IVIg therapy in brain inflammation: etiology-dependent differential effects on leucocyte recruitment

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Summary
Several studies have reported beneficial effects of intravenous immunoglobulin (IVIg) in diseases of the neuroaxis. However, IVIg effects on leucocyte recruitment, a hallmark feature of autoimmunity and acute inflammation, remain largely unexplored. Using intravital microscopy, we studied the effects of IVIg on leucocyte recruitment in experimental autoimmune encephalomyelitis, a model of multiple sclerosis. In IVIg-treated mice, a significant decrease in recruitment (rolling and adhesion) was observed prior to and following disease onset, and this was concomitant with improved clinical score. Since much of the recruitment is dependent upon \( \alpha_4 \)-integrin (ligand for VCAM-1) we used an in vitro flow chamber system and demonstrated a 60% decrease in \( \alpha_4 \)-integrin-dependent leucocyte adhesion to immobilized VCAM-1. Finally, we used leucocytes from multiple sclerosis patients and demonstrated that IVIg treatment decreased recruitment by 60% on human endothelium. However, when we visualized the role of IVIg in a second model of brain inflammation, cerebral ischaemia–reperfusion, IVIg actually promoted the formation of platelet–leucocyte aggregates in post-ischaemic cerebral vessels. In conclusion, we report a new mechanism of action of IVIg through interference of \( \alpha_4 \)-integrin-dependent leucocyte recruitment in both an animal model and human multiple sclerosis. We also report that IVIg will not be beneficial in all types of pro-adhesive states and may in fact be detrimental in a situation such as stroke.

Keywords: multiple sclerosis; stroke; intravital; rolling; adhesion

Abbreviations: EAE = experimental autoimmune encephalomyelitis; FcR = Fc receptor; HUVEC = human umbilical endothelial cell; Ig = immunoglobulin; IVIg = intravenous immunoglobulin; LSP = lipopolysaccharide; MOG = myelin oligodendrocyte glycoprotein; VCAM-1 = vascular cell adhesion molecule-1.
work but antibodies do not appear to play a role. One commonality and hallmark feature of inflammation and autoimmunity is leucocyte recruitment but the role of IVIg in this process is largely unknown.

The recruitment of leucocytes is dependent on a very set series of events dependent upon multiple proteins (Ley, 1996; Kubes and Ward, 2000). Intravital microscopy techniques have shed some light on leucocyte–endothelial cell interactions and showed that post-capillary venules are the major site of leucocyte recruitment. Tethering and rolling of leucocytes to endothelium are the first interactions where leucocytes move out of the main stream of blood and make contact with the endothelium. It is usually acknowledged that selectins are responsible for these events. The second interaction is the firm adhesion where leucocytes stop and begin emigration out of the blood vessels. Integrins play an important role in this second interaction. Disruption of either rolling or adhesion ultimately reduces leucocyte recruitment. It is becoming clear that not all tissues use the same molecular mechanisms for leucocyte recruitment (Liu and Kubes, 2003). Indeed, whereas selectins and integrins appear to play a limited role in leucocyte recruitment in lung or liver, they appear to be far more important in the brain where therapeutically targeting either family of adhesion molecules has been reported to reduce the extent of injury associated with multiple sclerosis and models of stroke (Suzuki et al., 1999; Miller et al., 2003; Ishikawa et al., 2004). Recently, use of α4-integrin antibody revealed benefit in both animal models of multiple sclerosis (Engelhardt et al., 1998) and in patients (Miller et al., 2003). Therefore, in the first series of experiments we used intravital microscopy and examined whether IVIg could affect α4-integrin-dependent leucocyte adhesion in vivo in experimental autoimmune encephalomyelitis (EAE) and in an in vitro model of α4-integrin-dependent adhesion.

