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# RELATIVE EFFICACY OF HUMAN MONOCYTES AND DENDRITIC CELLS AS ACCESSORY CELLS FOR T CELL REPLICATION*

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Two recent studies (1, 2) have identified cells in human blood that fully resemble the dendritic cells described previously in mice and rats (3). Among other similarities, the human equivalent is Ia positive and Fc receptor negative, occurs in trace numbers (<1% of blood mononuclear cells), and acts as a potent stimulator of T cell proliferation in vitro. For example, preparations enriched in dendritic cells are 10–100 times more active than monocytes or lymphocytes in stimulating the syngeneic and allogeneic mixed leukocyte reactions (MLR)[1] as well as oxidative mitogenesis—the proliferation of periodate-modified T cells (1, 2). Therefore the prevailing concept that monocytes are the principal accessory cells in man must be reexamined.

Monoclonal antibodies that distinguish macrophages from dendritic cells provide new probes for accessory or stimulator cells in the immune response. Selective depletion of murine dendritic cells, with specific antibody and complement, decreases accessory function dramatically (4–6). In man, antidendritic cell antibodies are not available, but alternative and useful reagents have been obtained. For example, 3C10 and 1D9 are related antimacrophage antibodies that do not react with dendritic cells, while 9.3F10 is an anti-HLA class II reagent (7) that reacts with both cell types. In this paper, we use these monoclonals to study the requirements for T cell proliferation. T cell growth is severely reduced when accessory cells are depleted with 9.3F10 and complement, or when an Fab fragment of 9.3F10 is added to the culture. Positive and negative monocyte selection experiments, with the fluorescence-activated cell sorter and with complement-mediated cytolysis, indicate that monocytes contribute little if at all to accessory function. In contrast, highly enriched and monocyte-depleted dendritic cells are potent stimulators of the syngeneic and allogeneic MLR and the response to soluble tetanus toxoid. Monocytes and dendritic cells express similar levels of Ia antigens, however, indicating that class II products need to be expressed on dendritic cells to induce several T cell-proliferative responses in man.

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[1] *Abbreviations used in this paper*: C′, complement; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; MLR, mixed leukocyte reaction; PBS, phosphate-buffered saline.

## Materials and Methods

*Monoclonal Antibodies.* The antibodies used in this study were described previously (7). 3C10 ($IgG_{2b}$) and 1D9 ($IgG_1$) are antimonocyte (7); BA-1 (IgM) and Leu-1 ($IgG_{2a}$) are anti-B and -T lymphocyte antibodies, purchased from Hybritech, Inc., La Jolla, CA and Becton, Dickinson & Co., Oxnard, CA, respectively; 9.3F10 ($IgG_{2a}$) reacts with class II major histocompatibility products or Ia-like antigens (7). Purified Ig preparations were used throughout.

*Cytotoxicity Assays.* A one-stage cytotoxicity protocol was used in which equal volumes of cells, antibody, and rabbit complement (C′) were incubated for 60 min at 37°C. All reagents were diluted in RPMI 1640 (Gibco Laboratories, Grand Island, NY), 0.3% bovine serum albumin, 25 mM Hepes buffer, and 10 μg/ml deoxyribonuclease (type I; Sigma Chemical Co., St. Louis, MO). The final concentrations of reagents were 1.6–2.5 × $10^6$/ml for cells, 1–10 μg/ml for antibodies, and 1:9 for rabbit serum reconstituted from lyophilized samples from two different rabbits. After treatment, the cells were washed three times and used as accessory cells or as responders in T cell proliferation assays. All cell numbers in Results are viable (trypan blue excluding) counts. Elimination of monocytes was monitored by cytology and nonspecific esterase staining (1).

*Cell Sorting.* Adherent blood mononuclear cells were cultured overnight and the released fraction (50% or more monocytes) was sorted into monocyte-rich and depleted components using fluoresceinated 1D9 antimonocyte antibody and a fluorescence-activated cell sorter (FACS II; B-D FACS Systems, Sunnyvale, CA), equipped with a Spectra-Physics 5 W argon-ion laser. Optical filtration was done by placing 520- and 530-nm "cut-on" filters (Ditric Optics, Hudson, MA) in series. The following instrument parameters were used in all cell sorting and analytical experiments: fluorescence-excitation wavelength, 488.8; laser power, 300 mW; photomultiplier voltage, 750 V; fluorescence preamplifier setting, 1–8; light scatter preamplifier setting, 2. Data were collected and displayed as dual parameter contour diagrams of fluorescence and light scattering intensity. Generally, the cells were sorted at ~2,200 cells per minute. The abort rate was <15% and total cell recovery was 80%. Directly fluoresceinated 1D9 was used to stain cells because, relative to indirect immunofluorescence, there were fewer dead cells (3 vs. ≥10%) and staining was simpler and faster. 1D9 antibody was conjugated to fluorescein isothiocyanate isomer 1 (FITC-celite, F-1628; Sigma Chemical Co.). 0.5 mg of 1D9 Ig and 0.5 mg of FITC-celite were mixed for 1 h at room temperature in 0.33 ml sodium carbonate, 0.1 M, pH 9.5. Unconjugated FITC was removed by gel filtration on a 7.5 × 600-mm LKB TSK-3000 column (LKB-Produkter AB, Bromma, Sweden) in 0.1 M $Na_2PO_4$, pH 6.5, with 0.02% $NaN_3$. The resulting reagent had a fluorescein/protein ratio of 4.8 and was used to stain cells at 5 μg for 5 × $10^6$ cells/ml RPMI 1640 with 10% horse serum, on ice for 45 min. The cells were washed twice in phosphate-buffered saline before sorting under sterile conditions.

*Cells.* Blood samples were obtained from normal volunteers in our laboratory, or were purchased as buffy coats from the Greater N. Y. Blood Center. Whole or unfractionated mononuclear cells were prepared from Ficoll-Hypaque columns. Adherent cells were selected after culture on 100-mm plastic petri dishes (Falcon Labware, Oxnard, CA) in RPMI 1640 supplemented with 5% fetal calf serum. The adherent cells represented 20–40% of total cells and typically were comprised of 50–70% monocytes, 20–40% B cells, 10–20% T cells, and 1–4% dendritic cells. After overnight culture, 60–80% of the adherent cells had detached, and these were either used as accessory cells directly or to prepare dendritic cells. Monocytes that remained attached to the dish after overnight culture were shown previously to be weak or inactive as accessory cells (1). Dendritic cells were enriched by a new technique described in the accompanying paper (7). Briefly, adherent cells were cultured overnight, treated with 3C10 and C′ for 1 h, washed, and treated again with 3C10 along with BA-1 and Leu-1 (anti-B and anti-T cell) antibodies and C′. After 2 h of additional culture, viable cells were retrieved by flotation on dense albumin columns. 0.1–0.2% of the starting mononuclear cells were recovered, and 70–80% were dendritic cells by cytologic criteria; contamination with monocytes and lymphocytes was <2%. Control low density populations were adherent cells that had been

exposed to C′ in the absence of antibody; these contained 60% monocytes and 5–10% dendritic cells. Monocyte-enriched fractions were also obtained from firmly adherent populations (see above and reference 1) detached with EDTA.

