INDUCTION OF ACUTE THROMBOCYTOPENIA AND INFECTION OF MEGAKARYOCYTES BY RAUSCHER MURINE LEUKEMIA VIRUS REFLECT THE GENETIC SUSCEPTIBILITY TO LEUKEMOGENESIS*

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Thrombocytopenia may accompany or follow a variety of viral infections including influenza (1), measles (2), rubella (3), chickenpox (4), Dengue (5, 6), and cytomegalovirus infections (7). Some human viruses have been shown to interact with circulating blood platelets (influenza, rubella).

Preleukemic thrombocytopenia has been observed in the latency period of leukemia after infection by several murine leukemia viruses, especially in Friend leukemia virus (8) and Rauscher murine leukemia virus (RMuLV) infections (9). In the RMuLV model, thrombocytopenia develops early after infection and lasts throughout the latency period. Platelet counts return to normal before the development of erythroleukemia, after which they fall again. In the case of spontaneous leukemia in AKR mice, thrombocytopenia has also been observed during the weeks preceding development of leukemia (10). It has been suggested that some thrombocytopenia-inducing viruses, especially leukemia viruses, could replicate in the cytoplasm of marrow megakaryocytes, cells from which circulating blood platelets are shed. Indeed, budding type C viral particles have been detected by electron microscopy after infection by Newcastle disease virus (11), Moloney's, Gross', Friend, and Manaker viruses (12), RMuLV (9), and BL/F virus (13).

In addition, it has been demonstrated that susceptibility to viral-induced leukemogenesis in mice is under genetic control and that the major histocompatibility complex (H-2) appears to be specifically involved (14-17). The gene(s) controlling the susceptibility to Friend leukemia virus seems to be related to the D region of the H-2 complex (18). It has also been shown that susceptibility to leukemogenesis is inherited as a dominant trait (14, 19). However, the mechanisms of action of this gene(s) and the nature of the gene product are not yet defined.

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Abbreviations used in this paper: CATCH, Ca++- and Mg++-free Hanks' balanced salt solution with adenosine, theophylline, trisodium citrate and Hepes buffer; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; R MuLV, Rauscher murine leukemia virus.
The aim of this work was first to determine if preleukemic thrombocytopenia induced by RMuLV was also under genetic control, and second to evaluate the possible expression of viral antigens on megakaryocytes and platelets in infected mice and to investigate the H-2 dependency of these antigenic expressions. The immediate interactions between platelets and RMuLV have been considered particularly in relation to the genetic susceptibility to leukemogenesis.

Materials and Methods

Mice. 6–8-wk-old female BALB/c and C57BL/6 mice were purchased from IFFA CREDO Laboratories, Centre de Recherche et d’Elevage des Oncins, St. Germain-sur-l’Arbresle, France. CBA, C3H, and DBA/2 mice were purchased from Charles River Breeding Laboratories, Inc., Elbeuf, France. BXSB mice were obtained from The Jackson Laboratory, Bar Harbor, ME. B10.D2, B10.BR, B10.G, B10.A(4R), B10.A(5R), B10.T(6R), B10.HTG, C57BL/10ScSn, and DBA/1 mice were purchased from Olac Laboratories, Oxon, England. (DBA/2 × BXSB) F1, (C3H × BXSB) F1, and (BALB/c × C57BL/6)F hybrids were obtained by local breeding.

Virus. Purified RMuLV-JLS V9 was obtained from Frederick Cancer Institute, Frederick, MD. The virus (10^12 viral particles/ml) was injected intravenously or intraperitoneally at dilutions varying from 1:5 to 1:2 × 10^5 in 0.2 ml sterile phosphate-buffered saline (PBS), 0.01 M, pH 7.2.

Antisera. Goat anti-Rauscher gp70 antiserum was a gift from Dr. J. H. Elder, Scripps Clinic and Research Foundation, La Jolla, CA. Fluorescein isothiocyanate (FITC) rabbit anti-goat IgG was obtained from Nordic, Lausanne, Switzerland.

