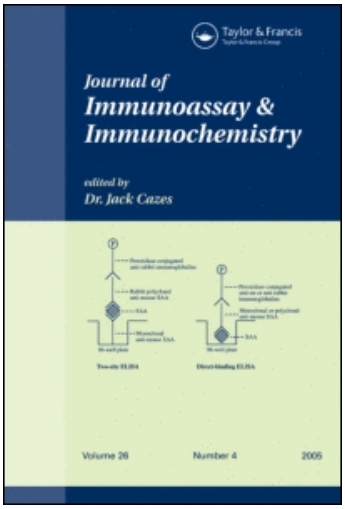

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CHARACTERISTICS OF A SPECIFIC RADIOIMMUNOASSAY FOR MEASUREMENT OF FERRITIN ON THE SURFACE OF PERIPHERAL MONONUCLEAR WHITE BLOOD CELLS IN CANCER PATIENTS

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ABSTRACT

Using ^{125}I-labeled rabbit anti-Hodgkin's spleen ferritin antibody (RHF), a simple radioimmunoassay has been developed for quantitation of ferritin on the surface of peripheral blood mononuclear white blood cells (PBM). This method makes use of a % specific binding determination (%SP) by measuring the amount of ^{125}I-labeled RHF bound to 1 x 10^6 PBM in the presence and absence of soluble ferritin. To standardize this procedure, artificial ferritin positive control cells were prepared by covalently coupling ferritin to cultured acute lymphoblastic luekemia cells. These cells were tested on a daily basis in parallel with patient PBM's to ensure inter and intra-assay precision and remained stable for over two years. Characteristics of ^{125}I-labeled RHF binding to control and patient PBM's were evaluated to determine the specificity of interaction and optimum binding parameters. %SP was linear in the range of 1 x 10^5 - 1 x 10^6 PBM's and was progressively inhibited by graded concentrations of soluble ferritin. $F(ab')_2$ preparations of RHF were equally as effective as intact RHF in blocking ^{125}I-labeled RHF binding confirming that ^{125}I-labeled RHF was not binding non-specifically to PBM Fc receptors. Additional experiments describing kinetics and methods of standardization of new lots of ^{125}I-labeled RHF are also described.

"KEY WORDS": Ferritin, Peripheral mononuclear white blood cells, Cancer, Radioimmunassay.

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INTRODUCTION

In a previous report, we described the results of the relationship of ferritin bound to the surface of peripheral blood mononuclear white cells (PBM) in malignancy when analyzed by a sensitive radioimmunoassay (RIA) procedure utilizing affinity purified ^{125}I-labeled rabbit anti-Hodgkin's spleen ferritin antibody (RHF) (1). That study found a positive correlation between percent specific binding of ^{125}I-labeled RHF (%SP) to PBM and clinically active metastatic disease. Previous work by others using antibody dependent complement mediated cytotoxicity have also demonstrated increased ferritin PBM's in cancer (2,3). This report presents results of studies designed to characterize the binding of ^{125}I-labeled RHF to human PBM's and further describes performance and precision characteristics of this assay.

MATERIALS AND METHODS

Ferritin positive and negative control cells

Ferritin positive and negative control cells were prepared as a stable reference system which would mimic binding to patient PBM's and control for both inter and intra-assay reagent stability and precision. These cells were also used to determine assay parameters prior to patient studies. Human acute lymphoblastic leukemia cells (CCL-119, American Type Culture Collection, Rockville, MD) were suspension grown in RPMI-1640 tissue culture medium (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum, 2mM glutamine and 100 ug/ml gentamycin. Maximum cell density achieved was 3×10^6 cells/ml.

Purified normal human spleen ferritin (NSF) (JBL Chemical Company, San Luis Obispo, CA) as a positive control (CCL-119F) and bovine serum albumin (BSA) as a negative control (CCL-119BSA) were covalently coupled to CCL-119 cells by a modification of the procedure of Rockoff et al (4). Briefly, to 2 to 3 x 10^7 cells/ml previously washed in Dulbeceo's modified phosphate buffered saline (DPBS) by centrifugation at 250 x g for 10 min at 4°, was added sufficient glutaraldehyde (25% w/w solution, Polaron Equipment Ltd., Watford, England) to give a final concentration of 1%. After gentle rocking for 30 min at 22°, cells were washed twice in DPBS and a sufficient volume of NSF or BSA added to give a final cell density of 3 x 10^7 cells/ml at a protein concentration of 50 to 80 ug/ml. Following protein coupling overnight at 4°, cells were washed twice in DPBS, aggregates removed by low speed centrifugation and a uniform suspension stored at 1 x 10^7 cells/ml in DPBS, 1% BSA, 0.2% sodium azide. Approximately 50 percent of the starting cell population was recovered by this technique. Electron microscopy confirmed that these cells were uniform and retained lymphoblastoid morphology. These standards were stable for greater than two years without any loss in surface ferritin as assessed by %SP of ^{125}I-labeled RHF.

^{125}I-labeled anti-Hodgkin's spleen ferritin antibody

Affinity purified RHF prepared as described previously (1), was labeled with Na ^{125}I, (Amersham, Chicago, IL) by the chloramine-T procedure of Greenwood et al (5). Organic fractions were separated from inorganic ^{125}I by elution from an ion exchange

column (Sephadex A-25, Pharmacia). The final specific activity of the labeled antibody was 1-2 uCi/ug.

