0273-2289/86/1302-0087$02.00

# Depletion of Human Lymphocytes from Peripheral Blood and Bone Marrow by Affinity Ligands Conjugated to Agarose–Polyacrolein Microsphere Beads

L. WEISS,* S. MARGEL, AND S. SLAVIN

*Department of Bone Marrow Transplantation and the Immunobiology Research Laboratory, Hadassah University Hospital, Jerusalem, Israel; and*
*The Department of Plastic Research, The Weizmann Institute of Science, Rehovot, Israel*

Received January 18, 1986; Accepted January 25, 1986

## ABSTRACT

Protein-A or goat anti-mouse-Ig (GAMIg) covalently bound to agarose-polyacrolein microsphere beads (APAMB) were employed for the removal of T cells from human peripheral blood leukocytes (PBL) and bone marrow (BM). The cell suspensions were treated with a monoclonal anti-T cell antibody (Leu-1) or monoclonal antilymphocyte antibody (CAMPATH-1) and passed through the conjugated APAMB columns.

Cell separation efficacy was determined by assaying the number and function of T cells in the final cell preparation in comparison with a sample of unseparated cells. The number of cells that form rosettes (E-RFC) with sheep red blood cells (SRBC) in a sample of PBL treated with anti-Leu-1 antibodies and subsequently passed once through GAMIg-conjugated APAMB dropped from a range of 41.5–86.0% to a range of 1.6–13.3%. The in vitro response to concanavalin-A (Con–A) dropped to a range of 0.7–27.2% (GAMIg) and a range of 1.2–21.8% (protein-A column) of the response of untreated PBL. Treatment with

*Author to whom all correspondence and reprint requests should be addressed.

CAMPATH-1 antibody and passage through a protein-A-conjugated APAMB reduced E-RFC from a range of 55.6–57.4% to a range of 3.2–3.9% and abolished the Con-A induced proliferative responsiveness to background levels.

Treatment of BM cells with CAMPATH-1 and passage of the cells through either GAMIg or protein-A conjugated APAMB columns resulted in reduction of E-RFC from a range of 12.4–17.7% to a range of 0–1% and from a range of 17.7–19% to a range of 1.6–3.2%, respectively. Viability of BM precursors, determined by the CFU-GM assay in semisolid medium, was not affected by these cell separation procedures.

The data suggest that protein-A or GAMIg-conjugated APAMB columns may be a useful tool for separation of BM cell suspensions into specific cell subsets that can be defined by monoclonal antibodies.

**Index Entries:** Cell fractionation using immunoadsorbent agarose-polyacrolein beads; bone marrow cells; depletion of T cells from human marrow; monoclonal antilymphocyte antibody (CAMPATH-1); monoclonal anti-T cell antibody (Leu-1); protein A; goal anti-mouse immunoglobulin; colony forming units (CFU-GM).

## INTRODUCTION

During the past few years, numerous techniques for cell separation, based mainly on cell size, lectins, density, and charge, have been devised (*1–4*). One of the simplest and most specific methods for separation of cells into subpopulations acording to their unique cell-surface properties is affinity chromatography. Separation of well-defined cell subsets can be accomplished with good purity and viability even when such cells comprise a small fraction of the total cell mixture. Cells can be separated by adsorption on an affinity adsorbent through interactions of cell-surface determinants or receptors with antibodies that can be bound directly or indirectly to a chromatographic column (*5–7*). In a previous publication, the separation of mouse T and B splenocytes by adsorption onto agarose-polyacrolein microsphere beads (APAMB) was described (*8*). In the present report, efficient removal of antibody-coated human T lymphocytes by protein-A or goat anti-mouse-Ig (GAMIg) covalently bound to APAMB is demonstrated. The efficacy of the procedure was established by assaying the capacity of the treated cells to form E-rosettes with sheep red blood cells (SRBC) and by studying proliferative response to concanavalin-A (Con-A) in vitro. Viability of the nonadsorbed bone marrow (BM) cells was unaffected, as determined by colony formation (CFU-GM).

Safe and high effective T cell depletion for prevention of graft versus host disease can be accomplished using a variety of techniques for purging of bone marrow cells *ex vivo* (*9–11*). It appears that the procedure presented here can be used for depletion of other defined cell subsets,

including tumor cells, for which more efficient techniques are not yet available. The potential clinical use of this technique for stem-cell purification in autologous BM transplantation in malignant hematological disorders still awaits further perfection of the technique, which is currently time consuming and involves substantial nonspecific cell loss.

