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Immunology

Fc (IgG) receptor distributions in homogeneous and heterogeneous cell populations by flow microfluorometry

(cell sorter/fluorescence activated cell sorter/immunofluorescence/lymphocytes/affinity crosslinked antibody)

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ABSTRACT **A flow microfluorometric method has been developed for quantitating the numbers of Fc receptors on individual cells. The cells were equilibrated at 0°C with radiolabeled, affinity-crosslinked rabbit IgG dimers, washed, and treated with fluorescent antibodies against rabbit IgG. The stained cells were analyzed for fluorescence emission by using a fluorescence-activated cell sorter and for bound dimer molecules by using a gamma counter. Standard curves relating fluorescence emission to numbers of dimer molecules bound to cells were used to determine Fc receptor distributions on P388D$_1$ cells, human peripheral blood lymphocytes, and normal mouse spleen cells. Essentially all of the P388D$_1$ cells bore Fc receptors, distributed in a skewed Gaussian profile having a peak at 2×10^5 receptors per cell. Human peripheral blood lymphocytes and mouse spleen cells contained positive and negative subpopulations. The percentage of positive cells in human lymphocytes from different donors ranged from 5 to 25; the receptor distributions of these cells were symmetrical and similar in all donors in shape and average receptor density (4.2×10^4 receptors per cell). Mouse spleen cells contained 55% positive cells with nonsymmetrical heterogeneous distributions of receptor densities. These cells peaked at 1 to 2×10^4 receptors per cell, but significant numbers of cells had receptor densities 10- to 20-fold greater.**

Receptors for the Fc regions of IgG molecules occur on a wide variety of cells (1). Depending on the type of cell, Fc receptors can be involved in a number of physiological responses; e.g., lysis of antibody-coated cells (2), phagocytosis of antibody-coated particles (3), or transport of IgG molecules across the intestinal mucosa (4).

The binding of monomeric IgG to Fc receptors is usually a relatively weak interaction (1) and therefore difficult to measure directly. Polymerized IgG molecules (immune complexes or heat and chemically aggregated immunoglobulins) bind more tightly than monomeric IgG, however, because they interact multivalently with cell surface receptors (1). In previous studies (5, 6), model immune complexes were prepared by crosslinking rabbit anti-2,4-dinitrophenyl (DNP) IgG antibody molecules at their combining sites by using bivalent affinity-labeling reagents. The covalently crosslinked IgG dimers and trimers were isolated by gel filtration and, after radioiodination, were used in cell-binding assays (7).

The radioactivity assay, however, measures average values of affinities and numbers of oligomer molecules bound per cell, which is an obvious limitation when the cell sample contains distinct subpopulations (e.g., mouse spleen cells and human lymphocytes). Thus, to characterize Fc receptors on cells from a heterogeneous source, the constituent subpopulations must first be isolated. For example, the binding of ^{125}I-labeled trimers to "purified" subpopulations of cells from human leukocytes has been measured (8), but some of the purified fractions still contained identifiable subpopulations of cells and, in addition, it was never clear that the isolation procedures did not alter the Fc receptors (9).

In contrast to radioactivity, immunofluorescence can be measured on individual cells (1). Fluorescence can be quantitated on large numbers of cells by flow microfluorometry (FMF), and has been used to study Fc receptors on murine cells (10–14). However, in those studies, the relationship between fluorescent signal and number of antibody molecules bound per cell was not known and therefore numbers of receptors per cell could not be determined. We have combined the radioactivity-binding assay with FMF analysis and can quantitate the Fc receptors on large numbers of single cells.

MATERIALS AND METHODS

Media. The following media were used: medium 1, RPMI 1640 containing 5% fetal calf serum, 0.03% glutamine, penicillin at 100 units per ml, and streptomycin at 100 μg/ml; medium 2, balanced salt solution containing 2% bovine serum albumin, 0.2% sodium azide, and 0.1 mM DNP-ε-aminocaproate (pH 7.4); medium 3, RPMI 1640 containing 20% fetal calf serum; medium 4, Hanks' balanced salt solution lacking phenol red but containing 2.5% bovine serum albumin; 0.2% sodium azide, and DNase at 0.01 mg/ml; medium 5, RPMI 1640 containing 20% pooled AB+ human serum, penicillin at 100 units per ml, streptomycin at 100 μg/ml, and 0.03% glutamine.

