Targeting Adhesion Molecules as a Potential Mechanism of Action for Intravenous Immunoglobulin

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Background—Intravenous immunoglobulin (IVIg) therapy has been shown to have therapeutic benefit in more than 50 inflammatory and immune-related diseases; however, the potential benefit of IVIg in cardiovascular disease is more limited, in part because our understanding of the mechanisms underlying the effects of IVIg in innate immunity is incomplete.

Methods and Results—in this study, a systematic assessment of the role of IVIg in leukocyte recruitment was completed with an in vitro flow-chamber system and in vivo intravital microscopy in a feline ischemia-reperfusion model system. IVIg treatment of blood resulted in a profound decrease in recruitment of either immobilized P-selectin or E-selectin due to direct effects of IVIg on the leukocyte (not substratum). Similar results were observed on endothelium treated with histamine, which induces P-selectin–dependent rolling and β2–integrin–dependent adhesion. IVIg reduced P-selectin glycoprotein ligand-1 (PSGL-1) antibody binding to PSGL-1 on leukocytes. Use of a β2–integrin–dependent static assay to bypass selectin–dependent recruitment revealed some inhibitory effectiveness (60%), which suggests that the majority of the effects of IVIg were due to selectin inhibition, with some inhibition of integrin function. In vivo intravital microscopy revealed a potent inhibitory effect of IVIg on P-selectin–dependent rolling and β2–integrin–dependent adhesion that led to reduced leukocyte recruitment and vascular dysfunction in postischemic microvessels.

Conclusions—Our data demonstrate that IVIg has direct inhibitory effects on leukocyte recruitment in vitro and in vivo through inhibition of selectin and integrin function. (Circulation. 2005;112:2031-2039.)

Key Words: endothelium • ischemia • reperfusion • leukocytes • immunoglobulin

Intravenous immunoglobulin (IVIg) is pooled IgG from thousands of donors. IVIg has been used in the treatment of many diseases, including a number of primary and secondary antibody deficiencies, systemic lupus erythematosus, multiple sclerosis, and rheumatoid arthritis.1–5 It has also been reported to be beneficial in a number of inflammatory conditions, including sepsis, systemic vasculitis, transplant rejection, and Kawasaki’s disease.1–3,5–10 Although the underlying molecular mechanisms of these diseases are quite different, IVIg appears to provide benefit in many of these pathologies. To date, our understanding of the role of IVIg in cardiovascular diseases such as ischemia-reperfusion injury is lacking. This is not trivial, because many cardiovascular diseases have an inflammatory component potentially amenable to IVIg treatment. However, not all patients respond positively to IVIg, and in rare instances, severe complications do arise.3 Improving our understanding of the mechanisms of action of IVIg would greatly improve our insights as to which disease states and which subsets of patients in a particular disease should be treated with IVIg.

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The molecular mechanisms by which IVIg may be effective include the modulation of Fcγ receptor expression, interference in the activation of the complement and cytokine network, provision of anti-idiotypic antibodies, and effects on the activation, differentiation, and effector functions of T cells and B cells.1–5 A key feature of each of the aforementioned inflammatory diseases is leukocyte recruitment. Yet to date, a systematic examination of the role of IVIg in the cascade of molecular events involved in leukocyte recruitment has not been performed.

Leukocyte recruitment is a multistep process that initially involves selectins expressed by both leukocytes (L-selectin) and endothelium (P-selectin and E-selectin) and their respective ligands.11,12 These molecules allow leukocytes to first tether and then roll along the endothelium, which will permit the endothelium to present proinflammatory molecules such as chemokines. Chemokines will cause activation of the integrins on the leukocytes, which allows for firm adhesion.12 Once adherent, the leukocytes can emigrate from the vascu-
lature via a number of adhesion molecules, including platelet and endothelial cell adhesion molecule-1 and CD99. It has been shown that inhibition of rolling and adhesion can reduce both the vascular dysfunction and tissue injury associated with ischemia reperfusion and the plaque formation associated with atherosclerosis. Whether IVIg can affect any of these molecular mechanisms is unclear at the present time.

