

# Role of LeuCAM Integrins and Complement in Platelet-Monocyte Rosette Formation Induced by Immune Complexes of Human Immunodeficiency Virus-Type 1-Immune Thrombocytopenic Purpura Patients

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Patients with human immunodeficiency virus-type 1-immune thrombocytopenic purpura (HIV-1-ITP) have elevated polyethylene glycol (PEG)-precipitable immune complexes (ICs) composed of IgG, IgM, and complement that are threefold to sevenfold higher than in healthy control subjects. These complexes contain anti-F (ab')<sub>2</sub> as well as anti-idiotypic antibodies versus anti-HIV-1 gp120. Because anti-F (ab')<sub>2</sub> and anti-idiotypic antibodies correlate with thrombocytopenia ( $r = .83$  [*J Clin Invest* 77:1756, 1986] and  $r = .90$  [*J Clin Invest* 89:356, 1992], respectively) we studied the binding of ICs to platelets and monocytes as well as their role in platelet-monocyte rosette formation. ICs bind to platelets in a saturation-dependent manner (optimum at 10  $\mu\text{g}/\text{mL}$ ; 0.5% of serum conc). Binding to platelets could not be inhibited with platelet saturating concentrations of aggregated IgG or with monoclonal antibody (MoAb) IV.3 versus FcR $\gamma$ II. Platelet binding could be inhibited with Fab anti-C3, anti-C1q, or anti-C4 by 57%, 40%, and 46% respectively, not with control Fab ( $P < .001$ ). Monocytes from HIV-1-ITP patients form rosettes with

normal platelets  $16.8 \pm 5.2$  rosettes/100 monocytes compared with  $4.8 \pm 0.8$  control monocytes plus normal platelets ( $P = .009$ ). Gel-washed HIV-1-ITP platelets formed  $19 \pm 2.0$  rosettes with U937 cells compared to  $6.3 \pm 1.0$  for normal platelets ( $P = 0.001$ ). Arming of U937 cells with HIV-1-ITP ICs (5  $\mu\text{g}/\text{mL}$ ) formed  $36.7 \pm 2.5$  rosettes compared with  $10.6 \pm 1.2$  for control ICs ( $P < .01$ ). Rosetting of armed U937 cells could be inhibited with MoAbs versus the  $\alpha$  chains of CD11a (LFA-1), 11b (Mac-1), or 11c (p150,95) by 67%, 70%, and 61%, respectively ( $P < .007$ ), whereas binding of ICs to U937 cells was unaffected. Isotype-matched control as well as MoAbs versus antigens on U937 cells (CD13, CD33) or the anti-FcR $\gamma$ II receptor had no effect. However, Fab fragments of polyclonal anti-C3 inhibited rosette formation by 78% ( $P < .01$ ); control Fab had no effect. Thus, platelet-monocyte rosette formation is not Fc dependent. It is complement receptor dependent and requires the cooperation of all three leuCAM integrins.

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**I**MMUNE thrombocytopenia is a common hematologic complication of human immunodeficiency virus type 1 (HIV-1) infection.<sup>1</sup> It shares laboratory and clinical features with classic autoimmune thrombocytopenic purpura (ATP) including increased levels of platelet-bound Ig, presence of normal or increased numbers of megakaryocytes in the bone marrow (BM), and improvement of thrombocytopenia following inhibition or ablation of the reticuloendothelial system (RES). HIV-1-immune thrombocytopenic purpura (HIV-1-ITP) differs from ATP in that levels of platelet-bound IgG, IgM, and complement are threefold to fourfold higher in patients with HIV-1-ITP compared to patients with ATP.<sup>2,3</sup> Polyethylene glycol (PEG) precipitates of sera from HIV-1-ITP patients are composed of immune complexes (PEG-ICs) that are threefold to sevenfold higher than levels obtained in ATP patients<sup>2,3</sup>; levels in ATP patients are no different from control subjects. PEG-ICs of HIV-1-ITP patients bind to platelets in a saturation-dependent manner.<sup>4</sup> These immune complexes contain IgG, IgM, C3, and anti-HIV-1 gp120 anti-idiotypic complexes.<sup>4,5</sup> Platelet-bound Ig from patients with HIV-1-ITP also contain IgG, IgM, and anti-HIV-1 gp120 anti-idiotypic complexes.<sup>4</sup>

We have studied the role of PEG-ICs as mediators of binding of platelets to cells of the monocytic lineage. Our previous work has indicated a role for the  $\beta$ 2 integrin Mac-1 in the binding of platelets to monocytes in the presence of serum IgG from patients with ATP.<sup>6</sup> Our current experiments suggest that LeuCAM integrins on cells of the RES and complement-dependent bridging of platelets to RES cells may be important in the pathogenesis of HIV-1-ITP.

## MATERIALS AND METHODS

**Patients.** Patients were recruited from New York University Medical Center. All patients had documented HIV-1 infection, confirmed by Western blot. Patients had been thrombocytopenic for a period of 3 to 24 months before blood donation. No patient had a

documented acquired immunodeficiency syndrome (AIDS)-defining illness.