$F(ab')_2$ fragments of RHF

In order to investigate the possible role of the Fc portion of ^{125}I-labeled RHF in binding to PBM's, $F(ab')_2$ fragments of RHF were prepared by pepsin digest of intact RHF. Following digestion for 8 hr at 37° of a 33% saturated ammonium sulfate fraction of RHF, $F(ab')_2$ fragments were isolated by column chromatography on Sephadex G-150 (Pharmacia). Following reduction in beta mercaptoethanol, $F(ab')_2$ fragments were analyzed by dissociating gel electrophoresis in the presence of sodium dodecyl sulphate. Proteins were transferred to nitrocellulose paper and incubated with ^{125}I-labeled goat anti-rabbit IgG (heavy and light chain specific) according to the procedure of Burnette (6). Following autoradiographic development, comparison with intact RHF and intact rabbit anti-mouse IgG showed complete absence of immunoglobulin heavy chains in the $F(ab')_2$ fractions confirming the absence of intact antibody in this preparation. Addition of various concentrations of the $F(ab')_2$ fractions to an IRMA assay for NSF, in which the solid phase antibody was preincubated with 1,000 ng/ml NSF, caused a dose-response inhibition of subsequent ^{125}I-labeled RHF binding proving that RHF $F(ab')_2$ fragments maintained their ability to specifically bind ferritin. RHF $F(ab')_2$ fragments were further purified by affinity chromatography as described above for subsequent use in studies to measure their ability to compete with ^{125}I-labeled RHF for ferritin on PBM's.

Patient sample preparation

PBM's were separated from fresh heparinized blood on Ficoll-Hypaque (Pharmacia) according to the procedure of Boyum (7). In some cases, red blood cells contaminated the PBM preparation. These were removed by lysis in ammonium chloride (0.83% ammonium chloride, 0.1 mM disodium EDTA, 1 mM potassium carbonate) for 5 min at 4° followed by the addition of DPBS and washing. Analysis of paired patient samples either exposed or not exposed to red blood cell lysing buffer showed no difference in %SP to patient PBM's.

RIA for cell bound ferritin

To each of two duplicate sets of 12 x 75 mm polystyrene tubes were added 100 ul of patient cells in balanced salt solution (1×10^6 cells/tube). To the first set of duplicates was added 100 ul of DPBS containing 0.1% BSA and 0.02% sodium azide (Bo buffer). To the second set of duplicate tubes was added 100 ul Bo buffer containing 3,000 ng of NSF to assess the extent of non-specific binding (NSB) of ^{125}I-labeled RHF to patient PBM's either by adsorption or interaction of antibody-antigen complexes with cellular Fc receptors. This was followed by addition of 100 ul of ^{125}I-labeled RHF (30-80 ng) in Bo buffer to all tubes. CCL-119F cells were set up in an identical fashion as patient cells except that 1×10^5 cells/tube were used. Tubes were incubated for 30 min at 22° followed by overnight incubation for a minimum of twelve hr at 4° with constant shaking to keep cells in suspension. This incubation schema resulted in equilibrium binding of ^{125}I-

labeled RHF to CCL-119F controls and patient cells. Following incubation, cells were washed once in 3 ml DPBS followed by centrifugation at 1,500 x g for 30 min. Supernatants were aspirated and radioactivity bound to cell pellets was measured in a gamma spectrometer.

To determine the effects of this incubation on cell viability, in some cases, an additional tube containing Bo buffer in place of ^{125}I-labeled RHF was set up and incubated identically to the other tubes. Following incubation, patient cells were assessed for viability by trypan blue dye exclusion.

Specificity studies

In some experiments, additional duplicate sets of tubes were run with various concentrations of either NSF, unlabeled affinity purified RHF (intact), or affinity purified $F(ab')_2$ fragments of RHF antibody. Additions were made in 100 ul Bo buffer to 1 x 10^6 PBM's followed by 100 ul of ^{125}I-labeled RHF. These studies were designed to investigate the specificity of ^{125}I-labeled RHF for cell surface ferritin and to study the possible role of Fc receptors on PBM's in the overall binding interaction. Fc receptors form a major component on the surface of lymphocytes, bind immunoglobins via their Fc region, and play a role in immune response (8). Additionally, ^{125}I-labeled RHF was preincubated with a 10-fold mol excess of either affinity purified goat $F(ab')_2$ antibody fragments directed against rabbit IgG $F(ab')_2$ region (GARFAB), or affinity purified goat $F(ab')_2$ antibody fragments directed against human fibriogen (GAHF) as control (Cappel Labs, West

Chester, PA). These $F(ab')_2$ goat antibody fragments were used as probes to assess their ability to interfere in the subsequent binding of ^{125}I-labeled RHF to PBM's and to evaluate non-specific immune complex binding to Fc receptors on PBM's. Following incubation of these antibodies for 30 min at 22° with ^{125}I-labeled RHF, the various preparations were utilized in the standard RIA for cell bound ferritin in place of ^{125}I-labeled RHF alone.

