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Evidence that platelet-derived microvesicles may transfer platelet-specific immunoreactive antigens to the surface of endothelial cells and CD34$^+$ hematopoietic stem/progenitor cells - implication for the pathogenesis of immune thrombocytopenias

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Abstract: The pathogenesis and tissue damage that accompanies destruction of platelets in immune thrombocytopenias (IT) is still not understood very well and in addition to platelets, other cells (*e.g.* endothelial cells, CD34$^+$ hematopoietic stem/progenitors) may also become affected. Based on our previous work that platelet antigens (*e.g.*, CD41) may be transferred by platelet-derived microvesicles (PMV) to the surface of other cells, we asked if platelet derived-antigens, especially those that are involved in the formation of anti-platelet antibodies in IT (*e.g.*, against antigen HPA1a) could be also transferred by similar mechanism. To address this issue normal human CD34$^+$ cells, human umbilical vein-endothelial cells (HUVEC) and monocytic cell line THP-1 were incubated with PMV derived from HPA1a$^+$ donors. We noticed that the HPA1a antigen is highly expressed on PMV-derived from the HPA1a positive platelets and is transferred in PMV-dependent manner to the surface of CD34$^+$ cells, HUVEC and monocytic THP-1 cells. These cells covered with HPA1a positive PMV but not by PMV derived from HPA1a negative platelets reacted with anti-HPA1a antibodies derived from the alloimmunized pregnant women. More importantly, human hematopoietic cells that were preincubated with HPA1a$^+$ PMV and subsequently exposed to anti-HPA1a serum and human NK cells, become subject to elimination by antibody dependent cell cytotoxicity ADCC. Thus, we postulate that PMV-dependent transfer of antigens may playing an important role in "expanding" the population of target cells that may be affected by anti-platelet antibodies and explain several pathologies that accompany IT (*e.g.* damage of endothelium, cytopenias).

Key words: MV - ITP - Stem/progenitor cells - Endothelium - ADCC

Introduction

IT develops as a result of immune-mediated destruction of platelets (PLT) by IgG antibodies that interact with some antigens expressed on their surface (*e.g.*, GPIIb-IIIa, GPIa-IIa) [8,9]. Pathogenesis of IT involves both lymphocytes reactive with PLT specific antigens [14,15] as well as autoreactive antibodies [16,18]. PLT covered with antibodies are eliminated by macrophages in Fc R-dependent mechanism [9]. In addition autoantibodies may also directly suppress megakaryopoiesis in bone marrow [7,17]. The IT develop both in older population [19] and in children [24].

Microvesicles (MV) are small fragments of cell membrane between 100 nm to 1 μm in size that are released by eukaryotic cells during cell membrane turnover as well as cell activation [5,22]. MV are present in blood in steady state conditions at a concentration of approximately 30 μg/ml. Our group presented the evidence that PMV can facilitate hematopoietic stem cells engraftment in myeloablated recipients [11]. We also showed that PMV could

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stimulate normal and malignant hematopoietic cells growth and survival [4].

Recently, it has been shown that MV can facilitate immune processes in various systems. It was shown that melanoma as well as ovarian cancer cell derived MV can harbor Fas ligand (FasL) on their cell surface. Both studies in a very elegant way showed that these MV could actively induce apoptosis of T cells expressing Fas receptor [1-3] and thus allow tumor cells to escape immunosurveillance. Furthermore, we have demonstrated that PMV may transfer CXCR4 receptor from PLT to other cells thus improving their homing after transplantation in bone marrow [11] or even render CXCR4 negative cells susceptible to HIV infection [23]. Finally, we showed that PMV may transfer several platelet-derived adhesion molecules to the lung [13] or breast cancer cells [12] and thus increase their metastatic potential.

Based on these data we hypothesized that PMV shed from IT-patient platelets may transfer platelet derived-antigens (*e.g.*, against antigen HPA1a) involved in the formation of anti-platelet antibodies to other cells and thus "expand" the population of target cells that may be affected by anti-platelet antibodies. This could explain several pathologies that accompany IT (*e.g.* damage of endothelium, cytopenias).