Blood Platelet Counts. Blood was obtained from the retroorbital plexus by using 20 μl microcapillaries and was immediately diluted 1:100 in Unopette kits (Becton, Dickinson & Co., Basel, Switzerland). The diluted blood sample was allowed to settle for 20 min in an “improved Neubauer” hemocytometer and platelets were counted under a Leitz phase-contrast microscope (E. Leitz, Inc., Rockleigh, N J) at 400 × magnification.

Bone Marrow Samples. Bone marrow was harvested from mouse femurs and tibias in CATCH medium (20), made of Ca++- and Mg++-free Hanks’ balanced salt solution (Gibco AG, Basel, Switzerland) containing 10^{-3} M adenosine, 2 × 10^{-3} M theophylline, 3.8% trisodium citrate, 25 mM Hepes buffer, and 3.5% bovine serum albumin fraction V (all from Sigma Chemicals, Zurich, Switzerland). This medium has been shown to prevent the rapid vacuolization and degranulation of megakaryocytes observed when other media are used (20). The marrow specimens were converted into a single-cell suspension by repeated aspiration-extrusion from a Pasteur pipette. For immunofluorescence studies, megakaryocyte-enriched populations were obtained by discontinuous gradient centrifugation in Percoll (Pharmacia Fine Chemicals, Zurich, Switzerland) (modified from Rabellino et al. [21]). For cell transfer experiments, megakaryocytes were further isolated by velocity sedimentation. Purity of the final cell suspension was 90%.

Immunofluorescence Studies on Megakaryocytes and Platelets. The megakaryocyte-enriched populations (3–6% of megakaryocytes) were washed twice (300 g, 10 min, 4°C) in CATCH medium and spun on a cytocentrifuge (Shandon Southern Instruments Inc., Sewickley, PA). The smears were fixed in acetone at −20°C for 10 min and incubated for 30 min at 37°C with goat anti-gp70 and rabbit anti-p30 antibodies. Normal goat serum, normal rabbit serum, or PBS were used as controls. The smears were washed three times in PBS 0.01 M, pH 7.2 and reincubated in the same conditions with the corresponding FITC conjugate. After three further washes in PBS, the slides were mounted and examined under a Leitz Orthoplan immunofluorescence microscope. Isolated platelets were obtained by differential centrifugation as follows: citrated blood (9 vol blood/1 vol 3.13% sodium citrate) was first centrifuged at 1,600 g for 4 min at room temperature. The platelet-rich plasma was then centrifuged at 2,200 g for 10 min at room temperature to obtain the platelet pellet. This pellet was then washed three times in EDTA-PBS (0.009 M Na2 EDTA, 0.0264 M Na2HPO4·2H2O, 0.14 M NaCl), pH 6.9. After fixation in
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Fig. 1. Thrombocytopenia after intravenous injection of RMuLV. H-2d (BALB/c, DBA/2, B10.D2), H-2k (C3H, CBA, B10.BR), H-2q (C57BL/6, BXSB, C57BL/10), and H-2k (DBA/2, B10.G) mice were injected intravenously with 0.2 ml RMuLV (1:20) on day 0. Blood platelets were counted at 1 h, 1 d, 4 d, and 9 d after the injection of RMuLV. Each point represents an individual mouse (B6 = C57BL/6; B10 = C57BL/10).

suspension by paraformaldehyde (1% wt/vol in PBS) and three further washes, the platelets were analyzed by indirect immunofluorescence, using anti-gp70 and anti-p30 antibodies.

Assay for gp70. Concentrations of gp70 in sera of mice injected with RMuLV were determined by their capacity to inhibit the binding of goat anti-feline leukemia virus antibody to 125I-labelled gp70 from RMuLV. The details of this radioimmunoassay were previously described (22).