**Preparation of PEG-ICs from patient and control sera.** PEG-ICs were prepared as described,<sup>7</sup> without 0.02% azide. These complexes contain IgG, IgM, C3,<sup>4,5</sup> C1q, and C4. The latter two components are present as determined by enzyme-linked immunosorbent assay (ELISA) with monospecific anti-sera on microtiter plates.

**Preparation of peripheral blood monocytes.** Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood on Ficoll-Hypaque (Pharmacia, Piscataway, NJ), and monocytes isolated by adherence to plastic as described.<sup>6</sup> Adherent cells (70% to 80% esterase positive) were adjusted to a concentration of  $10^6$  cells/mL for use in the adhesion assay. Highly purified monocyte preparations were obtained by lysis of contaminating B cells with anti-IgM and complement as described.<sup>6</sup>

**Cell culture.** U937 cells (human histiocytic lymphoma; American Type Culture Collection [ATCC], Rockville, MD) were grown in RPMI 1640 (GIBCO, Grand Island, New York) supplemented with 10% fetal calf serum (FCS; GIBCO) at 37°C in 5% CO<sub>2</sub>. The cells

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Submitted July 10, 1992; accepted December 15, 1992.

Supported by Grants HL01821 (K.H.) and HL13336 (S.K.) of the National Heart, Lung and Blood Institute, a grant from the Aaron Diamond Foundation (K.H.), and the Boulanger-Glazier Immunology Research Fund (S.K.).

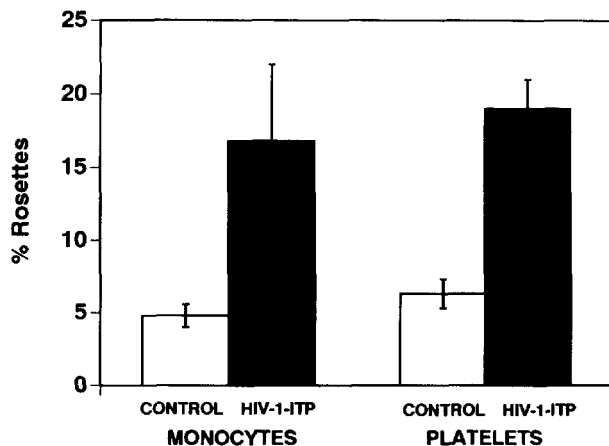
Presented at the 84th Annual Meeting of the American Society of Clinical Investigation, Baltimore, MD, May 1, 1992 (Clin Res 40: 138A, 1992).

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0006-4971/93/8109-0018\$3.00/0



**Fig 1.** Effect of HIV-1-ITP monocytes and control platelets ( $n = 6$ ) or HIV-1-ITP platelets and U937 cells ( $n = 4$ ) on monocyte-platelet rosette formation. Monocytes or platelets from HIV-1-ITP patients and control subjects were isolated, incubated at 37°C for 1 hour, and rosettes counted by phase microscopy.

were passed every 72 hours at a 1:10 dilution; cells were used in the rosette assay after 72 hours in culture. Cell viability was greater than 90% as determined by Trypan blue exclusion.

**Preparation of platelets.** Platelets were isolated from blood anticoagulated with 0.38% sodium citrate. Platelet-rich plasma (PRP) was obtained by centrifugation of blood at 320g and applied to a Sepharose 2B column (Pharmacia) equilibrated with phosphate-buffered saline (PBS)-citrate. Platelet concentration was adjusted to  $10^7$ /mL.

**Preparation of 'armed' U937 cells.** U937 cells were recovered from culture, washed twice in RPMI/FCS, adjusted to a concentration of  $10^6$ /mL, and incubated with PEG-ICs ( $5 \mu\text{g}/\text{mL}$ ) from HIV-1-ITP patients or control subjects for 1 hour at 4°C. The cells were washed twice in RPMI/FCS and resuspended at a concentration of  $10^5$ /mL.

**Preparation of aggregated IgG.** Diethyl aminoethyl cellulose (DEAE)-purified IgG was heated at 56°C for 60 minutes before utilization.

**Rosette assay.** Fifty microliters of monocyte or armed U937 cell suspension ( $10^5$ /mL) and 20  $\mu\text{L}$  of platelet suspension ( $2.5 \times 10^6$ /mL to give a monocyte:platelet ratio of 1:10) were combined in a microcentrifuge tube (Fisher, Pittsburgh, PA), centrifuged at 600g for 5 minutes, and incubated at 37°C for 1 hour. The supernatant medium was removed and replaced with 500  $\mu\text{L}$  fresh RPMI/FCS. An aliquot was examined in a hemocytometer under phase microscopy, and the percentage of rosettes (U937 cells or monocytes with two or more adherent platelets) formed by U937 cells or monocytes enumerated.

