Monoclonal Antibodies as Agents for Selective Radiolabeling of Human Neutrophils

M.L. Thakur, M.D. Richard, and F.W. White III

Department of Radiation Oncology and Nuclear Medicine, Division of Nuclear Medicine, Thomas Jefferson University, Philadelphia, Pennsylvania

Ten monoclonal antibodies (MAbs) have been evaluated as agents for selective radiolabeling of human neutrophils (PMNs). Four bifunctional chelating agents (BFCA) have been compared in order to determine the one that gives the best labeling of MAbs with radionuclides. Of these, the cyclic anhydride of diethylenetriaminepentaacetic acid (DTPA) has been chosen for routine use since it incorporated ^{111}In efficiently, and only minimally altered the immunoreactivity of the MAb. Two [^{111}In]DTPA MAbs (1 μg or less, anti-SSEA-1 and B.37.2.1) incorporated 80 ± 5% and 72 ± 6% of the added radioactivity with 10^7 separated PMNs in 0.5 ml plasma. In whole blood these values were 27 ± 5% and 38 ± 9%, respectively. Using the other three BFCAs with anti-SSEA-1, the corresponding values of 70%–80% and 3%–5%, respectively were obtained. Polyacrylamide gel electrophoretic examinations of the radioactivity that did not bind to blood cells revealed that when DTPA was used as the BFCA, 21% of the radioactivity was associated with a plasma protein of mol wt 60–80 kD and 57% remained bound to the MAb (mol wt 900 kD). When the other three BFCAs were employed, these values were >42% and <19%, respectively. When up to 4% of the PMN surface antigens were MAb bound, the PMNs physiologic function remained unaltered. For two MAbs, anti-SSEA-1, and B 37.2.1, the PMN specificity was found to be $1.6 \times 10^{-11}M$ and $2 \times 10^{-11}M$, respectively, and the number of cell surface antigens was estimated to be 5.1×10^5 and 7.8×10^5, respectively. For ^{99m}Tc labeling of MAbs, the use of four different reducing agents were examined. One agent, sodium-dithionite ($Na_2S_2O_4$), at a concentration of 0.2 mg/ml reaction mixture and with a molar ratio 3×10^3:1 to the MAb, gave us results that were comparable to those obtained with [^{111}In]MAb. We conclude that at least two of the ten MAbs, anti-SSEA-1 and B.37.2.1 are worthy of evaluation in humans.

J Nucl Med 29:1817–1825, 1988

The need for radioactive agents that will label neutrophils (PMNs), either cohertly in bone marrow before they are released, or even randomly, after they are released in circulation, was realized decades ago (*1–3*). Modest success was achieved using radioactive DNA precursors for cohert labeling and non-DNA agents for random labeling (*3*). A chemoattractant polypeptide such as N-formyl methionyl leucyl phenylalanine (FMLP) has also been used with some success (unpublished data). However, none of these agents has been successful enough to radiolabel neutrophils for imaging inflammatory processes in humans. Although neutrophils labeled with indium-111 (^{111}In) oxine or other lipophilic compounds are routinely employed in such applications, these agents are nonspecific, require phlebotomy, and separation of PMNs from other blood cells for in vitro labeling (*4*). This is undesirable and restricts the use of this otherwise excellent modality to only a limited number of medical centers.

Recent advances in hybridoma technology have provided investigators with the tools to produce monoclonal antibodies (MAbs) specific for neutrophil surface antigens. Such MAbs, when labeled with a radionuclide, should serve as ideal agents to label human neutrophils specifically in whole blood in vitro and possibly in vivo by injecting the radiolabeled MAb into the systemic circulation of a patient. This will eliminate the current in vitro procedure and greatly enhance the use of radiolabeled neutrophils in diagnostic applications.

This study was undertaken to investigate several "neutrophil specific" monoclonal antibodies, as poten-

Received Nov. 1, 1987; revision accepted Apr. 19, 1988.

For reprints contact: M. L. Thakur, PhD, Div. of Nuclear Medicine, Dept. of Radiation Oncology and Nuclear Medicine, 804 Main Building, Thomas Jefferson University, Philadelphia, PA 19107.

tial agents for radiolabeling human neutrophils. The preliminary results are reported herein.

MATERIALS AND METHODS

Antibodies

Ten murine antibodies, listed in Table 1 were obtained as generous gifts from various investigators and commercial companies. Some of the MAbs presented were already purified, but some were in ascites fluid. The pure antibodies were used without further processing. Those in ascites fluid were separated using a Protein A Sepharose Cl-4B affinity column (0.3 cm × 15 cm) and the procedure described by Pharmacia (*5*). The eluent was monitored by a uv detector and fractionated using an automatic fraction collector. The appropriate fractions were pooled and the MAb was concentrated using a Centricon-30 microconcentrator (*6*). The MAbs were washed in 0.9% NaCl adjusted to pH 7, collected, and their concentration determined spectrophotometrically. One hundred micrograms aliquots of the protein were then dispensed into a series of clean sterile microcentrifuge tubes and frozen until needed.

Labeling with Indium-111

Indium-111 solution with a high radioactive concentration (Atomic Energy Canada, 50 mCi/ml, 0.1*M* HCl) was chosen in order to minimize a quantity of metallic impurities. Four bifunctional chelating agents were evaluated. The cyclic anhydride of diethylenetriaminepentaacetic acid (DTPA), (not to be confused with the free acid DTPA), and ethylenediamine-di-o-hydroxy-phenylacetic acid (EDDHA) were obtained commercially (Sigma Chemical Corp.) (S)-4-[2,3-bis[bis(carboxymethyl)amino]propyl]alanine (aminobenzyl EDTA, ABE), and (S)-N-4 [2,3-bis[bis(carboxymethyl)amino] propyl] phenyl bromoacetamido (bromoacetamidobenzyl EDTA, BABE) were prepared after DeRiemer et al. (*8,9*). The purity of the newly synthesized ABE and BABE, before and after ^{111}In labeling was examined with high performance liquid chromatography (HPLC) (Water's NOVA-PAK C-18 column and a mobile phase containing 4% acetonitrile, 2% methanol, and 2% gl. acetic acid in water) equipped with both a uv monitor and a NaI (Tl) crystal detector. Small samples of ABE and BABE, generously supplied by Dr. Claude Meares (Dept. of Chemistry, University of California, Davis.) served as controls.