Data reduction and analysis

In order to normalize data, results were computed as %SP where:

$$\%SP = \frac{\text{mean CPM Bo - mean CPM NSB}}{\text{total CPM - mean CPM NSB}} \times 100$$

where Bo = CPM bound in the absence of excess soluble NSF; and NSB = CPM bound in the presence of 3,000 ng soluble NSF.

In order for the results of a daily assay to be acceptable, %SP to CCL-119F cells had to fall within 2 S.D. of the mean control cell binding. To ascertain that control cell binding related to actual patient cell binding, new ^{125}I-labeled RHF lots were first titrated with CCL-119F cells, adjusted to a dilution that gave equivalent binding to the old antibody lot and then tested in parallel with paired patient samples to ensure that lot to lot variability of labeled antibody was eliminated.

RESULTS

Patient %SP versus cell number

Since the parameter measured in clinical studies was %SP, it was essential to ensure that %SP be in a linear range of binding

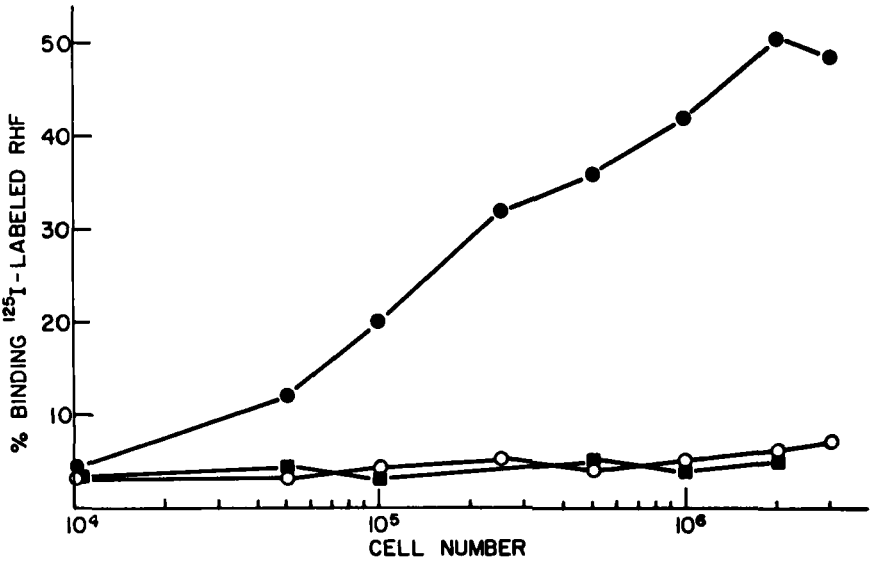

FIGURE 1. Linearity of ^{125}I-labeled RHF binding to control cells as a function of cell number. Various amounts of ferritin positive (CCL-119F) or negative (CCL-119BSA) control cells were incubated overnight at 4° with ^{125}I-labeled RHF. %SP to CCL-119F cells (●) was linear to 2 x 10^6 cells while % NSB (CPM NSB/Total CPM x 100) to CCL-119F cells in the presence of 3,000 ng NSF (○) was identical at each cell number tested. %SP to CCL-119BSA (■) was identical to % NSB of CCL-119F.

with both control and patient cells. Linearity of binding was necessary in order to accurately compare binding from patient to patient. If cell number levels were at saturation with regard to %SP for some patients and not for others, falsely low binding values would result. As shown in Figure 1, binding of ^{125}I-labeled RHF was specific for cells covalently coupled with NSF, while those having BSA coupled to their surface showed no %SP and had bound levels of ^{125}I-labeled RHF equivalent to CCL-119F cells

incubated in the presence of a large excess of soluble NSF. Binding of ^{125}I-labeled RHF to control cells was linear to 2 x 10^6 cells. As shown in Table 1, patient cells showed linearity of binding from 1 x 10^5 - 1 x 10^6 cells. In order to maximize the binding signal, patient cells were routinely tested at 1 x 10^6 cells/tube.

Kinetics of binding and cell viability

In developing incubation conditions for this assay, it was necessary to achieve equilibrium in order to standardize results. Figure 2 shows that when using CCL-119F control cells, equilibrium could be achieved in 2 hr at 22°. However, since viable patient cells exhibit capping phenomena followed by endo or exocytosis at elevated temperatures (9-11), incubations were conducted at 4° for a minimum of 12 hr, following a 30 min preincubation, a condition which also resulted in equilibrium binding. Extended incubation studies at 4° for selected patient samples showed no differences in % SP following 12 hr and 44 hr incubations (e.g., patient 1009, 3.3 and 3.9% S.P., respectively).

Cell viabilities via trypan blue dye exclusion were measured on freshly isolated PBM's and on aliquots taken through the standard overnight incubation procedure in order to determine the effect of incubation on cell destruction, release of intracellular ferritin, and its possible adsorption to viable cell surfaces. The mean viability of freshly isolated samples was 98% ±1.7% (S.D.) while after overnight incubation, viabilities were still quite high with a mean viability of 91% ±4.2% (S.D.) (n=185).

TABLE 1
%SP of ^{125}I-labeled RHF as a Function of Patient Cell Number

Isolated patient PBM's were adjusted to 1 x 10^6/ml. The standard binding assay as described in "Materials and Methods" was set up on each patient using 100, 50, 25 or 10 ul of cells. An appropriate volume of balanced salt solution was added to those tubes containing less than 100 ul of cells to adjust the final cell volume to 100 ul. The final assay volume after all reagent addition was 300 ul.