**Rosette inhibition experiments.** One microgram of monoclonal antibody (MoAb) to the  $\alpha$  chains of the Leu-CAM family of integrins (Mo 1 [anti-CD11b; Ortho Diagnostics, Raritan, NJ], LFA-1 [anti-CD11a; AMAC, Westbrook, ME], p150,95 [anti-CD11b, Becton Dickinson, Mountvale, CA]), or to the  $\beta 2$  chain of the same integrin family (60.3, anti-CD18, gift of Dr John Harlan, University of Washington, Seattle) was added to armed, washed U937 cells for 1 hour at 4°C before the addition of platelets as described above. In control experiments, identical amounts of antibodies with known affinity for cells of monocytic lineage, My7 and My9 (Coulter, Hialeah, FL), as well as isotype specific irrelevant monoclonals MOPC-21 (IgG1), MOPC-141 (IgG2b), and UPC-10 (IgG2a), (Sigma, St Louis, MO) were used. Fab fragments of anti-C3, anti-C4, and anti-Clq antibody were prepared from rabbit anti-C3 antisera (Sigma) or goat anti-C4

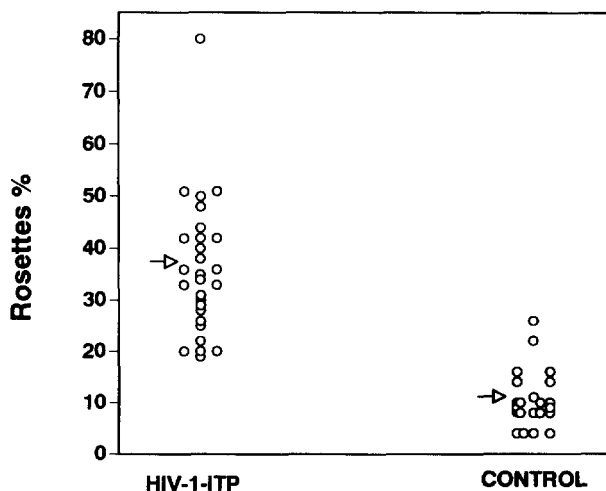
or Clq antisera (Atlantis Antibodies, Stillwater, MN) by DEAE cellulose chromatography, papain digestion,<sup>8</sup> and staphylococcal protein A affinity chromatography and used at 50  $\mu\text{g}/\text{mL}$ . Control Fab fragments were similarly prepared from nonimmune rabbit or goat sera. Anti-FcR $\gamma$ II MoAb IV.3 was a gift of Dr R.J. Looney (University of Rochester Medical Center, Rochester, NY).

**Binding of PEG-ICs to platelets.** Binding studies were performed as described previously with  $^{125}\text{I}$ -protein A.<sup>4</sup> In some experiments the PEG-ICs were incubated with Fab fragments of anti-C3, anti-C4, or anti-Clq antibody, 80  $\mu\text{g}/\text{mL}$ , or control Fab fragments at the same concentration for 1 hour at room temperature before addition to platelets. In other experiments PEG-ICs were added to platelets before and after the addition of aggregated IgG or MoAb IV.3 (anti-FcR $\gamma$ II). In another set of experiments saturating concentrations of heat aggregated IgG (40  $\mu\text{g}/\text{well}$ ) or an equal volume of buffer were added to the platelets adsorbed to plastic, before the addition of serial dilutions of  $^{125}\text{I}$ -PEG-ICs obtained from HIV-1-ITP patient sera.

**Binding of PEG-ICs to U937 cells.** U937 cells ( $10^6$ ) were incubated with PEG-ICs at concentrations of 5 to 100  $\mu\text{g}/\text{mL}$  for 1 hour at 4°C and washed in PBS. PEG-IC binding to U937 cells was assayed by indirect immunofluorescence in a flow cytometer (FACScan; Becton Dickinson) using fluorescein isothiocyanate (FITC)-labeled F(ab')<sub>2</sub> fragments of goat anti-human IgG (Sigma) and 1% formalin fixation. In other experiments MoAbs against CD11a, CD11b, CD11c, or isotype-matched controls (1  $\mu\text{g}/\text{mL}$ ), or Fab fragments of polyclonal anti-C3, anti-C4, or anti-Clq antibody (80  $\mu\text{g}/\text{mL}$ ), or control Fab fragments at the same concentration were added to the U937 cells for 1 hour at 4°C before the addition of the PEG-ICs. Results were expressed as an increase over background fluorescence by comparing mean peak fluorescence.