## MATERIALS AND METHODS

### *Reagents*

The following reagents were purchased from commercial sources: mouse Ig, GAMIg, Con-A, and poly-L-lysine HBr (Miles Yeda Rehovot, Israel); protein-A (Sigma, St. Louis, MO); agarose (Pharmacia, Uppsala, Sweden); trypan blue and acrolein (Aldrich, Milwaukee, WI); acrolein was distilled at atmospheric pressure before use. The GAMIg was affinity purified using mouse Ig coupled to APAMB (*12*). The monoclonal antibodies, monoclonal anti-T-cell (Leu-1) and monoclonal antilymphocyte (CAMPATH-1), were obtained from Dr. R. Levy, Stanford University School of Medicine, Stanford, CA and Dr. H. Waldmann, Cambridge University, Great Britain (*13,14*).

### *Synthesis of APAMB*

The APAMB were synthesized as reported previously (*8,12,15*). Briefly, a solution containing 0.96 g agarose in 16 mL distilled water was heated to 95°C until the gel melted into a clear solution. The temperature was then decreased to 70°C and 8 mL of Polyacroleins (PA) microsphere solution (12% w/v) (*12*) heated to 70°C were added. The solution was stirred for 30 min and then poured into 100 mL of peanut oil and stirred at 300 rev/min at 70°C. Ten minutes later the solution was cooled with ice. The APAMB formed were purified from the oil by several extractions with ether. Ether was removed by evaporation. The beads obtained ranged from 50–250 μm and 150–250-μm beads were obtained by passing the beads through appropriate sieves. The APAMB with diameters ranging from 150 to 250 μm were used for the removal of the T cells. The APAMB were stored at 4°C in distilled water containing 0.05% (w/v) sodium azide.

### *Binding of Poly-L-Lysine-Gluteraldehyde to the APAMB*

The APAMB (20 mL), in 35 mL of distilled water, were shaken for 24 h with 40 mg poly-L-lysine (mw 32,600, degree of polymerization 156). The poly-L-lysine-conjugated beads formed were washed free of unbound poly-L-lysine by repeated decantation. The conjugated beads (20 mL in 35 mL $H_2O$) were then shaken for 12 h with gluteraldehyde and washed with large amounts of distilled water.

### Coupling of Proteins to the APAMB

One milliliter of APAMB containing a spacer arm of poly-L-lysine gluteraldehyde was shaken for 24 h at 4°C with 10 mg of the appropriate protein, in this case, protein-A or GAMIg, in 10 mL PBS. Unbound protein was removed by repeated decantation with large amounts of PBS. Remaining aldehyde groups were blocked by shaking the beads with 1 mL of aqueous ethanolamine (pH 7.2) for 12 h. The conjugated beads were then equilibrated with sterile saline containing 5 U/mL penicillin.

### Cell Fractionation

The protein-A or GAMIg-conjugated APAMB were packed in a siliconized Pasteur pipet plugged with glass wool. The conjugated beads were washed with RPMI-1640 medium containing 0.3% human serum albumin (HSA). A sample containing $10 \times 10^6$ marrow cells or Ficoll-Hypaque-separated peripheral blood leukocytes (*16*), washed twice and suspended in 0.3% HSA RPMI-1640, was filtered through the beads (1 mL) column at room temperature at a rate of 1 drop/50–60 s. The nonadsorbed cells were recovered by centrifugation at 500*g* for 10 min and resuspended with RPMI-140 medium (Grand Island Biological Co., Grand Island, NY) containing 10% heat-inactivated human serum, 2 m*M* glutamine, 1 U/mL penicillin, and 1 μg/mL streptomycin. The conjugated beads were rinsed with glycin-HCl buffer and then stored in saline containing 2 U/mL penicillin and 2 μg/mL streptomycin until used.