In this study, we systematically examined the role of IVIg in an ischemia–reperfusion–induced multistep recruitment cascade of leukocytes. Intravital microscopy was used in a feline in vivo model of P-selectin- and integrin-dependent leukocyte recruitment of ischemia-reperfusion coupled with an in vitro laminar flow-chamber system. The results demonstrated that IVIg severely impairs P-selectin–dependent leukocyte rolling in both the in vivo and in vitro system. In the in vivo ischemia-reperfusion model, both P-selectin–dependent rolling and integrin-dependent adhesion was inhibited by IVIg, which translated to a very profound reduction in vascular dysfunction similar in degree to the antiadhesive effects.

**Materials**

**Reagents and Antibodies**

IVIg was a generous gift from Bayer Inc (Canada). Recombinant human P-selectin and E-selectin were purchased from R&D Systems Inc. Histamine was purchased from Sigma Chemical Co. The anti-β3-integrin antibody (mAb) was generously provided by Dr Paul Naccache (Laval University, Quebec City, Quebec, Canada). The anti-P-selectin glycoprotein ligand-1 (PSGL-1) antibody (KPL-1) was purchased from BD Pharmingen. Heparin was purchased from Sigma-Aldrich. Heparin was used to draw blood over the substrate. Whole blood was taken from healthy individuals, and 30 U/mL heparin sodium (1000 U) was added to prevent coagulation. Heparin has been shown not to affect leukocyte-endothelium interactions, whereas other anticoagulants, such as citrate, do affect interactions. The perfusion rate was set at 10 divisions/cm² for all flow-chamber experiments that involved soluble adhesion molecules and endothelial monolayers. Experiments were video recorded via a charge-coupled device camera (Hitachi Denishi) and a videocassette recorder (Panasonic) attached to the microscope. Rolling and adherent cell counts were made through playback video analysis.

Leukocyte recruitment was examined on immobilized E-selectin or P-selectin and histamine-stimulated endothelial monolayers. For the immobilized adhesion molecule experiments. Hanks’ balanced salt solution (HBSS; with Ca²⁺, Mg²⁺, and sodium bicarbonate) was perfused briefly over the coated coverslips, followed by whole blood, treated with or without IVIg (20 mg/mL, 30 minutes), and perfusion at 10 divisions/cm² for 5 minutes. The coverslip was once again perfused with HBSS to clear nonattached red blood cells and leukocytes, and 5 fields of view were recorded for 20 seconds each. Rolling and adhesion were determined by playback analysis as described previously. A leukocyte that remained stationary for at least 10 seconds was defined as adherent. Experiments without pretreatment of whole blood with IVIg were also performed wherein IVIg was added immediately before perfusion of whole blood.

**Flow-Cytometry Measurement**

To determine whether the IVIg could block surface expression of PSGL-1 and β3-integrin on leukocytes, we performed fluorescence-activated cell sorter (FACS) experiments. Briefly, a primary antibody directed against β3-integrin (IB4, 2.5 µg/mL) or PSGL-1 (2.5 µg/mL) was added to whole blood. After an antibody incubation of 30 minutes at 4°C, the red blood cells were lysed, and leukocytes were simultaneously fixed in 1% formalin and then labeled with FITC-conjugated mouse IgG and measured on a FACScan flow cytometer (Becton Dickinson). No primary antibody, an isotype, and no secondary antibody were used as controls for each set of experiments.

**Intravital Microscopic Studies**

The experimental preparation used in this study is the same as described previously. Briefly, cubs (1.2 to 2.4 kg) were fasted for 24 hours and initially anesthetized with ketamine hydrochloride (75
mg IM). The jugular vein was cannulated, and anesthesia was maintained by the administration of pentobarbital sodium. A tracheotomy was performed to support breathing by artificial ventilation. Systemic arterial pressure was monitored continuously with a chart recorder (Grass Instruments) with a Statham P23A (Gould) pressure transducer connected to a catheter in the left carotid artery. A midline abdominal incision was made, and a segment of small intestine was isolated from the ligament of Treitz to the ileocecal valve. The remainder of the small and large intestines was extirpated. Body temperature was maintained at 37°C with an infrared heat lamp. All exposed tissues were moistened with saline-soaked gauze to prevent evaporation. Heparin sodium (10 000 U) was administered; then, an arterial circuit was established above the superior mesenteric arterial and left femoral artery. Superior mesenteric arterial blood flow was monitored continuously with an electromagnetic flowmeter (Carolina Medical Electronics).

