DENDRITIC CELLS STIMULATE PRIMARY HUMAN CYTOLYTIC LYMPHOCYTE RESPONSES IN THE ABSENCE OF CD4+ HELPER T CELLS

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Cytolytic lymphocytes provide a host resistance mechanism that may be exerted against tumors, allografts, and cells infected with microbial agents, especially viruses and some bacteria. There are several types of killer cells (1): antigen-specific, MHC-restricted CTL, as well as lymphokine-activated killer (LAK)[1] and NK cells, which have less well-defined specificities.

Cytolytic lymphocytes are typically generated in tissue culture using heterogeneous populations of both responder lymphocytes and APCs, as well as some source of exogenous growth factors and other cytokines. Under such conditions, the proliferative and lytic responses by $CD8^+$ lymphocytes are enhanced by the lymphokine products of helper $CD4^+$ cells, especially IL-2 (2–5). However, murine lymphocytes depleted of $CD4^+$ T cells can also independently generate CTL directed against viral (6) and transplantation antigens (7–10). This development of CTL from helper-depleted populations is induced by dendritic cells and is accompanied by the release of IL-2 (9). In man the cellular requirements for cytolytic lymphocyte development have not been similarly analyzed, and it is often assumed that helper cells are essential. We will show here that it is possible to induce cytolytic cells from purified human $CD4^-$ lymphocyte populations in the primary MLR, as long as dendritic cells are used as the stimulator cells or APCs. The use of human cells has enabled us to describe several new features of the helper-independent response. Whereas in the murine MLR it is necessary to use heterologous serum in the cultures, and macrophages are inhibitory, the human response occurs in homologous serum, and monocytes are neither stimulatory nor inhibitory. We will also demonstrate that both antigen-specific CTL and antigen-nonspecific NK cells can be induced by human dendritic cells, and that the number of cells that transform into blasts and express

[1] *Abbreviations used in this paper:* CTLp, CTL precursor; $Er^{+/-}$, neuraminidase-treated sheep erythrocyte rosette-positive/negative lymphocytes; GVHD, graft-vs.-host disease; LAK, lymphokine-activated killer; NHS, normal human serum; TCGF, T cell growth factor.

activation antigens is very high, as many as 30–40% of the cultured cells at day 4–5 of the primary MLR.

Materials and Methods

Culture Medium, Serum, Buffers. Cells were cultured in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 1 mM glutamine (KC Biological, Lenexa, KS), 5 × 10^{-5} M 2-ME (Eastman Kodak, Rochester, NY), penicillin (100 Units/ml), streptomycin (100 μg/ml) (Gibco Laboratories), and 10% serum. FCS (HyClone Laboratories, Sterile Systems, Logan, UT) was heat inactivated for 30 min at 56°C. Normal human serum (NHS) was obtained by venipuncture from fasting, healthy, untransfused male volunteer donors, clotted in the presence of glass shards for 1 h at room temperature. The serum was clarified by centrifugation (1,500 g, 15 min), heat inactivated at 56°C for 30 min, and stored at −20°C until needed. Ca^{2+}- and Mg^{2+}-free saline buffers were used for all washes, either as PBS or as HBSS.

PBMC and Preparation of Leukocyte Subpopulations (11). Normal leukocyte concentrates (Greater NY Blood Program, New York, NY; specimens yielding positive serology for hepatitis B or HIV were not used) were layered over Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) and centrifuged at 1,000 g for 20 min at room temperature. The cellular interface was collected as a source of PBMC, washed in PBS or HBSS, and separated over continuous Percoll density gradients as previously described (11). Low density cells from this primary Percoll separation were used as a source of monocytes, and were further enriched if necessary by an adherence step on tissue culture plastic. The high density fraction was rosetted with neuraminidase-treated (*Vibrio cholerae* neuraminidase; Calbiochem-Behring Diagnostics, La Jolla, CA) sheep erythrocytes (Scott Laboratories, Fiskeville, RI), and separated over Ficoll-Paque into erythrocyte rosette–negative (Er$^-$) and erythrocyte rosette–positive (Er$^+$) fractions. The Er$^+$ fraction was further purified by passage over a nylon wool column (Fenwal Laboratories, Deerfield, IL) and used as a source of T cells. The Er$^-$ fraction was cultured for ∼36 h, panned over human Ig-coated plates to deplete contaminant monocytes, and then separated over a secondary Percoll gradient (11). Secondary low density cells were used as an enriched source of DCs, whereas the secondary high density fraction was predominantly B lymphocytes. This method routinely yielded an accessory cell population of dendritic cells enriched to 40–80% purity (11).

An alternative simpler approach was to omit the first Percoll separation. Bulk mononuclear cells were directly rosetted with neuraminidase-treated sheep erythrocytes for separation into Er$^-$ and Er$^+$ fractions as above. The Er$^-$ cells were cultured in RPMI–10% NHS. Er$^-$ cells that remained adherent after ∼36 h of culture, or that readhered to tissue culture plastic after ∼36 h culture, were used as a highly enriched source of monocytes with no difference in the functional results described below. Er$^-$ nonadherent cells were panned twice over human Ig-coated plates to deplete monocytes (11), then separated over a continuous Percoll density gradient to yield a DC-enriched low density fraction. The high density fraction from this separation again consisted primarily of small B lymphocytes.

Cell Phenotype Analysis. The following mAbs were used either as purified Ig or as hybridoma supernatants: anti-CD3 (OKT3/IgG2a, ATCC CRL 8001); anti-CD4 (Leu3a/IgG1, gift of Dr. Robert Evans [New York, NY]; OKT4/IgG2a, ATCC CRL 8002); anti-CD8 (OKT8/IgG2a, ATCC CRL 8014); "Simultest" anti-CD4-FITC/anti-CD8-PE (Leu3a/IgG1-FITC//Leu2a/IgG1-PE, Becton-Dickinson [B-D], Mountain View, CA); anti-CD11b (OKM1/IgG2b, ATCC); anti-CD14 (3C10/IgG2b, ATCC TIB 228); anti-CD16 (3G8/IgG1, gift of Dr. Jay Unkeless [New York, NY]; Leu11c/IgG1, B-D); anti-CD25 (anti-TAC/IgG2a, gift of Dr. T. Waldman [Bethesda, MD]); anti CD28 (9.3/IgG2b, gift of Dr. Paul Martin [Seattle, WA]); anti-CD45R (Leu18/IgG1, B-D); anti-HLA DR/DQ monomorphic epitope (9.3F10/IgG2a, ATCC HB 180); anti-TCR α/β chains (anti-TCR1/IgG1, B-D); anti-TCR δ chain, common region (IgG1; T Cell Sciences, Cambridge, MA). In some cases, the mAbs were directly conjugated with either FITC or PE; otherwise FITC-conjugated F(ab')$_2$ goat anti–mouse IgG + IgM (Grub, Scandic, Vienna, Austria) was used as a second-step reagent

for indirect staining. Stained cells were analyzed by cytofluorography on a FACScan instrument (Becton-Dickinson Immunocytometry Systems, Mountain View, CA).