ABE, BABE, and EDDHA were dissolved in 0.9% NaCl, pH 7. DTPA was suspended (1 mg/ml) in dry, metal free chloroform and sonicated for uniform particle size.

MAbs (100 μg each time) used were either IgM or IgG and their mol wt was assumed to be 900,000 or 150,000 D, respectively. For conjugation with EDDHA (MAb to EDDHA molar ratio 1:2.5) the Rodwell (*9*) carbiodiimide procedure, and for the conjugation with ABE (control) and BABE (MAb to BFCA molar ratio 1:10 to 1:3) the Meares (*8*) method was followed. The MAb to DTPA molar ratios of 1:1 to 1:5 were examined. The objective was to assess the ratio that would provide us with the optimal radionuclide yield and yet would not drastically alter the biochemical specificity of the protein as determined by the PMN labeling yields. Unreacted BFCA was eliminated by washing the protein twice with 2 ml 0.05*M* TRIS buffer pH 6.1 in a microconcentrator. The protein was then collected and a required quantity of ^{111}In acetate (0.1*M*, pH 6.1) was added (*10*). Following a 30-min incubation at 22°C, the labeled antibody solutions were washed as above and then tested for any unbound ^{111}In, with instant silica gel thin layer (ITLC-SG) chromatography (Gelman Sciences Inc. MI.) developed with 2*M* urea or with paper chromatography (Whatman No. 1) using (1:1) methanol and 0.1*M* ammonium acetate as the solvent. [^{111}In]MAb Rf = 0.0 and [^{111}In]DTPA Rf = 1:0). A further confirmation of the association of radioactivity with the protein was also obtained by HPLC (Water's Protein Pak—300 SW column, 0.05*M* phosphate buffer pH 7, flow rate of 1.0 ml/min.).

In each antibody labeling preparation 100 μg of MAb and 100 μg human serum albumin (HSA) not conjugated with BFCA served as controls for any nonspecific binding of the radionuclide. One microgram of each of these labeled MAbs (1 μg IgM = ~6.3 × 10^{11} molecules) was then incubated each with ~10^7 isolated human neutrophils suspended in 0.5 ml plasma. The number of MAb molecule was too small to saturate the antigenic binding sites available on neutrophil surfaces (see below). Following a 30-min incubation at 22°C, the radioactivity associated with the neutrophils in each preparation was determined and plotted as a function of the MAb to DTPA molar ratios.

TABLE 1
Monoclonal Antibodies Specific for Human Neutrophils

Name	Type	Form	Supplier
B.37.2.1	IgM	Purified	Wistar Inst.‡
MCA 161	IgG1	Ascites fluid	Serotec*
FMC 11	IgG1	Ascites fluid	Sera-Lab†
FMC 14	IgM	Ascites fluid	Sera-Lab†
MCA 87	IgG2a	Purified	Serotec*
MCA 149 A	IgG1	Ascites fluid	Serotec*
MCA 215	IgM	Purified	Serotec*
MCA 167	IgG2a	Ascites fluid	Serotec*
anti-SSEA-1	IgM	Purified	Wistar Inst.‡
B.6.2	IgG	Purified	NIH (NCI)

* Bioproducts for Science Inc., Madison, WI.
† Accurate Chemicals and Scientific Corporation, Westbury, NY.
‡ Philadelphia.

Determination of DTPA Molecules per MAb Molecule

The number of DTPA molecules attached per MAb molecule was estimated using the indium carrier method described previously (*10*). The radioactivity associated with the protein was determined, and the bound to free ratio and the number of indium ions bound per MAb molecule were calculated. The data were plotted according to Feldman in order to determine the number of DTPA molecules bound per MAb molecule (*11*).

Determination of Neutrophil Specificity

The antigenic specificity of the MAb and the number of antigenic sites present per human neutrophil were determined for B 37.2.1 and anti-SSEA-1 using ^{111}In or iodine-125 as tracers. Radioiodination was carried out as previously described (*10*), using Iodo-Gen (Pierce Chemicals) as the oxidizing agent. The unbound radioactivity was eliminated as before,

and the quantity of MAb was determined spectrophotometrically. Neutrophils were separated from 100 ml anticoagulated blood freshly drawn from normal volunteers and the cell concentration was determined using a ZM Coulter counter (*12*). The PMNs were then divided equally into eight 15-ml sterile polypropylene test tubes, each containing 2.8×10^7 cells in 0.5 ml plasma. To these was then added a known but increasing quantity of labeled MAb and the suspensions were allowed to incubate at 22°C for 30 min. The cells were then centrifuged, washed once with 2 ml plasma, centrifuged and the corresponding supernatants combined. The quantity of the radioactivity associated with the cells was determined. This allowed us to estimate the number of MAb molecules bound per cell. The experiment was repeated and data were subjected to Scatchard plots and regression analysis to determine the best fit for the intercepts at X and Y axis.

Labeling DTPA-MAb with Technetium-99m: The Choice of Reducing Agents

The major initial investigations were centered around reduction of technetium-99m (^{99m}Tc) for binding to DTPA-MAb. Stannous chloride, ascorbic acid, sodium borohydride and dithionite were chosen as reducing agents. To a known quantity of $^{99m}TcO_4^-$ solution in four groups of several test tubes in duplicate was added an increasing quantity of each agent and the mixtures were allowed to incubate at 22°C for 10 min under a nitrogen atmosphere. The contents from one group of these were then added to 100 μg each of DTPA coupled anti-SSEA-1 in 100 μl 0.05*M* TRIS buffer pH 6.1. The reducing agent to MAb molar ratios ranged from 8×10^2 to 1×10^4. Following a 15-min incubation at 22°C, the reaction mixture from each test tube was centrifuged, using the microconcentrator devices, and washed twice with 2 ml 0.05*M* TRIS buffer pH 6.1. The quantities of radioactivity associated with the protein, retained on the filter and washed through, were determined. The relative percentages of these were calculated and plotted as a function of the molar ratio. The protein determinations were verified using paper chromatography and HPLC.

