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DISTINCT FEATURES OF DENDRITIC CELLS AND ANTI-Ig ACTIVATED B CELLS AS STIMULATORS OF THE PRIMARY MIXED LEUKOCYTE REACTION

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Numerous experiments on the stimulatory requirements for antigen-sensitized T cells have yielded valuable information on the role of antigen processing in generating epitopes that are recognized by T cells in association with MHC molecules (1–6). These studies of immune recognition have used chronically activated or transformed T cells. Stimulation of the MLR is a distinctive system, because it permits an analysis of the activation requirements for small resting T cells and freshly sensitized lymphoblasts derived therefrom. Previously, it has been found that allogeneic MHC molecules on stimulator leukocytes are not sufficient to induce proliferation or lymphokine production from resting T cells. In particular, dendritic cells, small B cells, and macrophages all express MHC products, and can stimulate alloreactive, sensitized T lymphoblasts; yet, dendritic cells are at least 1–2 logs more active in stimulating resting T cells in the MLR (7, 8). Similar findings have been made for allograft responses in situ (9) and for T-dependent antibody responses in culture (10, 11).

The finding that small B cells are inefficient at stimulating resting T cells is of interest, since T cell help for antibody responses is classically delivered to B cells in an MHC-restricted fashion (12, 13). It follows that antigen-specific T and B cells should directly interact during the antibody response. It has previously been shown that preactivation of either the responder T cells, with allogeneic dendritic cells (7, 8), or the stimulator B cells, with LPS (14), leads to significant levels of antigen-specific T-B interactions. Also, there is evidence that antigen-specific B cells bind and process antigen via surface Ig to yield peptides that are presented to T cells in association with class II molecules on the B cell (15). We wondered, therefore, whether B cells that have previously encountered antigen and been activated through their surface Ig might be better than resting B cells as stimulators of primary T cells. We chose to study B cells that have been activated with anti-Ig coupled to Sepharose as a model for antigen-specific activation of B cells (16).

We describe here the properties of these anti-Ig activated B cells (anti-Ig blasts) in the primary MLR, comparing the blasts to small B cells and spleen dendritic cells. We find that allogeneic anti-Ig blasts can directly activate resting CD4$^+$ T cells, and that this process shares many properties in common with dendritic cells. However, there are some important differences between these two types of APCs,

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including their efficiency at binding alloreactive T cells and the molecular interactions required for this binding.

Materials and Methods

Mice

Mice of either sex were used between 7 and 10 wk old. [BALB/c × DBA/2]F$_1$ [C×D2 F$_1$; H-2^d, Mls$^{c×d}$], [C57BL/6 × DBA/2]F$_1$ [B6×D2; H-2$^{b×d}$, Mls$^{b×d}$], and C57BL/6 congenic for H-2^k [B6.H-2^k; Mls^b] were purchased from the Trudeau Institute, Saranac Lake, NY; CBA/J [H-2^k, Mls^d] were purchased from The Jackson Laboratories, Bar Harbor, ME; and C57BL/6 [B6; H-2^b, Mls^b] were purchased from Charles River Breeding Laboratories, Wilmington, MA.

Monoclonal Antibodies

A panel of mAbs, summarized elsewhere (17), were used in the form of hybridoma culture supernatants. For blocking experiments the mAbs were used at a final concentration of 25% (vol/vol); for complement depletion assays, optimal dilutions of mAbs were predetermined in microcytotoxicity assays; and for FACS analysis, the hybridoma supernatants were typically used at a 1:5 dilution.

Culture Medium

Culture medium in all cases was RPMI 1640 containing 10% heat-inactivated FCS (Hy-Clone Laboratories, Logan, UT; or Hazelton Biologics, Inc., Lenexa, KS), 50 μM 2-ME, glutamine, and antibiotics.