Rabbit Immunoglobulins. Rabbit IgG (15) and affinity-purified rabbit anti-DNP IgG antibody (5) were prepared as described. Affinity-crosslinked oligomers were prepared from rabbit anti-DNP IgG by using the bivalent affinity label bis(α-bromocetyl-ε-DNP-Lys-Pro)ethylenediamine [(BADL-Pro)$_2$ EDA; 5].

Goat Anti-Rabbit IgG Antibody. Goat anti-rabbit IgG antibody was raised by injecting a goat at multiple sites with a 1:1 emulsion of complete Freund's adjuvant and rabbit IgG (1 mg/ml in phosphate-buffered saline). The goat was boosted with the adjuvant emulsion after 10 and 13 weeks, serum was prepared (16), and the IgG fraction was precipitated with $(NH_4)_2SO_4$ (final concentration, 45%). The precipitate was dialyzed against borate (0.02 M) buffered saline (pH 8.5).

Rabbit IgG (150 mg) was coupled to 15 g of CNBr-activated

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Abbreviations: DNP, 2,4-dinitrophenyl; (BADL-Pro)$_2$EDA, bis(α-bromacetyl-ε-DNP-Lys-Pro)ethylenediamine; FACS, fluorescence activated cell sorter; FMF, flow microfluorometry; FITC–FGaR, fluorescein isothiocyanate-conjugated F(ab')$_2$ fragments of affinity-purified goat anti-rabbit IgG antibodies.

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Sepharose (Pharmacia) in 15 ml of 0.1 M $NaHCO_3$/0.5 M NaCl, pH 8.5 (17). The reaction was allowed to proceed for 2 hr with gentle agitation at 37°C. The IgG Sepharose was washed with 0.1 M NaOAc/0.15 M NaCl, pH 4.0 and then with 0.1 M sodium borate/0.15 M NaCl, pH 8.0 for three cycles. It was then put in a 2.5 × 12 cm column and washed with 3.5 M $MgCl_2$ and borate-buffered saline. About 95% of the added rabbit IgG was coupled to the Sepharose.

Ammonium sulfate-precipitated goat immunoglobulin was applied to the affinity column and washed with borate-buffered saline containing 0.02% NaN_3 until the absorbance at 280 mm of the eluate was less than 0.01. The bound antibodies were removed from the column with 3.5 M $MgCl_2$ and dialyzed into borate-buffered saline. The protein was concentrated by ultrafiltration and further purified by gel filtration.

$F(ab')_2$ Fragments. The affinity-purified goat IgG antibodies (10 mg/ml) were digested with pepsin (0.2% by weight) in 0.1 M NaOAc (pH 5.0) for 8–10 hr at 37°C (17). The digestion mixture was neutralized and fractionated on an Ultragel ACA-34 column. The concentrated $F(ab')_2$ peak gave a single band on $NaDodSO_4$/polyacrylamide gel electrophoresis (M_r = 100,000). It was rechromatographed on the rabbit IgG affinity column to remove any antibody inactivated by proteolysis.

Fluorescein-Labeled $F(ab')_2$ Fragments (FITC–FGaR). The concentrated $F(ab')_2$ fragments (0.5–4.5 mg/ml) were incubated for 2 hr at 37°C with a 5-fold molar excess of fluorescein isothiocyanate (FITC) in 0.2 M sodium borate/1.5 M NaCl, pH 9.0 (18). The unreacted FITC was removed by gel filtration and the protein was centrifuged at 50,000 × *g* for 20 min to remove any aggregates.

Cells. Cells from the murine macrophage-like tumor line $P388D_1$ were grown in spinner culture in medium 1 as described (19). Single cell suspensions of normal mouse spleens from 4- to 8-week-old C57BL/6 mice were prepared in medium 2 (7). Human peripheral blood lymphocytes were isolated from heparinized blood from normal donors as described (20) and cultured for 2 days at 1.5 × 10^6 cells per ml in medium 5 prior to use.