Cats were placed in a supine position on an adjustable Plexiglas microscope stage, and a segment of midjejunum was exteriorized through the abdominal incision. The mesentery was prepared for in vivo microscopic observation as described previously. The mesentery was draped over an optically clear viewing pedestal that allowed for transillumination of a 30-mm segment of tissue. The temperature of the pedestal was maintained at 37°C with a constant temperature circulator (model 80; Fisher Scientific). The exposed bowel was draped with saline-soaked gauze, whereas the remainder of the mesentery was covered with Saran Wrap (Dow Corning). The exposed mesentery was suffused with warmed bicarbonate-buffered saline (pH 7.4) that was aerated with a mixture of 5% CO₂ and 95% N₂. The mesenteric preparation was observed through an intravitral microscope (Optiphot-2; Nikon) with a 25 × objective lens (Wild L250/35; E. Leitz) and a 10 × eyepiece. The image of the microcirculatory bed (1400× magnification) was recorded with a video-camera (Digital 5100; Panasonic) and a video recorder (NV8950; Panasonic).

Single unbranched mesenteric venules (25 to 40 μm diameter, 250 μm length) were selected for each study. Venular diameter was measured either online or offline with a video caliper (Cardiovascular Research Institute, Texas A&M University). The number of rolling and adherent leukocytes was determined offline during playback analysis. Rolling leukocytes were defined as white blood cells that moved at a velocity less than that of erythrocytes in a given vessel. The number of rolling leukocytes (flux) was counted by frame-by-frame analysis. To obtain a complete leukocyte rolling velocity profile, the rolling velocity of all leukocytes entering the vessel was measured. A leukocyte was defined as adherent to venular endothelium if it remained stationary for >30 seconds. Adherent cells were measured at 10-minute intervals and expressed as the number per 100-μm length of venule. Red blood cell velocity (V_{rbc}) was measured with an optical Doppler velocimeter (Cardiovascular Research Institute, Texas A&M University), and mean velocity (V_{max}) was determined as V_{rbc}/1.6. Wall shear rate was calculated based on the newtonian definition: shear rate=(V_{max}/D_{v})×(8/time (seconds)), where D_{v} is the venular diameter.

**Experimental Protocol**

**In Vivo Experiments**

Baseline measurements of blood pressure, superior mesenteric arterial blood flow, V_{rbc}, and vessel diameter were obtained. Experiments were performed in untreated animals, IVIg (0.2 g/kg)-treated animals, and as a positive control, fucoidan-treated (25 mg/kg) animals in ischemia-reperfusion.

**Ischemia-Reperfusion Model**

In the first group of animals, the preparation was videotaped for 10 minutes, and then superior mesenteric arterial blood flow was mechanically reduced (Gaskell clamp) to 20% of control for 1 hour. The final 10 minutes of the ischemic period were videotaped, and the clamp was removed to restore intestinal blood flow. Video recordings were made at 10, 30, and 60 minutes of reperfusion. In the other series of animals, an identical protocol was completed, but the animals received an IVIg pretreatment (0.2 g/kg; Bayer) or a fucoidan pretreatment (25 mg/kg). The concentration of IVIg used is at the lower end of the dose administered to humans.** Another group of animals received 0.2 g/kg human albumin, which served as a control.

**Microvascular Permeability**

The degree of microvascular dysfunction was determined by vascular albumin leakage in cat mesenteric venules. Briefly, 25 mg/kg FITC-labeled bovine albumin was administered intravenously to animals 15 minutes before the start of the experimental procedure. Fluorescence intensity (excitation wavelength 420 to 490 nm, emission wavelength 520 nm) was detected with a silicon-intensified fluorescent camera (model C-2400-08, Hamamatsu Photonics), and images were recorded for playback analysis with a videocassette recorder. The fluorescent intensity of FITC-labeled albumin within a defined area (10×50 μm) of the venule under study and in the adjacent perivascular interstitium (20 μm from venule) was measured under control conditions at 60 minutes of ischemia and at 10, 30, and 60 minutes of reperfusion. This was accomplished with a video-capture board (Visionplus AT-OFG, Imaging Technology) and a computer-assisted digital imaging processor (Optimas, Bioscan). The index of vascular albumin leakage (permeability index) was determined from the ratio: (interstitial intensity—background)/(venular intensity—background), as reported previously.