Materials and methods

PMV preparation. PMV were prepared as described previously [4]. Briefly, platelets were activated by thrombin (0.1 U/mL) and collagen (4 µl/mL) for 30 min at 37°C with stirring. After activation platelets were centrifuged 2 times at 3000 pm (2000 g) for 15 min at 4°C and the PMV-enriched supernatants were collected. Supernatants were again centrifuged at 28000 g for 1 h at 4°C. PMV were washed and finally resuspended in Hepes buffer, pH=7.4. Quality of every PMV preparation was checked by flow cytometry analysis using phycoerythrin (PE)-conjugated anti-human antibodies against CD41 (Coulter-Immunotech, Marseille, France).

Isolation of human CD34$^+$ and human endothelial cells. Bone marrow samples were obtained according to procedures approved by Institutional Bioethics Committee. Light-density mononuclear cells (MNC) were separated by Ficoll-Isopaque density gradient centrifugation at 400 g for 30 minutes at room temperature. MNC were washed twice with PBS and CD34$^+$ cells were isolated by immunoaffinity selection with MiniMACS paramagnetic beads (Miltenyi Biotec, Germany) according to manufacturer's protocol. Briefly, cells were resuspended in isolation buffer and incubated for 30 minutes at 4°C with anti-human CD34 monoclonal antibody conjugated with microbeads, in a presence of FcR-blocking reagent. Labeled cells (CD34$^+$) were enriched on columns in the magnetic field. Unlabelled cells were washed out and after removal of the column from magnetic field CD34$^+$ cells were eluted with the plunger.

Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical vein using collagenase I (Sigma) digestion and propagated in M199 medium supplemented with 10% fetal calf serum, 1 mg/mL bovine brain extract, 1 ng/mL human epidermal growth factor, 1 mg/mL hydrocortisone, 10 U/mL heparin (all reagents from Sigma).

Binding and detection of PMV. Binding of HPA1a$^+$ PMV to target cells was evaluated using flow cytometry (THP1, CD34$^+$ cells) and immunofluorescent staining (endothelial cells). Nonadherent and adherent cells were incubated with PMV for 10 min to 2 h at 37°C in the presence of 2 mM $CaCl_2$ and nonadherent cells were washed twice at 1000 rpm (210 g) for 5 min to remove unbound platelets, the resulting cell pellets were resuspended in PMV binding buffer. Cover slips with adherent cells were washed three times. Next cells were incubated in presence of serum containing anti HPA1a antibodies at 1:4 dilution with PMV binding medium. Finally, cells were washed once or twice, resuspended in PBS and incubated with anti-human IgG FITC conjugated antibodies (DakoCytomation) to detect presence of HPA1a on the surface of target cells or with control anti-CD 41 MoAb (BD Sciences).

Cell proliferation by MTT Assay. The MTT assay was performed according to the manufacturer's recommendations (Promega). Briefly, THP1 cells were seeded in 96-well plates at 10^4/well in DMEM medium containing different dilutions of HPA1a$^+$PMV or HPA1a$^-$PMV serum (1/2, 1/4, 1/8, 1/16, 1/32). After 72 h 20 µl of CellTiter 96 Aqueous One Solution reagent were added to each well and plates were incubated for 3-4 h. Subsequently, plates were read at 490 nm using the ELx800 Universal Microplate Reader (Bio-tech) and analyzed with KC4 v3.0 with PowerReports software.

Cytotoxicity assay. Target cells (THP-1) were labeled with 1mM of Calcein AM in RPMI at 37°C for 20 min and washed twice with PBS. Labeled cells were incubated with HPA1a$^+$ PMV for 10 min to 2 h washed and incubated with serum containing anti-HPA1a antibodies. Cells were washed and incubated with the population of NK effector cells (human YT cell line) for 4 hours at several ratios (1:10, 1:25, 1:50 and 1:100). Dead cells were scored by FACS after propidium iodide staining.

CFU-GM colony formation. 0.2×10^4 CD34$^+$ cells were resuspended in 0.2 ml IMDM medium (Sigma Chemicals) containing 10 ng/ml IL-3 and 5 ng/ml GM-CSF. Subsequently 0.9 ml of Methocult medium (Stem Cells Technologies) containing growth factors was added, mixed gently and then plated in duplicate on 24-well culture plates (0.5 ml/well). Cultures were incubated at 37°C in fully humidified atmosphere supplemented with 5% CO_2. Colony-forming units-granulocyte-macrophage (CFU-GM) were identified and counted using an inverted microscope after 14 days of culture.

