Natural human polyreactive IgM induce apoptosis of lymphoid cell lines and human peripheral blood mononuclear cells

Sooryanarayana Varambally1,3, Yaron Bar-Dayan1, Jagadeesh Bayry1, Sébastien Lacroix-Desmazes1, Michael Horn1, Marc Sorel1, Yosefa Bar-Dayan1, Giovina Ruberti2, Michel D. Kazatchkine1 and Srinivasa V. Kaveri1

1INSERM Unité 430 and Université Pierre et Marie Curie, Institut des Cordeliers, 75006 Paris, France
2Institute of Cell Biology, National Research Council, Campus ‘A. Buzzati-Traverso’, 00016 Monterotondo Scalo (Rome), Italy
3Department of Pathology, University of Michigan Medical School, Ann Arbor, MI 48109, USA

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Abstract

Natural polyreactive IgM autoantibodies, encoded by unmutated germline Ig V genes, represent a major fraction of the normal circulating IgM repertoire. We have previously shown that therapeutic preparation of pooled IgM exerts immunomodulatory effects as assessed by in vitro and in vivo studies. Here, we show that the IgM preparation induces cell death in lymphoblastoid cell lines and in human peripheral blood mononuclear cells. The IgM-induced cell death involved classical features of apoptosis such as nuclear fragmentation and activation of caspases. Treatment of leukemic cells with IgM resulted in the cleavage of poly-(A)DP ribose polymerase, a substrate of caspase, and in a reduction in mitochondrial transmembrane potential during the early period of apoptosis induction. Natural IgM-induced apoptosis was inhibited by soluble Fas molecules and affinity-purified Fas antibodies from pooled IgM preparation induced apoptosis in lymphoblastoid cells, suggesting the involvement of the Fas receptor. Our results suggest a role for normal IgM in controlling cell death and proliferation, and imply a possible therapeutic role for IgM in autoimmune and lymphoproliferative disorders.

Introduction

Programmed cell death (apoptosis) is a physiological process of selective cell deletion that occurs during embryogenesis, metamorphosis, tissue atrophy and tumor regression (1–4). Tissue remodeling during embryogenesis and tissue homeostasis during adult life is governed by an equilibrium between proliferation, growth and apoptosis. Any disturbance of this balance leads to pathological situations, such as cellular accumulation in cancer. Apoptosis plays a critical role in maintaining peripheral lymphocyte homeostasis and for minimizing the expansion of autoreactive lymphocytes. It is also a mechanism for the induction of tolerance in autoreactive B and T cells, and, furthermore, excess cells after an immune response are removed by the induction of apoptosis (5). Recent work has clearly demonstrated that deregulation of apoptosis may underlie the pathogenesis of autoimmune diseases by allowing abnormal autoreactive lymphocytes to survive (6–8).

Natural antibodies (NAb) are those that are present in the serum of healthy individuals in the absence of deliberate immunization with the target antigen (9–11). The importance of NAb reactive with self-antigens has long been neglected, as tolerance to self was thought to be primarily dependent on the deletion of autoreactive clones during ontogeny. However, it is now well established that autoreactive antibodies and B and T cells are present in healthy individuals, and that autoreactive repertoires are predominantly selected during fetal life (11–13). Most NAb are encoded by their genes in germline configuration by B cells, which have not been subjected to somatic hypermutation and affinity maturation. It was initially considered that NAb are preferentially of the IgM isotype, since NAb were first reported in mice where IgM predominates over the other isotypes at the neonatal stage (14). It is now known that NAb belong to the IgM, IgG and IgA isotypes. In man and mouse, natural autoreactive antibodies (NAA) were...
showed to bind to a broad range of evolutionarily conserved cell-surface, intracellular and circulating antigens (15,16). Although several functions have been postulated for NAA under physiological conditions, the role of natural IgM antibodies remains as yet unclear.