Determining the Optimal Concentration of the Reducing Agent

Since the ^{99m}Tc concentration (mCi/ml) available for labeling may vary each time, the influence of the quantity of dithionite, the best observed reducing agent, on the protein labeling efficiency was studied.

In a series of six clean test tubes, 200 μCi of freshly eluted ^{99m}Tc was dispensed and the final volume of the solution was adjusted with 0.9% NaCl to 500 μl. Freshly prepared dithionite solution was then added to the test tubes in such a way that the reducing agent concentration ranged from 0.05 μg/μl to 0.6 μg/μl. Following a 10-min incubation at 22°C, the radioactivity was transferred to test tubes each containing 100 μg protein. The reaction mixture was then allowed to incubate for 15 min at 22°C and filtered through Centricon devices. The radioactivity in the filtrate, bound to the filter and associated with the protein was then measured, the percentages calculated, and the values were plotted as a function of the dithionite concentration.

Elimination of ^{99m}Tc-Colloid from the Final Product

Most reducing agents used during [^{99m}Tc]MAb preparation form ^{99m}Tc-colloid (*13*). Using the optimal dithionite concentration (0.2 μg/μl), as determined above, the following experiments were performed to ensure that the final product was free of colloidal ^{99m}Tc.

Samples from the preparations before Centricon purification were analyzed with HPLC. Absorbance and radioactivity elution peaks were monitored and fractions were collected to determine the quantity of radioactivity eluted. This was compared with standards consisting of samples equal in volume to that chosen for the HPLC injection. Similar analyses were also performed on the radiolabeled MAb samples recovered after the Centricon purification step, and on those that were purified by Centricon after filtration through 0.22 μm Millipore membrane.

Labeling Human Neutrophils, Separated Versus in Whole Blood

Antibodies were labeled with ^{111}In or ^{99m}Tc as described above. Anticoagulated fresh venous blood was obtained from normal volunteers. Depending upon the aim of the study, leukocytes, or neutrophils were separated (*12*) and their number determined. The cells were suspended in autologus plasma, or, for the gel electrophoresis experiments, in phosphate buffered saline (PBS) pH 7.4. The radiolabeled antibodies were then added to the cell suspension or to a known volume of whole blood in such a way that the MAb molecules would not saturate the neutrophil surface antigens. Following incubation, radioactivity associated the neutrophils, and supernatant, or in the case of whole blood, the neutrophils, erythrocytes, and plasma were determined. On each occasion, the labeled neutrophils were washed with fresh autologus plasma, to remove any unassociated radioactivity. The percent of the radioactivity associated with the cells was then determined.

Cell Viability Following MAb Labeling

The DTPA coupled anti-SSEA-1 was chosen for this study. Approximately 9×10^6 neutrophils were separated from human blood and suspended in 0.5 ml plasma in each of the five test tubes. Four of these received 0.5 to 5 μg of the labeled MAb. Assuming uniform and 100% interaction, the highest MAb concentration would bind 10% of all available neutrophil antigens. The cells in the fifth test tube served as control. Following a 30-min incubation, cells in all of the test tubes were centrifuged, washed free of unbound MAb, resuspended in 0.5 ml plasma and labeled with [^{111}In]Merc (2-mercaptopyridine-N-oxide), using our routine protocol (*14*). These were then subjected to a nylon wool adherence test as described previously (*15*). The experiment was repeated to obtain duplicate results. In another set of experiments, the control cells and those exposed to the MAb, were allowed to phagocytose [^{99m}Tc] antimony sulfide colloid (Cadema Med. Inc., Westtown, NY). The un-engulfed ^{99m}Tc-colloid was then eliminated using the method of Schroth et al. (*16*).

In each case the radioactivity associated with the neutrophils was determined and compared to the control cells.

Interaction of Labeled MAb with Neutrophils of Other Animal Species

Cross reactivity of the MAb with a laboratory animal neutrophils would permit the development of an experimental animal model for in vivo studies including the location of abscesses. In order to determine such a specificity, the anti-SSEA-1 and B.37.2.1 MAbs were labeled with ^{111}In in the

manner described above and evaluated for interaction with neutrophils separated from the blood of rats, cats, guinea pigs, dogs, rabbits, sheep, and pigs.

Gel Electrophoresis

The objective of the gel electrophoretic study was to determine the chemical form of radioactivity that remained in plasma or PBS following the incubation of ^{111}In-labeled antibodies with neutrophils in whole blood or in PBS. Ten percent polyacrylamide gel plates were prepared using the technique of Osterman (*17*). Twenty to fifty microliters of the supernatent was loaded into each of two wells. One hundred micrograms of (5 mg/ml PBS) transferrin was incubated with the PBS supernatant and analyzed similarly. In addition, [^{111}In] chloride, [^{111}In]DTPA, [^{111}In]transferrin, ^{111}In-labeled MAb, and other molecular weight markers such as the albumin and trypsin inhibitor, were loaded as reference in other wells.

Following electrophoresis, (SDS-Tris borate buffer pH 7.4, 250 V, 4 hr) autoradiography was performed, the gel was cut into 0.5-cm sections and the radioactivity associated was counted. The results were plotted as the percent of the total radioactivity versus the distance in cm from the lower end of the corresponding well.