Statistical analysis. Statistical analysis of the data was performed with Origin 5.0 using the Mann-Whitney test, with $p<0.05$ considered significant.

Results

Binding of HPA1a^{ve+}PMV to hematopoietic and endothelial cells

We have shown before that several antigens, including integrins and chemokines receptors, present on platelets surface can be transferred between PLT and other cells by PMV [1,11,23]. Here we evaluated if HPA1a antigen, that is involved in generation of PLT-destructing response could be also transferred by PMV to other cells such as normal human CD34$^+$ cells, human umbilical vein-endothelial cells (HUVEC) and monocytic (THP-1) cell line.

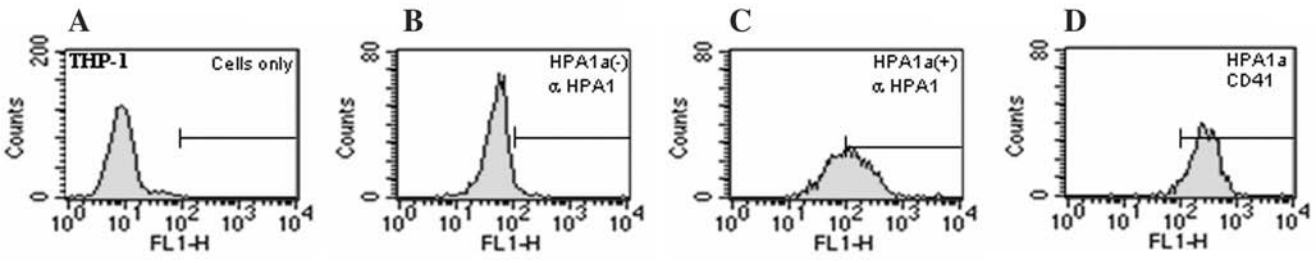

Fig. 1. Binding of HPA1a positive PMV to THP-1 and TF-1 cells. Detection of HPA1a$^+$ PMV and HPA1a- PMV on monocytic THP-1 cells (**A**) - cells only, (**B**) - HPA1a negative PMV, (**C**) - HPA1a positive PMV, (**D**) - positive control. Experiment was repeated 2 times with similar results. Representative data are shown.

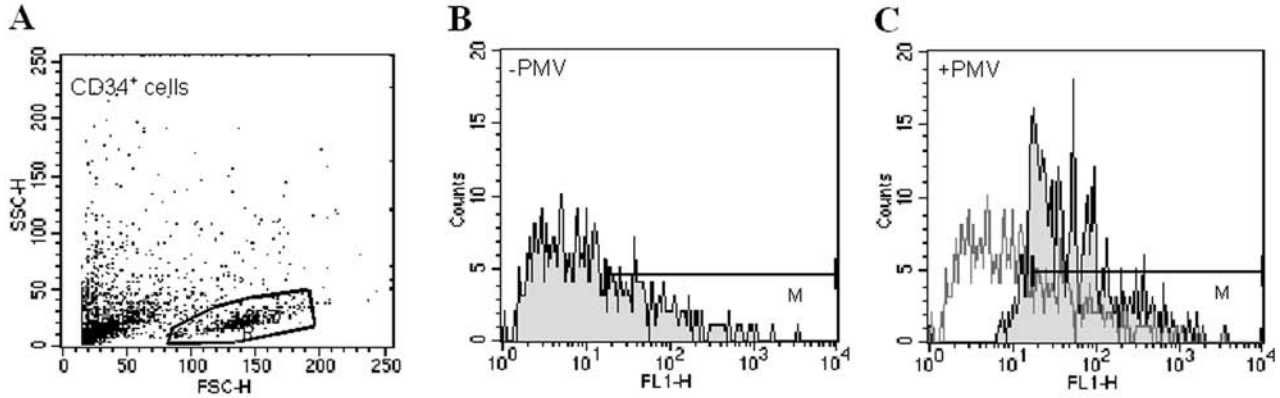

Fig. 2. Binding of HPA1a positive PMV to CD34$^+$ cells. **A**. Cytogram of CD34 positive cells. **B**. Detection of HPA1a antigen on CD34 incubated with anti-HPA1a serum and anti-human FITC conjugated Ab in absence of PMV. **C**. Detection of HPA1a antigen on CD34 cells preincubated with HPA-1a$^+$ PMV and than exposed to anti-HPA1a serum followed by anti-human FITC conjugated Ab. Experiment was repeated 3 times with similar results. Representative data are shown.