Purification of T Lymphocyte Subsets. Enriched populations of CD4$^+$ and CD8$^+$ T cells were purified by negative selection of antibody-coated lymphocytes, panned on goat anti-mouse IgG-coated petri dishes. Briefly, Er$^+$ nylon wool nonadherent lymphocytes at 5-7 $\times$ 10^6 cells/ml were coated with hybridoma supernatants on ice for 30-45 min, at concentrations previously determined to yield strongly positive fluorescence. The cell suspensions were washed three times in RPMI-5% FCS before panning. Pans were prepared using 60 or 100 mm bacteriologic grade petri dishes (Falcon 1007 or 1005; Becton-Dickinson, Lincoln Park, NJ), coated for 1 h at room temperature with 20 or 60 μg, respectively, of IgG fraction goat anti-mouse IgG (Fc fragment; γ chain specific, Cappel 0211-0121; Cooper Biomedical, Malvern, PA) in PBS. These dishes were gently rinsed four times with cold PBS just before use. The mAb-coated lymphocytes were resuspended in cold RPMI-5% FCS at 5 $\times$ 10^6 cells/ml, and either 3 or 8 ml was added to 60- or 100-mm dishes, respectively. The pans were centrifuged at 25 g at 4°C for 5 min in a swinging bucket rotor (Sorvall RC3B, rotor H6000A) with slow acceleration and no brake. The pans were swirled and rotated 180°, then centrifuged again in the same manner. Thereafter, nonadherent lymphocytes were gently washed from the pans, and their purity as a negatively selected population was monitored by cytofluorography (Fig. 1).

Panning was also used to deplete NK cells contained within a subpopulation of CD11b$^+$/ CD28$^-$ and CD16$^+$ cells comprising part of the CD4$^-$ fraction (Fig. 1). This subset could be depleted by adding the anti-CD11b mAb, OKM1, to the panning procedure. As shown in Fig. 1, the starting Er$^+$ nylon wool nonadherent cells ("bulk T cells," left column of Fig. 1) contained major CD4$^+$ and CD8$^+$ subsets and minor fractions of cells that were CD11b$^+$/CD28$^-$ and CD16$^+$. Inclusion of the anti-CD11b mAb OKM1, when removing CD4$^+$ lymphocytes by panning, yielded populations depleted of the minor CD11b$^+$/CD28$^-$, CD16$^+$ (right column of Fig. 1), and CD57$^+$ (not shown) subsets. The effect of OKM1 was specific since the CD11b subset was not depleted when anti-CD14/3C10 was used as a nonreactive, isotype-matched control mAb for anti-CD11b/OKM1. The phenotype of these starting cell populations was also maintained following stimulation in culture with allogeneic DCs (cf, Results, Fig. 5).

Mixed Leukocyte Reactions. Stimulatory APCs for the human MLR were either bulk mononuclear cells or one of the Er$^-$ subsets enriched in monocytes, dendritic cells, or small B lymphocytes, as described above. APCs were irradiated (3,000 rad, ^{137}Cs) and added to T lymphocytes at APC/T ratios of 1:10 or lower, as indicated in the respective experiments. MLRs were uniformly cultured in the presence of RPMI-10% NHS, either in 16-mm, 24-well, flat-bottomed (1.5 $\times$ 10^6 T cells), 96-well flat-bottomed (1.5 or 2 $\times$ 10^5 T cells), or 96-well U-bottomed (1 $\times$ 10^5 T cells) tissue culture plates (Costar, Cambridge, MA), according to the needs of the particular experiment. The cultures were maintained at 37°C in humidified 6% CO$_2$.

Proliferative activity in the MLR was measured by the incorporation of [^{3}H]TdR (New England Nuclear, Boston, MA) in the DNA of replicating cells. When MLRs were run in 16-mm macrowells, triplicate 100-μl aliquots were transferred to U-bottomed microwells, to which 1 μCi [^{3}H]TdR per well was added. MLRs cultured in microwells from the outset were pulsed similarly. After 6 h of incubation, the wells were harvested on glass fiber filters and counted. Responses have been reported as the mean cpm of triplicates.

Assay of T Cell Growth Factor in the MLR. Aliquots of MLR supernatants were harvested at representative time points, frozen, then thawed and added in serial dilutions to cells from the CTLL2 line in flat-bottomed microwells. 1 μCi [^{3}H]TdR was added to each microwell at 18-24 h, and DNA synthesis in the presence of the MLR supernatants was compared with that of CTLL2 cells cultured with graded doses of rIL-2 (reference 12; Biogen, Cambridge, MA). The amount of T cell growth factor in the MLR supernatants was then expressed in terms of units per milliliter of rIL-2.

Cytolytic T Lymphocyte Assay. Cytolytic activity derived from the MLR was measured in a standard 4-h ^{51}Cr-release assay. When the MLRs were cultured in 16-mm macrowells, the
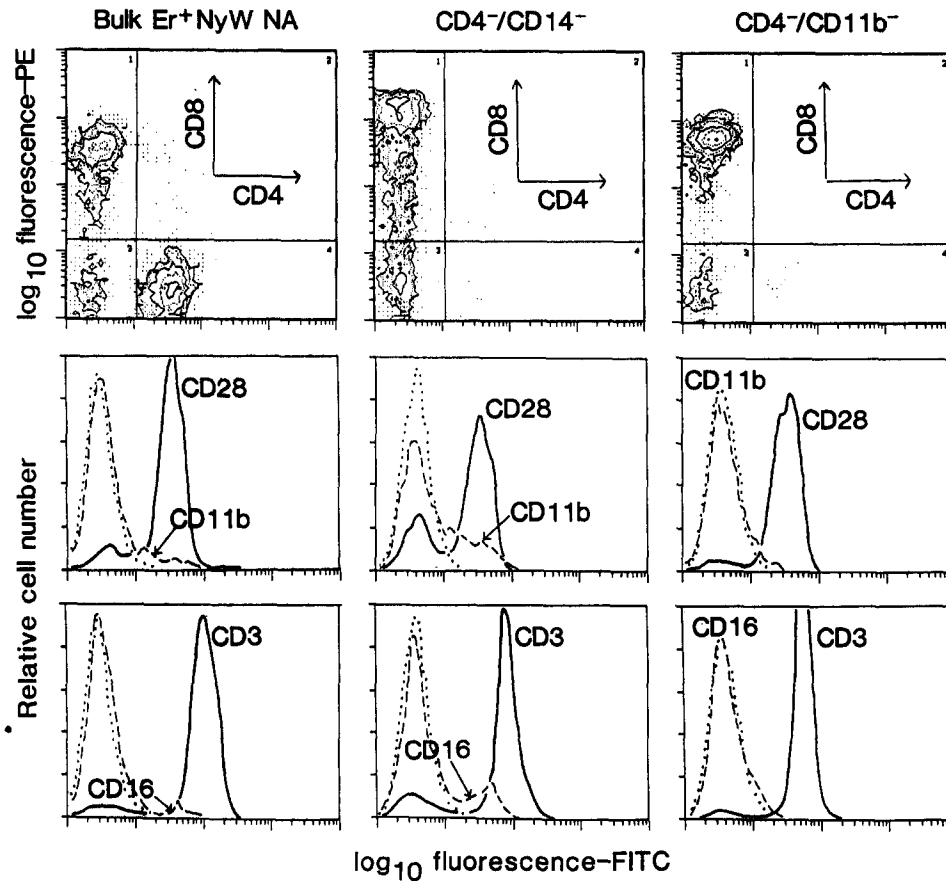
FIGURE 1. Phenotype of starting lymphocyte responders in the allogeneic MLR (cf., Fig. 5). Er+, nylon wool nonadherent [NyW NA] lymphocytes were used as bulk T cells. The two CD8+ enriched populations were CD4−/CD14− and CD4−/CD11b− lymphocytes negatively selected by panning with anti-CD4/OKT4 and either anti-CD14/3C10 (negative control) or anti-CD11b/OKM1. Each population was stained with the indicated mAbs and analyzed by cytofluorography. In the two-color analyses, anti-CD8/Leu2a-PE is shown along the ordinate, and anti-CD4/Leu3a-FITC is illustrated along the abscissa. In the single color histograms, either FITC-conjugated mAbs or two-step indirect staining with FITC-goat anti-mouse Ig and primary mAbs was used to determine $\log_{10}$ fluorescence along the abscissa. Major subpopulations of CD28+ and CD3+ lymphocytes are indicated (—) in the middle and bottom rows, respectively. Minor subpopulations of CD11b+ and CD16+ lymphocytes are indicated by (- - -) in the middle and bottom rows, respectively. Negative controls stained with FITC-goat anti-mouse Ig but no primary mAb are indicated by the unlabeled, dotted lines.