Cells

Dendritic Cells. Dendritic cells were prepared from spleen low density adherent cells obtained by flotation on dense BSA gradients (density = 1.08) and adherence to tissue culture dishes (Falcon Labware, Lincoln Park, NJ). Nonadherent cells were vigorously dislodged by pipetting over the monolayer at 2 h. The adherent cells were cultured overnight, whereupon the dendritic cells became nonadherent. The overnight released cells were depleted of contaminating Fc receptor–bearing B cells and macrophages by rosetting with SRBC coated with a mouse anti-SRBC antiserum (18). Alternatively, macrophages were depleted by readhering the cells to tissue culture plastic.

Splenic B cells. Single cell suspensions, depleted of RBC by NH$_4$Cl lysis, were passed over columns of Sephadex G-10 (Pharmacia Fine Chemicals, Piscataway, NJ) to remove macrophage and dendritic cells. T cells were then depleted by incubating the cells with anti-Lyt-1.1 (C3PO), anti-Thy-1.2 (HO-13.4, ATCC TIB 99), and anti-Lyt-2 (3.155, ATCC TIB 211) hybridoma supernatants on ice, then adding rabbit complement (Pel Freeze Biologicals, Rogers, AR) and incubating for 60 min at 37°C. We refer to these cells as small B cells, but these populations contain a small percentage of in vivo–activated, low density B cells (see Results).

B Blasts. B cells were cultured with 5 μg/ml affinity-purified goat anti–mouse Ig (Jackson ImmunoResearch, West Grove, PA) coupled to Sepharose (1 mg antibody per milliliter packed Sepharose; Pharmacia Fine Chemicals) at 2 × 10^6 cells/ml in upright 25-cm T flasks for 48 h. The Sepharose was removed by centrifugation on Ficoll-Paque (Pharmacia Fine Chemicals), and the activated B lymphoblasts were further enriched on discontinuous gradients of Percoll (Pharmacia Fine Chemicals) (19). We refer to such cells as anti-Ig blasts. B cells cultured for 48 h with 20 μg/ml LPS (*Salmonella typhosa*; Difco Laboratories, Inc., Detroit, MI) and purified as described for anti-Ig blasts yielded a population referred to as LPS blasts.

T Cells. Single cell suspensions from either mesenteric lymph node or mesenteric lymph node and spleen were depleted of RBC and then passed over nylon wool columns (Fenwal Laboratories, Deerfield, IL) to remove most dendritic cells, macrophages, and B cells. CD4$^+$ T cells were further enriched by incubating the nylon wool nonadherent cells with anti-Ia (B21-2, ATCC TIB229, anti-I-A^{b,d}; or 10-2.16, ATCC TIB 93, anti-I-A^k) and anti-Lyt-2 (3.155, ATCC TIB 213 and HO-2.2, ATCC TIB 150) plus rabbit complement, as described (7). In most experiments viable T cells were enriched by centrifugation through premixed solutions of 45% Percoll, 45% HBSS, and 10% FCS.

Mixed Leukocyte Reaction

Primary MLR. T cells (3-5 × 10^5) were cultured with graded doses of stimulators in 96-well flat-bottomed plates (microwells) in a total volume of 200 μl for the indicated times. Unless otherwise indicated, anti-Ig blasts and dendritic cells were treated with 3,000 rad and small B cells with 900 rad irradiation from a ^137Cs source before their addition to the cultures. Cultures were pulsed with 1 μCi/well [^3H]TdR (6.7 mCi/mmole; ICN Radiochemicals, Irvine, CA)] for the last 8-16 h of the culture.

Secondary MLR. Alloreactive T lymphoblasts were generated by culturing 5 × 10^6 T cells with 1-2% allogeneic dendritic cells in 24-well flat-bottomed plates (macrowells) (7). At 48 h, dendritic-T cell clusters were isolated by velocity sedimentation on Percoll gradients and then recultured at 3-5 × 10^5 cells/macrowell for 48 h more. Released lymphoblasts were harvested free of clusters by a second velocity sedimentation (7). Contaminating dendritic cells and CD8^+ T cells were removed with mAb (anti-Ia, anti-Lyt-2) plus complement. Alloreactive T blasts were restimulated at 3 × 10^4 blasts/microwell with graded doses of stimulators for an additional 24 h. Cultures were pulsed with [^3H]TdR for the last 8 h.