We also examined the effects of IVIg in a second model of brain inflammation, namely stroke that has also been shown to be dependent upon a pro-adhesive mechanism. A fundamental difference between multiple sclerosis and stroke is the involvement of platelets (Ishikawa et al., 2003) and α2-integrin-dependent neutrophil adhesion. Platelets have been reported as an essential bridge between endothelium and leucocytes in stroke but not multiple sclerosis. Although an inhibitory role of IVIg on platelets has never been demonstrated, the reverse effects (pro-adhesion) may also need to be considered. Indeed, thrombosis is the major complication of IVIg treatment and stroke has also been reported as a complicating factor of IVIg therapy. In this study, we used intravital microscopy of the cerebral microvasculature to directly visualize leucocyte recruitment in a stroke model with and without IVIg treatment.

Our results clearly demonstrate a direct positive impact of IVIg on α4-integrin-dependent leucocyte–endothelial cell interactions in EAE but fail to demonstrate any benefit in stroke. In fact, in our stroke model a far more severe thrombosis was observed with IVIg, providing some mechanistic insight for the few negative reports with respect to IVIg (Dalakas and Clark, 2003).

Methods

Female C57Bl/6 J mice were obtained from Charles River Breeding Laboratories (Montréal, Québec, Canada). Animals were maintained in an environment-controlled room with access to water and chow ad libitum. All procedures performed were in accordance with the University of Calgary Animal Care Committee and the Canadian Guidelines for Animal Research.

EAE was induced in mice as previously described (Kerfoot and Kubes, 2002). Briefly, peptide generated from myelin oligodendrocyte glycoprotein (MOG_{35–55}) was obtained from our peptide synthesis laboratory (University of Calgary, Calgary, Canada). Mice were immunized twice (days 0 and 7) s.c. with 50 μg of MOG_{35–55} in complete Freund adjuvant (Sigma-Aldrich, Oakville, Ontario, Canada) and also received pertussis toxin (200 ng, i.p.; List Biological Laboratories, Campbell, CA, USA) on days 0 and 2. Disease was monitored daily and scored according to the following Likert scale: 0, no signs; 1, tail paralysis; 2, tail paralysis and slight hind limb weakness; 3, severe hind limb weakness and righting problem; 4, hind limb paralysis; 5, complete hind limb paralysis and front limb weakness.

To study the effects of IVIg (Bayer, Ontario, Canada) on EAE time course, IVIg was injected (0.4 g/kg, i.p.) once daily starting on day 0. In a similar fashion, we started the treatment on day 7 in another series of experiments. In some experiments, the acute effects of IVIg (0.4 g/kg, i.v.) on cell trafficking in the pial vessels was visualized within 45 min after the injection. The concentration of IVIg was chosen as it falls within the range given to patients (0.2–2.5 g/kg). In all experiments, the sham group received a solution consisting of 10% human albumin (globulin free; Sigma, Ontario, Canada), 4 mM glycine and sucrose. Although there is no optimal control, this amount of albumin correlates with the amount of protein received with IVIg. However, in the experiments where we acutely injected a single dose of IVIg, Fab fragments (200 mg/kg) were also used as a control.

Intravital microscopy of the brain microvasculature

Intravital microscopy of the cerebral vessels was performed as previously described (Carvalho-Tavares et al., 2000). Briefly, we anesthetized mice with a cocktail of ketamine–xylazine (200 mg/kg, Rogar/STB; 10 mg/kg, MTC Pharmaceuticals) and cannulated the tail vein to administer rhodamine 6G or other required reagents. Rectal temperature was maintained at 37°C with a heating pad (Fine Science Tools Inc., Vancouver, British Columbia, Canada). Following craniotomy using a high-speed drill (Fine Science Tools Inc., Vancouver, BC, Canada), removal of the dura-mater exposed the pial vessels. The brain was then continuously superfused with artificial cerebrovascular fluid for the rest of the experiment.

Since the brain is not a translucent tissue, we used rhodamine 6G to label leucocytes and observe their interactions with brain endothelial cells in three superficial post-capillary venules (diameter of 30–70 μm) for each animal using a microscope (Axioskop, ×10 eyepiece and ×25 objective lens; Zeiss, Don Mills, Ontario, Canada) outfitted with a fluorescent light source (epi-illumination at 510–560 nm using a 590-nm emission filter). The image was projected to a monitor through a low light intensified charge-coupled device.
camera (Stanford Photonics, Palo Alto, CA, USA) mounted on the microscope. All experiments were recorded on VHS or digital tapes for playback analysis of rolling (cells moving at a velocity less than that of erythrocytes) and adhesion (cells that remain stationary for at least 30 s). It should be noted that despite the fact that we could not visualize the deep vessels of the brain, our intravitral microscopy parameters (leucocyte rolling and adhesion) correlate extremely well with leucocyte infiltration into brain performed by flow assisted cell sorting analysis (Kerfoot and Kubes, 2002).