cells were harvested and transferred in graded doses to U-bottomed microwells, to which 5 or 10 × 10³ radiolabeled target cells were added. Alternatively, when the MLRs were cultured initially in U-bottomed microwells, the cells were gently resuspended; and 5 × 10³ radiolabeled targets were added directly to the MLR microwells. CTL assays were run in triplicates for each experimental condition.

Cultured human macrophages were routinely used as allogeneic and syngeneic target cells for CTL assays. Monocytes were cultured in RPMI-10% NHS in Teflon beakers and labeled with ⁵¹Cr (New England Nuclear) as previously reported (13). The K562 cell line was ⁵¹Cr-labeled and used as targets for measurement of antigen-nonspecific lytic activity.

The microtiter plates were incubated at 37°C, 6% CO_2 for 4 h. Thereafter, the plates were spun at 15 g for 5 min at 37°C, and supernatant aliquots were harvested to measure ^{51}Cr release. Spontaneous release was measured from target cells cultured without effectors in medium only, and total release was measured from targets cultured in 0.1% SDS. Cytolytic activity has been reported as the percent specific release of ^{51}Cr, calculated in standard fashion as: percent specific release = 100 × [(mean cpm from triplicate test wells – spontaneous release)/(total release – spontaneous release)]. Spontaneous release was always <10–15% of the total release for the experiments reported here.

Results

Dendritic Cells Induce the Development of Antigen-specific CTL and NK Cells in the Absence of CD4⁺ Helper Lymphocytes. It is widely accepted that the standard population of stimulator cells in the human MLR, i.e., bulk mononuclear cells from peripheral blood, is unable to stimulate allogeneic CTL in the absence of CD4⁺ lymphocytes and their secreted "helper" lymphokines (14). Our initial goal was to test if, using dendritic cells as APC, one could elicit responses from the CD4⁻ population of human blood lymphocytes. This was indeed the case in that allogeneic dendritic cells could induce the formation of killers that would preferentially attack allogeneic vs. syngeneic monocytes as targets (Fig. 2). However, we noted that dendritic cells also stimulated the formation of nonspecific killers, as evidenced by lysis of K562 target cells (Fig. 2).

It has been reported that all mature CD4⁻ lymphocyte effectors derive from one of two precursor subsets characterized by reciprocal CD28⁺/CD11b⁻ "cytolytic" and CD28⁻/CD11b⁺ "suppressor" phenotypes (15, 16). We tested if OKM1, an IgG2b mAb against the CR3/CD11b epitope, could be used to deplete precursors of K562 lytic cells. Anti-CD4 and anti-CD11b mAb were used together to pan populations of nylon wool nonadherent lymphocytes (cf. Fig. 1 and Materials and Methods for the efficiency of the panning procedure). 3C10, a nonreactive IgG2b anti-CD14 mAb,
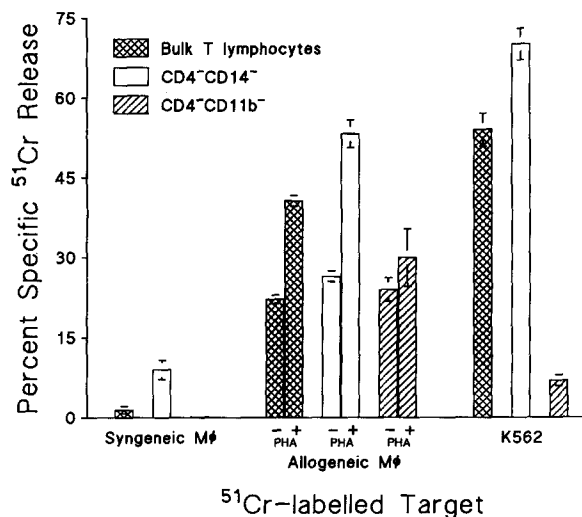

FIGURE 2. Cytolytic activity derived from primary MLRs stimulated by allogeneic blood dendritic cells. Lymphocyte responder populations were obtained as described under Materials and Methods and Fig. 1 (bulk T lymphocytes, ▨; CD4⁻/CD14⁻ lymphocytes, ▭; CD4⁻/CD11b⁻ lymphocytes, ▨) and stimulated by allogeneic blood dendritic cells (3,000 rad, ^{137}Cs) in U-bottomed microwells in RPMI-10% NHS (responder/ stimulator ratio of 25:1). After 4–5 d of culture, the cell targets indicated along the abscissa were ^{51}Cr-labeled, and 5 × 10³ targets were added directly to the microwells. PHA (10 μg/ml final) was added to a parallel cohort of cultures with ^{51}Cr-labeled allogeneic monocyte targets. The E/T ratio of the MLR cultures was ~30:1 at the time the targets were added. After a 4-h incubation, supernatants were harvested for determination of the percent specific release as shown along the ordinate.

was the isotype-matched control for the OKM1 mAb. Panning with OKM1 removed the K562 cytolytic precursors without significantly altering the level of antigen-specific killing (Fig. 2). Note that the addition of PHA during the CTL assay did not appreciably augment the measurable lysis by CD4−/CD11b− effectors from the allogeneic MLR (Fig. 2). However, PHA did enhance killing by a lectin-dependent mechanism by the bulk and CD4−/CD14− populations that contained nonspecific lytic cells. The CTL assay therefore efficiently detected all of the antigen-specific CTL response that developed from the allogeneic MLR, and these effectors developed primarily from the CD4−/CD11b− T cell precursors.