Results

H-2 Dependency of RMuLV-induced Thrombocytopenia. The occurrence of preleukemic thrombocytopenia after the infection of mice with RMuLV was first confirmed in BALB/c mice susceptible to RMuLV-induced leukemia. To study the kinetics of this thrombocytopenia in more detail, BALB/c mice were infected intraperitoneally with 0.2 ml RMuLV-JLS.V9 diluted 1:20 in sterile PBS. Blood samples for hematological parameters were collected sequentially. Thrombocytopenia developed within 4 d. A rebound phenomenon was observed 10–14 d after intraperitoneal injection. During that period, other cell counts were normal. The same experiment was performed on two strains of mice that are either susceptible (BALB/c) or resistant (C57BL/6) to leukemogenesis. Several dilutions (1:5, 1:10, 1:20, 1:200, 1:2 × 10³, 1:2 × 10⁴, and 1:2 × 10⁵) of RMuLV were injected intraperitoneally into BALB/c (H-2d) and C57BL/6 (H-2k) mice. Platelet counts taken 4 d after the injection showed that the degree of thrombocytopenia was dose dependent in BALB/c mice with virus dilutions from 1:20 to 1:2 × 10³. When virus was injected into BALB/c mice at dilutions of 1:5 and
Table I

Susceptibility to RMuLV-induced Thrombocytopenia in B10 Congenic and B10 Intra-H-2-recombinant Mice

<table>
<thead>
<tr>
<th>Strains</th>
<th>H-2-Tla complex</th>
<th>Qa</th>
<th>Percent decrease in platelet counts*</th>
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<td>K</td>
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<td>B10.A(3R)</td>
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<td>B10.HTG</td>
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<td>B10.BR</td>
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<td>B10.G</td>
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* Platelets were counted 1 d after intravenous injection of 0.2 ml RMuLV (diluted 1:20). Results are expressed as percentage of decrease in platelet counts compared with preinjection levels (mean of five to seven mice in each group). B10, C57BL/10.

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Fig. 2. Kinetics of expression of viral antigens, gp70 and p30, in bone marrow megakaryocytes of BALB/c mice. R MuLV (0.2 ml, 1:20) was injected intraperitoneally on day 0. Results are expressed as the percentage of positive megakaryocytes for either gp70 or p30 antigens (detected by indirect immunofluorescence). MKC, megakaryocytes.

1:10, the thrombocytopenia was not more pronounced than with 1:20, but the kinetics of the response were different; the thrombocytopenia occurred 1 d after the injection of the virus and the rebound phenomenon was observed after 4 or 5 d. In contrast, platelet counts remained unaffected at any dilution in C57BL/6 mice.

Thrombocytopenia after R MuLV injection was then evaluated in H-2d (BALB/c, DBA/2, and B10.D2) and H-2k (C3H, CBA, and B10.BR) mice, which are known to be susceptible to leukemia induction by R MuLV, as well as in H-2k (C57BL/6,
Fig. 3. (A) Immature megakaryocytes exhibiting peripheral staining for gp70 antigen (indirect immunofluorescence). Day 12 after intraperitoneal injection of 0.2 ml R MuLV (1:20) (X 630). (B) Mature megakaryocytes exhibiting reticular staining for gp70 antigen. Day 16 after intraperitoneal injection by R MuLV (same dose) (X 630). (C) Mature megakaryocytes releasing cytoplasmic fragments. Day 16 after intraperitoneal injection of R MuLV (same dose) (X 400). (D) Circulating blood platelets exhibiting gp70 antigen. Day 22 after intraperitoneal injection of R MuLV (same dose) (X 630).
BXSB, C57BL/10ScSn) and H-2^d (DBA/1, B10.G) mice, which are resistant to leukemia induction. Both intraperitoneal and intravenous injections were performed, using 0.2 ml 1:20 diluted RMuLV. The kinetics of thrombocytopenia differed according to the route of injection. After intraperitoneal injection, thrombocytopenia developed within 3–4 d; the rebound phenomenon occurred after 10–14 d in the H-2^d and H-2^k strains. However, intravenous injection induced thrombocytopenia within
a few minutes in H-2^d and H-2^k strains and platelet counts returned to pretreatment levels within 9 d (Fig. 1). Whatever the route of injection, no change in platelet counts was observed in the H-2^b and H-2^q strains examined. 0.2 ml buffer alone did not induce any decrease in platelet counts even within the first minutes after intravenous injection. No relation between Tla regions and thrombocytopenia was observed in these various strains of mice.