*T Cell Proliferation Assays.* Graded doses of viable, irradiated (3,000 rad) stimulator cells were added to $1.5 \times 10^5$ (6-mm flat microtest wells, 3596; Costar, Cambridge, MA.) or $2 \times 10^6$ (16-mm flat macrotest wells, 3524; Costar) responding T cells, obtained by passing nonadherent blood mononuclear cells over nylon wool as described (1). These responder cells were unrelated to the donor (allogeneic MLR), syngeneic to the donor (syngeneic MLR), or syngeneic and modified with 2 mM sodium periodate (oxidative mitogenesis). Cultures were maintained in RPMI 1640 supplemented with 10% human AB serum, 20 μg/ml gentamycin, 100 U/ml penicillin, $5 \times 10^{-5}$ M 2-mercaptoethanol, and 1 mM glutamine. Proliferation was measured with a 1 μCi pulse of [$^3$H]thymidine (6 Ci/mM; Schwarz/Mann Division, Becton, Dickinson & Co., Orangeburg, NY) at 96–114 h (MLR) or 48–64 h (oxidative mitogenesis).

Proliferative responses to soluble antigens were performed in cultures of 0.3 and $1.0 \times 10^5$ responders in round-bottomed wells (Linbro Chemical Co., Hamden, CT). 15% autologous plasma, rather than 10% AB serum was used, because it supported higher and more consistent proliferative responses. The antigens were tetanus toxoid (Massachusetts Dep't of Health, Boston, MA.) and Candida albicans extract (Hollister-Stier, Spokane, WA.). Cultures were pulsed with 1 μCi of [$^3$H]thymidine at 120–140 h. Responder cells were either unfractionated mononuclear cells or purified T cells prepared by passing nonadherent populations over nylon wool columns followed by further depletion of accessory cells with 9.3F10 (anti-Ia) antibody and complement.

*Blocking and Binding Studies with Anti-Ia or Anti-Class II Antibodies.* 9.3F10 is considered to be an anti-class II reagent because it precipitates typical 33,000 and 29,000 mol wt class II polypeptides from monocytes, and reacts with B cells, monocytes, dendritic cells, and B cell lines, but not T cells or HLA-DR-negative lines such as K562, CEM T, U937, HL60, and Jurkat (7). The precise specificities identified by 9.3F10 have not been defined. An Fab fragment was prepared by papain digestion of ascites-derived Ig as described (7). The Fab fragment was stored as a sterile solution of 500 μg/ml and used at a concentration of 6 μg/ml to block T cell proliferative responses in vitro. Control noninhibitory Fab fragments were 3C10 (antimonocyte) and 3G8 (antid-Fcγ receptor, kindly provided by Dr. Howard Fleit and colleagues, [8]). On a molar basis, 9.3F10 Fab was 10 times less effective than 9.3F10 Ig in blocking proliferative responses to tetanus toxoid. Quantitative binding studies with $^{125}$I-9.3F10 Fab were performed on $0.5–1 \times 10^5$ purified dendritic cells and monocytes, as described (7).

## Results

Monoclonal antimonocyte antibodies were used in three sets of experiments to identify the active stimulator for T cell growth in man. Specifically, the antibodies were used to deplete monocytes by C′-mediated cytotoxicity, to sort monocytes from other cell types on the FACS, and to help purify dendritic cells. Since Ia$^+$ monocytes proved to be weak accessory cells, we tested whether all proliferative responses were mediated by HLA class II determinants and compared the expression of class II antigens on monocytes and dendritic cells in quantitative binding studies.

*Selective Monocyte Depletion with Antibody and C′ Does Not Reduce Stimulatory Capacity for the MLR and Oxidative Mitogenesis.* 3C10 is an $IgG_{2b}$ monoclonal antibody that in the presence of rabbit C′ kills >95% of human monocytes but no other blood cells including dendritic cells (7). In the experiments described here, monocyte depletion was monitored by nonspecific esterase staining (see Fig. 1 and legends to Tables I–III). Monocyte-depleted mononuclear cells were fully capable of stimulating the allogeneic MLR and oxidative mitogenesis, even

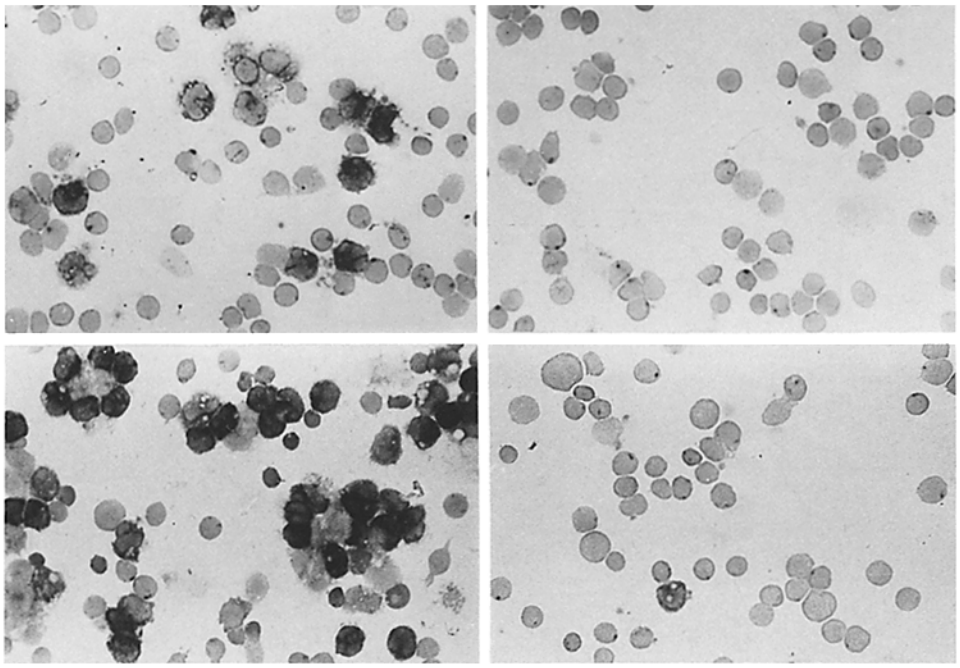

FIGURE 1. Elimination of monocytes with 3C10 and C′. Cells were exposed to C′ only (*left*) or 3C10 antibody and C′ (*right*), after which cytospin preparations were stained for nonspecific esterase. The top frames are unfractionated mononuclear cells and the lower frames adherent populations. Cells with dark cytoplasmic staining were classified as monocytes. Monocyte-depleted populations contain lymphoid cells with single, esterase-positive granules. × 400.

at limiting stimulator to responder ratios (Table I). In contrast, treatment with the anti-class II monoclonal antibody 9.3F10 (7) and C′ totally eliminated stimulating capacity (Table I). In most instances (e.g., experiments 2 and 3, Table I), treatment with anti-Ia alone was partially inhibitory.

The monocyte depletion experiments were repeated using adherent mononuclear cells as stimulators. These cells were enriched in stimulating capacity, relative to total mononuclear cells (compare the proliferative responses in Tables I and II). Removal of monocytes (Fig. 1) did not reduce accessory function and, in some cases, stimulatory function was actually increased (experiments 1 and 2, Table II). The latter can be attributed to the increased percentage of dendritic cells in the monocyte-depleted adherent fractions. In all cases, significant MLR and oxidative mitogenesis were induced even at limiting stimulator to responder ratios (1:12). At this dose, cultures stimulated with monocyte-depleted adherent cells had <1 monocyte per 2,000 T cells.