PATIENT CODE	%SP 1 x 10^6 CELLS	%SP 5 x 10^5 CELLS	%SP 2.5 x 10^5 CELLS	%SP 1 x 10^5 CELLS
15878	9.6	5.1	2.5	1.6
10625	9.3	4.5	2.2	1.5
10532	11.0	6.2	3.0	1.7
24430	7.5	4.1	2.0	1.0
26450	7.6	4.2	2.1	1.2
21244	14.7	10.2	5.9	2.7
24868	13.9	8.9	5.0	2.6
22589	5.0	2.4	1.5	0.6
20579	6.4	3.0	1.0	0.6
10285	4.5	2.7	1.9	1.3
14628	7.3	2.8	1.7	0.8
13425	5.5	2.9	1.3	0.7
20322	6.2	4.0	2.5	1.1
20778	6.2	2.7	1.3	0.6
24883	6.6	2.9	1.4	0.3
28558	5.5	2.4	1.5	1.0

Mean %SP 5 x 10^5: 1 x 10^6 cells = 0.53 ±.02 (S.E.)
Mean %SP 2.5 x 10^5: 5 x 10^5 cells = 0.53 ±.02 (S.E.)
Mean %SP 2.5 x 10^5: 5 x 10^6 cells = 0.28 ±0.02 (S.E.)

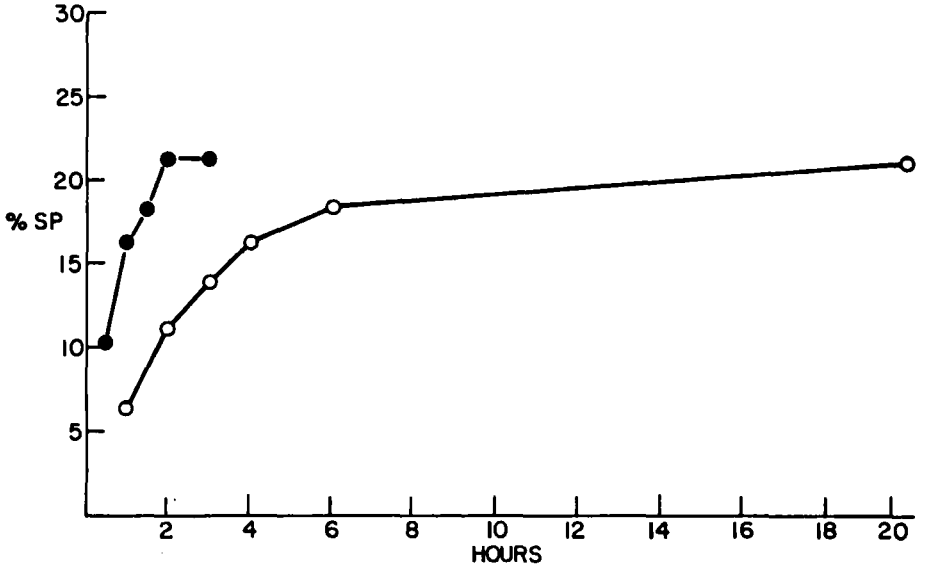

FIGURE 2. Kinetics of %SP of ^{125}I-labeled RHF to CCL-119F cells. 1 x 10^5 CCL-119F cells were incubated in the presence and absence of 3,000 ng NSF at 22° (●) and 4° (○). For 4° incubations, reactions were set up in an ice bath.

There was no correlation between decreased cell viability after overnight incubation and enhanced %SP of RHF suggesting that even if intracellular ferritin was released on cell death, it was not binding to the surface of intact cells and was removed in the final wash procedure.

Specificity of ^{125}I-labeled RHF binding to isolated PBM

In order to assess the specificity of binding of ^{125}I-labeled RHF to isolated PBM's, graded concentrations of purified NSF were used to competitively inhibit binding to both control and patient cells. As shown in Figure 3, incubation of CCL-119F with soluble