Because susceptibility to virus-induced leukemogenesis is known to be inherited as a dominant trait (4), the occurrence of thrombocytopenia after RMuLV injection was investigated in F_1 hybrid mice from “sensitive” and “resistant” parents. Intravenous injection of 0.2 ml RMuLV (diluted 1:20) was performed in (BALB/c X C57BL/6)F_1, (DBA/2 X BXSB)F_1, and (C3H X BXSB)F_1 female mice. Thrombocytopenia was observed in each of these three hybrids: (BALB/c X C57BL/6)F_1 RMuLV-injected: 0.59 ± 0.07 × 10^6 platelets/mm^3; control: 1.39 ± 0.08 × 10^6 platelets/mm^3; (DBA/2 X BXSB)F_1 RMuLV-injected: 1.03 ± 0.20 × 10^6 platelets/mm^3; control: 1.60 ± 0.07 × 10^6 platelets/mm^3; (C3H X BXSB)F_1 RMuLV-injected: 0.71 ± 0.03 × 10^6 platelets/mm^3; control: 1.41 ± 0.08 × 10^6 platelets/mm^3.

To further investigate the H-2 dependency of thrombocytopenia induction, RMuLV was injected into B10 intra-H-2-recombinant mice. Table I shows the origin of H-2 regions of the mice investigated, together with their susceptibility to RMuLV-induced thrombocytopenia. All the mice with allele d at the D-end developed thrombocytopenia. Each strain susceptible to thrombocytopenia induction differed from its resistant counterpart only at the D region of the H-2-Tla complex. For example, as in B10.T(6R) mice, even if the d allele is present only at the D-end and if the rest of H-2 complex is made of “resistant” alleles, thrombocytopenia developed with the same pattern as in H-2^d or H-2^k mice. In contrast, in B10.HTG, which have b allele at the D-end but d allele in the other regions of the H-2 complex, thrombocytopenia did not occur.

Expression of RMuLV Antigens on Megakaryocytes and its Relation to H-2 Complex.

The relationship between the occurrence of thrombocytopenia and possible viral antigen expression on marrow megakaryocytes was then investigated. RMuLV was injected intraperitoneally in BALB/c mice at a dose sufficient to induce thrombocytopenia (1:20 dilution, 0.2 ml/mouse). Then, at specific intervals thereafter, groups of five mice were killed and bone marrow was harvested from 4 to 35 d after infection. Megakaryocyte-enriched fractions prepared by discontinuous gradient centrifugation were smeared and analyzed by indirect immunofluorescence using antisera specific for gp70 or p30 antigens. On each slide, at least 2.5 × 10^3 megakaryocytes at different maturation stages were examined. Viral antigens were detectable from the 4th d of infection and the frequency of viral antigen-carrying-megakaryocytes increased progressively until day 35. In general, the appearance of gp70 antigen in megakaryocytes preceded that of p30 antigen (Fig. 2).

As can be seen in Fig. 3 A and B, gp70 antigen was detectable in both immature and mature megakaryocytes. However, the pattern of staining differed in immature and mature cells: young cells exhibited a peripheral pattern (cytoplasmic membrane) (Fig. 3 A), whereas fully mature megakaryocytes, with a well-developed demarcation membrane system, were stained with a reticular pattern throughout the cytoplasm (Fig. 3 B). The pattern of staining for p30 was essentially the same. This suggested the presence of viral antigens throughout the demarcation membrane system. It can also
be seen that cytoplasmic fragments of megakaryocytes (Fig. 3C), which would eventually give rise to circulating blood platelets, carried viral antigens. In fact, when the circulating blood platelets were examined for the presence of gp70 antigen, ~30% of them were found to be positively stained (Fig. 3D).