In some experiments, a murine model of stroke was used as described by others (Ishikawa et al., 2003). Briefly, following anaesthesia with ketamine–xylazine and external ventilation (Harvard Apparatus, USA), both carotids were clamped for 1 h. Rectal temperature was maintained at 37°C with a heating pad and a heating lamp as necessary. During the reperfusion period, the animals were prepared for intravitral microscopy as described earlier except that the bone was left in place. The bone was thinned as to become translucent using the high-speed drill. After 2 h of reperfusion, cell trafficking in pial vessels was recorded and IVIg (0.4 g/kg, i.v.) was then injected. One hour later, leucocyte–endothelial cell interactions were recorded again. In this particular series of experiments, a considerable increase in leucocyte and platelet trafficking and aggregation post-IVIg made cell counting virtually impossible. We therefore calculated the area of the venules occupied by the leucocytes and the platelets, before and after the treatment, using the NIH (National Institutes of Health) image 1.63 software for Macintosh on still images extracted from recordings of the experiments.

**In vitro flow chamber studies**

Whole blood (5 ml) from multiple sclerosis patients and control subjects was collected in polypropylene vials containing 30 U/ml heparin. Our laboratory previously showed that this treatment does not influence leucocyte adhesion (Reinhardt and Kubes, 1998). The whole blood was diluted 1:10 in Hank’s balanced salt solution and used within 2 h of collection.

Human umbilical vein endothelial cells (HUVEC) were harvested and cultured as previously described (Ibbotson et al., 2001). We mounted glass coverslips plated with HUVEC onto a polycarbonate chamber with parallel plate geometry. In another set of experiments, vascular cell adhesion molecule-1 (VCAM-1) was immobilized to the coverslip instead of HUVEC. While the chamber was sitting on the stage of an inverted microscope (Zeiss), we visualized the cells (×200) using phase contrast. We perfused the diluted whole blood at a shear rate of 2 dyn/cm² followed by Hank’s balanced salt solution. All experiments were recorded on videocassette (Panasonic, Secaucus, NJ, USA) using a charge-coupled camera (Hitachi Denshi, San Jose, CA, USA) for subsequent count of rolling or adherent cells. In the flow chamber assays, five fields of view were randomly chosen as previously described by our laboratory (Ibbotson et al., 2001).

**Demographic data**

The group of multiple sclerosis patients consisted of eight females and two males ranging in age from 27 to 62 years (median = 43 years). Nine subjects were diagnosed as having definite multiple sclerosis and one probable multiple sclerosis. Seven multiple sclerosis subjects had the relapse–remitting form, two had primary progressive and one had secondary progressive multiple sclerosis. The expanded disability status scale scores ranged from 1.5 to 6.5. None of the subjects were taking medication for their multiple sclerosis condition. The healthy subjects’ group comprised five males and three females with age ranging from 23 to 33 years (median = 29 years). We have previously observed that older individuals do not have altered leucocyte counts and males and females are generally the same (Ibbotson et al., 2001).

**Statistical analysis**

To eliminate inter-observer variability, only one person performed and analysed all experiments in mice (B.M.L.), whereas the in vitro experiments in patients were performed by L.M.H. and the VCAM-1 experiments by V.G. For analysis, all experiments were replayed on a monitor using a VCR or a computer and all rolling and adherent cells were manually counted by the observer frame by frame; a standard practice in laboratories using intravitral microscopy (Ley, 1996; Kerfoot and Kubes, 2002). We used a Wilcoxon rank-sum test to compare EAE score in the time course experiments. To detect acute effects of IVIg on cell trafficking, we performed a paired Student’s t-test. The same test was used to compare surface area occupied by leucocytes and platelets after IVIg treatment in ischaemia–reperfusion. Finally, experiments with human subjects were analysed with the Wilcoxon sign-rank test. In all cases, the alpha level was set at P < 0.05. All data were analysed with the statistical software SPSS 4.0 for Macintosh (Chicago, IL, USA).