In dose-response studies (Fig. 3) we noted that very low levels of dendritic cells were required to induce NK cell activity, whereas higher levels were required for the induction of CTL. Typically the required ratio of dendritic cells to T cells was 1:10 or 1:30. These doses are much higher than the number of dendritic cells present if bulk mononuclear cells were used as stimulators of CD4+ -independent CTL responses.

As in the mouse, helper-independent CD4− responses to histoincompatible dendritic cells usually peaked ∼24 h earlier than those of a bulk T cell population containing CD4+ helper lymphocytes (Fig. 4, A and B). The kinetics were identical regardless of the CD4− subset used as responders (data not shown). In syngeneic MLRs cultured in 10% human serum, only bulk T cells exhibited any measurable proliferation (Fig. 4 A), although it was markedly less than after allogeneic stimulation. FCS supported a greater syngeneic response by both T cell populations (data not shown), and we have therefore avoided its use in functional assays.

We conclude that allogeneic dendritic cells can induce the formation of antigen-specific and -nonspecific cytolytic cells in the absence of CD4+ helper lymphocytes. The precursors of nonspecific NK/LAK type cells can be removed by panning with an anti-CD11b mAb, OKM1, without altering the amount of antigen-specific CTL that develops in the allogeneic CD4− MLR. The kinetics of the CD4− response are rapid, and a syngeneic response is not observed by CD4− lymphocytes when cultured in human serum. These findings are representative of more than 15 experiments.

CD4+ T Lymphocyte Contaminants Do Not Contribute to the CD4− MLR. With even
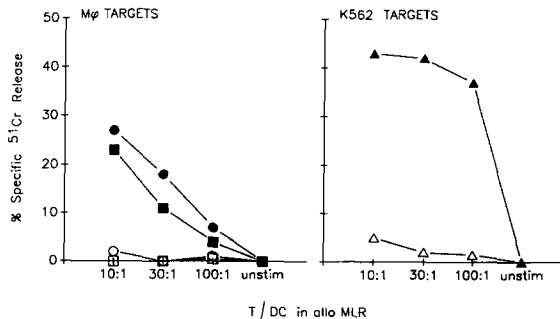

FIGURE 3. Cytolytic activity derived from primary MLRs stimulated by variable doses of allogeneic blood dendritic cells. Bulk T lymphocytes and CD4−/CD11b− T cells were stimulated by variable doses of allogeneic blood dendritic cells (3,000 rad, 137Cs) indicated by the T/DC ratios along the abscissa. MLRs were cultured in RPMI–10% NHS in U-bottomed microwells. On day 4 of the MLR, syngeneic and allogeneic monocytes and K562 cells were 51Cr-labeled, and 5 × 10^3 targets were added to each microwell. After a 4-h incubation, supernatants were harvested for determination of percent specific release. The cytolytic activity plotted against the ordinate is for the following E/T combinations: Bulk T lymphocytes anti-allogeneic Mø, ●; anti-syngeneic Mø, ○; anti-K562, ▲; CD4−/CD11b− T cells anti-allogeneic Mø, ■; anti-syngeneic Mø, □; anti-K562, △.
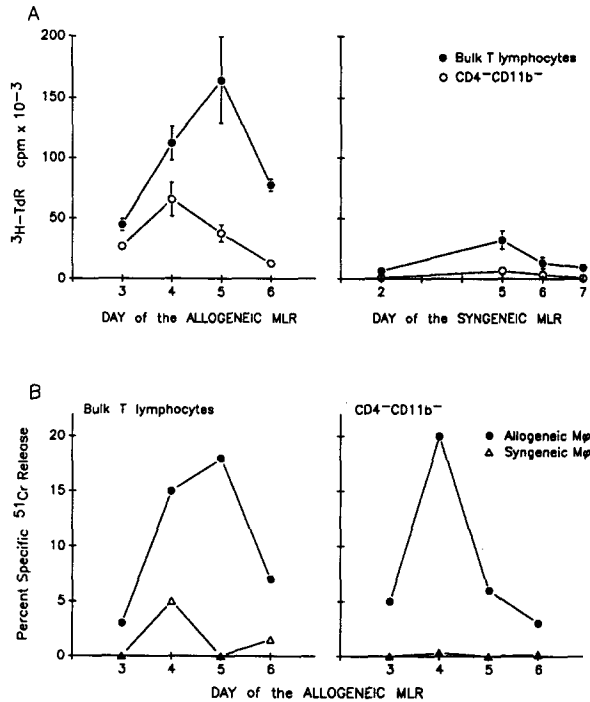
Figure 4. Kinetics of the primary human MLR elicited by blood dendritic cells. Bulk T lymphocytes and CD4⁻/CD11b⁻ T cells were stimulated in U-bottomed microwells (responder/stimulator of 10:1) by syngeneic or allogeneic dendritic cells (3,000 rad, ^{137}Cs) in the respective MLRs cultured in RPMI-10% NHS. On the day of culture indicated along the abscissa, the cultures were pulsed with 1 μCi/well of [^{3}H]TdR for 6-8 h and proliferation was measured in standard fashion (A: bulk T lymphocyte responders, ●; CD4⁻/CD11b⁻ responders, O). Concomitantly, 5 × 10³ ^{51}Cr-labeled syngeneic or allogeneic monocytes were added to separate MLR microwells, and supernatants were harvested and counted after 4 h for determination of percent specific ^{51}Cr release (B: allogeneic M⊘ targets, ●; syngeneic M⊘ targets, △).

low levels of CD4⁺ contamination (e.g., <2-3% as shown in Fig. 1), helper lymphocytes could proliferate disproportionately during the CD4⁻ MLR and contribute to the primary cytolytic responses illustrated above. We therefore phenotyped the responder lymphocytes after dendritic cell stimulation in the allogeneic MLR. Cytofluorographic data confirmed the continued purity of the starting CD4⁻/CD14⁻ and CD4⁻/CD11b⁻ populations (Fig. 5). Note the presence of a trace, but discrete subpopulation of δ chain-positive T cells, comprising ∼3% of both CD4⁻ lymphocyte populations. These cells were also present to a similar degree before stimulation (data not shown).