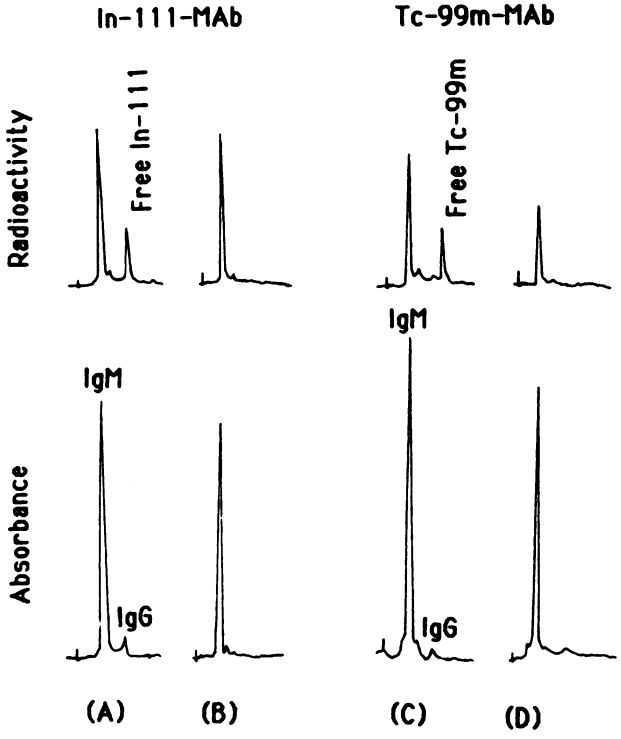

FIGURE 1
HPLC elution pattern of ^{111}In-and ^{99m}Tc-labeled anti-SSEA-1. (Protein Pak 300 SW, 0.05*M* phosphate buffer pH 7; flow rate 1 ml/min). A and B, and C and D are four separate injections for [^{111}In]MAb and [^{99m}Tc]MAb, respectively. A and C (top = radioactivity and bottom = absorbance for each injection) elutions indicate that the IgM, IgG, and unbound radioactivity can be separated and quantitated. The elution curves B and D are the injections of labeled MAb after purification from unbound radioactivity.

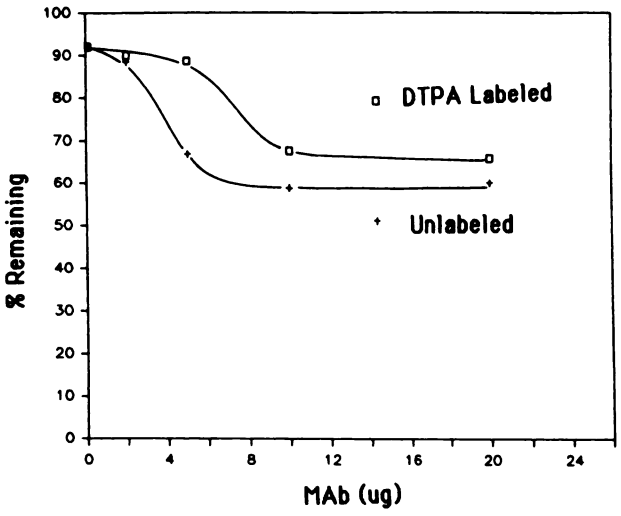

FIGURE 2
Percent of radioactivity displaced from the [^{111}In]DTPA-B.37.2.1 labeled human PMNs, following the challenge with DTPA-labeled and unlabeled B.37.2.1

RESULTS

The MAbs evaluated in this work are listed in Table 1. The use of Centricon's microconcentrator device (*18*) eliminated the need for the Sephadex column chromatographic procedure employed previously (*11*) for the separation of unbound DTPA or unlabeled ^{111}In. The device previously washed with 100 μl of 2% HSA solution, minimized the protein losses (2%–5% ^{111}In or ^{99m}Tc) and avoided dilution of the protein solution. This in turn enhanced the apparent labeling yields which were in the range of 70%–80%. The thin layer chromatographic and HPLC analysis of the final product revealed that only a negligible quantity of ^{111}In was unbound. The HPLC analysis of ^{111}In- or ^{99m}Tc-labeled protein was able to distinctly separate the IgM, any IgG impurity and the unbound radioactivity (Fig. 1). This clearly indicated that the final product contained <2% unbound radioactivity. The recovery of radioactivity through the HPLC column for [^{111}In]MAb was >98% and [^{99m}Tc]MAb before Centricon purification was ~80%. Since neither colloidal ^{111}In nor colloidal ^{99m}Tc are eluted through the HPLC column, one may be able to conclude that the unrecovered radioactivity may be a result of the formation of colloid, particularly in the case of ^{99m}Tc. Following membrane filtration and Centricon purification of the [^{99m}Tc]MAb, the recovery was almost quantitative. The amount of radioactivity bound nonspecifically to unconjugated anti-SSEA-1 averaged ~2%.

The DTPA to MAb molar ratios of 1:1 to 5:1 did not alter the immunoreactivity of the protein as measured by the quantity of the [^{111}In]DTPA-MAb associated with human neutrophils.

In all subsequent preparations therefore a DTPA to

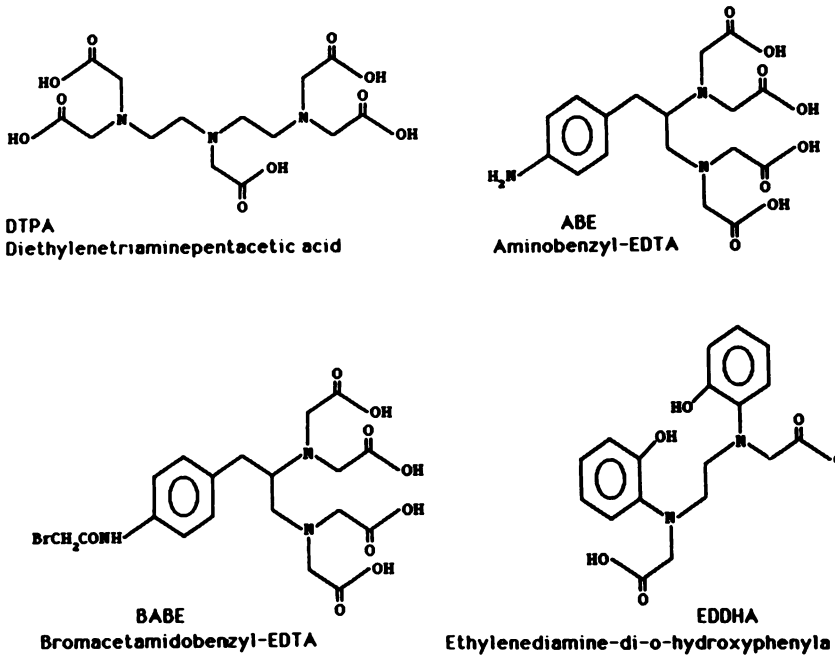

FIGURE 3
Structural formula of the four bifunctional chelating agents used.