### Proliferative Responses of Lymphoid Cells to Con-A

Proliferative responses to Con-A were assayed in triplicate by adding Con-A (final 25 μg/mL) to microculture wells containing $10^5$ cells in 0.2 mL RPMI-1640 medium (Grand Island Biological Co., Grand Island, NY) containing 5% human serum, using flat bottom microculture plates (Sterilin, England). The mixture was incubated for 3 d at 37°C in an incubator containing 5% $CO_2$ in air. $^3$H-thymidine ($^3$H-TdR) (1 μCi; specific activity 5 μCi/mmol, Negev, Israel) was added to the wells 16–20 h before harvesting. The cultures were harvested, the filter strips dried, and the samples counted in a liquid scintillation counter and reported as mean counts per minute (cpm) for each triplicate sample.

### Quantitation of T Cells by Rosette Formation with SRBC

T cells were quantitated by studying the proportion of lymphocytes rosetting with SRBC. Rosette-forming cells (E-RFC) were assayed by mixing 0.1 mL of 3–5 $\times 10^6$ cells/mL with 0.1 mL of 1% suspension of washed SRBC in 5% human AB serum in RPMI-1640 medium. The cells were centrifuged at 900 rpm at room temperature for 5 min and kept at 4°C overnight. Cell pellets were gently resuspended, and a drop of the suspension was mixed with one drop of 1% methyl violet (for staining all

the lymphocytes). The number of E-RFC (three or more SRBC surrounding a lymphocyte) was determined in triplicate and reported as mean RFC.

### *Tests for Colony Formation of Granuloid-Mononuclear Committed Cells (CFU-GM)*

Cell culture of marrow cells tested for CFU-GM was performed according to the method described by Pike and Robinson (*17*). Feeder layers containing $10^6$ leukocytes from a healthy donor were used as a stimulus for colony growth. Cells were prepared on the day of the experiment or one day before in McCay 5A medium (Gibco, Grand Island, NY) containing 0.5% agar (*12*). Bone marrow cells ($2 \times 10^5$ cells/mL/plate) were suspended in the same medium containing 0.3% agar and applied over the feeder layers. Culture plates were allowed to solidify at room temperature and then incubated for 14 d at 37°C in a 5% $CO_2$ humidified atmosphere. Colonies containing more than 40 cells were counted as positive.

## RESULTS

The efficacy of the removal of human T lymphocytes from peripheral blood leukocytes (PBL) treated with anti-Leu-1 or CAMPATH-1 antibodies and passed through a column containing protein-A or GAMIg-conjugated APAMB is demonstrated in Tables 1–4. Table 1 shows the percent of E-RFC in three different experiments. The untreated peripheral blood cells contained 41.5–86.0% E-RFC. Treatment with anti-Leu-1 antibodies alone did not eliminate all the E-RFC, but, rather, left 14.0–20.0% (data not shown). Passage of the anti-Leu-1 treated cells through protein-A-conjugated APAMB diminished the E-RFC to 8.5–16.6% (10–31.9% of the original RFC).

Depletion of T cells from PBL was also examined by assaying in vitro

TABLE 1
Removal of E-RFC from PBL by Treatment with Anti-Leu-1 and Passage Through GAMIg or Protein A-Conjugated APAMB

| Exp. | Untreated PBL, % RFC | Anti-Leu-1-treated PBL passed over GAMIg-APAMB | | Anti-Leu-1-treated PBL passed over protein-A APAMB | |
|---|---|---|---|---|---|
| | | % RFC | % Depletion | % RFC | % Depletion |
| 1 | 86.0 | 12.5 | 85.6 | 8.5 | 90.1 |
| 2 | 52.0 | 1.6 | 97.0 | 16.6 | 68.1 |
| 3 | 41.5 | 13.3 | 68.0 | 11.9 | 71.3 |
| Mean | 59.8 ± 16.4 | 9.1 ± 4.6 | 84.8 | 12.3 ± 2.9 | 86.9 |