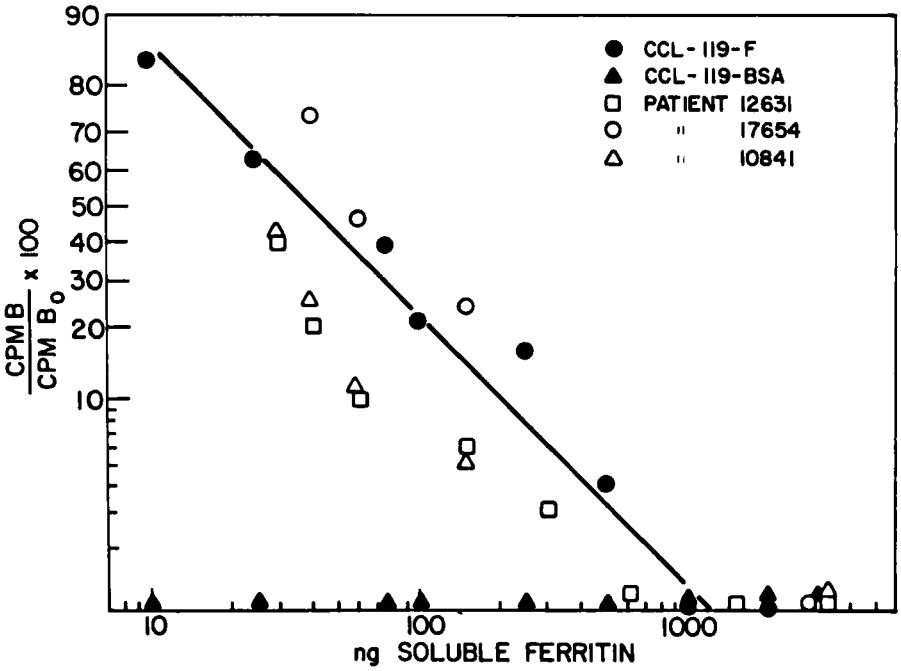

FIGURE 3. Positive and negative CCL-119 control cells (1×10^5) or isolated patient PBM's (1×10^6) were incubated in the presence of the indicated concentrations of soluble NSF. Results on the Y ordinate were calculated as the ratio of CPM bound at a given concentration of soluble NSF (B) divided by CPM bound in the absence of NSF (Bo) x 100.

ferritin could totally inhibit binding of ^{125}I-labeled RHF to cells with 50% inhibition of %SP at 40 ng NSF. In contrast, incubation of CCL-119BSA controls resulted in no change in binding beyond non-specific levels regardless of the concentration of soluble ferritin added. For CCL-119F cells, there was no change in binding inhibition from 3,000 ng down to 1,000 ng soluble NSF suggesting that the observed low binding levels at these concentrations was due to non-specific interactions and not to specific

antigen-antibody binding. When 1 x 10^6 patient cells were incubated with various concentrations of NSF, dose response curves similar to CCL-119F controls resulted. The dose response nature of competition by NSF for ^{125}I-labeled RHF binding to patient PBM's was ferritin specific.

The specificity of ^{125}I-labeled RHF for ferritin and not for Fc receptors on PBM's was demonstrated by the ability of unlabeled $F(ab')_2$ RHF fragments (Figure 4, right panel) to compete equally well for cell surface ferritin as did intact ^{125}I-labeled RHF. Increasing concentrations of $F(ab')_2$ competed as effectively as unlabeled intact RHF (Chart 4, left panel) versus ^{125}I-labeled RHF for ferritin on PBM's with 50% inhibition seen at approximately equimolar ratios for both unlabeled species.

Incubation of preformed immune complexes composed of ^{125}I-labeled intact RHF and GARFAB, when compared to uncomplexed ^{125}I-labeled RHF, demonstrated from 85% to 97% reduction in %SP to patient PBM's (Table 2) indicating that binding of ^{125}I-labeled RHF to PBM's was not a non-specific interaction due to Fc receptor binding of an immune complex. Preincubation with GAHF resulted in no inhibition in %SP suggesting that inhibition by GARFAB was not simply one of non-specific inhibition by goat antibody.

Inter and intra-assay precision and assay standardization

As the number of PBM's recovered from 20 ml of patient blood was highly variable with a range of from 4.0 x 10^6 to 4.0 x 10^7 cells and was consistently low from patients on chemo or radiation therapy, it was necessary to develop a testing procedure that

TABLE 2
Inhibition of %SP by $F(ab')_2$ Goat Antibodies
Directed Against the $F(ab')_2$ Region of ^{125}I-labeled RHF

Aliquots of ^{125}I-labeled RHF were preincubated for 30 min at 22° in the presence of a 10-fold mol excess of either GARFAB or GAHF as a control for goat $F(ab')_2$ fragments. Following preincubation, patient PBM's were tested in the standard RIA as described in "Materials and Methods" and %SP determined using ^{125}I-labeled RHF alone or the preincubated mixtures. Results for each %SP determination were the mean of duplicate determinations preformed in the presence and absence of 3,000 ng soluble NSF where the individual determinations differed by less than 5%.

PATIENT CODE	%SP NO ADDITIONS	%SP ^{125}I-LABELED RHF + GARFAB	%SP ^{125}I-LABELED RHF + GAHF
40129	4.6	0.4	4.1
40130	2.7	0.7	2.6
13745	7.9	1.1	7.0
211587	5.7	0.6	5.8
264456	8.9	1.1	8.0
294608	7.4	1.0	6.9
218851	4.7	0.7	4.4
40128	5.4	0.6	5.4
21749	13.5	0.8	12.4
213048	11.1	0.6	10.0
264342	7.2	1.1	6.8