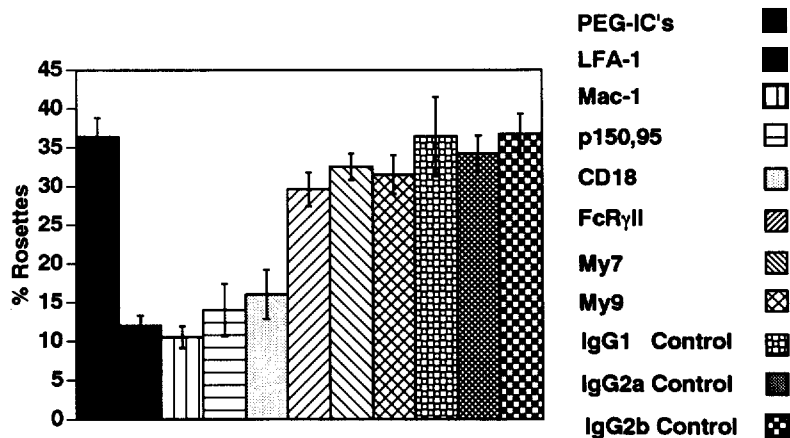
## RESULTS

**Platelet rosette formation with freshly isolated monocytes and platelets from HIV-1-ITP patients.** Freshly isolated monocytes from patients with HIV-1-ITP were incubated with washed, control platelets and rosettes were enumerated. Patient monocytes formed  $16.8 \pm 5.2$  rosettes/100 monocytes



**Fig 2.** Effect of PEG-ICs of HIV-1-ITP patients on arming of U937 monocytetes to form monocyte-platelet rosettes. U937 cells were armed with PEG-ICs ( $5 \mu\text{g}/\text{mL}$ ) from HIV-1-ITP patients ( $n = 28$ ) or control subjects ( $n = 22$ ), washed, and incubated with control gel washed platelets at a ratio of 10:1 (platelets: U937 cells). The cells were pelleted, incubated at 37°C for 1 hour, and rosettes counted by phase microscopy.

**Fig 3.** Effect of anti-LeuCam and anti-FcR $\gamma$ II receptor antibodies on U937 monocyte-platelet rosette formation. U937 cells were armed with HIV-1-ITP or control PEG-ICs and incubated with MoAbs against the  $\alpha$  chain of the LeuCAM integrins LFA 1, Mac-1, or p150 95 (CD11a, 11b, or 11c, respectively), the  $\beta$ 2 chain of the leu-CAM integrins (CD18), the subclass specific controls (MOPC-21, MOPC-141, and UPC-10), the myelomonocytic antigens (My7 and My9), n = 4, or the FcR $\gamma$ II receptor (IV.3), n = 3, before the addition of control gel-washed platelets.



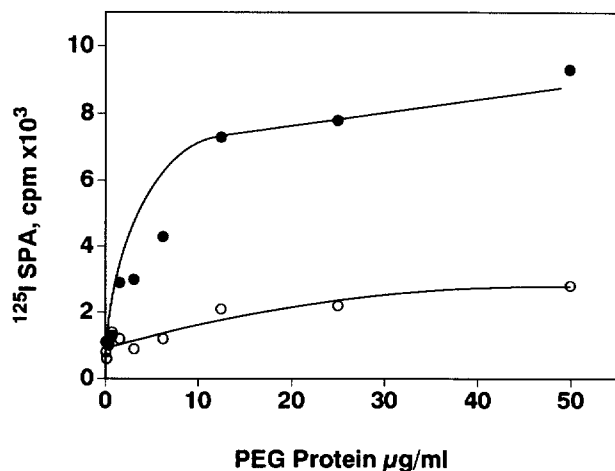
(mean  $\pm$  SEM, n = 6) while similarly prepared control monocytes produced  $4.8 \pm 0.8$  rosettes (n = 6, p = .009, Student's *t*-test) (Fig 1).

Freshly isolated gel-washed platelets from patients formed  $19 \pm 2.0$  rosettes/100 U937 cells, while control platelets formed  $6.3 \pm 1.0$  rosettes/100 U937 cells (*P* = .001, n = 4) (Fig 1).

Because these monocytes and platelets were obtained from patients with high levels of circulating immune complexes, we investigated if these complexes were involved in rosette formation.

**Dose response of PEG-ICs on rosette formation.** Armed U937 cells were incubated with platelets using concentrations of PEG-ICs ranging from 1 to 20  $\mu$ g/mL. Optimum rosette formation (45/100 U937 cells) occurred at a PEG-IC concentration of 5  $\mu$ g/mL (n = 4). This was 3.6-fold greater than the binding of PEG precipitable material from control subjects at 5  $\mu$ g/mL.