TABLE 2
Removal of Con-A Responding T Lymphocytes from PBL by Treatment with Anti-Leu-1 and Passage Through GAMIg-Conjugated APAMB

| Experiment | Cultured cells | $^3$H-TdR uptake (mean cpm ± SD) Controls | Con-A | % Reduction |
|---|---|---|---|---|
| 1 | Untreated PBL | 3669 ± 681 | 229,906 ± 35,414 | 99.3 |
| | Treated PBL | 540 ± 145 | 2176 ± 903 | |
| 2 | Untreated PBL | 506 ± 26 | 57,084 ± 2777 | 88.6 |
| | Treated PBL | 414 ± 98 | 6842 ± 825 | |
| 3 | Untreated PBL | 687 ± 208 | 34,396 ± 3994 | 72.8 |
| | Treated PBL | 304 ± 4 | 9492 ± 511 | |
| 4 | Untreated PBL | 131 ± 34 | 70,836 ± 7989 | 98.5 |
| | Treated PBL | 183 ± 113 | 1241 ± 126 | |
| 5 | Untreated PBL | 428 ± 213 | 29,803 ± 2744 | 79.3 |
| | Treated PBL | 366 ± 87 | 6444 ± 323 | |
| Mean of five experiments | | | | 87.7 |

proliferative responsiveness to stimulation with a T-cell mitogen. The proliferative response of nonadsorbed cells to Con-A compared to the original cell suspension was assessed by measuring the incorporation of $^3$H-TdR after 3 d of culture. The anti-Leu-1-treated cells subsequently passed through a GAMIg-conjugated APAMB column showed a median of 11.4% $^3$H-TdR uptake when compared to that of unseparated PBL (Table 2). Similarly, anti-Leu-1-treated cells passed through protein-A-conjugated APAMB resulted in a median of 18.8% $^3$H-TdR uptake, compared with unseparated PBL (Table 3).

The E-RFC of peripheral blood cells treated with CAMPATH-1 and passed through a protein-A-conjugated APAMB column diminished from 52.6 to 3.2% in one experiment and from 57.4 to 3.9% in another

TABLE 3
Removal of Con-A Responding T Lymphocytes from PBL Treated with Anti-Leu-1 and Passage Through Protein A-Conjugated APAMB

| Experiment | Cultured cells | $^3$H-TdR uptake (mean cpm ± SD) Controls | Con-A | % Reduction |
|---|---|---|---|---|
| 1 | Untreated PBL | 131 ± 34 | 70,836 ± 7989 | 81.2 |
| | Treated PBL | 192 ± 34 | 13,507 ± 628 | |
| 2 | Untreated PBL | 428 ± 213 | 29,803 ± 2744 | 78.2 |
| | Treated PBL | 1042 ± 934 | 7447 ± 570 | |
| 3 | Untreated PBL | 3669 ± 681 | 229,906 ± 35,414 | 98.8 |
| | Treated PBL | 1603 ± 156 | 4250 ± 1072 | |
| Mean of three experiments | | | | 86.1 |

TABLE 4
Removal of Con-A Responding T Lymphocytes from PBL by Treatment with CAMPATH-1 and Passage Through Protein A-Conjugated APAMB

| Experiment | Cultured cells | $^3$H-TdR uptake (mean cpm ± SD) Controls | Con-A | % E-RFC | % Reduction |
|---|---|---|---|---|---|
| 1 | Untreated PBL | 1174 ± 689 | 43,903 ± 4417 | 52.6 | 93.9 |
| | Treated PBL | 1405 ± 32 | 1167 ± 715 | 3.2 | |
| 2 | Untreated PBL | 1410 ± 362 | 47,936 ± 2559 | 57.4 | 93.2 |
| | Treated PBL | 3650 ± 2591 | 1895 ± 321 | 3.9 | |
| Mean of | Untreated PBL | | | 55.0 ± 1.9 | 93.6 |
| Mean of | Treated PBL | | | 3.5 ± 0.3 | |

(Table 4). The proliferative response to Con-A was completely abolished to background levels (Table 4).

The ability of depleted BM cells to form granulocyte–macrophage colonies CFU-GM was examined in vitro (Table 5). Three out of four experiments showed that treatment with the antibodies followed by passage through GAMIg or Protein-A-coated columns had no significant effect on CFU-GM. In one experiment, however, the number of CFU-GM was reduced to approximately 50%. The recovery of CFU-GM stem cells activity in the four experiments ranged from 36–53% of CFU-GM in unseparated BM.