In the first set of experiments THP-1 cells were incubated with PMV derived from HPA1a positive and negative platelets followed by incubation with HPA1a reactive serum from the alloimmunized pregnant women. The binding of anti-HPA1a immunoglobulins to cells preincubated with PMV were detected with FITC-conjugated anti-IgG Ab. As a control for equal binding of HPA1a positive and negative PMV we used staining with anti-CD41 MoAb. We noticed that the HPA1a antigen was highly expressed on PMV and was easily transferred to the surface of target cells in PMV dependent manner. PMV detection was highly specific because HPA1a antigen was only detected on the cells that were incubated with HPA1a positive PMV (Fig. 1C) and cells covered by PMV derived from HPA1a negative platelets did not bind anti-HPA1a antibodies (Fig. 1B). In control experiments both types of PMV transferred efficiently CD41 to the surface of the target cells (Fig. 1D).

Since HPA-1a antigen could be transferred by PMV to the other cells, we hypothesized that it could be also transferred to human CD34$^+$ cells. This could lead to destruction of CD34$^+$ stem/progenitor cells by anti-HPA-1a binding antibodies and thus contribute to the cytopenias which are seen in IT patients. To address this issue human bone marrow or cord blood derived CD34$^+$ cells were incubated with HPA1a$^+$ PMV and exposed to HPA1a reactive serum from the alloimmunized pregnant women. We found that highly purified CD34$^+$ cells were easily covered with PMV as evidenced by binding of anti-HPA1a serum (Fig. 2). This data suggests that CD34$^+$ cells could be a potential target for immune responses directed against platelet antigens.

Similar PMV-dependent mechanism could also contribute to destruction of endothelium in IT patients. To check this hypothesis that PMV may transfer immunoreactive HPA-1a to the endothelial cells we incubated HUVEC with HPA1a$^+$ PMV and subsequently exposed these cells to anti-HPA1a serum. By employing immunofluorescence microscope we confirmed PMV-mediated transfer HPA1a$^+$ to HUVEC (Fig. 3A). Again binding of anti-HPA1a Ab was very specific and anti-HPA1a serum did not bind to HUVEC incubated with HPA1a negative PMV (Fig. 3B). However binding of both HPA1a positive and

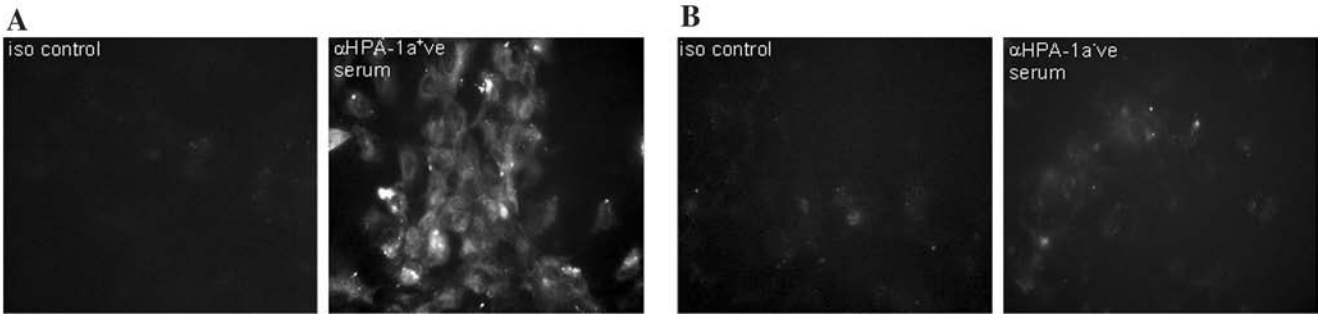

Fig. 3. Binding of HPA1a positive PMV to endothelial cells. **A**. Detection of HPA1a antigen on endothelial cells. HUVEC were incubated with HPA1a$^+$ PMV, washed and incubated with anti-HPA1a positive serum. Next, cells were washed again and anti-HPA1a antibodies were detected with anti-human IgG FITC conjugated Ab (HPA-1a$^+$ve serum). **B**. Detection of HPA1a antigen on endothelial cells was performed as in panel A except for the use of anti-HPA negative serum (HPA-1a-ve serum). Experiment was repeated 2 times with similar results. Representative data are shown.