One of the hallmarks of CD4⁺ lymphocyte function is the production of T cell growth factor[s] [TCGF], among which is IL-2. However, in the "helper-independent" CD4⁻ allogeneic MLR measurable amounts of TCGF were also found (Fig. 6). To exclude the production of significant amounts of IL-2 by trace contaminant CD4⁺ T lymphocytes, responder populations of bulk T lymphocytes and purified CD4⁻ lymphocytes were coated with anti-CD4 (Leu3a, 3 μg/ml final) and then stimulated with allogeneic DCs in the continuous presence of anti-CD4. Responder populations stimulated in the absence of anti-CD4 were cultured in parallel. We then compared supernatants harvested at serial time points from these MLRs with graded doses of rIL-2 for their ability to support the growth of CTLL2 cells (12). TCGF was readily detected in the allogeneic MLR when bulk T lymphocytes were stimulated by blood dendritic cells, and there was a 75% reduction when the bulk T cell responders were coated with anti-CD4. Smaller amounts of TCGF were present in the dendritic cell-stimulated CD4⁻ MLR, but there was *no* significant inhibition
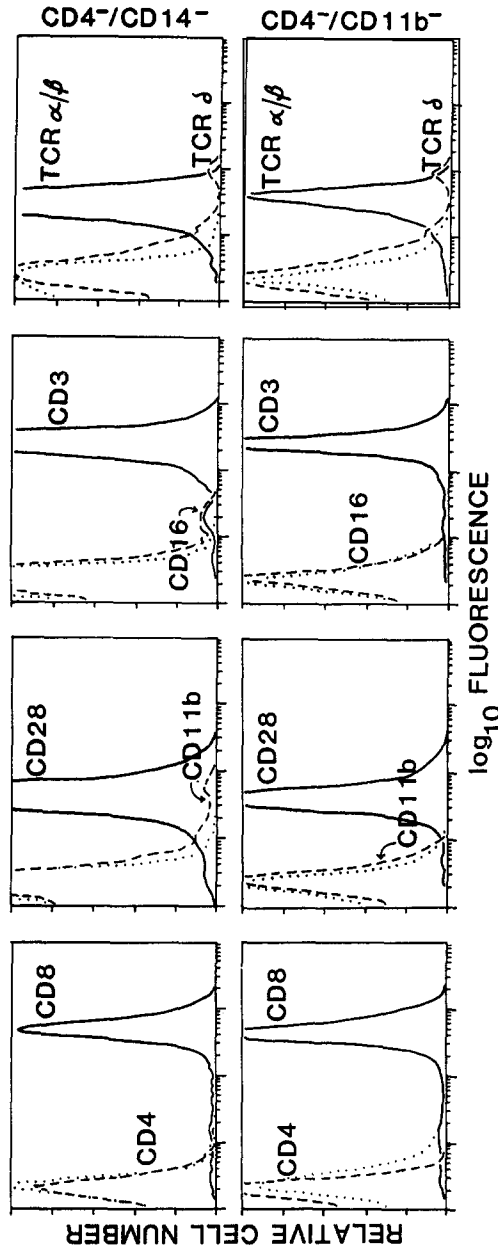
FIGURE 5. Phenotype of CD4⁻ lymphocyte responder subsets after stimulation in the helper-independent allogeneic MLR (cf. Fig. 1). CD4⁻/CD14⁻ and CD4⁻/CD11b⁻ lymphocyte subsets were stimulated by allogeneic dendritic cells (responder/stimulator of 10:1) in U-bottomed microwells for 4–5 d, which is the peak of the proliferative response. The MLR cultures were stained directly in the U-bottomed microwells with the indicated mAbs and analyzed by cytofluorography. (*Left to right*) Among both responder populations, the major subsets of CD8⁺, CD28⁺, CD3⁺, and TCR-α/β⁺ cells are shown (—); and the minor or absent subsets of CD4⁺, CD11b⁺, CD16⁺, and TCR-δ⁺ cells are shown (- - -). Negative controls stained with FITC–goat anti–mouse Ig but no primary mAb are indicated by the unlabeled, dotted lines.
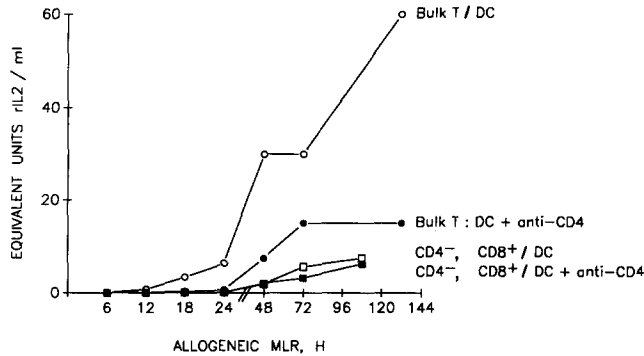
FIGURE 6. Production of T cell growth factor(s) by CD4$^-$ lymphocytes in the helper-independent allogeneic MLR. Bulk T lymphocytes (●) and CD4$^-$ lymphocytes (■) were coated with anti-CD4 (Leu3a, 3 μg/ml final) and then stimulated with allogeneic dendritic cells in the continuous presence of anti-CD4. Uncoated lymphocytes were stimulated and cultured alongside in the absence of anti-CD4 (bulk T, O; CD4$^-$, □). Supernatants were harvested at serial time points and tested for their ability to support the growth of the CTLL2 cell line (12). Activity is reported in units derived from a standard curve with graded doses of human rIL-2. In different experiments the absolute amounts of measurable TCGF were not always the same, but the results were qualitatively similar, i.e., anti-CD4 significantly reduced the amount of TCGF produced by bulk T cells but not by CD4$^-$ lymphocytes, regardless of the CD4$^-$ subset stimulated.

of these negatively selected responders by anti-CD4. [^{3}H]TdR incorporation by responder lymphocytes was also measured in allogeneic MLR cultures run in parallel. Anti-CD4/Leu3a in this concentration inhibited proliferation by bulk T cell responders by 45%, whereas it had no significant effect on [^{3}H]TdR incorporation by CD4$^-$ responders in the "helper-independent" allogeneic MLR. Similar findings have been obtained using the more highly purified CD4$^-$/CD11b$^-$ subset (data not shown).

Large Numbers of CD4$^-$/CD11b$^-$ Lymphocytes Display Phenotypic Markers of Activation after Stimulation by Histoincompatible DCs. We phenotyped responding lymphocytes in the MLR for activation markers such as the receptor for IL2 (17), the expression of class II MHC (18, 19), and the absence of the CD45R epitope (20, 21) on the large lymphoblasts. CD4$^-$/CD11b$^-$ T cells exhibited these phenotypic markers of activation only after primary stimulation by allogeneic dendritic cells (Fig. 7 A). Neither allogeneic monocytes (Fig. 7 B) nor syngeneic DCs (not shown) could elicit such activation markers, the staining for which was the same as the background staining of unstimulated CD4$^-$/CD11b$^-$ T cells (Fig. 7 C). Representative cytofluorographs of allogeneic dendritic cell-stimulated bulk T lymphocytes are shown for comparison (Fig. 7 D). We ascertained that large, IL-2R$^+$ cells comprised 43% of the alloreactive lymphocytes in the bulk T cell MLR and 33% of the alloreactive lymphocytes in the CD4$^-$/CD11b$^-$ cells MLR after a 4-5-d culture. This is representative of more than six experiments.