MAb (IgM) ratio of 5:1 was used. Using this proportion, we have obtained on the average, one DTPA molecule bound per MAb molecule. In a set of experiments where human neutrophils labeled with [^{111}In]DTPA-B.37.2.1 (DTPA:MAb: 5:1) were challenged with uncoupled or DTPA coupled B.37.2.1, indicated that uncoupled MAb were able to displace only ~5% more radioactivity from the cells than that with DTPA coupled MAb (Fig. 2). These results suggest that at least some MAb molecules conjugated with DTPA were unable to interact with the cells and exchange with the [^{111}In]DTPA-MAb molecules already bound to the cell surface antigens.

When a given quantity of [^{111}In] anti-SSEA-1, prepared using ABE, BABE, EDDHA, or DTPA (Fig. 3) as the bifunctional chelating agent was incubated with an equal number of isolated human neutrophils suspended in plasma, 70% to 80% of the radioactivity was cell associated (Table 2). In whole blood, however, the quantity of neutrophil associated [^{111}In]DTPA MAb averaged 27%, compared with that of only 3%–5% with [^{111}In]ABE, [^{111}In]EDDHA, or [^{111}In]BABE-MAb. In cases of the latter three, the quantity of radioactivity that remained in plasma was >75% as compared to ~40% with [^{111}In]DTPA-MAb (Table 3). Gel electrophoretic analysis of the radioactivity remaining in plasma indicated that ~21%, 42%, and 57%, for DTPA, ABE, and BABE, respectively, was associated with plasma proteins with mol wt 70–80kD, which is in the range of the mol wt of transferrin. The radioactivity that remained in plasma as mol wt 900kD (probably as [^{111}In]DTPA-anti-SSEA-1, [^{111}In]ABE-anti-SSEA-1 and [^{111}In]BABE-anti-SSEA-1) was 57%, 12%, and 19%, respectively. The results shown in Figure 4 also indicated that the transferrin added to the [^{111}In]ABE-anti-SSEA-1-PBS supernatant was able to bind ~70% of ^{111}In, compared with that of only ~15% from the [^{111}In] DTPA-anti-SSEA-1-PBS supernatant.

The cell labeling results obtained with other [^{111}In]

TABLE 2
Evaluation of Human Neutrophil (PMN) Specific Antibodies Coupled with DTPA and Labeled with ^{111}In

		Separated cells (%)			Whole blood (%)			
MAb	Class	PMN	Plasma	N	PMN	RBC	Plasma	N
Anti-SSEA-1	IgM	80 ± 5	20 ± 5	20	27 ± 5	28 ± 3	45 ± 2	12
B.37.2.1	IgM	72 ± 6	28 ± 7	6	38 ± 9	25 ± 4	37 ± 5	6
MCA 87	IgG2a	37 ± 5	63 ± 3	3	25 ± 3	15 ± 4	60 ± 4	3
MCA 149	IgG1a	15 ± 6	85 ± 3	3	14 ± 3	16 ± 4	70 ± 3	3
MCA 167	IgG2a	41 ± 4	59 ± 4	3	41 ± 4	14 ± 2	45 ± 4	3
MCA 215	IgM	17 ± 6	83 ± 3	3	14 ± 4	10 ± 3	76 ± 3	3
FMC 11	IgG1	39 ± 5	61 ± 5	3	–	–	–	
B.6.2	IgG	9 ± 5	91 ± 8	4	–	–	–	
MCA 161	IgG1	13 ± 4	87 ± 7	3	11 ± 2	13 ± 4	76 ± 4	3

TABLE 3
Results of Comparison of Four Bifunctional Chelating Agents

Agent	(Anti-SSEA-1) % ^{111}In and ^{99m}Tc associated with (N = 4) Neutrophils	Erythrocytes	Plasma
	In whole blood		
DTPA	27 ± 5 (30 ± 3)*	28 ± 2 (27 ± 3)	44 ± 2 (43 ± 4)
EDDHA	3 ± 2	18 ± 2	79 ± 3
ABE	4 ± 2	20 ± 4	77 ± 6
BABE	3 ± 1	15 ± 2	81 ± 5
	In separated cells		
DTPA	80 ± 5 (80 ± 4)	— (20 ± 3)	20 ± 5
EDDHA	70 ± 4	—	30 ± 2
ABE	73 ± 3	—	27 ± 3
BABE	80 ± 4	—	21 ± 3

*The numbers in parentheses are the results obtained using dithionite reduced [^{99m}Tc]DTPA-anti-SSEA-1.

DTPA labeled MAbs are given in Table 2. These too indicated higher labeling efficiencies in isolated neutrophils than in whole blood.

For labeling DTPA conjugated MAb with ^{99m}Tc, the use of dithionite gave the best results (Fig. 5). We routinely use 0.2 μg dithionite per μl of the reaction mixture (Fig. 6) for labeling 100 μg DTPA-IgM in 300 μl, giving us the reducing agent to MAb molar ratio of 3,000:1. At this concentration, ~75% of the added radioactivity binds to protein, 5% remains unreduced and 20% forms colloid. We also found that reducing ^{99m}Tc first enhances labeling, giving us approximately the same yield as with ^{111}In (70%–80%). [Antibodies labeled with ^{99m}Tc-labeled human neutrophils in whole blood with efficiencies (30 ± 3%)] equivalent to those with [^{111}In]DTPA-MAb (Table 2).

The neutrophil specificity and the number of surface antigens for B37.2.1 and anti-SSEA-1 were (Fig. 7A,B) $2 \times 10^{-11} M$, $1.6 \times 10^{-11} M$, and ~7.8×10^5 and 5.1×10^5 per cell, respectively. These compared closely to that with ^{125}I-labeled MAbs. When an average of 10% of the available surface antigens were bound to anti-SSEA-1, the phagocytic ability and nylon wool adherence of human neutrophils was ~70% and 80% of the respective control cells. At 4% or lower antigenic saturation, no apparent changes in cell function was observed.