*Antigen-induced Proliferative Responses in Monocyte-depleted Cultures.* It is known that large numbers of monocyte-enriched adherent cells (~5% of the culture) are required to reconstitute normal antigen-induced proliferative response by purified T lymphocytes (9–13). The number of monocytes surviving treatment with 3C10 antibody and C′ was so low (<1% of the culture) that we could ask whether selective monocyte depletion had any effect on proliferative responses to soluble protein antigens. Responses to tetanus toxoid and Candida albicans

TABLE I

*Accessory Function of Monocyte-depleted Blood Mononuclear Cells: Stimulation of the 1° MLR and Oxidative Mitogenesis*

| Treatment of mononuclear cells | | Proliferative response (cpm [$^3$H]thymidine) to graded doses of stimulators | | | | | |
|---|---|---|---|---|---|---|---|
| Antibody | Complement | Oxidative mitogenesis | | | 1° MLR | | |
| Experiment 1 | | 33% | 16% | 8% | 33% | 16% | 8% |
| None | None | 25,652 | 19,006 | 13,253 | 24,315 | 17,807 | 8,838 |
| None | Active | 25,811 | 18,604 | 13,828 | 43,292 | 26,914 | 12,443 |
| Antimonocyte | Heated | 24,255 | 15,371 | 11,525 | 42,217 | 19,604 | 10,666 |
| Antimonocyte | Active | 34,551 | 22,173 | 11,101 | 45,862 | 22,392 | 9,406 |
| No stimulators | | | 2,736 | | | 5,771 | |
| Experiment 2 | | | | | 33% | 16% | 8% |
| None | None | | | | 34,251 | 16,675 | 8,395 |
| None | Heated | | | | 17,693 | 9,134 | 5,412 |
| None | Active | | | | 17,560 | 13,348 | 4,281 |
| Antimonocyte | Heated | | Not tested | | 20,586 | 18,262 | 6,839 |
| Antimonocyte | Active | | | | 24,117 | 10,344 | 3,125 |
| Anti-Ia | Heated | | | | 6,447 | 3,915 | 2,858 |
| Anti-Ia | Active | | | | 1,340 | 384 | 275 |
| No stimulators | | | | | | 77 | |
| Experiment 3 | | 50% | 25% | 13% | | | |
| None | None | 30,928 | 24,404 | 15,804 | | | |
| None | Heated | 17,554 | 14,415 | 11,441 | | | |
| None | Active | 10,137 | 10,369 | 8,351 | | | |
| Antimonocyte | Heated | 11,971 | 12,694 | 13,536 | | Not tested | |
| Antimonocyte | Active | 28,570 | 14,309 | 6,017 | | | |
| Anti-Ia | Heated | 8,263 | 7,930 | 9,025 | | | |
| Anti-Ia | Active | 1,610 | 2,418 | 4,013 | | | |
| No stimulators | | | 673 | | | | |
| Experiment 4 | | | | | 33% | 16% | 8% |
| None | None | | | | 32,534 | 13,396 | 9,764 |
| None | Active | | Not tested | | 11,375 | 4,175 | 4,089 |
| Antimonocyte | Heated | | | | 44,630 | 18,740 | 12,089 |
| Antimonocyte | Active | | | | 34,413 | 16,610 | 6,833 |
| No stimulators | | | | | | 158 | |

$1.5 \times 10^5$ autologous periodate-treated (oxidative mitogenesis) or allogeneic (1° MLR), nylon-nonadherent T cells were cultured in triplicate microtest wells with graded doses of irradiated mononuclear cells that had been treated with antibody and C′ as shown. Rabbit C′ was either freshly reconstituted (active) or heat-inactivated at 56°C for 30 min (heated). The cells were pulsed with 1 μCi/well [$^3$H]thymidine at 54–72 h for oxidative mitogenesis or 128–144 h for the 1° MLR. The data is expressed as the mean cpm. Standard deviations were <15%. The percentage of monocytes in the stimulator populations, by diffuse nonspecific esterase staining, was:

| Experiment | No treatment | Antimonocyte + heated C′ | Active C′ only | Antimonocyte + active C′ | Anti-Ia + active C′ |
|---|---|---|---|---|---|
| 1 | 43 | 42 | 41 | 2 | Not done |
| 2 | 43 | 45 | 43 | 1 | 33 |
| 3 | 32 | Not done | 28 | 0.5 | 6 |
| 4 | 30 | 33 | 32 | 1 | Not done |

TABLE II

*Accessory Function of Monocyte-depleted Adherent Blood Cells*

| Treatment of adherent cells | | Proliferative response (cpm [$^3$H]thymidine) to graded doses of stimulators | | | | | |
|---|---|---|---|---|---|---|---|
| Antibody | Complement | 33% | 16% | 8% | 33% | 16% | 8% |
| | | Oxidative mitogenesis | | | 1° MLR | | |
| Experiment 1 | | | | | | | |
| None | None | 70,900 | 46,014 | 35,548 | | | |
| None | Active | 44,198 | 38,074 | 21,337 | | | |
| Antimonocyte | Heated | 68,677 | 56,199 | 38,171 | Not tested | | |
| Antimonocyte | Active | 136,857 | 95,795 | 73,876 | | | |
| No stimulators | | | 847 | | | | |
| Experiment 2 | | | | | | | |
| None | None | 41,679 | 25,379 | 20,149 | 52,983 | 35,866 | 27,033 |
| None | Active | 28,305 | 21,744 | 17,726 | 71,700 | 36,617 | 20,074 |
| Antimonocyte | Heated | 59,079 | 55,640 | 36,476 | 126,355 | 84,172 | 42,425 |
| Antimonocyte | Active | 84,663 | 89,190 | 61,279 | 144,122 | 139,980 | 81,070 |
| No stimulators | | | 2,091 | | | 1,581 | |
| Experiment 3 | | | | | | | |
| None | None | 35,689 | 25,567 | 16,797 | | | |
| None | Active | 47,071 | 31,329 | 24,002 | | | |
| Antimonocyte | None | 40,369 | 30,864 | 19,835 | Not tested | | |
| Antimonocyte | Active | 57,626 | 33,039 | 20,187 | | | |
| No stimulators | | | 1,421 | | | | |
| Experiment 4 | | | | | | | |
| None | None | | 29,951 | 19,955 | | 10,685 | 7,802 |
| None | Heated | | 32,934 | 21,514 | | 14,365 | 5,563 |
| None | Active | | 26,578 | 15,195 | | 11,560 | 6,649 |
| Antimonocyte | Heated | | 21,737 | 15,178 | | 10,725 | 7,523 |
| Antimonocyte | Active | | | 30,425 | | | 11,622 |
| Anti-Ia | Heated | | 1,717 | 1,737 | | 2,070 | 1,006 |
| Anti-Ia | Active | | 906 | 829 | | 993 | 927 |
| No stimulators | | | 1,109 | | | 373 | |

The experiments were constructed as in Table I except that irradiated adherent mononuclear cells were used as accessory cells or stimulators. The percentage of monocytes by nonspecific esterase staining was:

| Experiment | No C′ | C′ only | Antimonocyte + C′ |
|---|---|---|---|
| 1 | 40 | — | 0.2 |
| 2 | 60 | — | 0.5 |
| 3 | 53 | 50 | 4 |
| 4 | 53 | 51 | 1 |

remained intact in the virtual absence of monocytes even under culture conditions that were limiting in terms of antigen dose, cell dose, and time in culture (Table III). Treatment with 9.3F10 antibody (anti-class II or anti-Ia-like) and C′ dramatically reduced proliferative responses. Therefore monocytes seem unnecessary for proliferative responses to soluble antigens; treatment with anti-class II antibodies and C′ probably eliminates responses by killing another cell type such as the Ia$^+$ dendritic cell.