Here, we have examined the hypothesis that natural IgM exerts a role in the maintenance of immune homeostasis by inducing apoptosis of lymphocytes. As a source of natural IgM, we have used a therapeutic preparation of pooled IgM (IVIgM). We have previously shown that the IgM preparation exerts in vitro and in vivo immunomodulatory functions. IVIgM inhibits the activity of a large panel of autoantibodies and protects susceptible strains of animals from autoimmune diseases (17–20). In the present study, we have examined the apoptosis-inducing potential of IgM using different human lymphoblastoid cell lines, such as CEM, Jurkat (T cells) and Raji (B cells), as well as human peripheral blood mononuclear cells (PBMC). The apoptosis induced by natural IgM is associated with nucleosomal cleavage of DNA and expression of phosphatidylserine on the cell surface. The natural IgM-induced apoptosis is at least in part dependent on the Fas receptor and on the activation of caspases. Taken together, these observations provide novel insights into the role of normal circulating IgM in controlling proliferation and mortality of lymphocytes, and thereby a role in the maintenance of homeostasis of the immune system. Our results further imply a possible therapeutic role of IgM in autoimmune and lymphoproliferative disorders.

**Methods**

**Human IgM**

Normal IgM (IVIgM; a kind gift from the Laboratoire Français du Fractionnement et des Biotechnologies, Les Ulis, France) was processed from pooled plasma of over 2500 healthy donors by using a modified Deutsch–Kistler–Nitschmann’s ethanol fractionation procedure (17). The preparation contained >90% pure IgM.

**Cell lines**

Human lymphoblastoid cell lines CEM (T cells), Jurkat (T cells), Raji (B cells), HuT78 (Fas-sensitive human T cell lymphoma line) and HuT78.B1 (Fas-resistant subclone of the human T cell lymphoma line HuT78) (21) were maintained in RPMI 1640 medium (Gibco, Paisley, UK), as well as human peripheral blood mononuclear cells (PBMC). The preparation induced apoptosis of lymphocytes. As a source of natural IgM, we have used a therapeutic preparation of pooled IgM (IVIgM). We have previously shown that the IgM preparation exerts in vitro and in vivo immunomodulatory functions. IVIgM inhibits the activity of a large panel of autoantibodies and protects susceptible strains of animals from autoimmune diseases (17–20). In the present study, we have examined the apoptosis-inducing potential of IgM using different human lymphoblastoid cell lines, such as CEM, Jurkat (T cells) and Raji (B cells), as well as human peripheral blood mononuclear cells (PBMC). The apoptosis induced by natural IgM is associated with nucleosomal cleavage of DNA and expression of phosphatidylserine on the cell surface. The natural IgM-induced apoptosis is at least in part dependent on the Fas receptor and on the activation of caspases. Taken together, these observations provide novel insights into the role of normal circulating IgM in controlling proliferation and mortality of lymphocytes, and thereby a role in the maintenance of homeostasis of the immune system. Our results further imply a possible therapeutic role of IgM in autoimmune and lymphoproliferative disorders.

**Recombinant Fas molecules**

DAP.3 stable cell lines expressing FasΔExo3.4 and FasΔExo4 Fas splicing variants were used as a source of soluble Fas proteins as previously described (22). The FasExtra–GST fusion protein, previously described (22), was expressed in BL21 *Escherichia coli* by induction with 1 mM isopropyl-β-D-thiogalactopyranoside for 4 h at 37°C and purified from bacterial lysates with glutathione–Sepharose 4B (Pharmacia), according to the manufacturer’s instructions.

**Affinity purification of anti-Fas antibodies from IgM**

Purified human extracytoplasmic GST–Fas fusion protein was coupled to CNBr-activated Sepharose 4B. IgM (10 mg/ml; 0.01 mM) was incubated with 1 ml of GST–Fas affinity matrix overnight on a rocking platform at 4°C. After extensive washing with PBS containing 0.02% NaN₃ (PBS-Az), bound IgM was eluted using 0.2 M glycine–HCl, pH 2.8, and immediately neutralized with 2 M Tris and dialyzed against PBS.