Radioactivity Binding Assay. Binding was assayed as described (7, 15). Cells labeled with ^{51}Cr were equilibrated with increasing concentrations of ^{125}I-labeled IgG dimers in medium 2 with and without a large excess of unlabeled oligomeric IgG (21) and centrifuged through phthalate oils. These are immiscible with aqueous media and less dense than cells but more dense than media so that centrifugation through them can remove the cells from a medium without an aqueous phase wash step. Bound dimer was determined from the $^{125}I/^{51}Cr$ ratio in the cell pellet. The nonspecific controls (samples containing unlabeled oligomer) were generally 5–10% of the total binding. In experiments in which the cells were washed before sedimentation through oils, nonspecific-cell-associated dimer was a negligible fraction of total bound dimer.

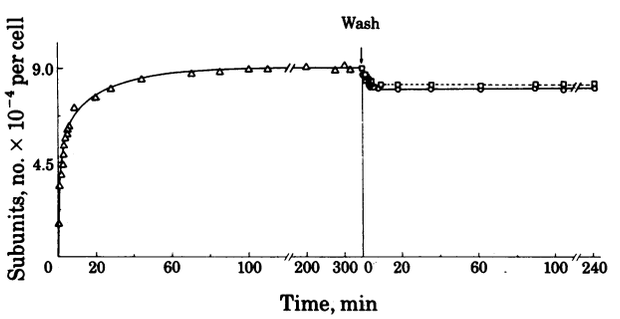

FIG. 1. Equilibration and dissociation of IgG dimers from $P388D_1$ cells at 0°C. Cells were incubated with 32 nM ^{125}I-labeled dimer, and aliquots were centrifuged through phthalate oils at specified times to determine the numbers of molecules of IgG bound per cell (△). After equilibrium was reached, the cells were washed with Hanks' balanced salt solution at 4°C and resuspended in medium 4 alone (○) or in medium 4 containing saturating amounts of FITC–FGaR (□). Cell-bound radioactivity was again monitored with time. Data are means of three replicates and SEM bars are smaller than the symbols.

Fluorescence Labeling of Cells. Cells (3 × 10^6) were equilibrated with dimer (radiolabeled or unlabeled) for 2 hr at 0°C in 250 μl of medium 4, then washed twice with balanced salt solution at 4°C and incubated in 100 μl of medium 4 containing saturating amounts of FITC–FGaR for 30 min at 0°C, and finally washed twice and resuspended in 1 ml of medium 4. Cells were maintained at 0°C before FMF analysis; more than 90% were viable by trypan blue exclusion.

Cell Analysis by FMF. The use and operation of the fluorescence activated cell sorter (FACS) has been described (22). FMF was performed by using a FACS II (Becton-Dickinson FACS Systems) and a PDP 11/40 computer (Digital Equipment Corporation). All signals were collected at the highest gain setting (amplification), at which point less than 2% of the cells fell above the dynamic range. At least 4 × 10^4 viable cells (23) were analyzed for fluorescence intensity. Offsets, including a "pedestal" voltage equivalent to 15 channels, were subtracted from all data presented in this paper. FMF data are displayed as cell-frequency histograms in which the fluorescence intensity of each cell has been assigned to one of 1024 equal channels. For comparisons of data, signals were corrected to the same gain (gain 16) after subtracting the offset. In some experiments (see Figs. 2, 4, 5, 6), the data were transferred to the National Institutes of Health PDP 10 computer and processed with the mathematical modeling program (MLAB) (24).

RESULTS

Binding of ^{125}I-Labeled Dimeric IgG to $P338D_1$ Cells. Oligomers of rabbit IgG bind with measurable affinity to the Fc receptors of a variety of cells (7, 8, 25), in contrast to the monomeric protein, which binds weakly and elutes rapidly. When $P388D_1$ cells were incubated with radiolabeled IgG dimers, the average number of molecules bound per cell increased with time until equilibrium was reached (Fig. 1). When the cells were then washed, a small amount (≤10%) of the dimer eluted