Lymphokine Bioassays

MLR supernatants were removed from microwells before pulsing (∼80 h of primary MLR) and assayed for IL-2, IL-4, and a B cell growth factor (BCGF).[1]

Interleukin 2. IL-2 was detected by its ability to maintain the growth of CTLL cells seeded at 5 × 10^3 cells per well in a final volume of 100 μl. Human rIL-2 was provided by Dr. Seth Rudnick (Biogen, Cambridge, MA) and served as a positive control. In a more extensive series of lymphokine studies (20) we reported that 40-60% of the CTLL proliferation maintained by primary MLR supernatants was inhibitable by anti-IL-2 mAb S4B6, while the remainder of the supernatant activity was not inhibitable by anti-IL-4 mAb 11B11.

Interleukin 4. IL-4 was detected by the costimulation assay in which IL-4 and anti-Ig together induce [^3H]TdR incorporation in high density or unfractionated murine B cells as described (20). As previously reported, this response is completely inhibited by 2-10 μg/ml 11B11 anti-IL-4 mAb (provided by Dr. E. S. Vitetta, Dallas, TX) (20). Murine rIL-4 was purified on an 11B11 affinity column from the conditioned medium of an IL-4 transfected HeLa cell line (generously provided by Dr. T. Honjo, Kyoto University, Kyoto, Japan) and served as a positive control.

BCGF. Anti-Ig-activated B lymphoblasts proliferate in response to a T cell–derived lymphokine(s) distinct from IL-2 or IL-4. We refer to this activity as B cell growth factor or BCGF (19).

APC–T Cell Binding during the MLR

During an MLR, dendritic cells and alloreactive T cells cluster (7). To measure the clustering activity of anti-Ig blasts and to compare them with dendritic cells, these stimulators were prelabeled with a nontoxic, nontransferable, fluorescent carbocyanine dye (diI; Molecular Probes, Eugene, OR) at 3 μg/ml for 60 min at 37°C, and then washed twice before use (21). The clusters were harvested by velocity sedimentation and the frequency of diI-labeled cells, as well as total cells, in the cluster was enumerated.

FACS Analysis

Cell suspensions (10^5-10^6 cells per sample) were stained on ice with optimal dilutions of rat mAbs. The cells were then washed three times with PBS containing 1% BSA and 0.02% NaN₃, stained with FITC-conjugated mouse anti-rat Ig (Boehringer Mannheim Biochemicals, Indianapolis, IN), washed, fixed in 3.7% formaldehyde, and analyzed on a FACScan (Becton Dickinson & Co., Mountain View, CA). Dead cells were gated out based on light scatter and 5,000-10,000 events were analyzed per sample.

Results

Purity and Phenotype of Dendritic and B Cell Populations. B cells, anti-Ig blasts, and dendritic cells were prepared from the spleens of age- and sex-matched mice. Cell

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[1] *Abbreviation used in this paper:* BCGF, B cell growth factor.

size (forward light scatter) and purity were monitored by flow cytometry. The dendritic cells had greater forward scatter than the B cells, but were roughly equal in *mean* scatter to the anti-Ig blasts (Fig. 1, *left*). The anti-Ig blasts and DC were equally rich in class II MHC products, having 5–10 times more I-A and I-E than small B cells (Fig. 1, *middle*). However, the phenotype of dendritic cells and B cell populations was otherwise distinct (*middle and right*). Small and activated B cells expressed Fc receptors (mAb 2.4G2), the B220 antigen (mAb RA 3–3A1), and the J11d antigen (mAb J11d), whereas purified dendritic cell populations reacted with none of these mAbs. Dendritic cells stained with the 33D1 mAb, while the B cell and anti-Ig blast preparations were negative.