**Assays for apoptosis**

Analysis of DNA fragmentation was performed using a previously described procedure (23). In brief, 2 × 10⁶ cells were harvested by centrifugation, washed with PBS, and lysed in 20 mM Tris, pH 7.4, 0.4 mM EDTA and 0.4% Triton X-100. Cell lysates were centrifuged at 10,000 g for 5 min. DNA fragments in the supernatants were precipitated with 0.5 M NaCl and an equal volume of isopropanol at −70°C. Samples were centrifuged at 10,000 g and the pellets were washed with 70% ethanol. Air-dried pellets were resuspended in 10–20 µl of TE (10 mM Tris±HCl, pH 7.4 and 1 mM EDTA) containing 0.1 mg/ml RNase and incubated at 37°C for 30 min. DNA samples from equivalent numbers of cells were then analyzed in 0.8% agarose minigels.

Expression of phosphatidylserines was determined by staining the cells with Annexin-V–FITC (2.5 µg/ml; Bender-Medisystems, Biowhittaker, Verviers, France) for 30 min on ice. As negative control, non-treated or HSA-treated (Biotransfusion, Paris, France) cells were used. Cells were washed with PBS-Az and resuspended in PBS-Az before adding propidium iodide (PI; Sigma, St Louis, MO) (50 µg/ml) to each sample (24). Samples were analyzed for green fluorescence (Annexin-V labeling) and for red fluorescence (PI uptake) using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

**Western blot analysis for poly-(A)DP-ribose polymerase (PARP)**

Cells (10⁶ cells) were treated for various times with IgM. Cell extracts were analyzed on 10% SDS-PAGE. Separated proteins were transferred onto PVDF membranes, blocked for 12 h in PBS containing 5% BSA and 0.1% Tween 20, and incubated for 2 h with rabbit anti-PARP antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were washed 5 times each with PBS containing 0.1% Tween 20 (PBS-T) and PBS, and incubated with horseradish peroxidase-conjugated secondary goat anti-rabbit IgG for 2 h. After washing the membranes 5 times with PBS-T and PBS, PARP was visualized using the ECL chemiluminescence system (Amersham, Arlington Heights, IL).

**Measurement of mitochondrial transmembrane potential (ΔΨₘ)**

IgM (0.01 mM)-treated cells (10⁶) were incubated for 20 min with CMTMRos (150 nM; Molecular Probes, Eugene, OR) in culture medium in 5% CO₂ at 37°C. The cells were washed once with PBS, centrifuged at 200 g and fixed in 2 ml PBS
containing 4% paraformaldehyde for 15 min at room temperature using a horizontal shaker at 150 r.p.m. Flow cytometric analysis of the fixed cells was carried out (Becton Dickinson) using excitation of a single 488-nm argon laser (25).

Statistical analysis
An unpaired Student’s t-test was used to determine the statistical significance of the data. A value of $P < 0.05$ was considered as the level of statistical significance.

Results

IgM induces cell death in autonomously growing cell lines with features characteristic of apoptosis

Cells of the T lymphoblastoid lineage CEM and the B cell line Raji were cultured with IVIgM. A dose-dependent loss of viability was observed in the presence of IVIgM in both CEM and Raji cell lines as assessed by MTT assay and uptake of PI (data not shown). For characterization of IgM-induced cell death, we tested whether the cells show typical characteristics of apoptosis such as DNA fragmentation and cell-surface expression of phosphatidylserines. As shown in Fig. 1(A), IVIgM induced typical DNA ladder formation in a dose-dependent manner in CEM T cells after 24 h of incubation with different concentrations of IgM, indicating evidence for apoptotic cell death in these cells rather than necrosis. Exposure of phosphatidylserines on the cell surface is an early event in apoptosis (26,27) and phosphatidylserines on the surface of cells can be detected with Annexin-V. Thus, CEM cells were incubated for 12 h in the presence of IgM, anti-Fas mAb CH-11 (IgM isotype, 100 ng/ml; Upstate Biotechnology, Lake Placid, NY), HSA or culture medium and subsequently stained with Annexin-V–FITC, a ligand of phosphatidylserine, as well as with PI, in order to detect dead cells. Early apoptotic cells will be single-positively stained for Annexin-V. As shown in Fig. 1(B), cells challenged with 0.05 mM IgM or 100 ng/ml mAb CH-11 show increased staining of Annexin-V (75 and 11% respectively) compared with non-treated or several fold higher concentration of HSA (0.15 mM)-treated cells (~5%). The expression of phosphatidylserines on the surface of the dying cells demonstrated that the cell death was apoptosis rather than necrosis.