**Statistics**

All data are reported as mean±SE. A Student t test was used to compare differences between groups, with a Bonferroni correction for multiple comparisons. Significance was set at P<0.05.

**Results**

**IVIg Can Directly Inhibit Leukocyte Interactions With Selectins**

Figure 1A shows that when whole blood was perfused at 10 dyne/cm² over P-selectin–coated coverslips, ~200 rolling leukocytes were observed. This rolling was a P-selectin–specific event, because a P-selectin antibody blocked all interaction (data not shown). There was no rolling observed on coverslips that were coated with a nonselectin protein (ie, BSA). Figure 1A also demonstrates that IVIg inhibited leukocyte rolling on P-selectin in a dose-dependent manner, with maximal inhibition at 20 mg/mL, which is within the range of IVIg concentrations achieved in patients. Addition of isolated plasma from a single normal human (3 to 10 mg/mL IgG) was insufficient to demonstrate any inhibitory effects (data not shown), yet IVIg at 1 mg/mL inhibited recruitment by 60%. Figure 1B demonstrates that addition of IVIg directly to the P-selectin–coated coverslip had a minimal effect on leukocyte–P-selectin interactions, whereas pretreatment of leukocytes with IVIg eliminated all interactions. This suggests that IVIg affects the leukocyte rather than the immobilized P-selectin. In all of the above experiments, we pretreated the blood or the P-selectin–coated coverslip with IVIg for 30 minutes. When blood was not pretreated with IVIg, there was no decrease in P-selectin–dependent leukocyte recruitment (Figure 1C). Similar results were observed with histamine-dependent rolling and adhesion (data not shown). Clearly, the 30-minute pretreatment was required.

In a second series of experiments, treatment of whole blood with IVIg followed by perfusion over immobilized E-selectin caused approximately an 80% inhibition of leukocyte recruitment, which suggests that IVIg can block interactions with multiple selectin substrata (Figure 1D). However, rolling on
vascular cell adhesion molecule-1 was not inhibited by IVIg, which suggests this is not a nonspecific effect (data not shown). PSGL-1 is the ligand for P-selectin and E-selectin. Figure 1E demonstrates that IVIg blocked 50% of the PSGL-1 expression on neutrophils but failed to block levels on lymphocytes (Figure 1F).

To study the effect of IVIg on a more complex system, we used primary passaged human endothelium to observe whether IVIg could inhibit leukocyte endothelial interactions in vitro on a physiological substrate. When histamine was used to induce P-selectin expression on endothelium, ≈80 leukocytes rolled on the stimulated endothelium (Figure 2A). When the experiment was repeated with blood treated with an optimal dose of IVIg, there was almost a complete inhibition of rolling leukocytes over histamine-stimulated endothelium (Figure 2A). Furthermore, when untreated blood was perfused over histamine-stimulated endothelium, ≈80 leukocytes (predominantly neutrophils) adhered, and similarly, the...
number of adherent leukocytes was significantly reduced if the blood had been pretreated with IVIg (Figure 2B). When histamine-stimulated endothelium was treated with IVIg followed by perfusion of whole blood, there was no inhibition in either leukocyte rolling or adhesion, which suggests that IVIg was having antiadhesive effects on leukocytes rather than the endothelium. When we removed the coverslips from the flow chamber, >95% of the adherent cells were neutrophils.

Although IVIg treatment of blood caused a decrease in both leukocyte rolling and adhesion on histamine-treated endothelium, this experiment failed to address the question of whether IVIg was directly inhibiting adhesion (via $\beta_2$-integrin) as well as rolling (selectin-dependent). Using a slightly modified flow-chamber assay in which blood flow is stopped (allowing for firm adhesion of leukocytes to endothelium independent of selectins), we observed an $\approx$60% decrease in leukocyte adhesion when blood was treated with IVIg compared with untreated blood (Figure 3A). Experiments with flow cytometry revealed that $\approx$30% of $\beta_2$-integrin expression on both neutrophils (Figure 3B) and lymphocytes (Figure 3C) was blocked by IVIg.