*Sorting of Monocytes Using Fluoresceinated Monoclonal Antibody.* Directly fluores-

TABLE III

*Monocyte Depletion Does Not Alter Responses to Soluble Antigens*

| Treatment of blood mononuclear cells | | Proliferative response (cpm [$^3$H]thymidine) | | | | | |
|---|---|---|---|---|---|---|---|
| | | $1 \times 10^5$ cells | | | $3 \times 10^4$ cells | | |
| Antibody | Complement | No antigen | Candida | Tetanus toxoid | No antigen | Candida | Tetanus toxoid |
| Experiment 1 | | | | | | | |
| None | None | 7,554 | 19,903 | 59,743 | | | |
| None | Heated | 7,803 | 18,935 | 48,078 | | | |
| None | Active | 8,963 | 17,030 | 54,236 | | | |
| Antimonocyte | Heated | 6,543 | 9,353 | 47,567 | Not tested | | |
| Antimonocyte | Active | 12,234 | 19,581 | 56,637 | | | |
| Anti-Ia | Heated | 11,178 | 24,732 | 46,219 | | | |
| Anti-Ia | Active | 139 | 325 | 4,906 | | | |
| Experiment 2 | | | | | | | |
| None | Heated | 5,205 | 15,052 | 10,649 | 983 | 1,541 | 3,733 |
| None | Active | 4,443 | 15,183 | 11,203 | 338 | 1,627 | 3,112 |
| Antimonocyte | Heated | 3,976 | 8,018 | 10,448 | 734 | 2,281 | 2,237 |
| Antimonocyte | Active | 3,493 | 13,592 | 15,809 | 238 | 1,567 | 3,130 |
| Anti-Ia | Heated | 879 | 4,410 | 7,115 | 538 | 1,545 | 1,929 |
| Anti-Ia | Active | 218 | 668 | 1,434 | 161 | 360 | 707 |
| Experiment 3 | | | | | | | |
| None | None | 1,552 | 6,053 | 15,868 | 1,143 | 1,223 | 3,745 |
| None | Heated | 3,883 | 4,354 | 17,138 | 567 | 1,208 | 5,279 |
| None | Active | 2,772 | 5,455 | 17,115 | 445 | 1,602 | 4,638 |
| Antimonocyte | Heated | 2,334 | 4,765 | 17,674 | 751 | 762 | 5,174 |
| Antimonocyte | Active | 1,832 | 4,485 | 19,679 | 524 | 461 | 5,177 |
| Anti-Ia | Heated | 921 | 2,376 | 14,738 | 702 | 573 | 3,786 |
| Anti-Ia | Active | 545 | 191 | 2,378 | 675 | 696 | 654 |

Mononuclear cells were obtained from Ficoll-Hypaque columns and treated with antibody and C′ as indicated. The cells were washed and cultured in 15% vol/vol isologous plasma at $1 \times 10^5$ or $3 \times 10^4$ cells per round-bottomed microtest well. Cultures were exposed on day 5–6 for 16–20 h with [$^3$H]thymidine at 0.5 μCi/well. Experiments 1 and 3 were from the same donor. Additional components to the experiments which are not shown include: (*a*) In experiment 2, a nine-fold lower dose of antigen was used. The responses were 50% of those shown, but the effects of antibody and C′ were unchanged qualitatively. (*b*) In experiment 2, a replicate experiment was cultured for 5 d. Proliferative responses were ~75% of those shown, but qualitatively the responses of the various treatment groups were unchanged. (*c*) In experiment 3, a replicate experiment was cultured in pooled AB serum, rather than isologous plasma. The use of AB serum reduced both background (no antigen; <1,000 cpm/culture) and antigen-stimulated responses. Qualitatively the various treatment groups gave results similar to those shown. The percentage of monocytes added to the cultures, determined by nonspecific esterase staining was:

| Experiment | Control | Antimonocyte/fresh C′ | Anti-Ia/fresh C′ |
|---|---|---|---|
| 1 | 30 | 0.5 | 6 |
| 2 | 20 | 0.6 | 18 |
| 3 | 15 | 0.7 | 10 |

ceinated 1D9 was used to sort $1D9^+$ (monocytes) from $1D9^-$ cells on the FACS. In establishing the gating conditions for sorting, we noted that 1D9 stained larger profiles in both adherent (Fig. 2*A*) and unfractionated mononuclear cells (not shown); also, 1D9 staining was blocked by the addition of excess nonfluoresceinated 3C10 (Fig. 2*B*) but not by 9.3F10 (not shown). The success of the sorting procedure under our gating condition (Fig. 2*C*) was monitored by cytology and by nonspecific esterase staining. The $1D9^+$ fraction was ≥97% monocytes by both criteria, while the $1D9^-$ fraction was 2% monocytes (Fig. 3, Table IV). The $1D9^-$ fraction consisted primarily of lymphocytes, but also contained most of the dendritic cells.

Adherent cells that had been sorted with fluoresceinated 1D9 were tested for functional activity. Control studies indicated that exposure to 1D9 did not significantly alter the capacity of adherent cells to stimulate the MLR or oxidative mitogenesis (Table V, experiments 1, 3), and that unsorted cells behaved similarly to mixtures of $1D9^+$ and $1D9^-$ cells (Table V, experiment 1). When the accessory functions of the sorted populations were compared, the $1D9^-$ or monocyte-depleted fraction was consistently at least four times more active (Table V). Also, at least 75% of the total accessory activity was in the monocyte-depleted fraction, since the $1D9^-$ cells represented 30–50% of the total. The $1D9^+$ or monocyte-enriched fraction weakly stimulated the allogeneic MLR and oxidative mitogenesis, and its stimulating capacity was comparable to that of nonadherent mononuclear cells. In most experiments, the syngeneic MLR was weak (<500 cpm), probably because dendritic cells were not greatly enriched by sorting; however, in one case (Table V, experiment 2), a significant syngeneic MLR was induced only by the monocyte-depleted populations. We conclude that monocytes are not the active accessory component of adherent cells from blood.

*Accessory Function of Highly Enriched Dendritic Cells from Blood.* To obtain evidence that dendritic cells were the principal accessory cells in adherent populations, we used monoclonal antibodies (3C10, antimonocyte; BA-1, anti-B; and Leu-1, anti-T cell) to prepare highly enriched dendritic cells that were severely depleted of monocytes and lymphocytes (7, and Materials and Methods). Control populations were exposed to C′ only and therefore contained large numbers of monocytes, some lymphocytes, and some (5–12%) dendritic cells.