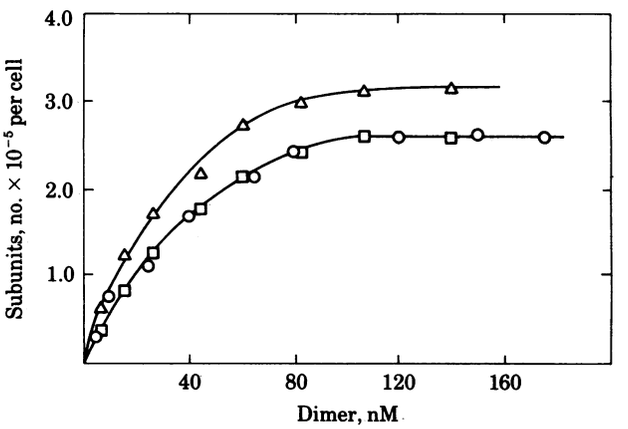

FIG. 2. Binding of dimeric rabbit IgG to $P388D_1$ cells as a function of free dimer concentration. Cells were equilibrated for 2 hr at 0°C. ^{125}I-labeled dimer subunits bound per cell without prior washing (△) and after washing (□). Cells equilibrated with unlabeled dimer, washed, and stained with FITC–FGaR (○). The mean fluorescence was determined by FMF, and the data were converted to subunits bound per cell using the standard curve (see Fig. 4).

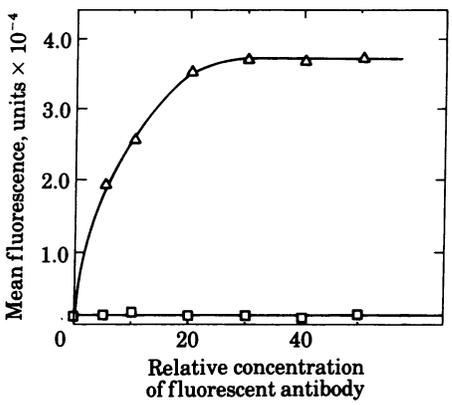

FIG. 3. Titration of bound rabbit IgG with fluorescent antibody. $P388D_1$ cells equilibrated with (△) and without (□) 0.15 μM dimeric IgG for 2 hr at 0°, washed, and then incubated with increasing concentrations of FITC–FGaR. Mean fluorescence was determined by FMF analysis.

rapidly from the cell surface, but the rest remained cell associated over a period of several hours, and repeated washing did not result in further loss of cell-bound dimer (data not shown). When the cells were equilibrated with increasing concentrations of radiolabeled dimer, and then either washed and sedimented through phthalate oils or were sedimented through the oils directly, binding was a hyperbolic function of dimer concentration and saturable (Fig. 2); however, the binding curve obtained from the washed cells was displaced downward to a small extent due to the component that elutes rapidly following washing. These data show that the amount of rapidly eluting dimer is small at all levels of saturation and that the binding curve obtained after a wash step is a reasonable approximation to the curve obtained without washing.

Fluorescence Labeling of Bound IgG Dimers. $P388D_1$ cells were equilibrated with 0.15 μM dimer or with medium alone, washed, incubated with increasing amounts of FITC–FGaR, and analyzed for fluorescence by FMF. The results showed that the mean fluorescence reached a plateau with increasing amounts of FITC–FGaR and that, in the absence of dimer, staining was negligible (Fig. 3). When saturating amounts of the staining reagent were added to cells bearing ^{125}I-labeled IgG dimers, there was no effect on the amount of radioactivity that remained cell associated. Moreover, mean fluorescence values were proportional to the number of dimer molecules bound (see Fig. 2).