Because evidence of H-2-dependent susceptibility to thrombocytopenia induction was provided by the previous experiments, the expression of gp70 and p30 antigens was investigated in various strains of mice. H-2d (BALB/c, DBA/2, B10.D2), H-2k (C3H, CBA, B10.BR), H-2b (C57BL/6, BXSB, C57BL/10ScSn), and H-2q (DBA/1, B10.G) mice received intraperitoneal injections of 0.2 ml 1:20 diluted RMuLV. The megakaryocytes harvested 16 d after injection were examined for the presence of viral antigens gp70 and p30 (Fig. 4). Only murine strains with either H-2d or H-2k haplotype exhibited positive staining for both viral antigens in their megakaryocytes, although some differences were observed in the magnitude of viral antigen expression. On the other hand, megakaryocytes from resistant strains of mice (H-2b or H-2q mice) did not express any viral antigens. In similar experiments, (BALB/c × C57BL/6), (C3H × BXSB), and (DBA/2 × BXSB)F1 hybrids also expressed gp70 and p30 antigens after injection of RMuLV, which correlates with the inheritance of susceptibility to thrombocytopenia.

The ability to transfer RMuLV infection with megakaryocytes isolated from infected mice was assessed. BALB/c mice were infected by intraperitoneal injection with RMuLV (1:20). 16 d later, mice were killed and bone marrow harvested. Megakaryocytes were isolated from bone marrow, and the megakaryocyte suspension

![Graph](https://example.com/graph.png)

**Fig. 4.** Expression of viral antigens, gp70 and p30, in bone marrow megakaryocytes of various strains of mice. The percentage of megakaryocytes positively stained for gp70 or p30 were evaluated 16 d after intraperitoneal injection of 0.2 ml RMuLV (1:20). MKC, megakaryocytes.
Fig. 5. Development of thrombocytopenia by injection of megakaryocytes from RMuLV-infected mice. Megakaryocytes were purified from bone marrow of BALB/c mice infected by intraperitoneal injection of 0.2 ml RMuLV (1:20) sixteen d before. 10⁸ MKC were injected intravenously into normal BALB/c mice on day 0 (○). As a control, 10⁸ MKC from normal BALB/c mice were injected into syngeneic mice (□). Each point represents individual platelet counts; bars represents means of platelet counts. MKC, megakaryocytes.

(90% pure) was injected after three washes in CATCH medium into the tail vein of five normal BALB/c mice (10⁸ megakaryocytes per mouse in 0.4 ml CATCH). Approximately 80% of the megakaryocytes in this preparation were positively stained for gp70 antigen. No other cell was positive for viral antigens. Megakaryocytes were similarly prepared from normal BALB/c mice and injected in other normal BALB/c mice as control. All the BALB/c mice that were injected with megakaryocytes isolated from infected animals developed thrombocytopenia within a week (Fig. 5). This thrombocytopenia lasted 6 mo, and during that time serum gp70 levels rose to 49.5 ± 11.9 µg/ml (normal values, 0.9 ± 0.2 µg/ml). During the 7th mo, erythroleukemia developed. In contrast, no change in platelet counts or in serum gp70 levels were detected, and leukemia did not develop in BALB/c mice injected with normal syngeneic megakaryocytes.

Discussion

In this study, we have shown that thrombocytopenia, which is known to occur in the preleukemic phase of R MuLV-induced disease (9), is under genetic control and is more specifically related to the H-2 complex. Both intraperitoneal and intravenous injection of R MuLV were used: in each case only H-2d and H-2k mice developed thrombocytopenia, whereas H-2b and H-2q mice did not. Unlike the thrombocytopenia due to Friend leukemia virus infection, which occurs only 7 d after intravenous injection (8), thrombocytopenia is observed within a few minutes after intravenous injection of R MuLV. Such a rapid development of thrombocytopenia is most likely due to platelet destruction in the periphery, possibly the result of direct interaction between platelets and viral particles.

The study of the response to R MuLV in various strains of mice, and most significantly in B10-congenic mice, suggests that the susceptibility to R MuLV-induced acute thrombocytopenia is controlled by a gene(s) closely linked to the H-2 complex. Furthermore, results using B10-intra-H-2-recombinant mice indicate that the gene(s) coding for susceptibility to thrombocytopenia is associated with the D region of the H-2 complex. The H-2Dd allele is associated with susceptibility to thrombocytopenia...
induction, whereas the H-2D<sup>b</sup> allele confers resistance. One should note that neither the QA<sub>a</sub> 3, 2, 1, nor the Tla regions are involved in conferring susceptibility to R MuLV-induced thrombocytopenia.