Relative Stimulatory Activity of Dendritic Cells and Anti-Ig Blasts in the MLR.   Small splenic B cells were weak stimulators of the primary MLR, but anti-Ig blasts were active (Fig. 2 *A*). In our initial experiments, the anti-Ig blasts were 3–10 times less active than dendritic cells in the primary MLR, and equal or more active in the secondary MLR, on a per cell basis (Fig. 2, *A* and *B*). Small B cells, in contrast, were poor stimulators in the primary MLR but were only 10 times less active than dendritic cells and anti-Ig blasts in the secondary MLR. Fractionation of these fresh B cells on Percoll discontinuous gradients produced a population of high density B lymphocytes (19), which were totally inactive in the primary MLR but still restimulated T blasts in the secondary MLR (data not shown), suggesting that a few activated B cells in the bulk population give rise to the responses seen at high stimulator doses. Stimulator cells of the wrong MHC haplotype were nonfunctional in restimulating the alloreactive blasts (Fig. 2 *B*) demonstrating the antigen specificity of the secondary MLR.

The weak ability of small B cells to stimulate a primary MLR suggested that the contamination of these preparations with mature dendritic cells, which are vigorous primary MLR stimulators, must be very low. However, in the 48-h anti-Ig activation of the B cells it was possible that relatively immature dendritic cells could mature and contribute to the primary MLR stimulation. To establish purity of the stimulators beyond the FACS studies of Fig. 1, we treated the stimulator populations with cell-specific mAbs and complement before use. The two mAbs used were 33D1, specific for the majority of dendritic cells in spleen (22), and RA3–3A1, specific for the B220 molecule on B cells (23). Depletion with 33D1 abrogated the stimulatory activity of dendritic cells but not anti-Ig blasts, while depletion with anti-B220 only depleted the function of anti-Ig blasts (Fig. 3).

Role of Mls Products in the Anti-Ig Blast Stimulation of the MLR.   In the course of our studies, several strain combinations, involving full MHC mismatches, with or without minor histocompatibility differences, were evaluated. In all cases, dendritic cells were very active as stimulators, but the activity of anti-Ig blasts varied in a strain-dependent fashion (data to be shown in Fig. 7, below). For example, CxD2 anti-Ig blasts were nearly as potent as CxD2 dendritic cells in stimulating B6.H-2^k T cells, while B6.H-2^k anti-Ig blasts were 30-fold less potent than B6.H-2^k dendritic cells at stimulating CxD2 T cells. Since CxD2 but not B6 mice carry strong Mls antigens (24), we suspected that the strong MLR stimulation by anti-Ig blasts in some strain combinations was due to the preferential expression of Mls antigens by those anti-Ig blast stimulators, as has been previously suggested for fresh populations of B cells (25).

FIGURE 1. FACScan profiles to demonstrate the purity and phenotype of the stimulator populations. Anti-Ig blasts (*top*), dendritic cells (*middle*), and small B cells (*bottom*) were stained with a panel of rat anti–mouse mAb, followed by FITC–mouse anti–rat Ig. The left column of panels are forward scatter profiles of the cell populations. (Note, all samples were analyzed at constant settings for forward and side scatter and fluorescence.) The remaining panels are fluorescence intensity vs. frequency histograms for a series of IgG2b and IgM rat anti–mouse mAbs, as indicated in the top panels. The IgG2b mAbs (*middle*) are: 2.4G2 anti-FcR, 33D1 anti–spleen dendritic cell (ATCC TIB 227), and M5/114 anti-Ia (ATCC TIB 120). The IgM mAbs (*right*) are: J11d (ATCC TIB 183), RA3-3A1/6.1 anti-B220 (ATCC TIB 146), and 3.155 anti-Lyt-2 (ATCC TIB 211). In all cases, the no-primary controls were PBS containing 1% BSA, 0.02% NaN₃. Note that RA3-3A1 and J11d staining of small B cells revealed a subpopulation of cells that did not stain with either mAb. These small leukocytes (as judged by light scattering and staining with mAb to leukocyte common antigen) typically represent 10–20% of our B cell preparations, but are negative for most common T, B, dendritic, and macrophage cell markers. Though we have not positively identified the population, e.g., NK cells, it is clear that these cells are not found after the 48 h culture to produce anti-Ig blasts.
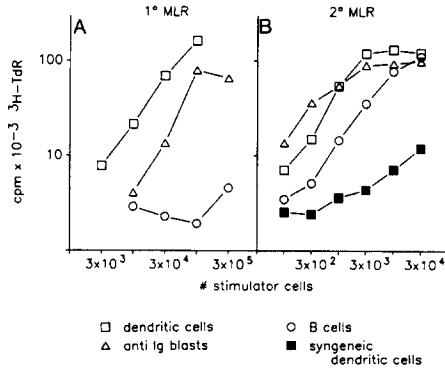
RELATIVE FREQUENCY