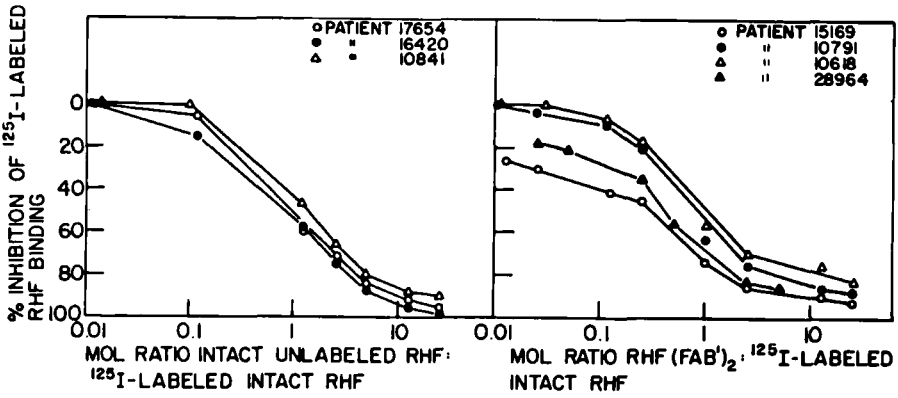

FIGURE 4. 1 x 10^6 PBM's in 100 ul balanced salt solution were incubated with either 100 ul Bo buffer, 100 ul containing 3,000 ng soluble NSF in Bo buffer, 100 ul of various amounts of affinity purified $F(ab')_2$ fragments (right panel), or unlabeled affinity purified intact RHF in Bo buffer (left panel) and ^{125}I-labeled intact RHF. % inhibition of ^{125}I-labeled RHF was computed as:

$$\frac{\text{mean CPM Bo - mean CPM unlabeled } F(ab')_2 \text{ or intact RHF}}{\text{mean CPM Bo - mean CPM NSB}} \times 100$$

utilized the minimum number of cells while at the same time gave a valid measure of cell bound ferritin. Because of this variability in cell number, it was not possible to routinely run multiple point dose response curves on every patient to develop a fully quantitative result as may be obtained by Scatchard analysis (12). Therefore, in order to control for ^{125}I-labeled RHF stability and interassay variability, CCL-119F was tested on a daily basis. As shown in Figure 5, interassay %SP was quite stable with a S.D. of only 1.7 %SP. All assays in which the %SP results for CCL-119F were beyond ±2 S.D. were discarded from the clinical study. Extended stability studies on CCL-119F showed no loss of binding after two years when stored at 4° (data not shown).

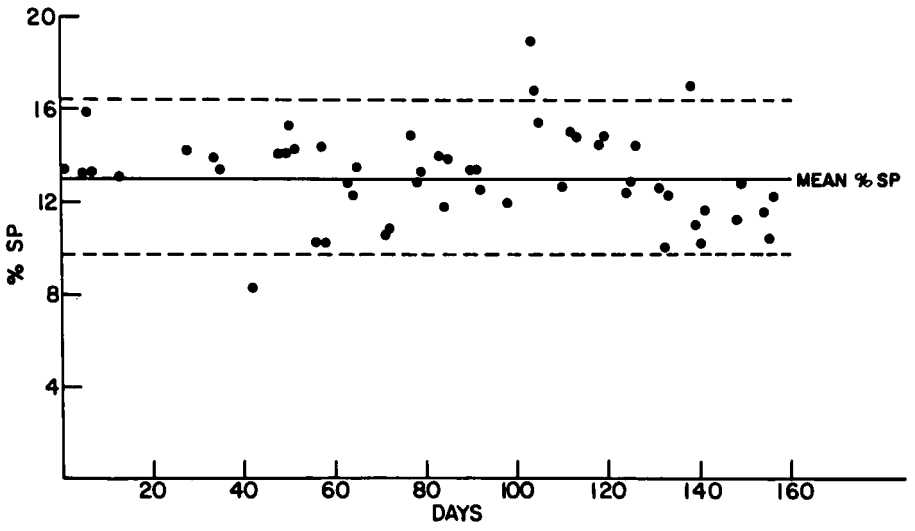

FIGURE 5. Interassay precision quality control chart utilizing CCL-119F positive standards. 1 x 10^5 CCL-119F cells were assayed on a daily basis in conjunction with patient samples to ensure proper functioning of all assay reagents. Test results from days 42, 103, 104 and 138 were discarded from clinical studies as they were outside the 2 S.D. (-----) range. The CCL-119F preparation used in this study is a different batch than that in Figures 1 and 2.

An additional criteria that must be met in order to use %SP as an absolute parameter and allow for interpatient and interassay comparison was adjustment of all reagents such that %SP was standardized to the mass of ^{125}I-labeled RHF and directly proportional to the mass of ferritin per unit cell number. As previously shown in Table 1, %SP was linear with cell number for all patients tested up to 1 x 10^6 cells. To ensure that the mass of ^{125}I-labeled RHF from each antibody iodination was consistently standardized, each new batch of antibody was adjusted prior to use in the clinical assay by a two step procedure. Each batch of labeled