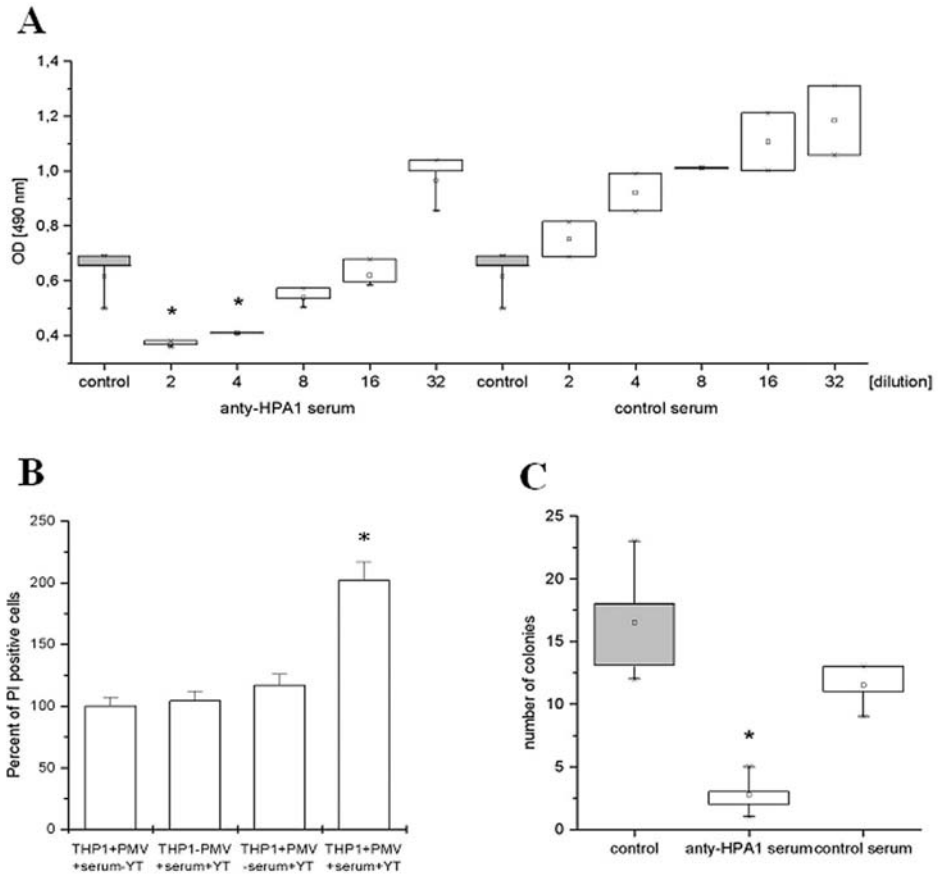

Fig. 4. ADCC assay with target cells coated with HPA1a positive PMV. **A**. THP-1 cells were incubated with HPA1a$^+$ PMV and subsequently with anti-HPA positive or negative serum (control). Proliferation of THP-1 cells was measured with MTT assay after 72 hours of incubation. Data from 2 independent experiments are pooled together; * $p<0.05$. **B**. THP-1 target cells coated with HPA-1a positive PMV were incubated with YT NK effector cell line in a presence or absence of HPA-1a positive serum. Data from 2 independent experiments are pooled together; * $p<0.05$. **C**. CD34$^+$ cells were coated with HPA1a$^+$ PMV and exposed to anti-HPA positive (n-2) or negative serum (n-2). CFU-GM colonies were scored after 11 days using inverted light microscope. Number of CFU-GM colonies /2×10^4plated cells is shown; * $p<0.05$. (A-C) each data is represented as a separate box. The horizontal lines in the box denote the 25th and 75th percentile values. The error bars denote the 5th and 95th percentile values. The square symbol in the box denotes the mean of data.

negative PMV was similar as assessed by level of CD41 antigen (data not shown).