**Results**

**Acute administration of IVIg blocks leucocyte–brain vessel interactions**

Since inflammatory cell infiltration is a prerequisite to EAE, we observed cell trafficking at three time points, specifically, 2 days before symptom onset, the day of symptom onset and 7 days after symptom onset—a time where disease severity reached its maximum. Accordingly, we observed that at these three times, both rolling (≥30 cells/min) and adhesion (≥5 cells/100 μm) were significantly increased compared with control animals which showed essentially no rolling and very few adhering cells (data not shown). When we administered acutely a single dose of IVIg, the number of rolling leucocytes decreased by ~50% at each time point (Fig. 1). Moreover, the number of adhering leucocytes also decreased by ~60% following IVIg administration. However, when we infused a solution containing an equivalent amount of human albumin or Fab fragments, no reduction in either leucocyte rolling or adhesion occurred (data not shown). Our results thus support the notion that IVIg has an anti-adhesive effect in EAE.

**Prolonged IVIg treatment reduces EAE-induced symptoms**

Since IVIg administration significantly decreased leucocyte trafficking in pial vessels, we sought to understand if this translated into ‘clinical significance’. To do so, we induced EAE with MOG on day 0 and treated the animals with human albumin (sham group) or the IVIg solution given i.p. on a daily basis. Disease appeared on days 11 and 12, respectively, for
each group, and peaked on day 20 (Fig. 2). The maximal EAE score for the sham group (3.7) was significantly higher than the experimental group (2.2). In fact, we detected a statistically significant difference in EAE score from day 13 to the end of the experiment. In both groups, once the maximal score was attained, it decreased slightly (≈10%) by day 30. In another experiment, IVIg treatment was started on day 7 after EAE induction. Again, disease apparition (day 12) was not different from the sham group while the maximal score was decreased (2.5). Clearly, IVIg did not interfere with the early initiating events of EAE. Hence, IVIg did not retard the time of apparition of the disease but nonetheless reduced its severity.

IVIg directly blocks \( \alpha_4 \)-integrin-dependent leucocyte recruitment on VCAM-1

\( \alpha_4 \)-Integrin is deemed extremely important in multiple sclerosis (Miller et al., 2003). Therefore, we examined whether IVIg could affect leucocyte recruitment on VCAM-1 (Fig. 3). In this simple system, the number of human leucocytes rolling was not affected by treatment of IVIg (9.2 cells versus 8.4 cells per field of view). However, the same treatment diminished the number of adherent leucocytes by 58%.

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**Fig. 1** IVIg decreases leucocyte trafficking in EAE. We measured the effects of IVIg (0.4 g/kg) on leucocyte rolling (A) and adhesion (B) in brain vessels of C56BL/6 mice sensitized with MOG. All post-IVIg values (45 min after injection) are significantly reduced (\( P < 0.05 \)) compared with the pre-IVIg values. \( n = 8 \) per group.

**Fig. 2** Daily IVIg administration (0.4 g/kg) influences EAE score. EAE score in C57BL/6 mice treated with IVIg (filled diamonds) or human albumin (open squares). *\( P < 0.05 \) relative to SHAM group. \( n = 8 \)–10 per group.
IVIg blocks leucocyte recruitment from multiple sclerosis patients

Under basal conditions wherein we did not activate HUVECs, almost no rolling or adhesion occurred when diluted blood from multiple sclerosis patients or healthy controls was perfused over untreated HUVEC monolayers (Fig. 4). Activation of HUVECs with TNFα increased the number of leucocytes rolling and adhering to ≈60 cells per field of view regardless of the source of leucocytes. Treatment of leucocytes from multiple sclerosis patients with IVIg significantly reduced these numbers of rolling and adherent cells by 60%. Interestingly, IVIg was less potent to reduce interactions when using leucocytes from healthy controls. In this case, rolling decreased by 45% and adhesion by 28%. These reductions in the number of rolling or adherent leucocytes are in agreement with the results obtained in EAE (see Fig. 1). These results are promising since a decrease in leucocyte recruitment in EAE translated into better clinical score.