A role for Fas in IgM-induced apoptosis

Fas, a member of the tumor necrosis factor receptor family, is constitutively expressed on tumor cells of hematopoietic and non-hematopoietic origin (28). Cross-linking of Fas by its natural ligand or by agonistic antibodies results in apoptosis of Fas+ cells (29,30). We investigated the role of Fas in IgM-induced apoptosis using two experimental approaches. In the first approach, soluble Fas molecules were used along with IgM to overcome the IgM-induced apoptosis. We used two different soluble Fas molecules obtained from cultures of DAP3 fibroblast clones transfected with expression vectors encoding soluble Fas molecules (DAP3.FasΔExo3.4 and DAP3.FasΔExo4). Culture supernatants obtained from DAP3 fibroblast clones transfected with the vector alone were used.
as negative control. CEM T cells were challenged with IgM (0.01 mM) in the presence of tissue culture supernatants of the following DAP3 clones: DAP3-FasΔExo3.4, DAP3-Fas ΔExo4 and DAP3-pCDNA3 empty vector. The percentage of IgM-induced cell death [i.e. (death in the presence of IgM and the supernatant) – (spontaneous death in the presence of supernatant alone)] was measured by PI uptake and shown as mean values ± SE obtained from three separate experiments carried out in duplicates. Statistical significance as determined by Student’s t-test is indicated (* P < 0.05).

In a second approach to investigate the role of Fas antibodies in natural IgM-induced apoptosis, Fas-sensitive and Fas-resistant cell lines were treated with IgM, and cell death was evaluated. HuT78 is a human T cell lymphoma line, highly sensitive to Fas-mediated apoptosis, whereas HuT78.B1 cells are resistant to Fas-mediated apoptosis due to the expression of a wild-type and a truncated Fas receptor (21). There was a significant reduction in IgM-mediated apoptosis in HuT78.B1 Fas-resistant cells compared with parental HuT78 cells, as measured by PI uptake (Fig. 2B).

**Fas-specific antibodies in IgM preparation**

To investigate the role of Fas receptor in IgM-induced apoptosis, Fas antibodies were purified from IVIgM by affinity chromatography using GST–Fas extracytoplasmic domain fusion protein immobilized on Sepharose beads. To test the biological activity of the affinity-purified Fas antibodies, CEM cells were incubated for 24 h with the antibodies at concentrations of 2 and 0.15 μM (Fig. 2C). The concentration of affinity-purified Fas IgM required to induce mortality was significantly lower than that of unfractionated IgM, as assessed by PI uptake. Similar results were obtained when Raji cells were treated with affinity-purified Fas antibodies (data not shown). To assess the potential therapeutic in vivo activity of the Fas IgM antibodies, we used freshly isolated PBMC. Both unfractionated IVIgM (0.01 mM) and affinity-purified Fas IgM (20 μM) induced apoptosis in purified PBMC (Fig. 3). Incubation of PBMC with commercial CH-11 mAb also resulted in apoptosis (data not shown). The overall extent of apoptosis of resting PBMC was, however, lower than that observed with transformed cells and consistent with the observations that transformed cells are more sensitive to apoptosis (31,32).
Induction of apoptosis is associated with the activation of caspases, a family of cysteine proteases (33–35). Here, we tested the involvement of caspase-3 in the IgM-induced apoptosis in Raji and CEM cells (Fig. 4 and data not shown). Upon activation, caspase-3 cleaves PARP, resulting in the appearance of an 85-kDa band on western blotting. Raji cells were challenged with IgM to induce apoptosis and the activity of caspase-3 in the Raji cells was evaluated by western blotting with a PARP antibody. Cleavage of PARP was detected at 18 h in the treated cells (Fig. 4, lane 2), while there was no degradation of PARP in the untreated cells (Fig. 4, lane 1). Similar results were obtained when CEM cells were subjected to IVIgM-induced apoptosis (data not shown).