Blood Dendritic Cells Are the Most Active Accessory Cells for Unsensitized CTL Precursors. We evaluated the APC requirements for a primary response by resting, naive CTL precursors (CTLp) in the absence of CD4$^+$ T cell help. Candidate APC populations were obtained as described (Materials and Methods; reference 11), irradiated (3,000 rad, ^{137}Cs), and added at different doses to negatively selected bulk CD4$^-$ lymphocyte responders in 16mm macrowells. At peak proliferation in the MLR on day 4-5, the cells were harvested and added to radiolabeled monocytes at the indicated E/T ratios; ^{51}Cr release was measured after 4 h (13). As shown in Fig. 8, den-
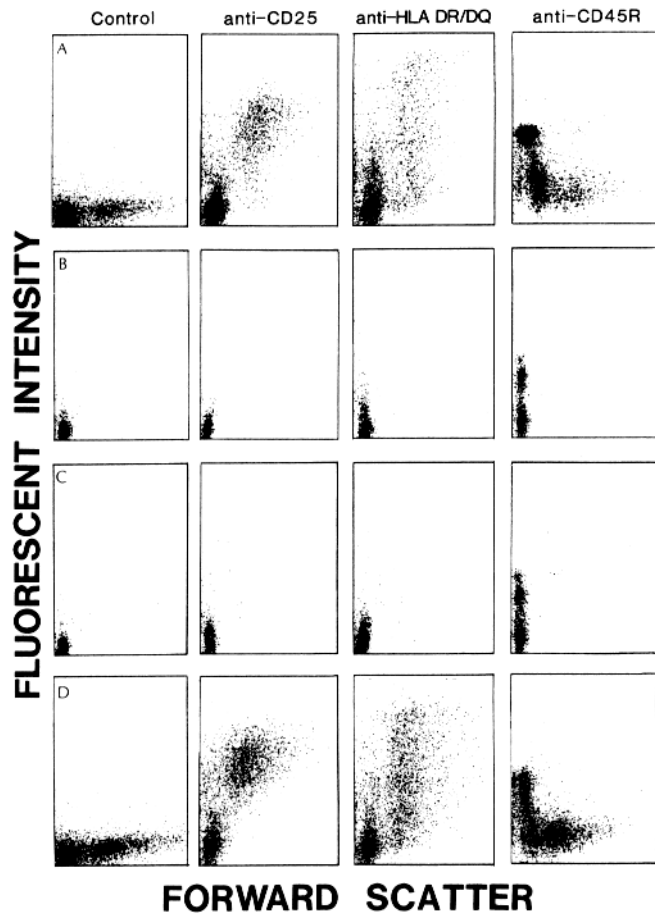
FIGURE 7. Large numbers of CD4⁻/CD11b⁻ lymphocytes display activation markers after stimulation by histoincompatible dendritic cells in the helper-independent MLR. CD4⁻/CD11b⁻ T cells were stimulated by allogeneic dendritic cells (A) or allogeneic monocytes (B) (responder/stimulator of 10:1) for 4–5 d in U-bottomed microwells. Unstimulated CD4⁻/CD11b⁻ T cells (C) and allogeneic dendritic cell–stimulated bulk T lymphocytes (D) (responder/stimulator of 10:1) were cultured simultaneously for comparison. At peak proliferation, the MLRs were stained directly in the U-bottomed microwells with the indicated mAbs and analyzed by cytofluorography. Negative controls stained with FITC–goat anti-mouse Ig but no primary mAb are illustrated in the far left column.

dritic cells were the most active APC for stimulating cytolytic effector function in a dose-dependent fashion and in the absence of CD4⁺ help.

We have previously reported that peritoneal macrophages could inhibit a primary CD8⁺ response in the murine system (9). However, this was not the case using primary leukocyte isolates from human blood. Monocytes did not inhibit the dendritic cell–stimulated response even at high doses (Fig. 8). Similar findings pertained when bulk T cells, comprised of both CD4⁺ and CD8⁺ responders, were assayed for primary cytolytic activity after allogeneic stimulation in the MLR (data not shown).

We have similarly examined the APC requirements for more highly purified CD4⁻ populations depleted of CD11b⁺ NK/LAK precursors, with regard to both proliferation and cytolytic activity. Blood dendritic were the most active APCs for stimulation of both proliferation and cytolysis (Fig. 9, A and B). The bulk Er⁻ population and the Er⁻FcR⁻ populations contained a lower proportion of dendritic cells, and these stimulators elicited lower but measurable responses at the higher dose. Monocyte depletion did not abrogate the observed responses, and adherent Er⁻ cells specifically enriched for monocytes did not display significant stimulatory capacity.
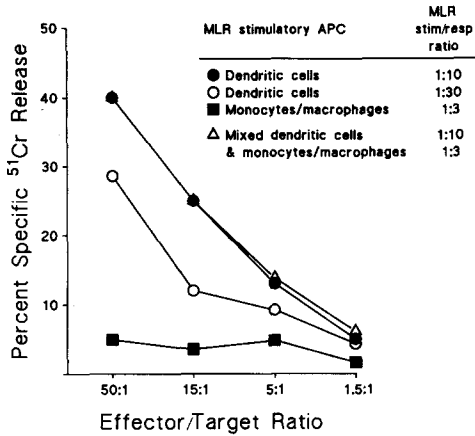
FIGURE 8. Comparison of APC activity in eliciting primary CTL from CD4⁻ allogeneic lymphocytes. CD4⁻ lymphocytes were cultured at 1.5×10^6 cells per 16-mm macrowell and stimulated with different APC populations at the indicated stimulator/responder ratios. At the end of day 4, the responding lymphocytes in the MLRs were harvested from the macrowells, counted, and added to triplicate U-bottomed microwells for assay of CTL activity against 10^4 ^{51}Cr-labeled allogeneic Mø from the original stimulator party. Supernatants were harvested after a 4-h incubation and percent specific release was calculated in standard fashion. Small B lymphocytes or bulk PBMCs (not shown) were no more active as stimulators in the helper-independent CD4⁻ MLRs than were monocytes.

Whether monocytes were used fresh or after 2 d of culture in 10% human serum did not affect their function as APCs for unsensitized precursors (data not shown). Bulk mononuclear cells or small B lymphocytes were similarly inactive in eliciting primary proliferative and cytolytic responses (data not shown).