Experiments in which both protein-A and GAMIg-conjugated APAMB columns were reused several times after removing adsorbed

TABLE 5
Effect of T-Cell-Depletion Procedures on BM CFU-GM

| Experiment | Experimental procedure CAMPATH-1 | Column | CFU-GM/2 × $10^5$ cells |
|---|---|---|---|
| 1 | − | Control | 146–156 |
| | + | GAMIg-conjugated APAMB | 125–138 |
| | + | Protein-A-conjugated APAMB | 117–126 |
| 2 | − | Control | 195–201 |
| | + | Protein-A-conjugated APAMB | 167–177 |
| 3 | − | Control | 65 |
| | + | Protein-A-conjugated APAMB | 80 |
| 4 | − | Control | 338–358 |
| | + | Protein-A-conjugated APAMB | 116–127 |
| | + | GAMIg-conjugated APAMB | 124–137 |

cells with glycine-HCl buffer showed that the efficiency of peripheral blood T lymphocyte removal was substantially reduced (by 30–50%), as measured by both E-RFC and $^3$H-TdR uptake (data not shown). This was the case for both anti-Leu-1- and CAMPATH-1-treated cells.

The most efficient antibody used in this study was the CAMPATH-1, which, in some preparations, removed most, if not all, of the E-RFC with the aid of a GAMIg-conjugated APAMB column (Tables 4 and 6). The second step of the treatment—adsorption of the antibody-coated cells on a column—showed that the GAMIg-conjugated APAMB column was more efficient than the protein-A-conjugated APAMB column [3.3% residual E-RFC compared to 13.1% residual E-RFC, respectively; (Table 6)].

TABLE 6
Removal of E-RFC from BM Cells Treated with CAMPATH-1 and Passed Through Protein A or GAMIg-Conjugated APAMB

| Experiment | Experimental procedure | | | % E-RFC | % Depletion |
|---|---|---|---|---|---|
| | | CAMPATH-1 | Column | | |
| 1 | Unseparated cells | − | — | 19.0 | |
| | Separated cells | + | Protein A-conjugated APAMB | 1.6 | 91.6 |
| 2 | Unseparated cells | − | — | 12.4 | |
| | Separated cells | + | GAMIg-conjugated APAMB | 1.0 | 91.9 |
| 3 | Unseparated cells | − | — | 17.7 | |
| | Separated cells | + | Protein A-Conjugated APAMB | 3.2 | 81.9 |
| | Separated cells | + | GAMIg-conjugated APAMB | 0.0 | 100.0 |
| Mean of 1 and 3 | Unseparated cells | − | — | 18.35 | |
| | Separated cells | + | Protein A-conjugated APAMB | 2.4 | 86.9 |
| Mean of 2 and 3 | Unseparated cells | − | — | 15.0 | |
| | Separated cells | + | GAMIg-conjugated APAMB | 0.5 | 96.7 |

## DISCUSSION

In the present communication, we have described the potential application of a new type of cell affinity chromatography using GAMIg- or protein-A-bound APAMB. The technique described was efficient in removal of functional T cells from BM, without affecting BM stem cells. Thus, this technique might also be applicable for removal of other subpopulations that can be defined by cell-surface specific antibodies. Potential targets for such an approach are tumor cells present in the marrow that can be removed in vitro, prior to autologous BM transplantation.

The reagents used in this work are potentially universal affinity adsorbents since most monoclonal antibodies are of murine origin and many antibody molecules can be bound to protein-A through the Fc segment. The APAMB display a number of advantages over conventional adsorbent beads. The APAMB fulfill the essential requirements of an effective insoluble support. They are stable, not toxic to the cells, and the nonspecific interaction between the cells and the conjugated beads is negligible (*8*). Agarose provides the strength and the high specificity of the beads. The encapsulated polyacrolein microspheres provide a high concentration of reactive aldehyde groups through which proteins are covalently bound in a single step under physiological pH to form the Schiff base products. The lack of leakage of proteins bound to the beads into the solution is explained by the polyvalent Schiff base bonds formed (*15*).

The APAMB-passed, T-cell-depleted BM precursors maintain their ability to form CFU-GM in vitro. Although these experiments indicate that CFU-GM are for the most part not affected by the cell separation procedures used, no firm conclusion can be made regarding CFU-GM recovery efficiencies because the number of colonies obtained in these experiments may represent the combined effect of some enrichment of stem cells as a consequence of T cell depletion and some loss of stem cells. In addition, stem cell activity may be altered in vitro as a result of the possible regulatory effects of T cells producing colony-stimulating activity (*18*). Nevertheless, despite the fact that CAMPATH-1 binds to B-lymphocytes as well as monocytes, the total cell recovery after passage through APAMB column was 40–44%. Recovery of CFU-GM stem cell activity was 36–53% of unseparated BM. The techniques described justify further experiments toward application of similar methodologies as part of the procedures required to deplete malignant cells bearing detectable cell-surface determinants in autologous BM transplantation.