IVIg Can Directly Inhibit Leukocyte Interactions and Vascular Dysfunction In Vivo

Next, an in vivo model of ischemia-reperfusion, previously shown to be mediated by both P-selectin and $\beta_2$-integrin-dependent leukocyte recruitment, was examined (same mechanism as for the histamine-treated endothelium). Under basal conditions (Figure 4A, top panel), very few cells rolled and adhered, whereas after ischemia-reperfusion, leukocyte rolling, adhesion, and emigration were increased greatly in the same vessel (Figure 4A, middle panel). Pretreatment with IVIg (0.2 g/kg) prevented the accumulation of rolling and adherent leukocytes at 30 minutes of reperfusion (Figure 4A, bottom panel; see video in Data Supplement). This concentration of IVIg is within the range that is used in human patients (0.2 to 2 g/kg).18,19 Quantification of the data is summarized in Figure 4B through 4D. The flux of rolling leukocytes under baseline conditions was $\approx$30 to 50 cells/min, and this value remained unchanged during the ischemic period (data not shown). Administration of IVIg did not affect this basal leukocyte rolling. During the reperfusion phase, the flux of rolling cells increased dramatically, ranging from 125 to 175 cells/min in untreated animals. IVIg treatment (0.2 g/kg) prevented the increase in the flux of rolling leukocytes during the reperfusion phase. In untreated animals, a 10-fold increase in leukocyte adhesion occurred during the reperfusion phase (Figure 4C). IVIg pretreatment of the animals reduced the adhesion of leukocytes at 10 minutes of reperfusion and essentially prevented all increases in leukocyte adhesion at 30 and 60 minutes. In fact, the number of adherent leukocytes in the IVIg-treated animals during the reperfusion phase was reduced to near-preischemic values. Figure 4D shows that in untreated animals, there was a significant increase in the number of emigrated leukocytes at 60 minutes of reperfusion. In IVIg-treated animals, a much more subtle increase in emigrated leukocytes was noted.

It has been reported previously that inhibition of leukocyte adhesion and emigration dramatically reduced the increase in microvascular dysfunction associated with reperfusion injury.22,23 Moreover, depletion of neutrophils also eliminated the vascular dysfunction.22,24 FITC-albumin was given intravascularly, and the leakage of protein from the mesenteric microvasculature was determined under control and reperfusion conditions in the same preparation. A very obvious increase in vascular protein leakage (reperfusion 30 minutes) could be seen in Figure 5A (middle versus left panel). When animals were pretreated with IVIg, there was a dramatic reduction in vascular protein leakage during the reperfusion phase. Computer-assisted quantification revealed a 6- to 7-fold increase in FITC-albumin leakage from venules during reperfusion after no IVIg administration or after albumin administration (control protein), whereas administration of IVIg to the experimental group increased microvascular dysfunction by $<2$-fold during reperfusion (Figure 5B). Because much of the vascular leakage is due to adhering and emigrating leukocytes, the decreases in the number of adher-
ent and emigrated cells by IVIg are likely responsible for the reduced vascular permeability. It has been shown that >80% of infiltrating leukocytes during ischemia-reperfusion are neutrophils.

Nevertheless, we decided to directly compare the effects of IVIg to antiadhesion (antiselectin) therapy. To inhibit leukocyte recruitment, we used a selectin antagonist, fucoidan, to inhibit leukocyte rolling, which has an impact on adhesion. Fucoidan treatment in the ischemia-reperfusion model showed a dramatic decrease in leukocyte rolling and a 60% decrease in adhesion at 60 minutes of reperfusion compared with untreated animals (Figures 6A and 6B). Similarly, there was also a 60% inhibition of vascular leakage in fucoidan-treated animals compared with untreated animals (Figure 6C). IVIg appeared to be at least as effective if not more effective (Figure 5 versus Figure 6) at preventing leukocyte adhesion and subsequent vascular permeability.

Discussion

Recruitment of leukocytes is a hallmark of many of the diseases in which IVIg has efficacy. Our data suggest that IVIg may function through interference of leukocyte recruitment. Using a simple system of observing leukocytes under flow conditions on immobilized protein (P-selectin or E-selectin), we found that IVIg inhibited leukocyte rolling.

