Immunogold Staining: Adaptation of a Cell-Labeling System for Analysis of Human Leukocyte Subsets

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We have assessed the Immunogold™ Cell-Labeling System (IGS) for potential use in the clinical laboratory. In this technique, cell suspensions incubated with monoclonal mouse antibodies are reacted with anti-mouse antibodies labeled with colloidal gold. Surface marker cells, bearing dark blue-black granules, are easily distinguished by light microscopy. The percentages of T cells, T cell subsets, B cells, monocytes, or granulocytes identified by IGS corresponds with numbers obtained by flow cytometry analysis or immunofluorescence studies. Results by IGS and flow cytometry were similar for samples from patients with aberrant lymphocyte populations (e.g., leukemias) or from transplant recipients. IGS may thus be a useful diagnostic technique for studying neoplasias or other immunologically mediated disorders. This technique can also be used to characterize the surface phenotype of leukemic cell lines. The sensitivity and accuracy of IGS can be evaluated by measurements of different cell lines mixed in predetermined ratios.

Additional Keyphrases: colloidal gold • immunoassay • monoclonal antibodies • leukocyte differentiation • leukemia • transplant rejection

The availability and use of monoclonal antibodies specific for functional leukocyte subsets or cell types is increasing in the clinical laboratory. At the same time, clinicians are realizing that quantification of cell subsets can influence subsequent therapy or aid in monitoring immunologically mediated diseases. The value of such information, however, depends on the reliability of the cell-labeling system used. The conventional labeling systems, immunoenzymic (1–4) and immunofluorescent (5–7) methods, have several disadvantages. Enzymic techniques tend to be time consuming, and often compromise cell morphology. Fluorescent-labeled antibodies require the use of specialized equipment, either a fluorescent microscope or a flow cytometer, and studies in which immunofluorescent microscopy is used to identify leukemic cells show this technique to lack sensitivity—because antigen density on neoplastic cells, particularly in B cell chronic lymphocytic leukemias, is less than on normal cells (7, 8). Besides sensitivity, other factors that determine the reliability of a tagging system include accuracy, precision, reproducibility, simplicity, and flexibility.

Here we report our study of the utility of another cell-labeling system, immunogold staining (IGS), which has been used previously to identify lymphocytes (9–11).1 We have successfully adapted it for routine use in identification of monocytes, granulocytes, and leukemic cell-lines, as well as lymphocytes, in both normal individuals and patients. In addition, we have compared IGS with other systems: flow cytometry and immunofluorescence microscopy.

Materials and Methods

Cell Lines

Cell lines were cultured in RPMI 1640 growth medium supplemented with, per liter, 100 mL of heat-inactivated fetal bovine serum (Gibco, Grand Island, NY) and 1 mmol of glutamine, plus antibiotics, and maintained at 37 °C, in a CO2-enriched (50 mL/L) atmosphere. HL-60 cells, a promyelocytic cell line (12), and Jurkat cells, a human T-cell leukemia cell line (13), were obtained from Dr. John Lee (SmithKline and French, Swedeland, PA). Mixtures of cell lines were stained by IGS in several experiments to examine the linearity and accuracy of the technique.

Reagents

Monoclonal antibodies with specificity for T cells, T helper cells, T suppressor/cytotoxic cells, B cells, leukocytes, or granulocytes (see Table 1) were obtained from Ortho Diagnostics, Raritan, NJ: OKT11 (14); from Becton Dickinson, Mountain View, CA: Leu 4, Leu 2a, Leu 3a, Leu 6, Leu 11b, Leu 12, HLA-DR, HLe-1 (15–19); and from Coulter Immunology, Hialeah, FL: B1 (20). Monoclonal antibody specific for granulocytes, My-1 (21), was a generous gift from Dr. Curt I. Civin, Johns Hopkins School of Medicine, Baltimore, MD. For control reagents we used Leu 6 (Becton Dickinson), an IgG monoclonal antibody specific for thymocytes but unreactive with normal peripheral T or B cells; IgG-purified supernates or ascites fluids from NS-1 myeloma cells (CappeL Laboratories, Malvern, PA); and normal polyclonal mouse IgG (Pel-Freez, Rogers, AR). Goat anti-mouse antibody bound to colloidal gold particles of 40 nm diameter, GAM G40, was obtained from Geometric Data, Wayne, PA.

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<tr>
<th>Table 1. Specificity of Anti-Human Monoclonal Antibodies</th>
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1 Nonstandard abbreviations: IGS, immunogold staining; D-PBS, Dulbecco's phosphate-buffered saline; B-CLL, B cell chronic lymphocytic leukemia.