We then investigated whether IgM-induced apoptosis involves alteration of the $\Delta \Psi_m$. Changes in the $\Delta \Psi_m$ occur in early phases of programmed cell death (36). To examine the role of mitochondria in IgM-mediated apoptosis, Raji cells were challenged with IgM (0.01 mM) and the $\Delta \Psi_m$ was monitored by the fluorescence of the cationic lipophilic dye CMTMRos. Reduced $\Delta \Psi_m$ was measured by the change in the fluorescence (Fig. 5). Indeed, there was a weak shift in the mean fluorescence after 2 h of incubation indicating a reduction in $\Delta \Psi_m$. Prolonged incubation of Raji cells with IgM (up to 8 h) further reduced the $\Delta \Psi_m$. These results indicate an immediate effect of IgM on the mitochondrial membrane potential of mitochondria. Mitochondrial changes associated with apoptosis include permeability transition pore formation in the mitochondrial membranes (37). The exact mechanism involved in the pore formation is still not well understood, but members of the Bcl-2 family of proteins play an important role in the pore formation (38). Similar results were obtained with IVIgM-treated CEM cells (data not shown).

Discussion

In this study, we have characterized the apoptosis-inducing effects of normal human IgM on human lymphoid cell lines as well as freshly isolated PBMC. We have observed that the IgM-induced cell death was dose dependent. The cell death was associated with DNA ladder formation, loss of membrane integrity and cell-surface exposure of phosphatidylserine—characteristic features of apoptosis. We demonstrate that IgM-induced cell death involves the Fas receptor, activation of caspase-3 and a reduction in mitochondrial transmembrane potential during the early phase of apoptosis induction. Our results suggest a role for normal IgM in controlling cell survival and death, and imply a possible therapeutic role of IgM in autoimmune and lymphoproliferative disorders.

Defective apoptosis is associated with the pathogenesis of several autoimmune diseases (39–43). It has been proposed that too little apoptosis would lead to persistence of autoreactive cells resulting in the production of autoantibodies (40). The importance of Fas-mediated apoptosis in lymphoproliferative disorders is well documented. MRL mice homozygous for lpr (lymphoproliferation) or gld (generalized lymphoproliferative disease) develop a systemic autoimmune disease resembling systemic lupus erythematosus (SLE), Sjögren’s disease and rheumatoid arthritis (44). Induction of Fas-mediated apoptosis by IVIgM preparations indicates the possibility to use IgM in the treatment of autoimmune and lymphoproliferative diseases.

We demonstrate that IgM induced apoptosis of lymphocytes at least partially mediated by Fas receptor cross-linking as suggested by two different approaches. The Fas-sensitive
HuT78, a human T lymphoblastic cell line, was compared for its sensitivity to IgM-mediated apoptosis with that of a Fas-resistant variant, HuT78.B1. HuT78.B1 contains a wild-type and a truncated Fas receptor (21). In addition, these cells are defective in eliciting some early signal transduction events upon Fas cross-linking such as activation of acidic sphingomyelinase (45). Our results indicate that the Fas-resistant cell line displayed reduced sensitivity to IgM-mediated apoptosis. The latter data, together with the observation that IgM-mediated apoptosis is inhibited by soluble Fas molecules, provided strong evidence for a role of Fas cross-linking in IgM-induced apoptosis. However, induction of apoptosis of HuT78B1 cells with IgM indicates the involvement of additional apoptotic pathways.

It is possible that IgM binds to other cell-surface molecules including cytokine receptors and possibly growth factor receptors. It is conceivable that such stimulations could lead to apoptosis. Thus, cross-linking of the B cell antigen receptor with IgM antibodies initiates a cascade of events that culminates with growth arrest and cell death in B lymphocytes (46). Several studies have shown that the caspase cascade plays a central role in this process with or without the involvement of mitochondria (47–49). The use of the caspase inhibitor z-VAD-fmk or overexpression of Bcl-2 prevents apoptosis, but not growth arrest induced by anti-IgM antibody treatment (47). Results from the present study suggest that IgM-mediated apoptosis involves the activation of caspase-3 and a reduction in the $\Delta \Psi \text{m}$. Fc receptors for IgM on B lymphocytes and macrophages have been characterized (50). Although the role of Fc receptors for IgM in IgM-mediated apoptosis has not been elucidated, it is likely that the interactions initiated through the binding of Fc portions of antibodies or immune complexes to cell-surface Fc receptors on hematopoietic cells lead to a variety of immune responses that may include apoptosis (51,52). The ability of natural human IgM to induce apoptosis of neuroblastoma cells through interaction with a 260-kDa surface antigen on neuroblastoma cells has been demonstrated (53,54). Abnormal and modified glycolipid and glycoprotein synthesis by tumor cells is a common feature of malignant cells, and results in the expression of these modified structures on the surface (55,56). It is likely that natural IgM antibodies bind most likely to epitopes on carbohydrate structures of specific receptors.