Blood Dendritic Cells, but not Monocytes, Stimulate the Development of Nonspecific Cytolytic Lymphocytes. Antigen-nonspecific NK cells are dependent on lymphokine for their activation and development (22–24). We therefore compared the capacity of blood dendritic cells and monocytes to sensitize NK cell precursors in a bulk T cell popu-
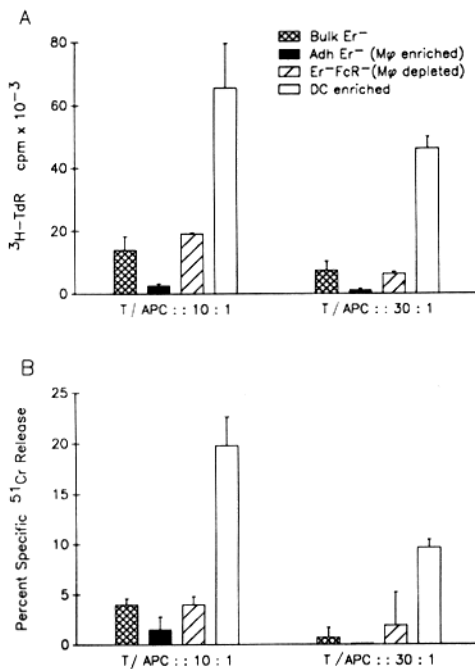
FIGURE 9. Comparison of APC activity in eliciting primary responses by CD4⁻/CD11b⁻ T cells in the helper-independent allogeneic MLR. CD4⁻/CD11b⁻ lymphocytes were stimulated in U-bottomed microwells by the indicated APC populations [Bulk Er⁻, ▓▓; adherent Er⁻, ■; Er⁻FcR⁻, ▨▨; dendritic cells, ▢]. The responder/stimulator doses illustrated are 10:1 (*left*) and 30:1 (*right*). At the end of day 4, the microwells were pulsed with 1 μCi/well of [³H]TdR for 6–8 h and incorporation was measured as an index of proliferation (*A*). Concomitantly, 5×10^3 ^{51}Cr-labeled allogeneic Mø targets were added directly to separate MLR microwells for a standard 4-h CTL assay (*B*).

lation where the amount of lymphokine would not be limiting. As shown in Table I, the percent specific lysis of K562 targets correlated directly with the stimulatory dose of histoincompatible dendritic cells and the amount of proliferation. Monocytes were neither active by themselves, nor inhibitory in combination with dendritic cells, in stimulating lytic effectors against K562 targets.

Discussion

Similar Features of the Murine and Human CD4$^-$ MLRs. As in the mouse (7, 9), resting human lymphocytes that are extensively depleted of CD4$^+$ cells can be induced by blood dendritic cells to synthesize DNA and develop cytolytic activity. In both species CD4$^+$ lymphocyte depletion is sufficiently complete that helper cells are not detectable at the onset or during the CD4$^-$ MLR, and the responses are not inhibited by anti-CD4 mAb. T cell growth factors are produced in both human and murine CD4$^-$ MLRs. However, the magnitude and longevity of lymphokine production and T cell growth are less than observed in populations containing CD4$^+$ lymphocytes.

Other types of APCs in human blood, as in mouse spleen, are weak or inactive in stimulating the CD4$^-$ MLR. Typically, the required ratio of dendritic cells to T cells (e.g., 1:10 or 1:30) is much higher than the number of dendritic cells that are present when bulk mononuclear cells are used as stimulators. This probably accounts for the fact that CD4$^+$-independent cytolytic responses have not been previously described in the human.

The significance of the CD4$^-$ MLR is enhanced by experiments in vivo in the mouse that have shown that resistance to some infectious agents (6) and tumors (25) can develop in the apparent absence of CD4$^+$ T cells. For example, elimination of CD4$^+$ function by in vivo administration of anti-CD4 mAb has little effect on an animal's capacity to develop CTL and to survive infection with ectromelia virus (6),

TABLE I

Blood Dendritic Cells, but not Monocytes, Stimulate Development of K562 Lytic Cells in the Primary Allogeneic MLR

	Dose of allogeneic stimulators [3,000 rad, ^{137}Cs] added at time 0 to 1.5 × 10^5 BULK Er$^+$ NyW NA lymphocytes			
DC MØ	1.5 × 10^4 0	1.0 × 10^4 0.5 × 10^4	0.5 × 10^4 1.0 × 10^4	0 1.5 × 10^4
Percent specific ^{51}Cr release against K562 targets, day 4 of the MLR	44%	42%	36%	5%
[^{3}H]TdR × 6 h, day 4 of the MLR	55,674	53,874	48,079	5,086

1.5 × 10^5 bulk T (Er$^+$ NyW NA) lymphocytes were stimulated in U-bottom microwells by dendritic cells (DC) or adherent Er$^-$ monocytes (MØ). The DC or MØ were added either alone or in combination at the doses indicated. On day 4, 5 × 10^3 ^{51}Cr-labeled K562 cells were added to the microwells, and supernatants were harvested after a 4-h incubation for determination of percent specific release. A parallel group of cultures was pulsed for 6–8 h also on day 4, with 1 μCi/well of [^{3}H]thymidine to measure proliferation. DC, blood dendritic cells. MØ, blood monocytes. Er$^+$ NyW NA, SRBC rosette-positive, nylon wool nonadherent lymphocytes.

even though the neutralizing antibody response to ectromelia is markedly suppressed. One unknown in interpreting these data is whether dendritic cells are capable of presenting peptides derived from ectromelia in association with class I. It is possible that antigen presentation on class I molecules by dendritic cells efficiently primes a CD8$^+$ response in the absence of helper cells. If other APCs are the only source of class I MHC–peptide complexes during an immune response, there may be a greater need for helper cells reactive with class II MHC–peptide complexes.

There are new features and implications, however, that have become evident in the human CD4$^-$ MLR that were not apparent in the murine system. We will discuss several of these.

Antigen-specific CTL and Antigen-nonspecific NK Cells Generated in the Dendritic Cell-stimulated, Helper-independent CD4$^-$ MLR. During the CD4$^-$ MLR, lytic cells develop that kill allospecific targets from the original stimulator as well as nonspecific targets such as K562. The latter are standard targets for NK cells. Precursors of these nonspecific lytic effectors can be depleted by panning with an anti-CD11b mAb, a known marker of NK cells (26, 27). Anti-CD11b, but not a control anti-CD14 mAb, removes a minor CD4$^-$ subset of CD11b$^+$/CD28$^-$, CD16$^+$ cells that is distinct from the major CD11b$^-$/CD28$^+$, CD16$^-$ subset. Negative selection by panning with anti-CD11b, however, does not significantly abrogate the antigen-specific CTL that develop in the helper-independent CD4$^-$ MLR.

It is possible that the NK cells that develop in the CD4$^-$ MLR do so because dendritic cells express epitopes that are also carried by standard K562 targets. Alternatively, the amount of IL-2 secreted in the dendritic cell–stimulated CD4$^-$ MLR may be sufficient to support NK cell proliferation as a bystander event. NK cells are known to expand in response to IL-2; but it is thought that expansion requires relatively high doses of lymphokine, since NK cells predominantly express the p75 intermediate affinity IL-2R (22–24).