**Effect of PEG-ICs of HIV-1-ITP patients versus control subjects on arming of U937 monocytes to form monocyte-**

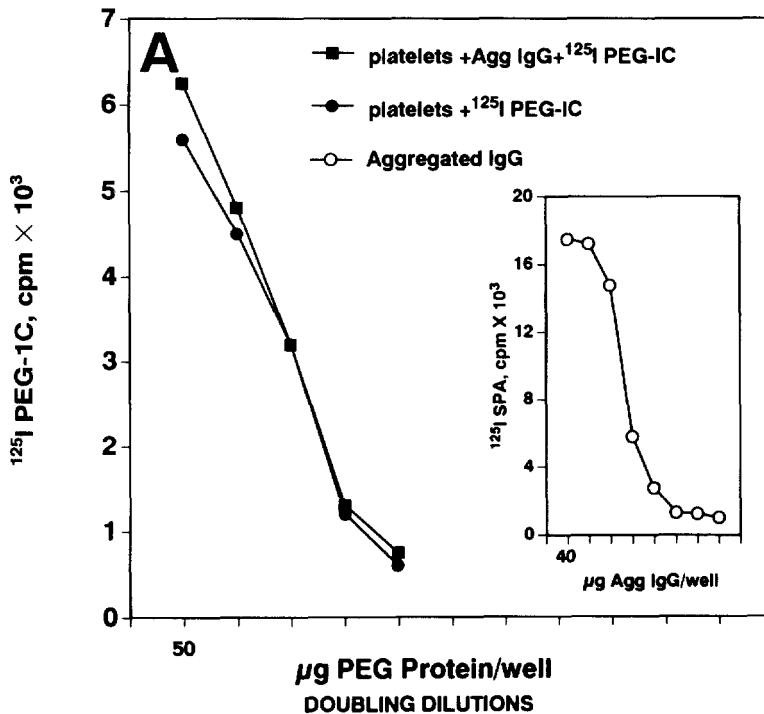


**Fig 4.** Binding of PEG-ICs to platelets. HIV-1-ITP patient (● — ●) or control (○ — ○) PEG-ICs were serially diluted from 50 to 1  $\mu$ g/mL and incubated with control gel-washed platelets adsorbed to plastic microtiter plates. The adsorbed platelets were washed, and bound IgG detected by  $^{125}$ I-staphylococcal protein A.

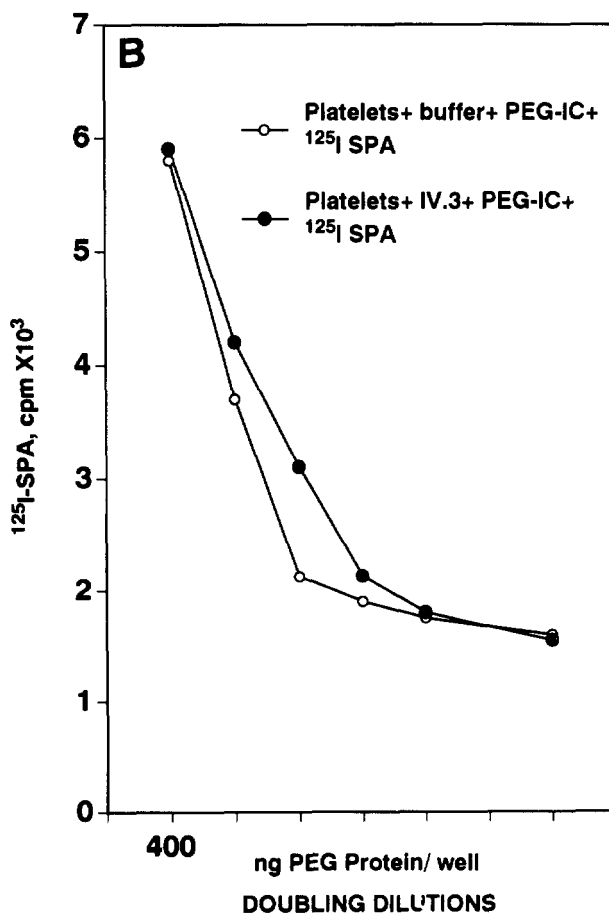
**platelet rosettes.** To determine whether PEG-ICs of HIV-1-ITP patients were qualitatively different from PEG-precipitable material of control subjects, both were incubated with U937 cells and platelet rosette formation enumerated. U937 cells armed with PEG-ICs (5  $\mu$ g/mL) from patients with HIV-1-ITP formed  $36.7 \pm 2.5$  rosettes/100 U937 cells (n = 28) with control gel-washed platelets from 12 different control subjects, whereas U937 cells armed with control PEG-ICs at the same concentration formed  $10.6 \pm 1.2$  rosettes/100 U937 cells (n = 22, *P* < .01) (Fig 2). Thus, PEG-ICs of HIV-1-ITP patients are qualitatively different from PEG-precipitable material of control subjects with respect to their ability to bind to platelets.