It should be noted that IVIg was given directly to the leucocytes in this series of experiments suggesting that the IVIg targeted the leucocytes. IVIg did not promote benefit when it was administered to the endothelium. In fact, in some instances increased adhesion was noted (data not shown).

Untoward effects of IVIg in brain ischaemia–reperfusion

As previously described by others (Ishikawa et al., 2003), 1 h of ischaemia followed by 2 h of reperfusion of the brain increased significantly leucocyte rolling and adhesion compared with the sham group. In this setting, platelets were also recruited (Fig. 5). Much to our surprise, IVIg triggered an additional increase in leucocyte and platelet trafficking. The magnitude of the accumulation was such that it precluded any direct count of leucocytes or platelets. Instead, we measured the area of the pial vessels occupied by leucocytes and platelets to quantify this change (Fig. 6). Before IVIg injection, platelets and leucocytes were interspersed throughout the vessels constituting 17% of the vessel area. After IVIg injection, platelets and leucocytes formed large aggregates occupying more than 40% of the area of brain vessels.

Discussion

Using intravital microscopy, we directly visualized the effects of IVIg on α4-integrin-dependent leucocyte recruitment in superficial brain vessels of the mouse. We observed that IVIg potently reduced leucocytes rolling and adhesion in EAE and that IVIg lessened the EAE score, an indicator of disease severity. Using an in vitro flow chamber assay we were able to directly demonstrate that IVIg disrupted α4-integrin-dependent leucocyte–VCAM-1 interactions. Finally, we noted that IVIg could decrease leucocyte recruitment from multiple sclerosis patients on HUVEC. This is in line with previous studies reporting some beneficial effects for IVIg in diverse autoimmune diseases, including EAE and multiple sclerosis (Dalakas, 1997; Larroche et al., 2002). By contrast, when brain inflammation was induced by ischaemia–reperfusion, IVIg significantly enhanced the number of leucocytes and platelets in brain vessels. These cells formed large aggregates decreasing the lumen of the vessels. Clearly, IVIg decreases leucocyte–endothelial cell interactions in EAE but not in a model of stroke suggesting some important fundamental differences.

Although there are many studies on the efficacy of IVIg, the mechanisms of action in multiple sclerosis and EAE are not completely understood. The main theme revolves around cytokine production. For example, TNFα levels are decreased in EAE rats treated with IVIg (Achiron et al., 1994). In line with this, it was shown that IVIg induces a shift in the pattern of cytokine production with lower production of Th1-type cytokines by T cells (Pashov et al., 1997). Although MBP-specific T cells are found in mice treated with IVIg, the T cells may be in an anergic-like state since EAE was not induced by adoptive transfer of T cells from IVIg-treated animals (Pashov et al., 1998). Our data identify a different, although not necessarily mutually exclusive mechanism for IVIg, i.e. an
inhibition of leucocyte rolling and adhesion in brain vessels. Indeed, cytokines are known to increase adhesion on endothelium and so it is possible that IVIg, by blocking cytokines, might inhibit leucocyte recruitment. There is also the possibility that the decrease we observed in vivo is due to an anti-proliferative effect of IVIg on lymphocytes. However, our data suggest that IVIg has direct anti-adhesive effects, in as much as IVIg blocked leucocyte recruitment on VCAM-1 (no cytokine used) and when leucocytes from multiple sclerosis patients were treated with IVIg, a reduction in leucocyte adhesion was also noted post cytokine delivery. Finally, following in vivo acute administration of IVIg to already inflamed microvessels in EAE, an almost immediate reversal of rolling and adhesion occurred, making cytokine inhibition or inhibition of proliferation an unlikely candidate in this anti-adhesive process.