Our results suggest that the gene(s) controlling susceptibility to R MuLV-induced thrombocytopenia is the same gene(s) (or closely linked to the gene) that controls the susceptibility to leukemogenesis. This hypothesis is supported by the following evidence. First, R MuLV induces thrombocytopenia only in leukemia-susceptible H-2<sup>d</sup> and H-2<sup>b</sup> mice but not in leukemia-resistant H-2<sup>b</sup> and H-2<sup>a</sup> mice. Second, as in the case of the gene controlling leukemogenesis (14, 19), the susceptibility to thrombocytopenia is inherited as a dominant trait, as suggested by the results in F<sub>1</sub> hybrids.

Type C viral particles have been detected by electron microscopy in the cytoplasm of marrow and spleen megakaryocytes after injection by R MuLV (12, 23, 24). We have shown in this study that viral antigens are expressed on megakaryocyte cell membranes. The glycoprotein of the virus envelope (gp70), as well as the core protein (p30), are detectable on the surface and throughout the demarcation membrane system of megakaryocytes. The presence of p30 antigen together with gp70 antigen is indirect evidence of viral replication inside these cells, because core proteins are produced during active viral replication. More directly, the presence of infective virus in megakaryocytes is suggested by the fact that isolated megakaryocytes from infected mice, when injected in normal syngeneic mice, can trigger thrombocytopenia and induce erythroleukemia. It is striking that in the initial phase of R MuLV infection, megakaryocytes are the only marrow cells exhibiting viral antigens. These observations suggest that megakaryocytes may be among the first sites of R MuLV replication.

The infection of megakaryocytes by R MuLV appears to be governed by the same gene that controls susceptibility to thrombocytopenia, because only those megakaryocytes from mice developing thrombocytopenia express viral antigens. The preferential expression of viral antigens in megakaryocytes suggests the existence of a receptor-like molecule for R MuLV on megakaryocyte cell membranes. This molecule would be the gene product of the H-2 linked gene that confers susceptibility to R MuLV-induced thrombocytopenia as well as leukemogenesis. In view of the fact that platelets and megakaryocytes share most of their membrane components, it is probable that platelets would also bear this H-2-linked receptor-like molecule on their membrane. This would be consistent with the rapid in vivo effect of R MuLV on platelets of susceptible mice after intravenous injection. This mechanism could be of relevance in the development of virus-induced leukemia in mice.

Summary

Acute thrombocytopenia and megakaryocyte infection have been investigated during the preleukemic phase of the disease induced by the Rauscher murine leukemia virus (R MuLV) in mice. Injection of R MuLV, either intravenously or intraperitoneally, rapidly induced thrombocytopenia, possibly as a result of direct interaction between platelets and viral particles. The susceptibility to this acute thrombocytopenia was genetically controlled and was inherited as a dominant trait. Murine strains with H-2<sup>d</sup> or H-2<sup>b</sup> haplotype, which are susceptible to the induction of leukemia by R MuLV, developed thrombocytopenia, whereas leukemia-resistant H-2<sup>b</sup> and H-2<sup>a</sup> strains of mice failed to develop thrombocytopenia. Using B<sub>10</sub> H-2-congenic and intra-H-2-recombinant mice, it was shown that the susceptibility to R MuLV-induced
thrombocytopenia was controlled by gene(s) in or closely linked to the D region of the H-2 complex.

Megakaryocytes may be one of the first sites for the replication of RMuLV. Indeed, among bone marrow cells, only megakaryocytes expressed viral antigens gp70 and p30 during the initial phase of RMuLV infection. In addition, megakaryocytes from infected mice were able to transfer preleukemic thrombocytopenia as well as leukemia in syngeneic mice. The infection of megakaryocytes by RMuLV appears to be genetically controlled in a manner similar to the induction of thrombocytopenia, since only the megakaryocytes from mice developing thrombocytopenia were infected by RMuLV. These results indicate that the gene(s) governing the induction of thrombocytopenia by RMuLV may be the same gene(s) (or closely linked to the gene) that controls the susceptibility to leukemogenesis, and would be consistent with the expression of the gene product, presumably a receptor-like molecule for RMuLV, on platelet and megakaryocyte membranes.

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References