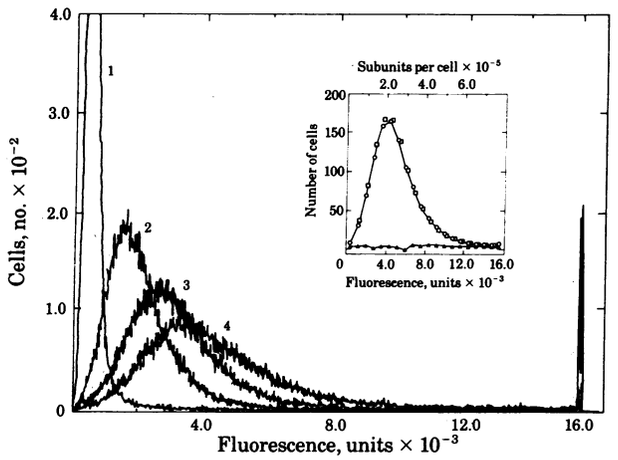

FIG. 4. FMF analysis of $P388D_1$ samples treated with various concentrations of IgG dimers. Profiles 1–4 are from cells equilibrated with 0, 10, 50, and 120 nM dimer, respectively. Cells were subsequently washed, labeled with fluorescent antibody, and analyzed with the FACS II. (*Inset*) FACS profiles from $P388D_1$ cells incubated with 220 nM (○) or 110 nM (□) dimer (both of which are sufficient to saturate the IgG Fc receptors; see Fig. 2). Data points represent averages of 50 channels over the 1024-channel range. △, Points were calculated by taking the difference between profiles.

Table 1. $P388D_1$ samples used to construct Fig. 5

Free dimer, nM*	Sorting range (corrected channels × 10^{-4})†	Mean fluorescence × 10^{-4}‡	Subunits, no. × 10^{-5} per cell§	Median Coulter volumes, μl^3
9	0.23–0.64	0.58	0.88	602
9	0.26–0.90	0.82	0.99	602
9	0.96–1.68	1.33	1.68	635
12	0.30–0.92	0.80	0.99	602
12	0.45–1.69	1.15	1.50	618
12	0.49–2.24	1.77	2.36	667
35	0.58–2.96	1.72	2.19	667
65	0.85–3.57	2.07	2.57	684
120	0.30–1.48	1.08	1.35	618
120	0.50–3.47	2.06	2.85	700
120	2.08–2.99	2.36	2.98	651

* Concentration of dimer with which cells were equilibrated.
† Lower and upper channel numbers (corrected to gain 16) of sorting range.
‡ Determined by reanalysis of the sorted sample. The mean fluorescence (0.12×10^4) of cells treated with FITC–FGaR but not dimer has been subtracted.
§ As determined from bound radioactivity and Coulter counts.

Standard Curve. To determine whether the number of IgG molecules bound was proportional to the fluorescence emission over the entire dynamic range of the FACS, we compared radioactivity with mean fluorescence units for cell populations containing different levels of bound ^{125}I-labeled dimer. Cells were equilibrated with increasing concentrations of radiolabeled dimer and stained with FITC–FGaR. Control experiments in which cells were incubated with either ^{125}I-labeled dimer or unlabeled dimer showed that the level of staining was not altered by the isotope.

Different samples of cells were sorted according to fluorescence emission by using the FACS II, and aliquots of each cell fraction were tested for (*i*) cell number and volume in a Coulter counter, (*ii*) bound radioactivity with a gamma counter, and (*iii*) mean fluorescence in the FACS. The data (Table 1) show that the mean fluorescence values and the average numbers of dimer molecules bound per cell varied according to the fluorescence intensity used in the sorting and that the cell volumes showed, at most, a slight positive correlation with bound dimer or mean fluorescence.

A standard curve constructed by plotting mean fluorescence values against the number of IgG subunits bound per cell (Fig. 5) shows a linear relationship between the two parameters; the best fit straight line, generated by linear regression analysis, passes through the origin, within experimental error. Dividing the channel number (corrected for background fluorescence) by

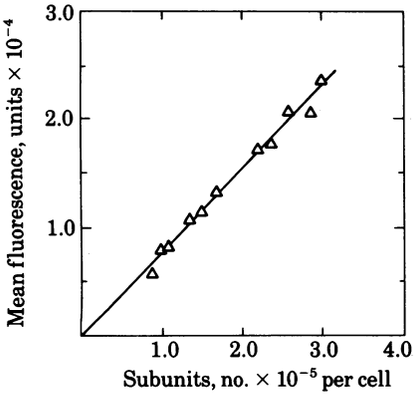

FIG. 5. Mean fluorescence as a function of IgG subunits bound per cell. Cells were equilibrated with different concentrations of radiolabeled dimer and stained with FITC–FGaR. Each cell preparation was then sorted in the FACS by increasing fluorescence emission, and the sorted samples were assayed for bound radioactivity and mean fluorescence (see Table 1).

the slope of the standard curve gives the number of IgG molecules bound per cell, for the cells in that channel. If the receptors are saturated with dimer (see Fig. 4, *Inset*), then the number of bound subunits equals the number of Fc receptors per cell, providing that each subunit is free to interact with a receptor. The slopes of the standard curves remained constant from day to day; for one preparation of FITC–FGaR, the SEM of the slopes from seven different curves was about 4%.