FLUORESCENCE INTENSITY

FORWARD SCATTER

anti-Ig BLASTS

DENDRITIC CELLS
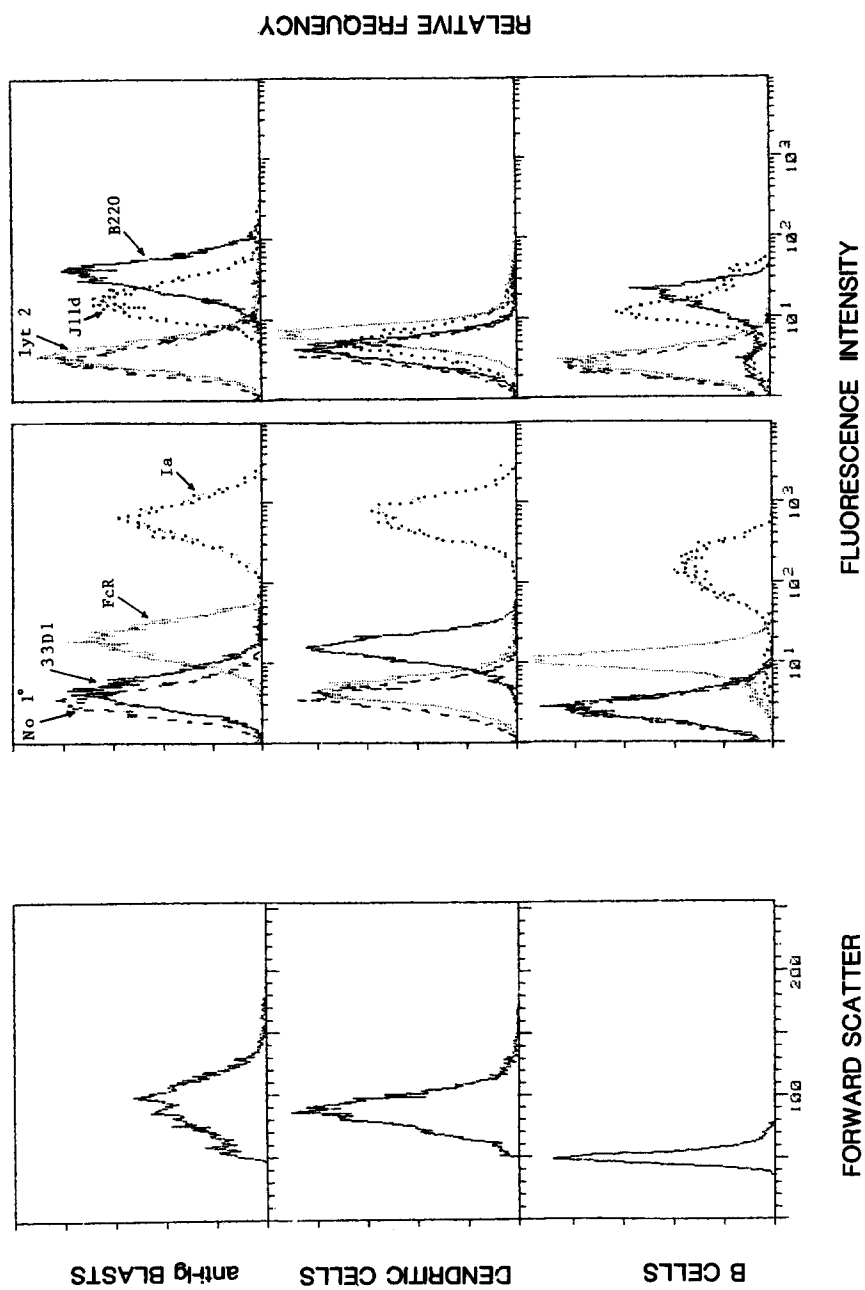
B CELLS

FIGURE 2. The relative capacity of dendritic cells, anti-Ig blasts, and small B cells to stimulate the MLR. (A) 3 × 10⁵ CD4⁺ CBA/J T cells were stimulated in microwells with graded doses of stimulators from CxD2 spleens. Cultures were pulsed with 1 μCi/well [³H]TdR from 85–93 h of the MLR. Proliferation of T cells cultured alone was <2,000 cpm. (B) 3 × 10⁴ CBA/J anti-CD2 T blasts (see Materials and Methods) were restimulated in microwells with graded doses of allogeneic irradiated stimulators, as above. Control stimulators were syngeneic spleen dendritic cells. Cultures were pulsed from 18–24 h of the secondary MLR. [³H]TdR incorporation of T blasts alone was <3,000 cpm.

The Mls refers to a set of non-MHC–linked loci, which elicit strong proliferative responses from resting T cells in mixed leukocyte cultures (24, 26). To pursue the role of Mls, we compared the ability of anti-Ig blasts and dendritic cells to stimulate Mls-mismatched but MHC-matched CD4⁺ T cells. CBA dendritic cells stimulated both Mls-mismatched (B6.H-2ᵏ) T cells and syngeneic (CBA) T cells