The MAb, MCA-167, also cross-reacted with human platelets and, therefore, was not considered further for the PMN work. The most promising antibodies, anti-SSEA-1 and B.37.2.1, were tested for their affinity with the neutrophils of several different animal species. In none of these cases was there more than 3% of the radioactivity PMN associated.

DISCUSSION

The aim of this investigation was to evaluate ^{99m}Tc- or ^{111}In-labeled MAbs as agents that will label human PMNs selectively in whole blood, in vitro, and perhaps in the future, in vivo. In order to be able to make a fair assessment and reach a suitable practical judgement several related parameters were carefully examined.

Of the ten MABs studied, antibody raised against stage specific embryonic antigen-1 (SSEA-1) was most promising in terms of its availability in purified form, high specificity, and the high degree of interaction with human neutrophils in plasma as well as in whole blood.

Out of the four bifunctional chelating agents tested, the association constants for [^{111}In]DTPA are equivalent to those of [^{111}In]transferrin, which are higher than those of [^{111}In]EDTA and its analogs (*19*). Our results, in which on the average not more than one BFC molecule was bound to one MAb molecule, show that the incorporation of ^{111}In with human neutrophils in whole blood was higher (27%) when DTPA was used as the

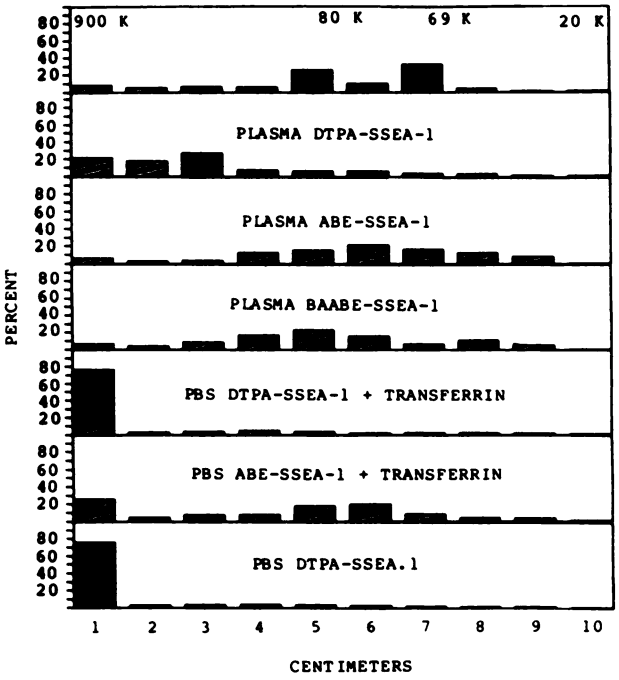

FIGURE 4
Percent radioactivity distribution as a function mol wt in each of the seven (horizontal) polyacrylamide gel electrophoretic lanes. The lane representing the histograms on the top of the figure contained radiolabeled compounds ranged from mol wt 900 k to 20 kD. The histograms in lanes 2, 3, 4, and 7 show the distribution of radioactivity remained in the supernatant following cell labelings (see brief description). Lanes 5 and 6 represent distribution of radioactivity following the incubation of [^{111}In]DTPA-anti-SSEA-1 and [^{111}In]ABE-anti-SSEA-1 with transferrin. Note that in the case of the latter, a large proportion of radioactivity has migrated to protein with mol wt 60–80 D. In the case of the former (lane 5) >80% of radioactivity remained with anti-SSEA-1 (mol wt 900 k).

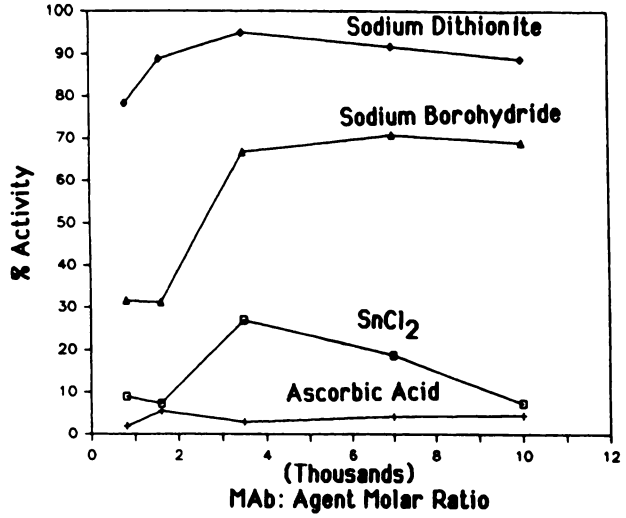

FIGURE 5
Influence of the reducing agent to MAb molar ratios on the preparation of [^{99m}Tc]DTPA-MAb. Percent radioactivity is the proportion of the total radioactivity (apparently) associated with protein.

bifunctional chelate than when EDTA analogs were used (Table 3). Our results (Fig. 4) also indicated that of the [^{111}In]DTPA remaining in plasma, 21% was bound to protein of mol wt 70–80kD compared with that of 42% and 57% of [^{111}In]ABE and BABE. This is surprising but may be due to the lower stability constants of [^{111}In]ABE and BABE than those of [^{111}In] DTPA (*19*). The actual cell labeling efficiency with [^{111}In]DTPA-MAb may be higher than 27% observed, since only ~70% of PMNs can be separated from whole blood by the methods used.

