Disparities in Estimates of IgG Bound to Normal Platelets

By Neil Blumberg, Debra Masel, and Mark Stoler

Estimates of the number of IgG molecules bound to normal platelets have ranged from several hundred to several tens of thousands. The lower estimates were generated from direct binding assays and stoichiometric assumptions. The higher values derive from competitive binding assays, in which platelet-associated IgG (PAIgG) is calculated from a standard curve using soluble IgG standards. Using a kinetic-ELISA (enzyme-linked immunosorbent assay) antiglobulin assay, we measured normal platelet IgG to be $21,200 \pm 9,400$ molecules per platelet when a competitive assay and soluble IgG standards were used. Direct measurement of bound antiglobulin by kinetic-ELISA and stoichiometric assumptions yielded a measurement of $259 \pm 117$ IgG molecules per platelet. Soluble IgG and PAIgG are not comparable in their ability to bind anti-IgG. Disparities in estimates of normal PAIgG are probably due to methodological differences. The estimate most likely to be correct is several hundred IgG or less per normal platelet.

MATERIALS AND METHODS

Competitive assay for PAIgG using calibration with a soluble IgG standard curve. In this assay, PAIgG or soluble IgG standards compete with solid-phase IgG for binding to an alkaline phosphatase-linked anti-IgG antiglobulin reagent. The principle of the assay is that increased concentrations of soluble IgG or PAIgG will compete for anti-IgG and reduce anti-IgG binding to the solid-phase IgG. The solid-phase is a 96-well microtiter plate (Immulon II, Dynatech, Inc., Alexandria, Va) that has been previously washed three times with distilled water. All washes are performed by machine (Miniwash, Dynatech). A 100-μL solution of 100 ng purified human IgG (Pelfreeze Biologicals, Rogers, Ark) in coating buffer (1.59 g/L Na2CO3, 2.93 g/L NaHCO3, pH 9.8) is placed in each well and incubated overnight at 4 °C. Two wells are coated and incubated with 100 ng of purified bovine serum albumin (BSA) (Sigma Chemical Co, St Louis) in coating buffer as negative controls. No binding of anti-IgG-alkaline phosphatase conjugate should occur in these two wells.

Platelets are prepared from EDTA anticoagulated normal donor blood collected in evacuated collection tubes (Sherwood, St Louis). Platelet-rich plasma is made by centrifugation at 250 g for ten minutes, and three washes are performed with phosphate-buffered saline (PBS)-EDTA (3.0 g/L Na2 EDTA, 3.75 g/L Na2HPO4, 8.2 g/L NaCl, pH 7.0). Platelets are resuspended at a count of 50,000/μL in PBS-Tween (PBS as earlier, without EDTA, but with 0.5 ml/L Tween 20 [Sigma, St Louis]). Removal of possible platelet fragments was not attempted.

A series of dilutions of purified IgG is prepared containing 25, 50, 100, 250, 500, 750, and 1,000 ng in 100 μL of PBS-Tween. These serve as soluble standards to compete with the solid-phase IgG for alkaline phosphatase–anti-IgG conjugate.

The microtiter plate coated with IgG is washed four times with PBS-Tween before use. The soluble IgG standard curve dilutions are added to individual wells in triplicate, as are 100 μL of 50,000/μL solutions of washed platelets (total 5 × 10⁶ per well). Several duplicate wells are used as negative controls. The BSA-coated wells receive 100 μL of PBS-Tween. Two IgG-coated wells receive 100 μL of PBS-Tween to serve as zero points for competitive binding (ie, maximal binding of anti-IgG due to lack of competing soluble IgG). Two wells coated with IgG receive neither soluble IgG, platelets, nor anti-IgG, to serve as controls for spontaneous substrate conversion.

PBS-Tween (200 μL) is added instead. Once all soluble IgG standards and the platelets have been added, 100 μL of a 1:300 dilution (in PBS-1% BSA) of alkaline phosphatase coupled to affinity-purified antihuman IgG (Sigma) is added to each well, except as noted earlier. The plate is then gently rotated (Microshaker, Dynatech) for one minute, covered with a plastic seal, and incubated at 37 °C for one hour. The contents are then dispelled by inversion, and the plate washed four times with PBS-Tween. Substrate is added to each well (200 μL of 1 mg/mL p-nitrophenylphosphate (Sigma) in 5.3 g/L Na2CO3, 0.2 g/L MgCl2-6H2O, pH 9.8).
The plate is rotated for 18 minutes, and determination of bound anti-IgG conjugate is performed automatically by a through-the-plate spectrophotometer (Dynatech MR600) coupled to an Apple IIe computer (software program available from Dr. Jonathan Cowles, 149 Tobey Rd, Pittsford, NY 14534). Bound anti-IgG conjugate is determined kinetically as ΔA405nm/min × 1,000. Bound anti-IgG has been shown to be linearly proportional to this slope in a kinetic-ELISA (enzyme-linked immunosorbent assay). A standard curve of slope (ΔA405nm/min × 1,000) vs IgG in the soluble standards is constructed to calibrate the assay. This is linear over the range of the assay when a log-log plot is used (see Fig 1). The quantity of IgG bound to 5 × 10⁶ platelets is calculated by using the measured mean ΔA405nm/min × 1,000 and the standard curve. Values are reported as 10⁻¹⁵ g (g) IgG per platelet. These are converted to molecules of IgG per platelet assuming a molecular weight (mol wt) of 150,000 daltons.