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Transport or stabilizing medium (RPMI 1640 supplemented with gentamycin, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid, 25 mmol/L, pH 7.2-7.6, and antibiotics) were a gift from Dr. Paul Wallace (Smith/Kline Clinical Labs, King of Prussia, PA). This and other stabilizing media such as McCoy's medium can maintain T and B cell numbers for as long as three days of storage (22).

**Procedures**

**Immunogold staining.** Peripheral blood was collected into EDTA-containing tubes (Vacutainer Tubes; Becton Dickinson, Rutherford, NJ). Although the technique can be applied to whole blood, to enrich for leukocytes and reduce cell counting time, we obtained buffy coat cells by centrifuging for 15 min at 1500 rpm, and prepared samples of leukocyte-enriched buffy coat cells.

We incubated 20 μL of the buffy coat suspension for 15–30 min at 4 °C with 5 μL of monoclonal antibody. After washing these cells twice in IGS wash buffer (Dulbecco's phosphate-buffered saline (D-PBS; Gibco) plus 10 mL of bovine serum albumin (Sigma Chemical Co., St. Louis, MO), 20 mL of heat-inactivated human AB serum, and 2 g of Na3PO4 per liter), we added 15 μL of GAM G40 and let the mixture react for 15–30 min at room temperature. After washing these once in IGS wash buffer, we added 5 μL of autologous human plasma to each cell pellet and incubated at 37 °C for 30–45 min to enhance "patching" of the goat anti-mouse-bound colloidal gold. After gentle mixing, we prepared blood smears (Miniprep®, Geometric Data), then fixed and counterstained them by either of two methods: (a) fixation in glutaraldehyde, 10 mL/L of absolute ethanol for 10 min at room temperature, washing in running tap water, then counterstaining for 20 min with methyl-green pyronin (0.5 g of methyl-green (Merck 1714) (E. Merck, Darmstadt, F.R.G.) and 0.2 g of pyronin G (Carlo Erba, Italy; no. 45005) in 100 mL of acetate buffer, 0.1 mol/L, pH 4.4), rinsing in tap water, then air drying; or (b) automatically counterstaining slides with Wright's Stain in a Hemastainer® (Geometric Data). We then examined the cell preparations by light microscopy. Positive cells are easily distinguished by the presence of blue-black gold aggregates on the cell surface. We considered cells to be labeled when we observed five or more surface granules.

**Immunofluorescent staining.** After obtaining buffy coat cells we partitioned the remaining peripheral blood into leukocytes, mononuclear cells, or granulocytes. Mononuclear cells were collected from the interface after centrifugation over ficoll–hypaque gradients (LSM; Alltech, Boothington, MD), and washed twice in D-PBS. Granulocytes, recovered from the gradient pellets after lysis of erythrocytes, were washed twice with D-PBS. To obtain whole leukocyte populations, we lysed 100-μL aliquots of whole blood by incubation with an aqueous buffer, pH 7.2–7.4, containing 37 mg of disodium EDTA, 1 g of KHCO3, and 8.3 g of NH4Cl per liter, then washed with D-PBS containing bovine serum albumin, 10 mL/L. More than 95% of the unlysed cells were granulocytes. Leukocyte pellets—mononuclear cells, or granulocytes, each 107 cells/mL—in 100 mL of D-PBS plus bovine serum albumin were incubated with 5 μL of monoclonal antibodies in 12 × 75 mm polystyrene tubes for 30 min at 4 °C. After washing these twice with D-PBS we reacted the cells for 30 min at 4 °C with fluorescein-labeled goat F(ab')2 anti-mouse IgG (Cappel Labs). We washed the cells, then resuspended them (1 × 106 to 2 × 106/mL in D-PBS plus 10 mL of bovine serum albumin and 19 g of paraformaldehyde per liter) for analysis by flow cytometry (Coulter Epics V) by identifying the different regions where lymphocyte or monocyte populations are found, based on forward and right-angle light scatter. Alternatively, we prepared the washed cells for immunofluorescence by fixing them in paraformaldehyde (7 g/L) for 5–7 min, washing them, resuspending the cell pellets in buffered glycerol, then mounting them onto slides, and examining them with an Orthoplan microscope (Leitz, Rockleigh, NJ).