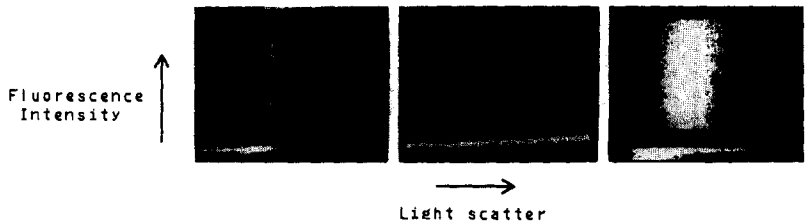


FIGURE 2. FACS profiles of adherent cells stained with fluoresceinated 1D9 antimonocyte antibody. (*A*) The fluorescence vs. light scatter profile shows that 1D9 stains most of the large cells. (*B*) A specificity control in which the same cell populations were stained with fluoresceinated 1D9 in the presence of a 20-fold excess of nonfluoresceinated 3C10, another antimonocyte antibody that competes with 1D9 (7). (*C*) Gates used to sort $1D9^+$ cells (upper rectangle) from $1D9^-$ cells (lower stripe); these gating conditions discarded cell aggregates and dead cells, but also 15% of viable single cells.

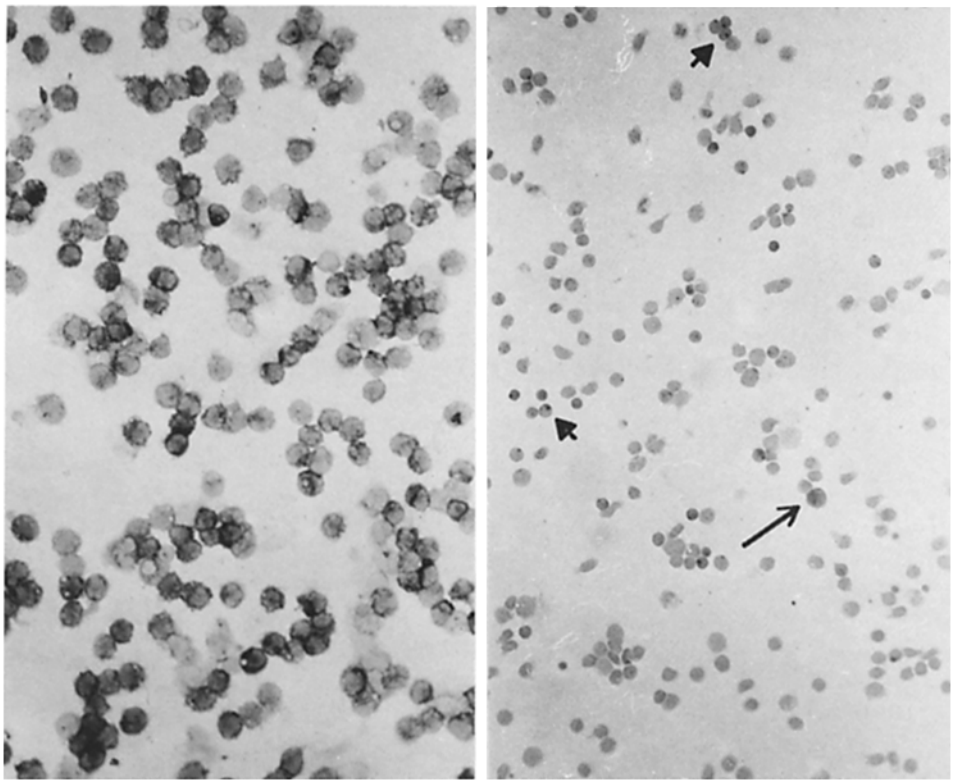

FIGURE 3. Sorting of adherent blood mononuclear cells with fluoresceinated 1D9 antimonocyte antibody. Most of the 1D9$^+$ cells (*left*) stain for nonspecific esterase. The 1D9$^-$ fraction (*right*) has ≤2% monocytes (arrows), but has lymphoid cells with single, esterase-positive granules (arrowheads). × 160.

TABLE IV

*Cell Sorting of Mononuclear Cells with Fluoresceinated 1D9*

| Population sorted | No. of expts. | Unfractionated | | 1D9$^+$ cells | | 1D9$^-$ cells | |
|---|---|---|---|---|---|---|---|
| | | Fluor$^+$ | NSE$^+$ | Fluor$^+$ | NSE$^+$ | Fluor$^+$ | NSE$^+$ |
| | | | | % | | | |
| Peripheral blood mononuclear cells | 1 | 21 | 22 | 97 | 95 | 0.5 | 0.5 |
| Adherent mononuclear cells | 5 | 37 ± 5 | 37 ± 6 | 98 ± 1 | 97 ± 2 | 1 ± 1 | 3 ±3 |

Unfractionated mononuclear cells or plastic-adherent cells were stained with fluoresceinated 1D9 and separated into 1D9-positive and 1D9-negative fractions on the FACS II, or left unseparated (unfractionated). The three populations were then evaluated for the percent (mean ± standard error) fluorescent cells (fluor$^+$) and percent cells staining diffusely for nonspecific esterase (NSE$^+$).

TABLE V

*Accessory Function of Adherent Blood Mononuclear Cells: Cell-sorting Studies with Fluoresceinated Antimonocyte Antibody*

| Stimulator population | Response (cpm [$^3$H]thymidine) to graded doses of stimulators | | | | | | |
|---|---|---|---|---|---|---|---|
| | 33% | 16% | 8% | 33% | 33% | 16% | 8% |
| | Oxidative mitogenesis | | | Syngeneic MLR | Allogeneic MLR | | |
| Experiment 1 | | | | | | | |
| Monocyte enriched (1D9$^+$) | 7,651 | 5,946 | 4,821 | | | | |
| Monocyte depleted (1D9$^-$) | 50,216 | 31,281 | 16,884 | | | | |
| Unseparated, 1D9 treated | 36,598 | 23,042 | 15,769 | Not tested | | Not tested | |
| Unseparated, not treated | 34,409 | 20,078 | 11,968 | | | | |
| Sorted, then reconstituted | 34,177 | 24,258 | 12,641 | | | | |
| No stimulators | | 334 | | | | | |
| Experiment 2 | | | | | | | |
| Monocyte enriched (1D9$^+$) | 17,675 | 13,517 | 12,685 | 140 | 32,329 | 25,993 | 14,052 |
| Monocyte depleted (1D9$^-$) | 96,404 | 64,138 | 30,339 | 2,079 | 101,548 | 59,520 | 28,235 |
| Unseparated, 1D9 treated | 66,313 | — | — | 1,448 | 44,212 | 20,965 | — |
| Nonadherent cells | 16,963 | 11,675 | 9,064 | 173 | 14,569 | 5,671 | 2,130 |
| Whole blood mononuclear cells | 38,648 | 23,450 | 13,316 | 303 | 36,609 | 27,929 | 14,612 |
| No stimulators | | 6,138 | | 99 | | 182 | |
| Experiment 3 | | | | | | | |
| Monocyte enriched (1D9$^+$) | 2,763 | 2,378 | 1,589 | 101 | 717 | 1,325 | 1,842 |
| Monocyte depleted (1D9$^-$) | 20,755 | 9,201 | 3,040 | 223 | 13,898 | 6,430 | 1,102 |
| Unseparated, 1D9 treated | 20,643 | 10,575 | 4,996 | 563 | 12,680 | 7,094 | 3,664 |
| Unseparated, not treated | 26,433 | 13,776 | 6,217 | 515 | 17,442 | 10,033 | 6,730 |
| Nonadherent cells | 464 | 341 | 406 | 92 | 106 | 97 | 73 |
| Whole blood mononuclear cells | 17,954 | 7,063 | 2,955 | 94 | 8,813 | 3,200 | 312 |
| No stimulators | | 74 | | 74 | | 224 | |