HPA1a positive PMV render hematopoietic cells susceptible to immune responses

To address if PMV-transferred antigens can activate the immune responses to platelet antigens, we focused on activation of an Ab-dependent response that involves activation of complement cascade (Fig. 4A) as well as NK cell-mediated cellular response (Fig. 4B) to THP-1 cells covered or not-covered with PMV.

We noticed a significant decrease in THP-1 cell proliferation covered by HPA-1$^+$ PMV after exposure to reactive serum being both source of antibodies and complement proteins (Fig. 4A). Similarly viability of THP-1 cells covered by HPA-1$^+$ PMV was decreased in a presence of NK cell line YT as

evidenced by an increase in number of PI positive cells which prior to cytotoxic assay were covered by HPA-1$^+$ PMV (Fig. 4B).

Because we noticed that PMV transferred HPA-1 antigen to CD34$^+$ cells (Fig. 2) we looked at the influence of anti HPA-1 serum on CD34$^+$ cells colony formation. Accordingly, CD34$^+$ cells were coated with HPA-1$^+$ PMV and exposed to YT cells in a presence of anti HPA-1- or control serum. We noticed significant decrease of CFU-GM colony formation by CD34$^+$ cells which were covered by HPA-1$^+$ PMV and subsequently exposed to anti HPA-1 serum (Fig. 4C).

Discussion

PMV play an important role in intercellular crosstalk and as reported they may (i) stimulate target cells directly by surface-expressed ligands acting as a kind of "signaling complex" [22], (ii) transfer surface receptors from one cell to another [4,11,23], (iii) deliver proteins, mRNA, bioactive lipids into target cells [4,21], and finally, (iv) serve as a vehicle ("Trojan horse" mechanism) to transfer infectious particles between cells (*e.g.*, HIV or prions) [10,20,23]. Here we describe for the first time evidence that PMV may transfer immune-reactive antigen HPA-1a from the platelets to the membranes of hematopoietic cells. We postulate that this mechanism may play an underappreciated role in pathogenesis of IT-related cytopenias as well as in endothelium damage.

Evidence accumulates that the decrease in platelet count that is seen in IT patients is related to (i) clearance of IgG covered- PLT by immune system and (ii) damage of megakaryocytes [7,17]. It is well documented that the HPA-1a antigen expressed on the surface of the platelets may trigger an immune response that lead to the formation of anti-HPA-1a immunoreactive antibodies [6]. However, the exact mechanisms that lead to the tissue damage and cytopenias in IT patients are still unknown. In this paper we provide an evidence that hematopoietic cells covered by PMV bearing IT-related antigen HPA1a may bind anti-HPA1a immunoreactive antibodies and thus become a target for destruction by humoral (complement dependent) and cellular (NK dependent) immune mechanisms.

The fact that PMV transferred HPA1a antigen to the surface of the target cells is in agreement with our earlier data showing that several antigens expressed on platelet membranes (*e.g*, CD41 or CXCR4) could be transferred by PMV from platelets to the surface of normal and malignant hematopoietic cells as well as to the cancer cells [4,11-13,23]. We reported that platelet-derived surface molecules transferred to the target cells may play an important role in regulating adhesion of these cells [4,11] and susceptibility for HIV infection [23]. This supports a concept that platelet-derived surface molecules are functional after transfer and incorporation to the membranes of the target cells. This assumption is relevant for our study because in order to trigger the immune response, PMV-transferred HPA1a antigen has to be properly expressed on the target cells - what is required for its recognition and binding by the anti-HPA1a specific antibodies.

In order to demonstrate that cells that express PMV-derived HPA1a could trigger immune response we employed two different models. In the first model we showed that anti-HPA-1 Ab may trigger immune reaction against target cells by employing Ab-mediated complement-dependent response. In the second one the same Abs triggered NK-cell mediated cytotoxicity.

These results in overall suggests that in addition to observed destruction/elimination of platelets in IT patients, an underappreciated and important role in pathogenesis of this disorder may also play damage of both vascular endothelium and pool of hematopoietic CD34$^+$ stem/progenitor cells due to the transfer of platelet-related immune-reactive antigens by PMV. The clinical significance of this phenomenon is currently studied in our laboratories.

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