Fc Receptors on Heterogeneous Cell Populations. Two heterogeneous normal cell populations—human peripheral blood lymphocytes and mouse spleen cells—were incubated with saturating concentrations of IgG dimer, stained with FITC–FGaR, and analyzed by FMF (Figs. 6 and 7). In these figures, fluorescence units (abscissas) have been converted to Fc receptors per cell by using the standard curve (see Fig. 6, *Inset*). Incubations

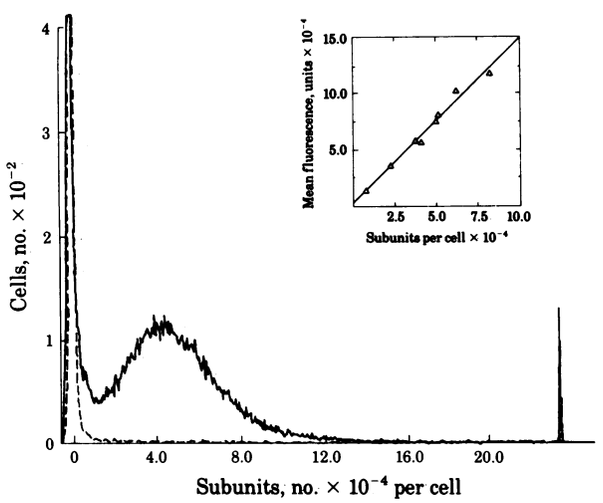

FIG. 6. Distribution of Fc (IgG) receptors on normal human peripheral blood lymphocytes. Fc receptors on lymphocytes from a single donor were saturated with rabbit dimer (0.15 μM) at 0°C, washed, and labeled with FITC–FGaR. Profiles represent fluorescence emission histograms of cells saturated with dimer and incubated with FITC–FGaR (——) or incubated with FITC–FGaR alone (-----). (*Inset*) Standard curve obtained from human peripheral blood lymphocytes by using procedures analogous to those in Table 1. Numbers of dimer molecules bound per cell were calculated from the fluorescent channel numbers and the slope of the standard curve. The mean fluorescent channel of the control (---) was equated with zero receptors per cell.

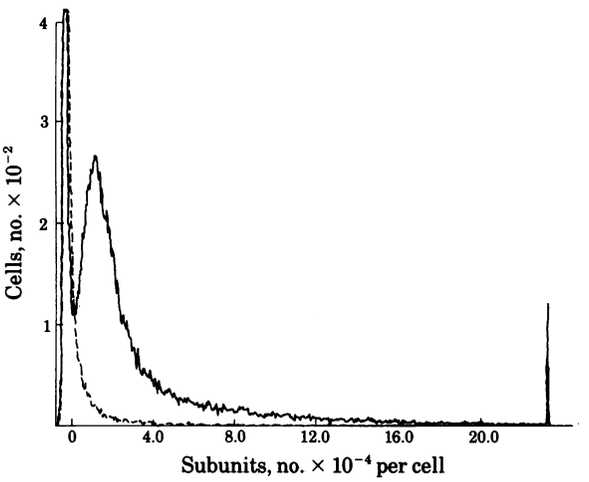

FIG. 7. Distribution of IgG Fc receptors on C57BL/6 mouse spleen cells treated as in Fig. 6. Profiles represent fluorescence histograms of cells saturated with dimer and incubated with FITC–FGaR (——) or incubated with FITC–FGaR alone.

in which radioactive dimer was used showed that fluorescence emissions of stained spleen cells fell on the same curve. The profiles show that cells from both sources have at least two distinct subpopulations: cells having fluorescence equivalent to background levels ("negative cells") and cells having fluorescence clearly above background levels ("positive cells").