The radioactivity associated with platelets was <2%

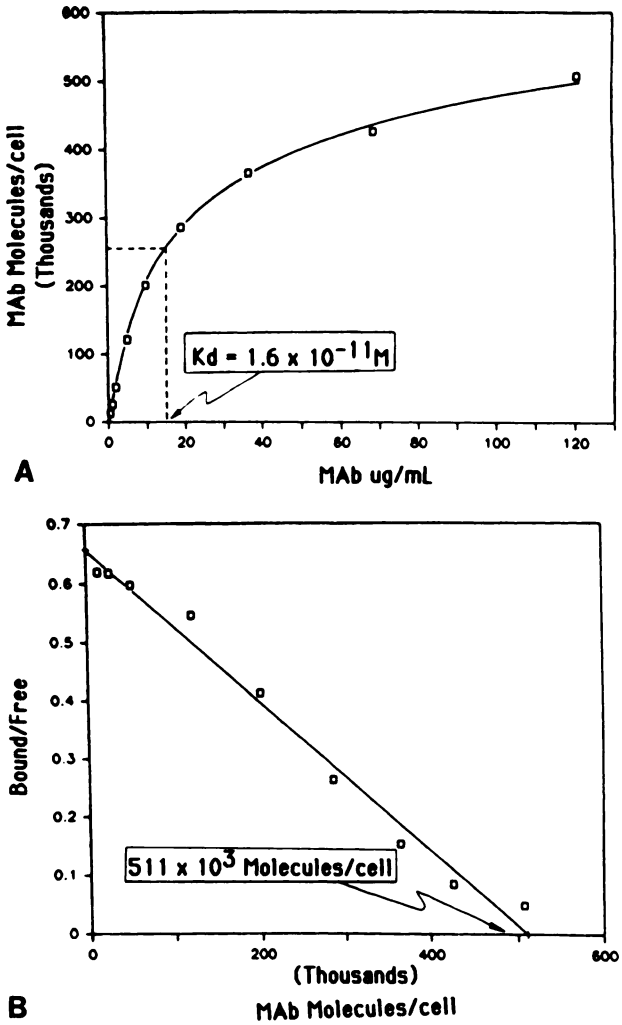

FIGURE 7
A,B: Scatchard plots determining the Kd value of [^{111}In] DTPA-anti-SSEA-1 for human PMNs and the number of the MAB specific antigens on the surface of each PMN.

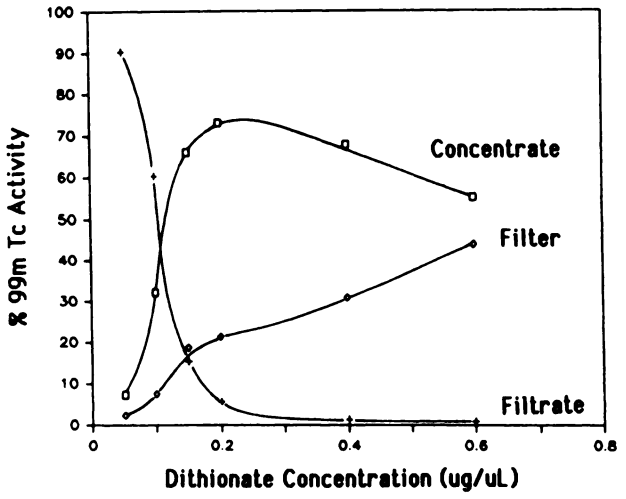

FIGURE 6
Influence of Dithionite concentration on the DTPA-MAb labeling. (The best results were obtained in the dithionite concentration of 0.2 μg/μl reaction mixture).

for all MAbs (with the exception of MCA-167) and that with the erythrocyte fraction was ~30%. When neutrophils were isolated, the [^{111}In]DTPA-anti-SSEA-1 labeling efficiency increased to ~80%. It is interesting to note that Locher et al. (*20*), by injecting iodine-123 (^{123}I) labeled anti carcino embryonic antigen MAb-47 (CEA-A47) into humans had successfully detected occult abscesses, and yet had reported that only 40% antibody was associated with isolated neutrophils.

In labeling MAb with ^{99m}Tc, we observed that dithionite was the agent of choice not only because it resulted in the higher association of radioactivity with the protein and the PMNs but also it is water soluble and has potential for inclusion in a possible kit. For imaging occult abscesses, the use of ^{99m}Tc may be more desirable due to its ready availability and shorter half-life than ^{111}In. However, ^{99m}Tc, unlike ^{111}In, may be spontaneously eluted from labeled cells in vivo and may

subsequently prove to be unacceptable. Such advantages or disadvantages can be assessed more reliably only after controlled studies of both agents have been performed in humans. This is particularly true since anti-SSEA-1 or B37.2.1 did not cross-react with the neutrophils obtained from several commonly available laboratory animal species. Unlike the screening of MAb specific for certain human tumors, which can be grown in animals such as the nude mice, the in vivo evaluation of MAb specific for human neutrophils is limited to only human subjects unless the MAb also interacts with neutrophils of the laboratory animal species.

The previous experience using MAb specific for human PMNs is limited to ten patients in whom ^{123}I-labeled CEA-A47, was administered (*21*). In these studies, abscesses were imaged but up to 60% of the radioactivity was estimated to be in the bone marrow. This made the visualization of abscesses ambiguous and called for SPECT imaging. The anti-SSEA-1 is an IgM class of MAb produced by immunizing mice with murine embroyal carcinoma F9 cells (*22*). A survey of normal human tissues indicated that the SSEA-1 antigen is expressed on granulocytes, their precursors, brain, stomach, colon, breast, adrenal gland, salivary gland, bladder, and kidney (*23*) but not on the liver, erythrocytes, or lymphocytes (*24*). Using immunoperoxidase microscopy, Ballou et al. (*25*) have found that SSEA-1 is also present on several normal mouse tissues especially kidney and the brain (but not neutrophils) and yet there was no significant retention of the radiolabeled anti-SSEA-1 injected in vivo. Liebert et al. (*23*) have reported that the peptic fragments (F[ab']2μ) of the anti-SSEA-1 has poor ($10^{-6}M$) affinity for single human cell in vivo. These findings, together with our observation that the anti-SSEA-1 gave us the best human neutrophil specificity ($10^{-11}M$), make the agent most desirable for labeling human neutrophil in vivo.

When a number of MAb molecules sufficient to saturate 10% of the available neutrophil antigens were incubated, the phagocytic ability and nylon wool adherence of the PMNs was 70% and 80%, respectively of the unexposed control cells. When 4% of the antigenic receptors were MAb bound no difference in the above PMN functions was observed. If the circulating PMN pool in an average normal human adult is estimated to be $\sim 3\times10^{10}$, there would be a total of $\sim 1.5\times10^{16}$ antigens available for the anti-SSEA-1 binding. At a rate of 4% saturation, a mg of the MAb could be administered, we plan to inject only $\sim$100 μg of the labeled MAb in human trials.