**Effect of anti- $\beta$ 2 integrin and anti-FcR $\gamma$ II antibodies on rosette formation.** Because previous studies had shown a role for the  $\beta$ 2 integrin Mac-1 in the binding of platelets to monocytes in ATP patients in the presence of their serum IgG,<sup>6</sup> we examined the role of  $\beta$  integrins in HIV-1-ITP immune complex-induced rosette formation. Armed U937 cells were incubated with MoAbs against the  $\alpha$  chains of the Leu-CAM family of integrins (CD11a, 11b, and 11c), the  $\beta$ 2 chain of the LeuCAM family of integrins (CD18), the FcR $\gamma$ II (CDw32) receptor (IV.3) or against the 'control' myelomonocytic antigens My7 and My9 (CD13 and CD33), or against isotype-matched control MoAbs (MOPC-21 [IgG1], MOPC-141 [IgG2b], and UPC-10 [IgG2a]) before the addition of control gel-washed platelets. Antibodies against CD11a, CD11b, and CD11c reduced rosette formation by 67%, 70%, and 61%, respectively (*P* < .007), for each positive control rosette formation (Fig 3). Surprisingly, IV.3, the MoAb against FcR $\gamma$ II had no effect (Fig 3). Isotype-matched control MoAbs reduced rosette formation by less than 3%. Anti-CD13 and CD33 (antibodies against non-integrin antigens found on U937 cells) reduced rosette formation by 14% and 33%, respectively (*P* = .1, n = 4, data not shown). Thus, LeuCAM receptor occupancy inhibits rosette formation, whereas FcR $\gamma$ II occupancy does not.

**Effect of antibody against C3/C3bi.** Because PEG-ICs of HIV-1-ITP patients contain C3<sup>4,5</sup> and because Mac-1 and p150,95 are receptors for C3bi, we examined the role of C3 receptor binding in rosette formation. U937 cells that had been armed with control and HIV-1-ITP PEG-ICs were in-



**Fig 5.** Effect of aggregated IgG or MoAb IV.3 (against FcR $\gamma$ II) on binding of PEG-ICs to platelets. (A) Platelets were adsorbed to microtiter plates as described and then incubated with buffer or aggregated IgG at 40  $\mu\text{g}/\text{well}$  for 1 hour at room temperature before the addition of serial dilutions of  $^{125}\text{I}$ -PEG-ICs, starting at 50  $\mu\text{g}/\text{well}$ . Inset documents saturation binding of aggregated IgG to platelets at 40  $\mu\text{g}/\text{well}$  (monitored with  $^{125}\text{I}$ -staphylococcal protein A [SPA]). (B) Platelets were adsorbed to microtiter plates and then incubated with buffer or a saturating concentration of IV.3 (5  $\mu\text{g}/\text{mL}$ ) before the addition of serial dilutions of PEG-IC starting at 400  $\text{ng}/\text{well}$ . Binding was monitored with  $^{125}\text{I}$ -SPA. Control binding of  $^{125}\text{I}$ -SPA plus platelets or platelets plus IV.3 was  $1.4 \times 10^3$  cpm.



cubated with Fab fragments of polyclonal anti-C3 (containing anti-C3bi). Platelet-U937 rosette formation of  $36 \pm 3$  was inhibited by 80% ( $P < .01$ ,  $n = 4$  compared to rosette formation with Fab fragments of nonimmune rabbit IgG,  $39 \pm 7$ ). Thus, rosette formation requires the interaction of C3 in PEG-ICs with C3 receptors on platelets and/or monocytes (see below).

To better understand the mechanisms of platelet-monocyte rosette formation, we investigated the interaction of PEG-ICs with platelets and with U937 cells.

**Binding of PEG-ICs to platelets.** Binding of PEG-ICs to platelets is concentration-dependent and saturable with optimum binding occurring at a PEG-IC concentration of 10  $\mu\text{g}/\text{mL}$  (Fig 4). Because the serum concentration in HIV-1-ITP patients is 0.7 to 1.5  $\mu\text{g}/\text{mL}$ ,<sup>2,3</sup> the saturating concentration is pathophysiologically relevant.

**Binding of PEG-ICs to platelets is not Fc receptor dependent.** Because of the unexpected finding that anti-FcR $\gamma$ II receptor antibody did not inhibit rosette formation, we elected to independently confirm that PEG-IC binding to platelets was not Fc receptor dependent. Figure 5A shows the lack of inhibition of  $^{125}\text{I}$ -PEG-IC binding to platelets by a platelet-saturating concentration of aggregated IgG (inset of figure). Similar negative results were obtained with a platelet-saturating concentration of MoAb IV.3 against the platelet FcR $\gamma$ II receptor (Fig 5B).

**Inhibition of binding of PEG-ICs to platelets with anti-C3, anti-C1q, and anti-C4 antibody.** Because anti-C3 antibody inhibited rosette formation, we elected to determine whether its effect was on binding of PEG-ICs to platelets. Figure 6 shows that incubation of PEG-ICs with Fab anti-C3 at ratios