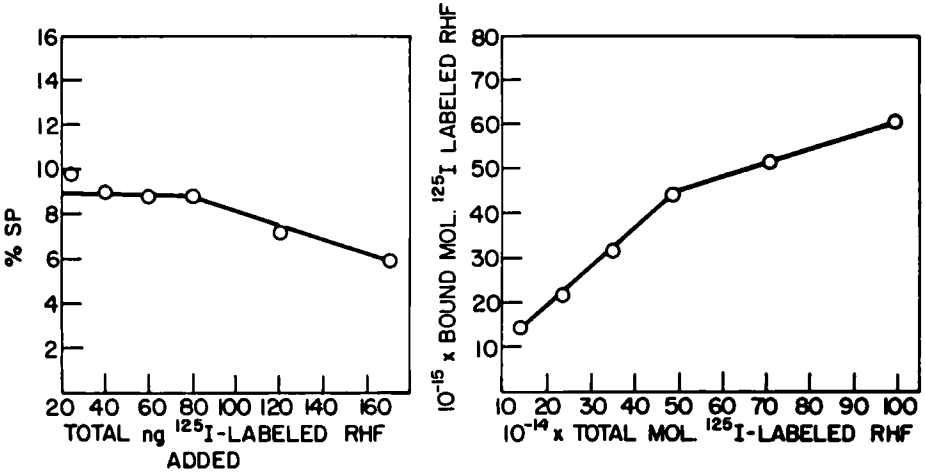

FIGURE 6. Left Panel - Titration of ^{125}I-labeled RHF with CCL-119F control cells. 1 x 10^5 CCL-119F cells were incubated in the presence and absence of excess NSF and various amounts of ^{125}I-labeled RHF in order to determine %SP and standardize the mass of each new lot of labeled antibody. Each new lot was adjusted to give %SP identical to a previous lot. Right Panel - Mol of ^{125}I-labeled RHF bound to CCL-119F cells as a function of total mol ^{125}I-labeled RHF added. Data are the same as in left panel, replotted to show the relationship of %SP to mol ^{125}I-labeled RHF bound to CCL-119F. %SP remained unchanged as long as the ratio of bound:free antibody was linear. As saturation of cell binding sites was approached, %SP decreased as unbound antibody increased.

antibody was first titrated against CCL-119F control standards to a level giving a %SP identical to a previous lot currently in use in the clinical studies. Figure 6 (left panel) depicts a typical titration curve of %SP versus antibody mass. Note that %SP remained unchanged with regard to antibody mass in a range of approximately 40-80 ng of ^{125}I-labeled RHF antibody added. When antibody mass was increased above that level, excess unbound antibody existed and the ratio of cell bound to total antibody decreased. When expressed as a function of mol antibody bound to

total mol of antibody added (Figure 6, right panel), it becomes clear that %SP remained unchanged as long as the ratio of mol ^{125}I-labeled RHF bound to mol added remained linear. As saturation of PBM binding sites was approached, the relationship of bound to total mol ^{125}I-labeled RHF became non-linear and %SP decreased.

In practice, the mass of antibody routinely utilized was just at the inflection point of the %SP titration curve allowing for selection by functional criteria of the same antibody mass for each iodinated preparation. Calibration of each labeled antibody lot eliminated potential errors found in direct estimation of antibody mass from specific activity calculations which are difficult to determine with absolute precision on radioiodinated antibodies purified by ion exchange chromatography. This approach also compensated for reproducibility problems such as batch to batch variations in immunoreactivity of radioiodinated antibodies without the necessity of having to run multi-point standard curves for each patient tested.

Paired comparisons on the same patient cells using different lots of antibody were routinely made prior to introduction of a new lot of ^{125}I-labeled RHF into clinical studies to ensure that this mode of standardization directly reflected the status of binding to patient PBM's. As shown in Table 3, paired patient studies on seven preparations of labeled antibody showed excellent correlation with regard to %SP results over the entire clinical range of values, suggesting that when standardized in the method described, %SP may be used as an accurate parameter of measurement of ferritin on isolated patient PBM's.

TABLE 3
Paired Patient Comparisons of Various Lots of ^{125}I-Labeled RHF
After Adjustment of Antibody Mass Using CCL-119F Control Cells

New radioiodinated lots of RHF were titrated against CCL-119F control cells to adjust antibody mass prior to paired patient comparison with a currently used lot of antibody to ensure standardization of antibody.

RHF CODE LOT 1	RHF CODE LOT 2	NO. PATIENTS PAIRED	MEAN %SP LOT 1 (±S.D.)	RANGE %SP LOT 1	MEAN %SP LOT 2 (±S.D.)	RANGE %SP LOT 2	REGRESSION SLOPE LOT 1:LOT 2	CORRELATION COEFFICIENT (r)
628	720	28	4.3 ±2.2	1.4 - 13.4	3.4 ±1.7	1.4 - 10.7	1.14	0.886
720	805	9	5.0 ±1.8	2.3 - 7.3	4.7 ±1.8	2.6 - 6.7	0.949	0.962
805	916	7	6.0 ±3.0	1.7 - 10.8	5.5 ±3.6	1.6 - 12.8	0.778	0.940
201	228	29	7.2 ±2.2	3.0 - 12.5	8.2 ±2.6	4.0 - 17.4	0.770	0.918
228	405	16	8.2 ±3.9	2.7 - 16.0	9.4 ±4.6	3.0 - 18.7	0.856	0.994
405	630	17	5.4 ±2.5	1.8 - 10.1	5.8 ±2.5	2.2 - 11.9	0.955	0.994