Separation of specific cell subsets that can be defined by monoclonal antibodies by cell affinity chromatography has several advantages over cytolysis, using xenogeneic sera as a source of complement, mainly because of their potential toxicity to stem cells. In addition, several classes of antibodies may not result in adequate lytic effect. Likewise, certain cell

types, particularly tumor cells, may not be as readily lysed as normal lymphoid cells.

There is one drawback to the procedure in its present form and that is the impractibility of removing cell subsets from large volumes of cells resulting from the slow elution rate. Application of this methodology for transplantation in large animals requires changes that would result in a relatively short overall procedure time.

## ACKNOWLEDGMENTS

We wish to thank Dr. H. Waldmann for providing the CAMPATH-1 and for valuable assistance in the preparation of this manuscript and Ms. Offarim for assistance in the preparation of APAMB. This research was supported by grants from: the National Institutes of Health (CA 30313); Ministry of Health (Israel); Lady Tata Memorial Trust; Israel Cancer Research Fund; and the Bergman Foundation.

## REFERENCES

1. Dicke, K. A., and Van Bekkum, D. W. (1971), *Transplant. Proc.* **3,** 666.
2. Shortman, K. (1974), *Cont. Topics in Mol. Immunol.* **3,** 161.
3. Sabalovic, D., and Serron, B., (eds.) (1976), in *Techniques of separation and characterization of human lymphocytes.* Editions INSERM, Paris.
4. Reisner, Y., Linker-Israeli, M., and Sharon, N. (1976) *Cell Immunol.* **25,** 129.
5. Chess, L., MacDermott, R. P., and Schlossman, S. F. (1974), *J. Immunol.* **113,** 1113.
6. Manderino, G. L., Gooch, G. T., and Stavitsky, A. B. (1978), *Cell Immunol.* **41,** 264.
7. Marshak-Rothstein, A., Fink, P., Gridley, T., Raulet, D. H., and Bevan, J. (1979), *J. Immunol.* **122,** 2491.
8. Margel, S., Offarim, M., and Eshhar, Z. (1983), *J. Cell. Sci.* **62,** 149.
9. Reisner, Y., Kapoor, N., Kirkpatrick, P., Pollack, M. S., Cunningham-Rundles, S., Dupont, B., Hodes, M. Z., Good, R. A., and O'Reilly, R. S. (1983), *Blood* **61,** 341.
10. Waldmann, H., Polliack, A., Hale, G., Or, R., Cividalli, G., Weiss, L., Weshler, Z., Samuel, S., Manor, D., Brautbar, C., Rachmielewitz, E. A., Slavin, S. (1984) *Lancet* **2,** 438.
11. Slavin, S., Waldmann, H., Or, R., Cividalli, G., Naparstek, E., Steiner-Salz, O., Michaeli, J., Galun, E., Weiss, L., Samuel, S., Morecki, S., Bar, S., Brautbar, C., Weshler, Z., Hale, G., Rachmilewitz, E. A., and Reisner, Y., (1985), *Transplant. Proc.* **17,** 465.
12. Margel, S., Beitler, U., and Offarim, M. (1982), *J. Cell Sci.* **56,** 157.
13. Engleman, E. G., Warnke, R., Fox, R. J., and Levy, R. (1981), *Proc. Natl. Acad. Sci.* **78,** 1791.
14. Hale, G., Bright, S., Chubley, G., Hoang, T., Metcalf, D., Mumra, A. J., and Waldmann, H. (1983), *Blood* **62,** 873.
15. Margel, S., Offarim, M. (1983), *Anal. Biochem.* **128,** 342.
16. Boyum, A. (1968), *Scand. J. Clin. Lab. Invest. Suppl.* **21,** 77.
17. Pike, B. L., and Robinson, W. A. (1970), *J. Cell Physiol.* **76,** 77.
18. Cline, M. J., and Golde, D. W. (1974), *Nature* **248,** 703.