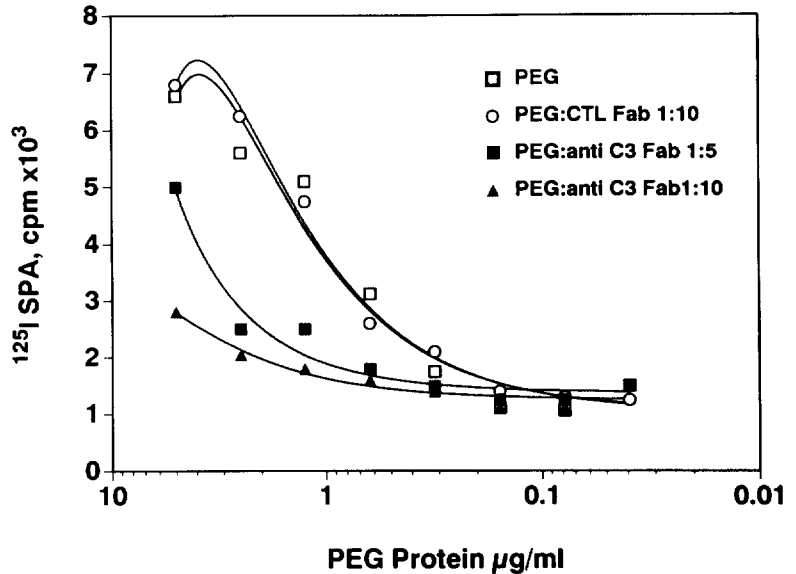


Fig 6. Effect of preincubation of PEG-ICs with Fab anti-C3 antibody on binding to platelets. HIV-1-ITP PEG-ICs were applied to control gel-washed platelets adsorbed to plastic microtiter plates alone (□ - □) or following preincubation of the PEG-ICs with Fab fragments of control rabbit IgG at a 1:10 weight ratio (PEG-ICs: Fab fragments) (○ - ○), or Fab fragments of rabbit IgG anti-C3 at 1:5 (■ - ■) or 1:10 (▲ - ▲) ratios. The adsorbed platelets were washed, and bound IgG detected by <sup>125</sup>I-staphylococcal protein A (n = 2).

of 1:5 and 1:10 (by weight) decreases platelet binding by 24% and 57%, respectively, while incubation with control Fab fragments had no effect. Similar inhibition was noted with anti-Clq and anti-C4 Fabs of 40% and 46%, respectively, at 1:5 ratios (*P* < .001, n = 2, data not shown).

*Detection of binding of PEG-ICs to U937 cells via fluorescence flow cytometry.* Binding of patient PEG-ICs to U937 cells increased in a linear fashion over the range of concentrations used, while control PEG-IC binding reached optimum binding at 10 μg/mL. Binding of patient PEG-ICs to U937 cells was threefold greater than control PEG-IC binding at 50 μg/mL and 3.5-fold greater at 100 μg/mL (*P* < .01 and < .005 respectively; n = 4, Table 1).

*Effect of monoclonal antibodies versus CD11a, 11b, and 11c on binding of PEG-ICs to U937 cells.* Because rosette formation was inhibited by all three LeuCAM integrins, we elected to determine whether inhibition was caused by blocking of binding of HIV-1-ITP PEG-ICs to U937 cells. Preincubation of U937 cells with anti-CD11a, 11b, or 11c had no effect on binding of PEG-ICs to U937 cells at 5 and 20 μg/mL (two to five experiments, data not shown).

*Effect of Fab anti-C3 and anti-C4 on binding of PEG-ICs to U937 cells.* Because Fab anti-C3 inhibited rosette formation as well as binding of HIV-1-ITP PEG-ICs to platelets, we elected to determine whether anti-C3 as well as anti-C4 would also inhibit binding to U937 cells. Table 1 shows 44% to 42% inhibition of binding of PEG-ICs to U937 cells following preincubation of ICs with Fab anti-C3 or anti-C4, respectively (*P* < .001, n = 4). Negligible inhibition was noted with nonimmune Fab.

DISCUSSION

These studies provide the first direct evidence of increased platelet-monocyte interaction mediated by the unique immune complexes produced by patients with HIV-1 infection. PEG-ICs from HIV-1-ITP patients are 3.4-fold more potent in inducing platelet U937 cell rosette formation than control PEG-precipitates of normal subjects. Because these complexes are present in threefold to sevenfold greater concentration in the sera of HIV-1-ITP patients compared with control subjects<sup>2,3</sup> and the optimum in vitro concentration for platelet-monocyte rosette formation is 70- to 150-fold less than

Table 1. Effect of Anti-C3 and Anti-C4 Fab on Binding of Peg-ICs to U937 Cells

|                            | PEG Concentration, μg/mL |           |           |              |           |           |
|----------------------------|--------------------------|-----------|-----------|--------------|-----------|-----------|
|                            | 0                        | 5         | 10        | 20           | 50        | 100       |
| Control                    | 1                        | 1.6 ± 0.5 | 2.1 ± 1.7 |              | 1.8 ± 1.3 | 2.2 ± 1.5 |
| Patient                    | 1                        | 1.6 ± 3.3 | 2.3 ± 0.6 |              | 5.4 ± 0.4 | 7.7 ± 0.1 |
| Patient + anti-C3          |                          |           |           | 0.56 ± 0.05* |           |           |
| Patient + anti-C4          |                          |           |           | 0.58 ± 0.04* |           |           |
| Patient + control antibody |                          |           |           | 0.86 ± 0.08  |           |           |
| Control + anti-C3          |                          |           |           | 0.96 ± 0.14  |           |           |
| Control + anti-C4          |                          |           |           | 0.85 ± 0.06  |           |           |
| Control + control antibody |                          |           |           | 1.18 ± 0.04  |           |           |