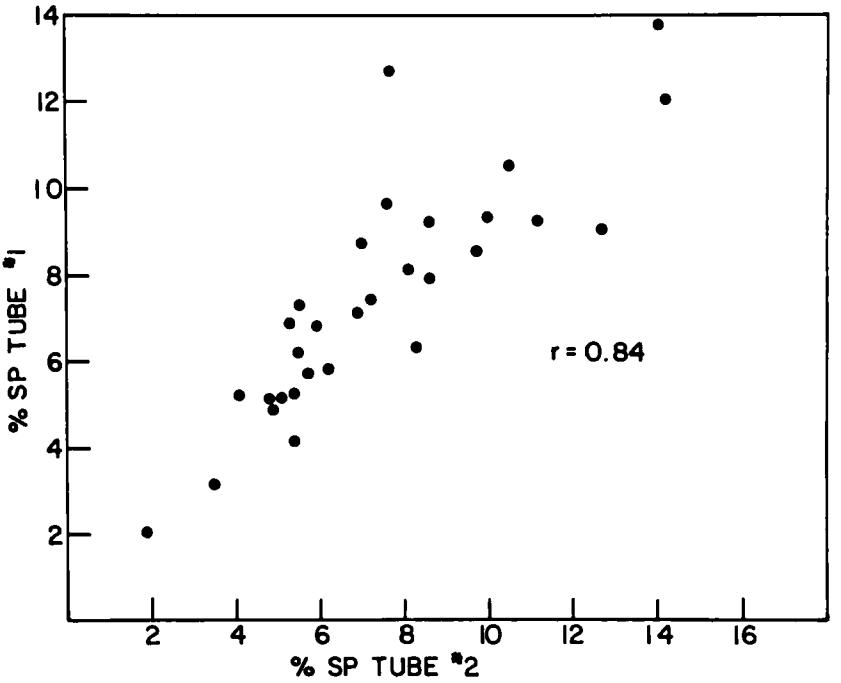

FIGURE 7. Precision of Ficoll-Hypaque PBM isolation. Duplicate tubes of blood from individual patients were prepared separately with regard to PBM isolation, followed by incubation of cells with ^{125}I-labeled RHF in the presence and absence of excess soluble NSF to assess %SP. The correlation coefficient for %SP of paired patient samples as determined by linear regression analysis was high (r=0.84, n=30) suggesting that the PBM isolation procedure was not responsible for differences in patient binding.

Precision of PBM isolation on ficoll

To evaluate overall assay precision, duplicate tubes of blood collected simultaneously from the same patients were handled separately with regard to PBM isolation and subsequent %SP analysis. As shown in Figure 7, duplicate samples showed excellent correlation in terms of %SP. Patients with high binding values had elevated levels in both tubes, while patients with low %SP values maintained low levels in both samples. Of 30 patients

paired in this fashion, 83% had differences in %SP of less than 2. These results indicate that differences in binding values between patients were not due to artifacts of sample handling and PBM isolation, but rather were due to intrinsic differences in the amount of ferritin on the cell surface.

DISCUSSION

Based on the studies described, a simple RIA for analysis of ferritin on the surface of isolated patient PBM's has been developed. This interaction is specific for ferritin and does not appear to be due to binding of ^{125}I-labeled RHF to Fc receptor sites located on PBM's. Fc receptors on mononuclear white cells preferentially bind immune complexes with much greater affinity than monomeric immunoglobulins (13). It is, therefore, of interest that results of experiments performed in this report using two different immune complexes, where the Fc portion of immunoglobulin available for binding to PBM Fc receptor resided on ^{125}I-labeled RHF, resulted in decreased rather than enhanced binding to PBM's. These results suggest that the antigen combining region was blocked, thereby preventing ^{125}I-labeled RHF from binding to cell surface ferritin. For one of these immune complexes, specific soluble antigen (ferritin) was bound to its corresponding combining site on ^{125}I-labeled RHF. In the other complex, the antigen combining site on ^{125}I-labeled RHF was sterically blocked by the goat $F(ab')_2$ fragment directed against the $F(ab')_2$ portion of RHF. These results, in conjunction with the ability of an RHF molecule from which the Fc portion had been enzymatically cleaved to com-

pete with ^{125}I-labeled RHF for cell bound ferritin, provide strong evidence that the binding of ^{125}I-labeled RHF to PBM's is highly specific for ferritin.

Unlike other methods of measurement of cell surface antigens such as immunofluorescence or antibody dependent complement mediated cytotoxicity, this procedure does not suffer from labor intensive procedures such as sample fixation and manual counting of cells under a microscope. While cytofluorographs have recently been developed to quantitatively assess cell surface antigens, thereby eliminating much of the error inherent in manual cell counting procedures, these instruments are prohibitively expensive for routine clinical use.

In recent years, the measurement of markers on the surface of PBM's has drawn increased attention as a new source of easily accessible material providing information of clinical relevance. The most dramatic of these is the use of monoclonal antibodies to define T and B cell subsets in classification of leukemias and other diseases characterized by alterations in immune competency (14). Binding studies using radiolabeled hormones have been used to measure insulin and growth hormone receptors on the surface of PBM's (15). Such techniques are now used to classify certain diabetic states. While most studies of this nature use multiple point competitive binding curves between labeled and unlabeled hormone to develop quantitative data with regard to receptor number and affinity constants, in practice, yields of PBM's are insufficient to conduct these studies unless large volumes of

blood are utilized. This is especially true when cancer patients undergoing active therapy are the population being investigated.

In order to conduct a large scale clinical study on the relationship of ferritin bearing PBM's in malignancy and to adequately assess a large number of diseases (1), it was necessary to develop a simplified immunoassay procedure utilizing a minimal number of patient cells. The results of this study demonstrate that by careful control and standardization of reagents, measurement of the differences in binding of ^{125}I-labeled RHF in the presence and absence of soluble ferritin can be used as a quantitative measure of ferritin bound to PBM.

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