates that the cellular antigen of BHK recognized by these mAbs is expressed on the cell surface. The 8-F1-B6 recognized cytoplasmic antigen of BHK cells. We have repeated the binding assay using K562 cell and obtained the same pattern of binding with these mAbs (data not shown). Individual clone has its own characteristic features with regard to the binding of P1, NS-1, platelet, or BHK cells, as summarized in Table 1. This suggests that cross-reactivity between dengue virus NS-1 protein and self-antigens does exist.

*The effect of anti-platelet antibody on platelet function.* When the platelet was incubated with monoclonal antibody, we did not observe the platelet degranulation by $^3$H-serotonin release assay (data not shown). However, in the presence of complement, 13-F4-G5, 11-F6-C3, and 15-B11-D10-binding platelets would be lysed (Fig. 6). Moreover, anti-platelet mAb would enhance the ADP-induced platelet aggregation. At suboptimal amount of ADP (5 μM), the platelet aggregation was enhanced by 13-F4-G5 in a dose-dependent manner (Fig. 7). 13-F4-G5 alone or together with goat anti-mIgG antibodies did not cause the platelet aggregation spontaneously. Based on the data above, we conclude that anti-dengue virus, especially anti-NS1, antibodies would cross-react with platelet as well as cellular self-antigen, and cause their dysfunction.

**Self evaluation**

We have found that the dengue infection can be used as a model to study the molecular mimicry between autoimmunity and virus infection.