We realize that such research at the intersection of many disciplines and yet unknown biochemical and physiologic parameters may be challenging, but believe that it will be a worthwhile exercise, since, if successful, it has so much to offer in clinical care as well as in biomedical research.

ACKNOWLEDGMENTS

The authors thank Dr. Ballou, of the University of Pittsburgh, Drs. Trinchieri, and Solter of the Wistar Institute, Dr. Colcher of NIH, Mr. Owens of the Bioproducts for Science Inc., and Mr. Rosenberg Jr. of Accurate chemicals and Scientific Corporation for the supply of antibodies. They also thank Dr. C. Meares of U.C., Davis for supplying the reference sample of ABE and BABE, Dr. Alvarez of Cytogen for his interest and Miss Debra Jones for typing the manuscript. The work was supported by the DOE grant DE-FG02-85ER 60295 and in part by the NIHCA 05137-10.

REFERENCES

1. Perry S, Goodwin HA, Zimmerman TS. Physiology of granulocytes. Part I. *JAMA* 1968; 203:937–944.
2. Cooly H, Gardner PH. The use of selenomethonine (75 Se) as a label for canine and human platelets. *Am Soc Clin Inv* 1965; 44:1036–1037.
3. Thakur ML, Gottschalk A. Role of radiopharmaceuticals in nuclear hematology 341-360, Radiopharmaceuticals II. Soc. Nucl. Med., NY, 1979.
4. Thakur ML, Coleman RE, Welch MJ. Indium-111 labeled leukocytes for the localization of abscesses. Preparation, analysis, tissue distribution, and comparison with Ga-67 citrate. *J Lab Clin Med* 1977; 89:217–228.
5. Fredriksson UB, Fagerstam LG, Cole AWG, et al. Protein A-Sepharose Cl-4B affinity purification of IgG monoclonal antibodies from mouse ascites. S-751 82 Uppsala, Sweden, 1985.
6. Centricon-Microconcentrator. Amicon Publication No. 1-259B. Amicon, Danvers—Massachusetts, 1986.
7. DeRiemer AH, Mears CF. BLEDTA II: Synthesis of a new tumor visualizing derivative of Co(III-Bleomycin). *J Labeled Comp Radiopharm* 1981; XVIII:1517–1534.
8. Meares CF, McCall MJ, Reardan DT. Conjugation of antibodies with bifunctional chelating agents: isothiocyanate and bromoacetamide, the agents, methods of analysis, and subsequent addition of metal ions. *Anal Biochem* 1984; 142:68–78.
9. Rodwell JD, Alvarez VL, Lee C, et al. Site-specific covalent modification of monoclonal antibodies: in vitro and and in vivo evaluation. *Proc Natl Acad Sci USA* 1986; 83:2632–2636.
10. Thakur ML, Thiagarajan P, White III F, et al. Monoclonal antibodies for specific cell labeling: consideration, preparation and preliminary evaluation. *Nucl Med Biol* 1987; 14:51–58.
11. Feldman HA. Mathematical theory of complex ligand-binding system at equilibrium. *Anal BioChem* 1972; 48:317–338.
12. Thakur ML, Lavender JP, Arnot RN, et al. Indium-111-labeled autologus leukocytes in man. *J Nucl Med* 1977; 18:1012–1019.
13. Paik CH, Eckleman WC, Reba RC. Translocation of Tc-99m from low affinity sites to high affinity sites of antibody. *Nucl Med Biol* 1986; 13:359–362.
14. Intenzo CM, Desai AG, Thakur ML, et al. Comparison of leukocytes labeled with indium-111-2 mercapto N-oxide and indium-111 oxine for abscess detection. *J Nucl Med* 1987; 28:438–441.
15. MacGregor RR, Spagnolo BG, Lentnek AL. Inhibi-

tion of granulocytes adherence by ethanol, predinsone and aspirin, measured with an assay system. *N Engl J Nucl Med* 1974; 291:602–606.
16. Schroth HJ, Oberhausen E, Berberich R. Cell labeling with colloidal substances in whole blood. *Eur J Nucl Med* 1981; 6:469–472.
17. Methods of protein and nucleic acid research. Chap. I. Osterman LA, ed. New York: Springer Verlag, 1984.
18. Thakur ML, White F III, Tricheri G, et al. Specific radiolabeling of human neutrophils: evaluation of In-111 labeled antibodies B.37.2.1 [Abstract] *J Nucl Med* 1986; 27:947.
19. Meares CF, Kinetic agents or binding of metal ions and antibodies. *Nucl Med Biol* 1986; 13:311–318.
20. Locher JT, Seybold K, Andres RY, et al. Leukocyte imaging using I-123 monoclonal antibodies. Abstracts of Scientific Communication. 4th World Congress in Nucl. Med. and Biol. Buenos Aires, Argentina, 1986.
21. Locher JT, Seybold K, Andres RY, et al. Imaging in pulmonary and infectious lesions after injection of radioiodinated monoclonal anti-granulocyte antibodies. *Nucl Med Commun* 1986; 7:659–670.
22. Solter D, Knowles BV. Monoclonal antibodies defining a stage-specific mouse in ionic antigen (SSEA-1). *Proc Natl Acad Sci USA* 1978; 75:5565–5569.
23. Leibert N, Jaffe R, Taylor RJ, et al. Detection of SSEA-1 on human renal tumors. *Cancer* 1987; 59:1404–1408.
24. Fox N, Damajanov I, Knowles BV, et al. Immunohistochemical localization of mouse stage specific in ionic antigen-1 in human tissue and tumor. *Cancer Res* 1983; 43:669–678.
25. Ballou B, Jaffe R, Taylor RJ, et al. Tumor Radioimmuno location: differential antibody retention by antigenic normal tissue and tumor. *J Immunol* 1984; 132:2111–2116.