at equivalent levels, so that the dendritic cells did not appear capable of presenting Mls (Fig. 4, top left, right). However, CBA anti-Ig blasts were 100 times more potent in stimulating B6.H-2ᵏ vs. CBA T cells (Fig. 4, top left, right). Fresh B cells did stimulate an Mls response, but at a 30-fold lower efficiency than activated B cells, consistent with a low level contamination of fresh B cells with in vivo–activated B cells (data not shown). In the absence of an Mls stimulus, dendritic cells were 10–30-fold more potent than anti-Ig blasts at stimulating allogeneic T cells (Fig. 4, bottom). For the remainder of our studies, except where noted, we chose to study the activity of these different APCs in the absence of strong Mls responses because the nature and significance of this locus remains unknown, in contrast to the MHC, which is critical in transplantation and antigen presentation.

Common Features of Dendritic Cells and Anti-Ig Blasts as MLR Stimulators. One feature known to be shared by dendritic cells and LPS-induced B blasts is that stimulatory activity is resistant to substantial doses of ionizing irradiation (27). Stimulatory
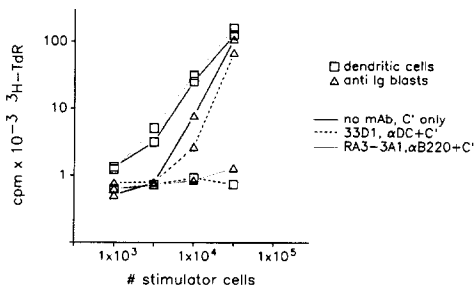