It is well established that natural IgM play an important role in the first line of defense against infectious agents (57). Results from the present study demonstrate that not only transformed lymphoblastoid cells, but also resting PBMC, are sensitive to IgM-induced apoptosis, suggesting that natural IgM antibodies might have a role in the regulation of proliferation of immune cells and in the first-line defense against infections, and in the immunosurveillance against transformed malignant cells (58). However, we believe that the apoptotic effect of natural antibody preparations is not indiscriminate since all lymphocytes are not equally sensitive to antibody-mediated apoptosis. In fact, transformed cells are more sensitive to apoptosis than the non-transformed cells (31,32) and naive PBMC are less sensitive to apoptosis than activated PBMC (59). Thus, it is possible that such Ig preparations might be beneficial in restraining an immune system that is abnormally activated due to polyclonal activation.

Serum IgM plays an important role in controlling the expression of IgG autoreactivity (60,61). The addition of purified IgM to autologous IgG suppresses IgG-associated autoreactivity (61). IgM-dependent regulation of IgG autoreactivity in serum is defective in autoimmune conditions, such as autoimmune thyroiditis and SLE (61,62). In addition, loss of detectable IgG autoantibody activity in the serum of patients in remission of ANCA vasculitis was shown to be associated with the generation of IgM anti-idiotypes directed against the patient’s acute-phase IgG autoantibodies, indicating that IgM may suppress pathogenic autoantibodies of the IgG isotype in remission of autoimmune disease (62). Mice that lack secreted IgM developed elevated levels of IgG autoantibodies to double-stranded DNA and histones, had abundant deposits of immune complexes in the glomeruli, and succumbed to the disease at an earlier age. Similarly, the absence of secreted IgM also resulted in an accelerated development of IgG autoantibodies in normal mice. These findings suggest that secreted IgM, including IgM autoantibodies produced naturally or as part of an autoimmune response, may lessen the severity of autoimmune pathology associated with IgG autoantibodies (63). We have previously shown that infusion of (Lewis × Brown-Norway) F1 rats with IVIgM protects the animals against experimental autoimmune uveitis induced by immunization with the soluble retinal S antigen (17). IVIgM was shown to suppress anti-acetylcholine receptor (AChR) autoantibody production in vivo and to prevent the loss of AChR at the muscle endplates in a reconstituted SCID mouse model of myasthenia gravis (18). As apoptosis plays a critical role in maintaining peripheral lymphocyte homeostasis and for minimizing the expansion of autoimmune lymphocytes, our results suggest that the beneficial and protective role of IgM in various models of diseases may involve induction of apoptosis of autoantibody-producing lymphocytes and inhibition of cellular proliferation. Thus, together with IVIg containing exclusively IgG, therapeutic IgM may be of potential benefit in several conditions, including cancer, in which defective apoptosis is implicated in the pathogenesis (64–69).

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Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>$\Delta \Psi \text{m}$</td>
<td>mitochondrial transmembrane potential</td>
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<td>IVIgM</td>
<td>i.v. IgM</td>
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<td>NAA</td>
<td>natural antibodies</td>
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<tr>
<td>NAA</td>
<td>natural autoreactive antibodies</td>
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<tr>
<td>PARP</td>
<td>poly(ADP-ribose) polymerase</td>
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<tr>
<td>PI</td>
<td>propidium iodide</td>
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<tr>
<td>PBS-Az</td>
<td>PBS containing 0.02% NaN3</td>
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<tr>
<td>PBS-T</td>
<td>PBS containing 0.1% Tween 20</td>
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<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cell</td>
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<tr>
<td>SLE</td>
<td>systemic lupus erythematosus</td>
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