The experiments were constructed as in Table I except that irradiated adherent mononuclear cells were used as accessory cells after cell sorting with fluoresceinated 1D9 antimonocyte antibody. The percentage of fluorescent (fluor$^+$)- and nonspecific esterase-positive (NSE$^+$) cells are tabulated below. In two of the experiments, the percentage of myeloperoxidase-positive cells was also measured and recorded in parentheses (after a day in culture, monocytes are not all peroxidase positive).

| Experiment | 1D9$^+$ | | 1D9$^-$ | | Unseparated | |
|---|---|---|---|---|---|---|
| | Fluor$^+$ | NSE$^+$ | Fluor$^+$ | NSE$^+$ | Fluor$^+$ | NSE$^+$ |
| 1 | 97 | 97 | 1 | 1 | 22 | 25 |
| 2 | 98 | 96 (69) | 1 | 2 (2) | 40 | 42 (36) |
| 3 | 99 | 98 (50) | 1 | 0.6 (0.7) | 35 | 38 (17) |

The monocyte- and lymphocyte-depleted adherent cells (primarily dendritic cells) were highly enriched in MLR-stimulating capacity, and were active at stimulator to responder ratios from 1:24 to 1:100 (Fig. 4).

Purified dendritic cells were then compared to monocyte-enriched populations as stimulators of the proliferative response to soluble tetanus toxoid antigen. Purified T cells, depleted of $Ia^+$ cells with 9.3F10 and C′, were used as responders. Accessory function in the dendritic cell fraction was clear-cut even at stimulator to responder ratios of 1:320 (Fig. 5). Monocyte-enriched populations were much less active (4–10-fold) and this activity may well have been due to contaminating dendritic cells. For example, 5–20% monocyte-enriched cells were required to elicit significant tetanus toxoid responses (Fig. 5), yet previous studies had shown that monocytes could be depleted to a level of 1% with no loss of function (Table III). We conclude that dendritic cells, in the virtual absence of monocytes and lymphocytes, are potent stimulators of proliferative responses to soluble antigens.

*Contribution of Class II (Ia-like) Molecules to Accessory Function.* Treatment with the anti-class II antibody 9.3F10 and C′ eliminates accessory function (Table I–III) but does not affect T cell responsiveness (Fig. 5). Class II molecules could either be a marker for active accessory cells such as dendritic cells (1–4), and/or could contribute directly to function. Evidence for the latter was obtained in experiments in which the Fab fragment of 9.3F10 was present continuously. T cell proliferation in the allogeneic MLR, the syngeneic MLR, oxidative mitogenesis, and the tetanus toxoid response was blocked by 9.3F10 Fab, in most cases by 80–90% (Fig. 6). No inhibition was seen with two other monoclonal Fab fragments, the anti-Fc receptor reagent 3G8 (8) and the antimonocyte antibody

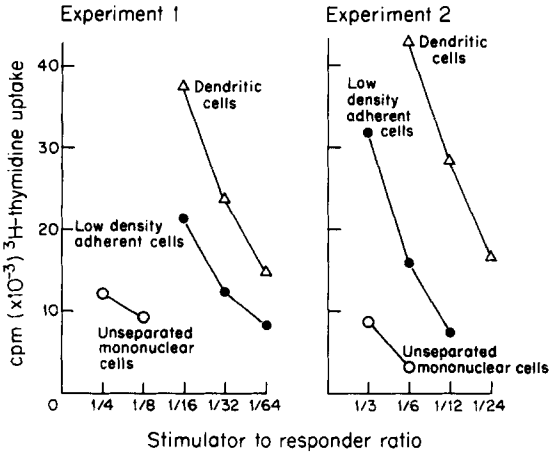


FIGURE 4. Allogeneic MLR-stimulating capacity of enriched dendritic cells. Dendritic cells were enriched from adherent mononuclear cells by eliminating monocytes and lymphocytes with monoclonal antibodies and C′ (see Materials and Methods). The viable cells, which were 65–75% dendritic cells, were retrieved by flotation on dense albumin columns. Low density cells were also obtained from adherent populations exposed to C′ only; these control cells were 60% monocytes, and 5–12% dendritic cells. In both experiments, stimulation by whole blood mononuclear cells is presented for comparison; however these cells were not exposed to Ab, C′, or albumin columns. [$^3$H]thymidine uptake of T cells in the absence of stimulators was 151 and 224 cpm, respectively.

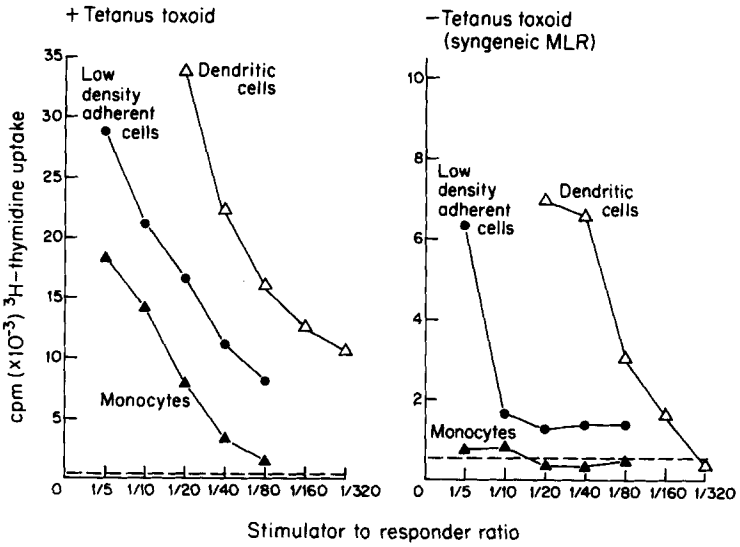


FIGURE 5. Stimulatory capacity of dendritic cells in the proliferative response to tetanus toxoid antigen and in the syngeneic MLR (no antigen). As in Fig. 4, adherent cells were treated with cytolytic 3C10, BA-1, and Leu-1 antibodies to provide dendritic cells that were 75% pure. Control low density adherent cells were exposed to C′ only and were primarily monocytes. Monocytes were also obtained from firmly adherent cells (1); the latter contained small numbers of dendritic cells and were not exposed to antibodies, C′, or albumin columns. Dotted lines show the uptake of [$^3$H]thymidine in the absence of stimulator cells.