To our knowledge, no studies have directly assessed the importance of IVIg upon leucocyte adhesion under dynamic flow conditions and only one study to date (that focused on oxidant production) mentioned, in passing, a reduction in neutrophil binding to endothelium with IVIg, under static conditions, when the neutrophils were stimulated with LPS for 1 h (Jonas et al., 1995). Other indirect evidence for a role of IVIg in adhesion has included a reduction in CD11a/CD18 (LFA-1) expression on neutrophils. Since LFA-1 is not known to be altered on neutrophils this may suggest that IVIg ‘coats’ the neutrophil preventing its interaction with substratum. However, the fact that IVIg specifically inhibited adhesion but not rolling on VCAM-1 argues against non-specific coating of the leucocytes. In vivo, McCuskey and colleagues have used intravital microscopy and demonstrated that systemic administration of TNF or LPS induced hepatic inflammation and that IVIg reduced leucocyte recruitment into the liver (Ito et al., 1999). Although these authors proposed that this could be due to IVIg’s impact to reduce endotoxin levels and Kupffer cell function, we propose that an alternative explanation could be that IVIg directly inhibits leucocyte adhesion. One final study worth mentioning is that IVIg could reduce αIIb/α3 integrin mediated platelet aggregation (Teeling et al., 2001). This is worth noting, as platelets can contribute quite

Fig. 4 IVIg reduces interactions of human leucocytes with HUVECs. We measured the ex vivo rolling and adhesion of leucocytes (black bars, sham group; open bars, IVIg-treated group) to HUVECs. (A) Rolling of leucocytes from multiple sclerosis patients. (B) Adhesion of leucocytes from multiple sclerosis patients. (C) Rolling of leucocytes from healthy controls. (D) Adhesion of leucocytes from healthy controls. *P < 0.05 relative to the TNF group. n = 9–10 per group.
significantly to the inflammatory process, at least in part, as a pro-adhesive tether or bridge between the endothelium and circulating leukocytes.

In the stroke model where platelets are recruited, IVIg actually showed a detrimental effect. Administration of IVIg is usually considered a safe procedure where minor side effects (e.g. headache, skin reaction) are uncommon. However, in recent years, thrombosis has been reported in some patients in a number of studies. In fact, stroke has been the one complicating factor undermining IVIg safety profile (Dalakas and Clark, 2003). Why a few patients suffer thrombosis with IVIg treatment remains completely unclear. We used a subtle stroke model to induce a pro-adhesive state for platelets and found that this led to IVIg greatly exacerbating leucocyte and platelet recruitment. It is possible that the platelet is the key player in this inappropriate IVIg effect. First, it has been shown that platelets are recruited in this stroke model (Ishikawa et al., 2003). Secondly, platelets obtained post-stroke were shown to have increased expression of Fc receptor (FcRIIa) (Calverley et al., 2002). In this specific situation where FcRs are already present on platelet surface, IVIg might be the precipitating agent that triggers leucocyte–platelet interactions possibly by binding to FcRs on the platelets.

At first glance some notable differences between our in vivo and our in vitro results can be seen. First, in EAE, both rolling and adhesion were affected by IVIg, whereas in vitro, on immobilized VCAM-1, only adhesion was altered by IVIg. However, this observation may provide further important insight. In vitro, our conditions were such that rolling and adhesion are entirely mediated by α4-integrin/VCAM-1, whereas in vivo, much of the adhesion is dependent upon α4-integrin, but the majority of the rolling is dependent upon selectins (Kerfoot and Kubes, 2002). Clearly, our data demonstrate that IVIg only affects the adhesion aspects of α4-integrin. The in vivo data suggest that some of the IVIg effects may extend to selectins. Indeed the leucocyte recruitment on TNFα-stimulated endothelium is dependent upon both selectins and integrins, and IVIg again had some effect upon both the rolling and adhesion of leucocytes.

This is a demonstration that IVIg may inhibit inflammation in vivo by reducing leucocyte adhesion. Furthermore, we observed that the VCAM-1–α4-integrin interactions are specifically affected by IVIg. This adds to our knowledge of the effects of IVIg on the immune system and could explain its efficacy in many clinical settings. However, in the particular case of the central nervous system, our results showed a dichotomy, in the effects of IVIg, related to the etiology of inflammation.

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