A Large Number of Lymphocytes Are Activated during the CD4$^-$ MLR by Allogeneic Dendritic Cell Stimulation. Several changes in surface phenotype are known to accompany human T cell activation. These include the appearance of the p55 IL-2R (CD25) (17) and MHC class II molecules (18, 19). Correspondingly, activated T lymphoblasts do not express a CD45R epitope (20, 21). Using mAb to these antigens to monitor the extent of T cell stimulation at the single cell level, we find that at the peak of the CD4$^-$/CD11b$^-$ MLR, as many as 33% of the cells are enlarged T lymphoblasts expressing the activation phenotype CD25$^+$/HLA-DR,DQ$^+$/CD45R$^-$. Activation antigens and an increase in cell size do not develop if the stimulator cells are allogeneic monocytes or syngeneic dendritic cells.

The frequency of CD8$^+$ T cells that respond to allogeneic dendritic cells seems high, but can be explained if 1–10% of the CD8$^+$ cells respond to allogeneic class I MHC Ag and undergo two to five cell divisions in 4–5 d. This figure is consistent with the number of T cells that can respond to a full allogeneic MHC difference in the rat (28, 29).

Allogeneic Monocytes Are Unable to Stimulate, but also Do Not Inhibit the Helper-independent CD4$^-$ MLR. Other candidate APCs, notably monocytes and small B cells, do not elicit a primary MLR by CD4$^-$ responders. Similar observations have been made in the mouse, but in that species peritoneal macrophages also inhibit the function of allogeneic dendritic cells (9). In man, blood monocytes are not inhibitory even

at high monocyte/dendritic cell ratios (3.3:1). Sprent and Schaefer have described a particular set of experimental conditions whereby certain populations of mouse macrophages can stimulate DNA synthesis in CD4$^-$ lymphocytes (30). It is unlikely that their populations were contaminated with dendritic cells, since the stimulators did not induce a CD4$^+$ MLR. However, in man the blood monocyte population is consistently without APC activity for the primary MLR, whether used freshly isolated from blood or after 2 d of culture on Teflon-coated surfaces in human serum. These apparent species differences may reflect a potential role for macrophage-derived cytokines that may have been present in the experiments of Sprent and Schaefer.

Monocytes are capable of class I alloantigen presentation since they can be recognized as specific targets by sensitized CD8$^+$ CTL (13, 31) elicited by dendritic cells. However, while naive, unsensitized CTLp form discrete aggregates with allogeneic dendritic cells that are easily seen by microscopy within the first 12–24 h of culture, we observe little if any monocyte-T cell cluster formation in our cultures. All of the specific CTL response in the allogeneic MLR develops from these clustered CTLp–dendritic cell aggregates, which can be separated from the nonclustering, nonreactive lymphocytes by velocity sedimentation (our unpublished data). The lack of primary stimulatory function in monocytes may therefore be at the level of contact formation between the APC and the resting, or unprimed CD8$^+$ T cell. These observations are consistent with many prior studies of CD4$^+$ T cell responses in which one of the distinct features of dendritic cells as APCs is their capacity to identify and bind antigen-specific lymphocytes from a pool of resting precursors (32, 33). We have tested whether our culture conditions may limit monocyte-T cell contact by virtue of the fact that monocytes adhere to tissue culture plastic. However, we have cultured CD4$^-$ MLRs in small Teflon beakers to which monocytes do not adhere, and we have still not observed clustering or a productive CD8$^+$ response (our unpublished data).

Implications of CD4$^+$-independent Cytolytic Lymphocyte Responses for Transplantation. CTL directed against major and minor histocompatibility differences have been implicated in graft-vs-host disease (GVHD) in both animal and human models (34–39). Murine CD4$^+$ (class II MHC restricted) and CD8$^+$ (class I MHC restricted) cells can both contribute to GVHD, although the relative contribution of each subset varies with the minor histocompatibility antigens under study (40). Despite apparent HLA identity and MLR compatibility, GVHD and graft rejection remain well-recognized obstacles to the successful use of human allogeneic bone marrow transplantation (41–44). The potential causes are legion, but include class I major and possibly minor histocompatibility differences that would be undetected by available serologic reagents or by standard MLR testing in which bulk mononuclear cells are used as stimulators.

Since alloreactive CD8$^+$ T cells are detected in high frequency when dendritic cells present allogeneic class I molecules, it may be more efficient to detect subtle class I MHC incompatibilities using dendritic cells rather than bulk mononuclear cells as stimulators. We have recently made several improvements in our dendritic cell enrichment protocol so that yield and purity similar to that obtained from leukocyte concentrates can be derived from 50–100 ml peripheral blood samples (our unpublished data). The use of CD4$^-$ responder populations would also focus the analysis on class I MHC incompatibilities that might otherwise be obscured by a

significant syngeneic response to class II MHC antigen by CD4$^+$ T cells. Human CTL responses to minor transplantation antigens could also contribute to GVHD, for which ample precedent exists in animal models where minor histocompatibility differences are better defined (34, 35, 40, 45). Again the use of dendritic cells might help detect minor histoincompatibilities in the human, particularly if both primary and secondary MLRs are examined. Toward these ends we are initiating a clinical study in which we are enriching dendritic cells for comparison of APC function with blood mononuclear cells, to determine if it is possible to identify major and minor histocompatibility differences undetected by standard tissue typing approaches.

Summary

Cytotoxic lymphocytes are typically generated from unfractionated suspensions of human lymphocytes by stimulating with heterogeneous APCs and exogeneous growth factors. We have found that human blood dendritic cells can directly stimulate allogeneic human CD8$^+$ T cells to proliferate and express antigen-specific cytotoxic activity. These primary responses, which are accompanied by the release of T cell growth factor(s), are induced in the absence of CD4$^+$ helper T cells and are not inhibited by anti-CD4 mAb. Both antigen-specific CTL as well as nonspecific NK cells can be elicited by dendritic cells. The NK cell response can be depleted at the precursor level by panning with an anti-CD11b mAb, which removes a CD11b$^+$/CD28$^-$, CD16$^+$ subset from the starting CD4$^-$ responders. Allogeneic blood monocytes are neither stimulatory nor inhibitory of these primary CD4$^-$ MLRs, even though monocytes present alloantigen in such a way as to be recognized as specific targets for CTL that have been sensitized by dendritic cells. The number of CD8$^+$ cells that are blast transformed and express an activated phenotype (i.e., HLA DR/DQ$^+$, CD25/IL-2R$^+$, CD45R$^-$) reaches 30–40% of the culture at day 4–5, the peak of the helper-independent response. We conclude that antigen-presentation by dendritic cells is sufficient in itself to prime cytolytic precursors. We speculate that using dendritic cell stimulators and CD4$^-$ responders in MLRs may be more efficient than standard tissue typing approaches for the detection of subtle, but important class I MHC-restricted histoincompatibilities in human transplantation.

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