FIGURE 3. Purity of dendritic cell and anti-Ig blast stimulator populations. CxD2 spleen dendritic cells and anti-Ig blasts were prepared and the cells were then split into three aliquots and incubated on ice with 33D1 (anti-dendritic cell mAb), RA3-3A1/6.1 (anti-B220 mAb), or control medium. Rabbit complement was added and further incubation was continued at 37°C for 60 min. After washing, cells were resuspended to concentrations based on precomplement depletion numbers, and added in graded doses to 3 × 10⁵ CD4⁺ CBA/J T cells. Percent specific kill (relative to control medium) in the experiment shown was 33D1: dendritic cells, 100%; anti-Ig blasts, 22%; RA3-3A1: dendritic cells, 0%; anti-Ig blasts, 95%. Cultures were pulsed from 100–112 h of the MLR.
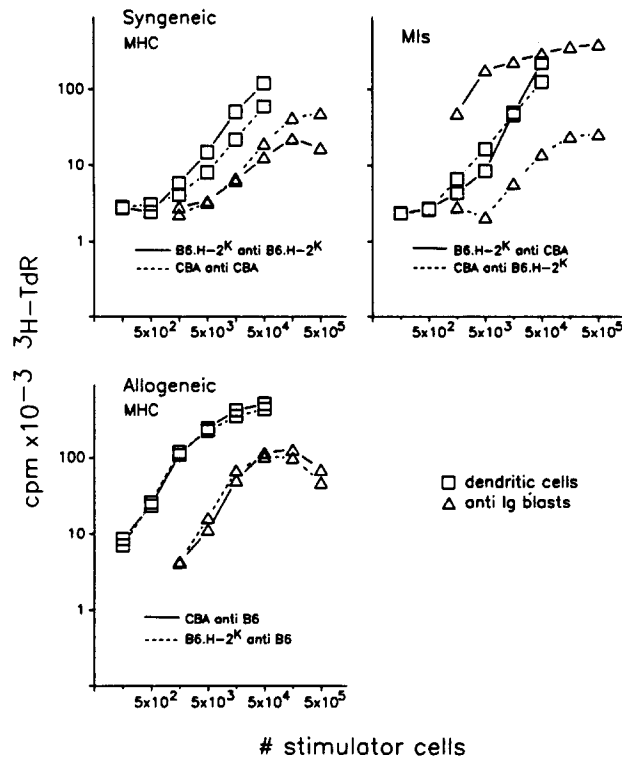
FIGURE 4. Role of Mls vs. H-2 antigens in the primary MLR stimulated by dendritic cells vs. anti-Ig blasts. 5×10^5 CD4$^+$ T cells were stimulated with graded doses of anti-Ig blasts or dendritic cells in the strain combinations indicated. Anti-Ig blasts and dendritic cells received 3,000 rad irradiation before use as stimulators. Cultures were pulsed from 73–85 h of the primary MLR. (*Left*) Syngeneic MLR. B6.H-2^k stimulators with B6.H-2^k responders and CBA/J stimulators were cultured with CBA/J responders. (*Right*) Mls stimulation. CBA/J stimulators were cultured with B6.H-2^k responders (Mls strong) and B6.H-2^k stimulators were cultured with CBA/J responders (Mls weak). (*Bottom*). Allogeneic MLR. B6 stimulators were cultured with CBA/J or B6.H-2^k responders.

activity of anti-Ig blasts was comparable whether the cells were exposed to 900 or 3,000 rad of ^{137}Cs, or mitomycin C (Fig. 5 *A*). In contrast, the weak stimulation by small B cells was sensitive to 3,000 rad ^{137}Cs (Fig. 5 *A*) as reported (28).

The MLR induced by either dendritic cells or anti-Ig blasts was blocked by mAb to the appropriate class II MHC products (Fig.5 *B*), as expected for the CD4$^+$ T cell MLR. mAbs to an irrelevant MHC haplotype, or to several non-MHC antigens on the stimulator surface (anti-FcR, anti–leukocyte antigen) failed to block the MLR (data not shown).

Both dendritic cells and anti-Ig blasts induced the production of several lymphokines in the primary MLR (Table I), extending our previous observations on dendritic cells as primary MLR stimulators (20). Three factors were evaluated with specific bioassays: IL-2, IL-4, and a BCGF that supports the growth of anti-Ig blasts but is distinct from IL-2 and IL-4. All three activities were detected, although, on a per cell basis, the dendritic cells were ~3–10 times more active than the anti-Ig blasts in lymphokine induction (Table I). The relative levels of lymphokine production correlated well with the relative levels of T cell proliferation induced by the different stimulator cells (Fig. 5 *A*). Small B cells were far less active than either anti-Ig blasts or dendritic cells at inducing these factors, consistent with the minimal T cell proliferation stimulated by these cells in the primary MLR.