U937 cells (10<sup>6</sup>/mL) were incubated with various concentrations of patient or control PEG-ICs and binding detected by fluorescence flow cytometry with FITC-labeled F(ab')<sub>2</sub> fragments of goat anti-human IgG. In some experiments 100 μg of Fab fragments of anti-C3 or anti-C4 were incubated with PEG-ICs in 100 μL for 1 hour at 4°C before the addition of 0.9 mL of 10<sup>6</sup> U937 cells for an additional 1 hour at 4°C. Relative fluorescence and SEM is given for 0, 5, 10, 50, and 100 μg/mL. The relative percentage of positive or negative control is given for 20 μg/mL, n = 4. \**P* < .001.

the serum concentration,<sup>2,3</sup> these complexes are both qualitatively and quantitatively favored to mediate platelet-monocyte interaction in vivo, which could contribute to thrombocytopenia. This hypothesis should be interpreted with caution because there is no correlation between platelet count and PEG-precipitable immune complex level or platelet-associated IgG.<sup>3</sup> It is possible that the presence of these immune complexes more directly reflects the disorder of immune regulation rather than the thrombocytopenia. However, it is also possible that the qualitative composition of the immune complex may be more directly related to the thrombocytopenia. This appears to be the case. We have recently demonstrated the presence of anti-idiotypic-anti-HIV-1gp120 antibody in the PEG-immune complexes and platelet eluates of HIV-1-ITP patients. Serum anti-idiotypic HIV-1gp120 antibody correlated strongly with thrombocytopenia,  $r = .9$ ,  $P < .001$  in a group of 31 HIV-1-infected patients (21 thrombocytopenics, 10 non-thrombocytopenics).<sup>4</sup>

The binding of HIV-1-ITP PEG-ICs to platelets is surprisingly not Fc $\gamma$ RII-mediated. This was established by demonstrating that binding was not inhibited by aggregated IgG or MoAb IV.3 against the Fc $\gamma$ RII receptor, under saturating conditions of binding of aggregated IgG and IV.3 to platelets. Rather, the binding of HIV-1-ITP PEG-ICs to platelets appears to be complement-mediated, because anti-C3 Fab fragments inhibited platelet-monocyte rosette formation, and anti-C3b, anti-C4, and anti-Clq Fab inhibit binding of PEG-ICs to platelets. It is of interest in this regard that platelets have a C3b/C3bi (gp 45 to 70) receptor,<sup>9,10</sup> a Clq receptor,<sup>11</sup> as well as a recently described complement receptor 4 (CR4).<sup>12</sup>

The binding of HIV-1-ITP PEG-ICs to U937 monocytes is also not Fc $\gamma$ RII-mediated because IV.3 did not inhibit rosette formation. Rather, the binding appears to be complement-mediated because anti-C3b and anti-C4 Fab inhibited binding of PEG-ICs to U937 cells.

Increased expression of  $\beta$ 2 integrins has been observed in U937 monocytes after HIV-1 incubation.<sup>13</sup> Therefore, it is of particular interest that occupancy of all three  $\beta$ 2 receptors with either of the specific anti-functional antibodies for LFA-1, Mac-1, or p150,95 or an MoAb reacting with the  $\beta$ 2 subunit of all three receptors inhibited rosette formation. Yet binding of PEG-ICs to U937 cells was not inhibited by any of these MoAbs. Thus, neither Mac-1 nor p150,95 is acting as a complement receptor for the PEG-ICs. The mechanism of inhibition of rosette formation by LeuCAM receptor occupancy is yet to be elucidated. It is unlikely that all three receptors are required on U937 cells for binding to platelets. Indeed, the counter-receptor for LFA-1 (ICAM-1) is not present on platelets.\* It is more likely that receptor occupancy has an inhibitory effect on U937-monocyte function. Gresham et al<sup>14</sup> have reported elevated cyclic adenosine monophosphate (AMP) levels in immune complex-stimulated neutrophils treated with anti-CD11b (Mac-1) or CD18 ( $\beta$ 2 subunit). They

also noted that inhibition of phagocytosis of opsonized erythrocytes with anti-CD18 could be overcome with a cyclic AMP inhibitor, HA1004, which inhibits protein kinase A. It is likely that similar effects may be occurring with U937 cells treated with anti-LeuCAM antibodies.

Regardless of the mechanism, it is clear that platelet-monocyte rosette formation induced by PEG-ICs can be regulated by LeuCAM receptor occupancy and requires complement for platelet-monocyte bridging. Modulation of LeuCAM integrins or complement receptors on platelets or cells of the RES may provide therapeutic approaches for the treatment of HIV-1-ITP.

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\* Hymes K, Karpatkin S: Unpublished fluorescence flow cytometry data (n = 2).