**Direct binding assay for PAIgG.** The alternative method for measuring PAIgG involves stoichiometric calculation of the activity of the alkaline phosphatase-anti-IgG conjugate (number of anti-IgG molecules added per test) and measurement of the proportion of the conjugate that actually binds to 5 × 10⁶ platelets. The calculations of the number of anti-IgG added are shown in the next section.

The measurement of percentage binding to 5 × 10⁶ platelets is performed as follows: The microtiter plate is not coated with IgG, since it serves only as a reaction vessel. Washed platelets (5 × 10⁶) (100 μL) are incubated with 100 μL of a 1:300 dilution of the same alkaline phosphatase-anti-IgG conjugate used in the competitive assay. The incubation conditions are identical: 37 °C for one hour. The platelets are sedimented at 500 g for ten minutes and washed at assay. The incubation conditions are identical: 37 °C for one hour. The quantity of IgG bound to 5 × 10⁶ platelets is calculated by using the measured mean ΔA405nm/min × 1,000 and the standard curve. Values are reported as 10⁻¹⁵ g (g) IgG per platelet. These are converted to molecules of IgG per platelet assuming a molecular weight (mol wt) of 150,000 daltons.

**Results**

The mean normal PAIgG using the competitive assay, calibrated by standard curves of soluble IgG to convert ΔA405nm/min × 1,000 to mass units, was 5.3 ± 2.36 fg per platelet (n = 324) (± 1 SD). Using a mol wt of 150,000 daltons for IgG, this converts to approximately 21,300 IgG molecules per platelet. PAIgG of patients with immune thrombocytopenia are usually fivefold to 100-fold higher than these levels (Fig 2). Patients with acute leukemia and thrombocytopenia are usually fivefold to 100-fold higher than these levels (Fig 2). Patients with acute leukemia and thrombocytopenia are usually fivefold to 100-fold higher than these levels (Fig 2). Patients with acute leukemia and thrombocytopenia are usually fivefold to 100-fold higher than these levels (Fig 2). Patients with acute leukemia and thrombocytopenia are usually fivefold to 100-fold higher than these levels (Fig 2).

The proportion of conjugate binding to normal platelets in the direct assay, as quantitated by the ratio ΔA405nm reacted/ΔA405nm unreacted conjugate, was 0.31% ± 0.14% (n = 5). That this low-level binding is not due to inadequate amounts of anti-IgG in the 1:300 dilution of antilglobulin conjugate is evidenced by the fact that binding increases many fold when platelets heavily coated with IgG are used in the assay as described. Conversion to number of IgG molecules per platelet is by multiplying the mean percentage binding (0.31%) by the maximum theoretical number of anti-IgG molecules reacted per platelet (83,400) = 259 molecules per platelet.
assumptions and are not likely to be incorrect by more than a factor of two. As discussed in Materials and Methods, the assumptions used are, in fact, more likely to overestimate PAIgG. Although most PAIgG assays use a two-stage antiglobulin procedure, we used a typical competitive one-stage immunoassay. There are no theoretical or practical reasons why this should have affected our results.

Methods for PAIgG determinations calibrated using soluble IgG rest on the assumption that PAIgG and soluble IgG standards compete equally well for anti-IgG. Ware et al's data argue strongly against the accuracy of this assumption. One possible explanation for this disparity is that the location on the platelet of PAIgG might favor bivalent binding kinetics for an anti-IgG reagent, rather than the univalent binding kinetics that apply to solution reactions between anti-IgG and IgG. The difference between univalent and bivalent affinity constants can be as much as tenfold to 100-fold. This possibility could be studied by using anti-IgG reagents with univalent binding properties [e.g., F(ab) fragments].

In summary, use of a standard curve of soluble IgG to measure levels of PAIgG overestimates the actual number of molecules per platelet by approximately 80-fold in our hands. Results of PAIgG estimates using soluble IgG standards should perhaps be reported as "fg per platelet in soluble IgG equivalents." We believe that our data and those in the literature are most consistent with a maximal estimate of <300 molecules of IgG per normal platelet.

ACKNOWLEDGMENT

We thank Drs Joanna Heal and Dean Arvan for helpful discussions. Carol Cole provided exemplary secretarial assistance. Kathy Lopez, Debby Polidori-Schwarz, Gail Smyth, and Glenda Spencer assisted in the normal range study.

REFERENCES

Disparities in estimates of IgG bound to normal platelets

N Blumberg, D Masel and M Stoler