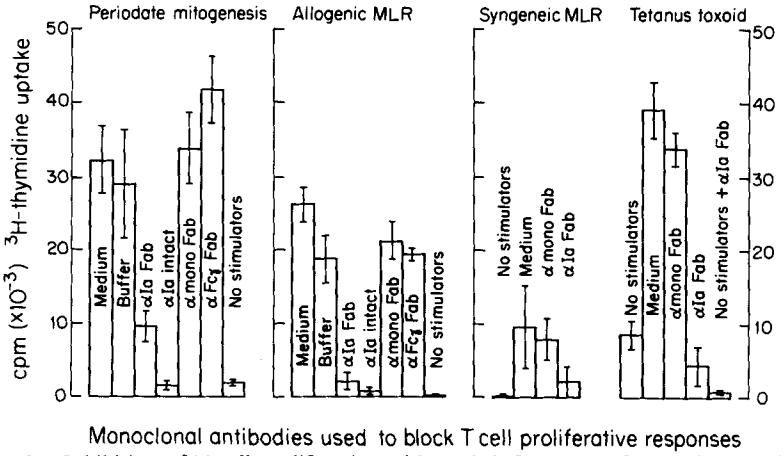


FIGURE 6. Inhibition of T cell proliferation with an Fab fragment of an anti-HLA class II (anti-Ia) monoclonal antibody. Four T cell proliferative responses were carried out in the presence of intact anti-Ia (9.3F10, 6 μg/ml), Fab fragments (6 μg/ml) of 9.3F10, and two controls, antimonocyte 3C10 and anti-Fcγ receptor 3G8.

3C10 (7).

Purified monocytes and dendritic cells were then evaluated for reactivity with 9.3F10 (Table VI). Most if not all monocytes and dendritic cells were stained by indirect immunofluorescence. In quantitative binding studies with $^{125}$I-9.3F10 Fab, monocytes and dendritic cells both expressed ~150,000 binding sites per

TABLE VI

*Quantitation of HLA-class II Molecules on Monocytes and Dendritic Cells*

| Cells | Percent $Ia^+$ | ng bound/ $10^5$ cells | 9.3F10 binding sites/cell |
|---|---|---|---|
| Experiment 1 | | | |
| Dendritic cells | 81 | 0.99 | 118,000 |
| Low density adherent cells | 99 | 1.23 | 149,000 |
| Monocytes | 97 | 1.28 | 159,000 |
| Experiment 2 | | | |
| Dendritic cells | 65 | 0.90 | 108,000 |
| Monocytes | 95 | 1.06 | 131,000 |

Dendritic cells (80 and 62% pure by cytologic criteria) were prepared by the antibody-mediated cytolysis method. Control or low density adherent cells were treated with C′ only, and this population was 71% monocytes, 12% dendritic cells, and 17% lymphocytes. Highly enriched monocytes were obtained from the persistently adherent population and were >90% monocytes. The number of $Ia^+$ cells in each population was determined by immunofluorescence with 9.3F10. Binding of $^{125}$I-9.3F10 Fab (specific activity of $6 \times 10^6$ cpm/μg) was determined in duplicate for two saturating concentrations (2.5–10 μg/ml). The value shown is the mean of the determinations with a standard error of <15%. Data are expressed per $Ia^+$ cell.

cell. We conclude that class II products are needed but must be expressed on dendritic cells to stimulate the T cell proliferative responses studied in this paper.

## Discussion

It is well known that accessory cells from human blood adhere to glass or plastic. For example, stimulator cells for the primary MLR and for oxidative mitogenesis are enriched in an adherent fraction that represents 20–30% of total mononuclear cells; the nonadherent population, which contains most of the T cells and many of the B cells, is weak or inactive (e.g., experiments 2, 3, Table V). The monocyte is the predominant adherent cell and is often assumed to be responsible for accessory function. Small numbers of dendritic cells also are present in adherent populations (1, 2). If one is to determine how accessory cells initiate immune responses, it is essential to analyze the capacities of each adherent cell type, no less so than analyzing the different kinds of lymphocytes that mediate immunity. However, the characterization of dendritic cells in man has been more demanding than in mouse or rat. Human blood has a large (~20-fold) excess of monocytes relative to dendritic cells. Also, the two cell types do not differ as much in physical properties (buoyant density and capacity to adhere firmly to glass or plastic) as do their rodent counterparts. Therefore has not been clear whether monocytes can function as accessory cells, or if the function of dendritic cell-enriched preparations has been due to dendritic cells alone. In this paper, we have used monoclonal antimonocyte and anti-HLA class II antibodies to further characterize accessory cells in man.

*Selective Depletion of Monocytes.* The first approach was to eliminate most (>95%) of the monocytes with specific antibody and rabbit C′ (Fig. 1, Tables

I–III). This treatment did not kill dendritic cells or other cell types (7). Elimination of monocytes did not reduce stimulation of the MLR and oxidative mitogenesis, and did not reduce proliferative responses to the soluble antigens, Candida albicans and tetanus toxoid. It would be difficult to establish whether treatment with 3C10 and C′ removed every monocyte, and often it is reasoned that one must remove virtually every macrophage to render lymphocytes accessory cell dependent. However, this hypothesis is inconsistent with many dose-response studies in which it has been observed that much larger numbers (5–30% of the culture) of adherent cells are required to fully reconstitute lymphocyte function after depletion of adherent cells (9–15). It is possible that small numbers of monocytes exert a trophic effect in vitro, but our data suggest that the key accessory cell that must be removed and replenished is the dendritic cell rather than the monocyte.

Previous studies have made relatively little use of specific antimacrophage antibodies and C′ to deplete accessory function. Raff et al. (16) described the "Mac-120" monoclonal, which can kill ~50% of monocytes and reduce stimulator function for the syngeneic MLR and for antigen-induced proliferative responses. It has not been established if treatment with Mac-120 alters the function of dendritic cells. This possibility must be entertained since 3C10 killed >95% of monocytes but did not reduce accessory function even under limiting assay conditions (Tables I–III).

*Positive Selection of Monocytes.* The second approach in this paper was to separate adherent mononuclear cells into monocyte-rich and -poor populations using fluoresceinated antimonocyte antibodies and the FACS (Figs. 2, 3, Tables IV, V). Sorting provided, in one step and in good yield, populations that were ≥97% and ≤2% monocytes by esterase staining and by cytology. The monocyte-depleted fractions, which contained the dendritic cells, had the bulk (~75%) of the accessory activity. The monocyte-rich fraction exhibited only weak activity, comparable to that seen with nonadherent mononuclear cells. Rosenberg et al. (17) noted that monocytes, selected with the 63D3 antimonocyte monoclonal antibody, restored pokeweed mitogen responses in sparse cultures. However, dose responses comparing monocytes and dendritic cells in this assay were not presented, and it was not clear whether both dendritic cells and/or monocytes had to be removed to render lymphocytes accessory cell dependent. The 1D9 sorting experiment (Table V) represents the first time in which macrophages and dendritic cells have been separated from one another using a specific antibody. Analogous experiments have been performed with mouse spleen adherent cells using a one step readherence method. The dendritic cell-rich component contained most of the stimulatory capacity for the MLR (3, 18), and for the development of hapten-specific cytolytic T cells (19).