Furthermore, we found that IVIg could interfere in a more complex in vitro system, such as histamine-treated endothelium, and in an in vivo system in which IVIg interfered with not only leukocyte rolling but also leukocyte adhesion and transmigration. Although there have been a number of proposed mechanisms by which IVIg works, there has been no study that examined directly the role IVIg has on selectin- and integrin-dependent leukocyte adhesion under flow conditions. One study to date has showed that IVIg caused a reduction in the binding of lipopolysaccharide-treated neutrophils to endothelium in a static adhesion assay. There is also indirect evidence that IVIg could reduce the expression of lymphocyte function-associated antigen-1 (LFA-1) on neutrophils, thereby reducing leukocyte adhesion. In vivo studies using intravital microscopy have demonstrated that IVIg could reduce leukocyte recruitment into the liver after systemic administration of tumor necrosis factor-α or lipopolysaccharide. However, those authors proposed a mechanism that involved macrophage activity. The present study shows for the first time that IVIg can directly inhibit selectin-dependent leukocyte rolling in human systems in vitro and in animal models of ischemia-reperfusion in vivo. Moreover, the present data suggest that IVIg could be considered as a form of treatment in certain vascular pathologies associated with ischemia-reperfusion injury.
We have demonstrated that IVIg inhibits leukocyte rolling on selectins in vitro. It appears that IVIg is targeting the P-selectin/P-selectin ligand interaction more than the \( \beta_2 \)-integrin/intercellular adhesion molecule-1 interaction. Additionally, under in vivo conditions in which recruitment is largely P-selectin dependent (ischemia-reperfusion model), IVIg also inhibited leukocyte recruitment. Using the ischemia-reperfusion model, we have previously demonstrated that when anti-P-selectin therapy is used, rolling must be inhibited by \( >90\% \) before any decrease in leukocyte adhesion is observed.\(^{14} \) The present data showed that in the human in vitro system, IVIg did block leukocyte rolling by this amount, which likely explains the dramatic impact on adhesion. By contrast, there was only approximately a 50\% decrease in leukocyte rolling with IVIg treatment in the present in vivo model. Surprisingly, we observed an 80\% decrease in the number of adherent cells. These data suggest that IVIg in the present in vivo system may be directly inhibiting both the process of rolling and adhesion of leukocyte recruitment.

Clearly, the present data suggest that in vitro, there is a more minor effect of IVIg on \( \beta_2 \)-integrin–dependent adhesion, whereas in vivo, the data suggest a very significant (80\%) impact of IVIg on \( \beta_2 \)-integrin–dependent adhesion. There are a number of possible explanations for this. In vitro, flow was stopped completely to allow neutrophils to adhere, and under these conditions, IVIg had only a minor effect. In vivo, the situation is more dynamic, and in the presence of the initial shear, it is possible that IVIg can impact more dramatically on neutrophil adhesion. In other words, the small (40\%) reduction in vitro may translate into a more profound effect in vivo. Additionally, mast cells and other cells have been implicated as contributors to neutrophil recruitment by releasing proinflammatory mediators.\(^{28,29} \) These cells are not present in the in vitro system used in the present study, and if IVIg affects these cells, then the effect would only be seen in vivo. Finally, other possible differences include macrovascular endothelium in vitro versus microvascular endothelium in vivo and potential species differences.

There was a very profound effect on vascular permeability with IVIg treatment. Previously, it has been shown that vascular leakage is entirely dependent on adhering neutrophils.\(^{24} \) Indeed, the prevention of leukocyte adhesion or the depletion of neutrophils prevents the vascular leakage seen after ischemia (Figure 6). IVIg treatment inhibited leukocyte adhesion and subsequent emigration and in turn led to the decreased vascular permeability observed in treated animals. Clearly, IVIg could have very beneficial effects in numerous cardiovascular diseases in which infiltrating leukocytes and associated edema and vascular dysfunction play a large role in pathology.

The exact mechanism by which IVIg prevents leukocyte–endothelial cell interactions remains unknown, but it was
In summary, the present data clearly demonstrate that IVIg directly inhibits leukocyte–endothelial cell interactions. The mechanisms involve direct inhibition of leukocyte interactions with selectins, with a lesser effect with β2-integrins. Furthermore, these data suggest therapeutic potential for IVIg in the treatment of cardiovascular disease associated with reperfusion injury.

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References


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