Human peripheral blood lymphocytes from eight donors were examined. The positive cells from the eight donors varied from 5% to 25% of the total population; i.e., over a 5-fold range. However, both the widths and symmetries of the profiles of the positive cells of the various donors were remarkably similar. Thus, the average (±SEM) of the fluorescence values of the positive cells from the eight samples was 279 ± 8 fluorescent units, corresponding to (4.15 ± 0.12) × 10^4 receptors per cell. In fact, the shapes of the receptor density profiles from the positive cells of human peripheral blood lymphocytes do not differ greatly from those obtained from $P388D_1$ cells (Fig. 4), even though $P388D_1$ is a homogeneous cell line.

In contrast to human lymphocytes, spleen cells from different C57BL/6 mice varied only to a minor degree in percentages (57 + 5 from seven mice) of positive cells. However, the Fc receptor distribution of the positive cells from mouse spleen was much less symmetrical than that for human lymphocytes and showed greater heterogeneity in density (see Fig. 7). A peak in cell number usually occurred at 1 to 2 × 10^4 receptors per cell, but significant numbers of cells had receptor densities 10- to 20-fold greater than the peak value. Unlike human lymphocytes, the positive cells from different mouse spleens exhibited significant variations in receptor density profiles.

DISCUSSION

We have described a method for measuring the numbers of IgG molecules bound to Fc receptors on large numbers of individual cells. The method uses FMF in conjunction with techniques for quantitating Fc receptors (5, 7). It has been shown that IgG oligomers prepared by using the bivalent affinity-labeling reagent, $(BADL\text{-}Pro)_2EDA$, can saturate Fc receptors on a variety of cells at relatively low concentrations and that Fc, but not Fab fragments, can inhibit binding (7, 21). Crucial to the current study is the observation (see Fig. 1) that, once bound, the oligomers remain cell-associated for long periods after the free oligomer has been removed by washing. When maintained at 0°C, the oligomers presumably stay on the cell surface because they

can be labeled with fluorescent antibody. Treatment with fluorescent antibody has no effect on the amount of bound dimer (see Fig. 1) and, under conditions where cells are saturated with the staining reagent (see Fig. 3), fluorescence emission is linearly related to the number of IgG molecules bound per cell (see Fig. 5).

Dimers that have been crosslinked by using $(BADL\text{-}Pro)_2EDA$ elute from cell surfaces at much lower rates than dimers formed with other reagents, [e.g., bis-DNP-pimelic ester (6) or dimethylsuberimidate (21) (S. K. Dower, personal communication)]. The tendency of $(BADL\text{-}Pro)_2EDA$ dimers to remain on cell surfaces for long periods of time at 0°C following a wash step is necessary if the FMF technique is to give an accurate measure of receptor densities. The rapidly eluting component (Figs. 1 and 2) is fortunately small and so the errors in receptor density determinations are also small. Another potential source of error in estimating receptor densities is monomeric binding of the dimers to the Fc receptors, leading in the worst case to two-fold overestimates of numbers of receptors per cell. Because monomeric rabbit IgG rapidly elutes from cells and cannot be detected on cell surfaces after washing (7), it is likely that dimer binding is stabilized by bivalent interactions in which each IgG subunit binds to an Fc receptor. Thus, errors due to monovalent binding are likely to be small.

The percentages of positive and negative cells obtained in this study with mouse splenocytes and human peripheral blood lymphocytes are in good agreement with other results (1). From other studies, it seems likely that the negative cells in mouse spleen are largely T lymphocytes, and the positive cells consist of B lymphocytes, some macrophages, and perhaps a subpopulation of T cells (1, 11–14). The marked heterogeneity in receptor numbers of the positive cells (see Fig. 7) suggests that the various subpopulations of mouse spleen cells have receptor numbers differing by more than an order of magnitude. The positive cells from human peripheral blood lymphocytes most likely consist of B cells, null cells, and T cells (1). It is interesting that, despite the expected heterogeneity in cell types, the positive cells of human lymphocytes (see Fig. 6) have a symmetrical distribution in receptor number with a relative width no greater than that of the $P388D_1$ cell line (see Fig. 4). This suggests that, in human lymphocytes, the major subpopulations of positive cells have similar numbers of receptors.

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