In interpreting positive and negative selection experiments, one must consider the fact that monocytes can inhibit lymphocyte responses in vitro (e.g., 20–22). Typically, however, the addition of monocytes to dendritic cells (Table IV in reference 1) or to monocyte-depleted adherent cells (this paper, Table V, experiment 1) does not reduce accessory function. In our experience, inhibition of T cell responses has been apparent only at high doses, corresponding to 30–50% monocytes. The experiments reported here were performed at relatively

low monocyte to T cell ratios, where inhibition likely was insignificant. It is of interest that one can generate responses in monocyte-depleted cultures, so that it should now be possible to obviate monocyte suppression whenever it does occur.

*Function of Purified, Monocyte-depleted Dendritic Cells.* Antimonocyte antibodies were used in a third type of study that considered the capacity of purified dendritic cells to stimulate T cells in the primary MLR and tetanus toxoid response (Figs. 4, 5). In our previous work (1), dendritic cells were purified entirely by "physical" techniques. These cells lost the capacity to adhere to plastic after overnight culture (unlike most monocytes) and had a low buoyant density (unlike most lymphocytes). A dendritic cell-enriched fraction could be obtained by selecting adherent mononuclear cells that, after culture, were low density and loosely adherent. Yet these preparations contained at least 10% monocytes and lymphocytes, which could have contributed to function. Contaminating cells could be removed by rosetting methods (erythrocytes treated with neuraminidase for T cells; with antibody for Fc receptor-bearing monocytes; or with anti-human Ig for B cells), but only with a considerable loss of dendritic cells. In contrast, elimination of monocytes and lymphocytes with specific cytolytic antibodies (3C10, BA-1, Leu-1) provided dendritic cells that were highly enriched, in good yield, and depleted of contaminating cells by standard criteria. These enriched preparations of dendritic cells were strong stimulators of T cell proliferation to alloantigens and soluble proteins (Figs. 4, 5). Activity was detected at stimulator to responder ratios of 1:100 or less.

The identification of accessory cells could be made more rigorous if one could relate activity in any cell population with the precise content of dendritic cells. This is not yet possible for three reasons. First, low frequencies of dendritic cells (0.1–3.0%), as occurs in unfractionated blood and monocyte-enriched populations, cannot be enumerated rigorously. Second, the purification of dendritic cells, monocytes, and responding T cells requires procedures that often cannot be applied uniformly to every population under study. Third, small numbers of dendritic cells in either the stimulator or responder populations may enhance responses to other cells. Thus mouse spleen dendritic cells enhance cytolytic T cell responses to hapten and class I alloantigens on T cells and on $Ia^-$ splenocytes (19, 23). Given the strong stimulatory capacity of dendritic cells, the weak capacity of monocytes and lymphocytes, and the failure to observe any loss of function with extensive monocyte and lymphocyte depletion (1, 2, this paper), we would conclude that the dendritic cell is the principal accessory cell in blood.

*Contribution of HLA-class II Molecules to T Cell Growth.* An extensive literature documents the fact that class II products of the major histocompatibility complex act as restriction elements for T cells. We have used a new monoclonal, 9.3F10, to study the contribution of HLA-class II molecules to the MLR, oxidative mitogenesis, and the tetanus toxoid response. Although the determinant identified by 9.3F10 is not known, the antibody precipitates a 33,000/29,000 mol wt doublet typical of class II products, and reacts with most monocytes, B cells, dendritic cells, and $Ia^+$ cell lines. Conceivably, 9.3F10 recognizes a specificity common to products of many class II loci, since it has the notable capacity to block T cell proliferation even when used as an Fab fragment (Fig. 6). Our

working hypothesis is that 9.3F10 allows one to quantitate those Ia molecules needed for the proliferation of most class II-restricted T cells. Strikingly, both dendritic cells and monocytes bind comparable amounts of 9.3F10 Fab (Table VI). Since monocytes are weak or inactive in stimulating T cell growth, it seems that class II products must be present on dendritic cells to initiate replication.

Most likely, 9.3F10 blocks replication by inhibiting the recognition of dendritic cell class II products. Studies in mice and guinea pigs indicate that monoclonal anti-Ia antibodies can block function at the level of the accessory cell (e.g., 24, 25). It has been reported that anti-Ia can also inhibit the T cell response to interleukins (26). However, interleukin-mediated human T cell growth, monitored as described recently in a murine model (5), was not inhibited by 9.3F10 (J. M. Austyn, personal communication).

It is not clear why $Ia^+$ human monocytes and dendritic cells have such different functional capacities. Comparable observations have been made in studies of $Ia^+$ macrophages in mice (3–6, 18, 19, 27). We favor the idea that dendritic cells are differentiated to induce responses in unprimed, resting or memory T cells, as were studied in this paper. The dendritic cell probably acts directly to initiate the response of class II-restricted cells, as well as indirectly on other T cells by controlling the release of soluble mediators like T cell growth factor (5). $Ia^+$ monocytes may very well interact directly with class II-restricted activated T cells or their products during the effector limb of the immune response. Yet the monocyte does not mediate the formation of sensitized cells, which appears to be the function of specialized dendritic cells.

## Summary

Monocyte-specific monoclonal antibodies (7) were used to compare the efficacy of monocytes and dendritic cells as accessory or stimulator cells for human T cell replication. Both unfractionated and plastic-adherent mononuclear cells were first treated with a cytolytic antimonocyte antibody that kills >95% of monocytes but not dendritic cells. When tested as stimulators of the mixed leukocyte reaction (MLR) and of oxidative mitogenesis (the proliferation of T cells modified with sodium periodate), the monocyte-depleted cells had normal or enhanced stimulatory capacity. Monocyte-depleted mononuclear cells also proliferated normally to soluble antigens (Candida albicans, tetanus toxoid), even under limiting conditions of cell dose, antigen dose, and culture time. Adherent blood mononuclear cells were next separated into monocyte-enriched and -depleted components using fluoresceinated antimonocyte antibody and the cell sorter. The depleted fraction (<2% monocytes by esterase staining and by cytology) contained the dendritic cells and exhibited at least 75% of the accessory activity. The monocyte-rich fraction (~97% esterase positive) stimulated the MLR and oxidative mitogenesis weakly, and was comparable in potency to nonadherent cells. Cell-specific antibodies and complement were also used to prepare dendritic cells that were thoroughly depleted of monocytes and lymphocytes. The dendritic cells (70–80% pure) were potent stimulators of the allogeneic MLR, syngeneic MLR, and tetanus toxoid response, being active at stimulator to responder ratios of 1:100 or less. Taken together with previous studies (1, 2), these experiments indicate that the dendritic cell is the major stimulator of T cell replication in

man.

The contribution of class II products of the major histocompatibility complex (7) was then evaluated with a new monoclonal, 9.3F10. Accessory function was dramatically inhibited if cells bearing class II antigens were killed with 9.3F10 and complement, or if class II molecules were blocked by the addition of 9.3F10 Fab to the culture medium. The expression of 9.3F10 class II products was therefore studied on purified monocytes and dendritic cells. Most if not all cells in both populations reacted with 9.3F10, and each population exhibited ~150,000 $^{125}$I-Fab 9.3F10 binding sites per cell. Since Ia$^+$ dendritic cells are active accessory cells, but Ia$^+$ monocytes are not, class II products are necessary but not sufficient for the stimulation of T cell proliferation in man.

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