**Results**

**Comparison Studies**

**IGS vs flow cytometry for analysis of lymphocyte subsets in normal and patient populations.** We determined by IGS and flow cytometry the percentages of T cells (OKT11+), T helper cells (Leu 3a+), T suppressor cells (Leu 2a+), and B cells (B1) or total lymphocytes (HLe-1+) in cell suspensions obtained from normal healthy individuals. Figure 1 illustrates the correlation between the two techniques for markers on T or B cells, and for the shared leukocyte surface antigen recognized by HLe-1. The difference of the means of surface-marker-positive cells analyzed by a t-test were as

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Fig. 1. IGS vs flow cytometry for 12 normal donors (left) and for 12 patients (right)

Results indicate the percentage of positive lymphocytes stained for the surface markers indicated. Each data point was for cells from the same donor source and was based on manual enumeration of 200 lymphocytes by IGS and 10,000 lymphocytes by flow cytometry.
follows: OKT11, \( p < 0.01 \); Leu 2a, \( p < 0.05 \); Leu 3a, \( p < 0.01 \);
B1, \( p < 0.01 \); HLE-1 \( p < 0.2 \). Thus significantly \( p < 0.05 \)
more cells are identified by the IGS technique than by flow
cytometry. With HLE-1 as the monoclonal antibody, the IGS
technique labels almost 100% of the lymphocytes and more
than 95% of the monocytes, results similar to those by flow
cytometry.

Results for samples from patients with B cell chronic
lymphocytic leukemia (B-CLL), acute myelomonocytic leuk-
emia, myelomonocytic leukemia, or infectious mononucleo-
sis and kidney transplant recipients were analyzed by both
methods (Figure 1). Despite the highly increased percentage
of B cells in the B-CLL patients or the diminished T helper/T
suppressor ratio in the transplant recipients, the correlation
between the two techniques was highly significant \( r = 0.98,\
p < 0.001 \). In some instances, more cells were labeled by
the IGS technique than by flow cytometry, once again suggest-
ing that the IGS technique may be more nearly accurate. To
support these results, we are now acquiring more patients' samples to permit thorough statistical analysis by various
tests.

**IGS as immunofluorescence microscopy for analysis of
granulocytes.** To compare the IGS technique with immuno-
fluorescence for analysis of granulocyte suspensions, we used
three different monoclonal antibody probes: My-1 (reactive
with myeloid cells), Leu 11b (specific for Fc receptors
for IgG on granulocytes), and HLE-1. As Table 2 clearly
indicates, IGS can be applied to studying myeloid markers
on granulocytes.

**IGS and Leukemic Cell Lines**

In examining surface markers on a T cell line (Jurkat)
and a promyelocytic line (HL-60) by IGS, we found that 95% of
the Jurkat cells were OKT11 positive, about 70% were
Leu 3a positive, and less than 10% were Leu 2a positive.
More than 95% of the HL-60 cells were My-1 positive.

Taking advantage of the homogeneity and purity of the
cell lines, we examined the accuracy of IGS labeling by
mixing known quantities of the two cell lines together,
maintaining a constant total number of cells. Three such
sets of measurements are shown in Figure 2; in these and in
all other measurements thus far, the percentage of OKT11-
positive cells observed has been very nearly that expected.
Results were similar when we analyzed OKT11 positive or
My-1 positive cells simultaneously in another mixing
experiment (Figure 3). Thus IGS can be used to study surface
markers on either lymphoid or myeloid leukemic cells.

**Specificity of IGS Labeling**

Because lymphocytes and monocytes have high numbers
of Fc receptors, we determined the extent, if any, of nonspe-
cific binding or Fc receptor binding of monoclonal antibodi-
es. The binding of irrelevant mouse IgG polyclonal antibody

| Table 2. Comparison of IGS with
| immunofluorescence for analysis of
| granulocytes |
|-----------------|------------------|
| Monoclonal antibody | IGS | Immunofluorescence |
| My-1 | 94.3 (93–96) | 95.4 (94–97) |
| Leu 11b | 98.7 (97–100) | 98.3 (97–100) |
| HLE-1 | 96.3 (92–99) | 95.0 (92–99) |
| Control | 3.0 (0–6) | 1.1 (0–2) |

*Mean percentage (and range) of positive granulocytes from three separate
donors, with 200 cells counted for each determination.

Fig. 2. IGS and leukemic cell lines
Jurkat cells (T cell leukemia cells) and HL-60 cells (promyelocytic cells) were
mixed in various proportions with a constant cell count of 5 × 10^6 cells per
determination, then stained for OKT11 by IGS. Results, expressed as the
percentage of marker-positive cells in 200 cells visually examined for each
determination, represent data from three separate sets of measurements.

Fig. 3. IGS and leukemic cell lines stained for either OKT11 or My-1
surface antigens
Results are expressed as the percentage of marker-positive cells of 200 cells
examined for each data point.

or IgG monoclonal antibodies of different sources was less
than 3–4% (Table 3). The presence of these mouse IgG
antibodies had little or no effect on binding of Mab to all
lymphocytes (HLE-1), to B cells (B1), or T cells (Leu 4).
Therefore, with our experimental protocol, Fc binding does
not contribute to or diminish identification of T or B cells.

**Stabilizing Medium and IGS Labeling**

A cell-labeling technique like IGS would have great
practical value if a sample could be analyzed hours or days
after collection and labeling. To determine whether storage
of the samples affected the reliability of the IGS procedure,
we stored leukocytes at 4 °C, room temperature, or 37 °C. In
the absence of any supplements, the viability and morpholo-
gy of the cells deteriorated at 4 °C and room temperature
by 24 h, and by 48 h in samples stored at 37 °C. This was
accompanied by a simultaneous decline in the number of
labeled cells observed. However, when we diluted the samples with an equal volume of stabilizing medium, there was little change in the percentages of T cells, T cell subsets, or B cells at room temperature up to 72 h after collection (Figure 3). Morphologic detail became obscured, however, at room temperature after 48 h, although cell viability was still 90% or greater after 72 h. Conversely, storage at 4 °C resulted in unacceptable cell morphology after 24 h—the percentages of positive cells, especially T cells, having declined rapidly by this time—and viability was less than 90% by 48 h. Samples stored at 37 °C had acceptable cell morphology, and maintained T and B cell percentages, but viability declined to 73% by 24 h and dropped to 68% after 72 h. Thus enumeration by IGS and identification of samples held in stabilizing medium at room temperature is still possible for at least 24 h after collection.

Discussion

The IGS technique is an attractive cell-identification system, requiring no cell separation: leukocytes can be labeled in whole blood as well as in buffy coat preparations, Ficoll-gradient-purified mononuclear cell preparations (9), or enriched populations of granulocytes or cell lines. Moreover, small sample volumes will suffice, and the technique is applicable for at least 24 h to blood samples mixed with stabilizing medium. Finally the IGS technique provides a permanent sample, which facilitates short- or long-term studies of patients, e.g., with leukemia, in whom effects of chemotherapy or remission status are monitored over time.

Although these aspects describe the convenience of the IGS technique, the true value of the procedure is the quality of information obtained. Simultaneous correlation of surface phenotype with morphologic detail can be useful in discrimination of cell subsets within populations with similar physical features (e.g., lymphocytes). Once a difficult task, classification of hematologic neoplasias by morphology alone has become easier through use of cell-type-specific monoclonal antibodies. In particular, chronic lymphocytic leukemias can now be classified as being of B cell or T cell origin by the use of T cell- (OKT11, Leu 4) or B cell-specific (B1, Leu 12) monoclonal antibody probes. A diminished epitope density of the cell surface marker that reacts with B1 monoclonal antibodies on leukemic cells from B-CLL patients has been described in other studies (2,9). However, for all the cases of B-CLL we studied, there was no difficulty in discriminating between positive and negative cells. Our optimization of the IGS method, which facilitated patching and visual identification of positive cells, yielded accurate results even in the most extreme cases, such as in B-CLL, where immunofluorescent techniques may underestimate the number of labeled cells. In fact, the statistical data presented in this paper also suggest that the IGS technique may be more nearly accurate than flow cytometry. However, larger sample sizes are necessary to support these results. These findings, combined with the comparison with results by flow cytometry in normal as well as leukemic patients, further demonstrate that IGS can be sensitive and accurate enough for diagnosis of bloodborne neoplasias.

The ability to determine surface phenotypes of cells and accurately label cell lines by IGS also suggests that the method can be used to screen hybridoma supernates from hybridoma preparations. For example, one could define cellular binding specificity for clones producing monoclonal antibodies that were reactive only with leukemic cells, but not with normal peripheral lymphocytes. At present, the arduous screening of antibodies from hybridomas is usually done by indirect immunofluorescence or enzymatic techniques.

In conclusion: The IGS technique could be easily adapted to use in a clinical laboratory routinely performing cellular analyses of normal or abnormal blood smears. In a more extended application, IGS could be used for studying surface markers or cell receptors in a research environment. Finally, IGS is ideally suited for automated quantification of absolute and relative numbers of leukocyte subsets. To this end, the technique has already been adapted for use in an automated cell-counting instrument, Hematrak®.

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References