Capacity of Different APC to Cluster T Cells. Cellular aggregates, which are selectively enriched in dendritic cells and antigen-specific T lymphocytes, are the sites in which primary MLRs are initiated in mouse (7, 21) and man (29). We noted
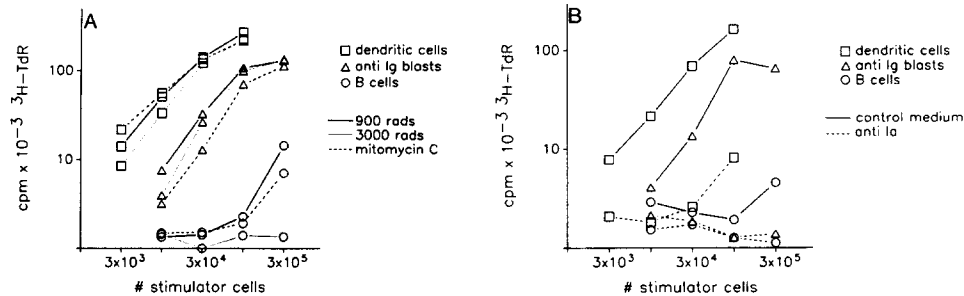
FIGURE 5.   Comparable features of anti-Ig blasts and dendritic cells as MLR stimulators. (*A*) Radioresistance of MLR stimulatory function. B6xD2 dendritic cells, anti-Ig blasts, and B cells were prepared and added in graded doses to 3×10^5 CD4$^+$ CxD2 T cells. Before addition, the stimulators were irradiated with 900 or 3,000 rad from a ^{137}Cs source, or treated with mitomycin C (Sigma Chemical Co.) at 40 µg/ml for 30 min at 37°C. Cultures were pulsed from 72 to 90 h. In a parallel secondary restimulation of alloreactive T blasts, only the stimulation by freshly isolated B cells was abolished by high dose ionizing radiation (data not shown). (*B*) mAb to I-A/I-E blocks MLR stimulation. CxD2 spleen-derived dendritic cells, B cells, and anti-Ig blasts were prepared and added in graded doses to 3×10^5 CD4$^+$ CBA/J T cells. 25% (vol/vol) anti-Ia hybridoma supernatant (M5/114 ATCC TIB 120) or control medium was added at the start of the MLR. M5/114 reacts with both I-A and I-E of the H-2^d haplotype. Cultures were pulsed from 85 to 93 h of the MLR. A control isotype-matched hybridoma supernatant had no effect on MLRs by all three stimulators (data not shown).

TABLE I

Lymphokine Production in the MLR Induced by Different Populations of Allogeneic Leukocytes

| Stimulator | | [^{3}H]TdR uptake (cpm $\times$ 10^{-3}) at graded stimulator:responder ratios | | | | | | | | | | | | |
| | | IL-2 | | | | | IL-4 | | | | BCGF | | | |
Cells	Treatment	1:1	1:3	1:10	1:30	1:100	1:1	1:3	1:10	1:30	1:1	1:3	1:10	1:30
DC	900 rad	—	235	252	235	88	—	51	37	15	—	21	17	12
	3,000 rad	—	225	234	193	71	—	50	28	21	—	26	16	10
	Mitomycin C	—	211	213	207	140	—	39	30	20	—	20	19	12
α-Ig B	900 rad	189	205	117	34	—	31	26	19	18	14	15	9	7
	3,000 rad	214	213	82	30	—	45	34	26	22	17	14	9	7
	Mitomycin C	206	194	62	17	—	32	19	21	17	15	14	8	8
Sm B	900 rad	81	22	10	14	—	13	16	11	11	5	6	6	6
	3,000 rad	12	10	6	5	—	15	13	15	16	4	4	5	4
	Mitomycin C	62	30	10	7	—	16	13	15	16	6	7	7	8

The MLR cultures are identical to those set up to measure proliferative responses in Fig 5A. At 72 h of the ongoing primary MLR, 100 µl was removed from each microwell and tested for IL-2, IL-4, and BCGF.

IL-2 Assay.   Supernatants were tested in the CTLL proliferation assay at 20% vol/vol. The [^{3}H]TdR incorporation by CTLL cultured with supernatants from unstimulated T cells was 6,333 cpm. The maximum response with human rIL-2 (50 U/ml) was 142,907 cpm.

Anti-Ig Costimulation Assay for IL-4.   Supernatants were tested at 25% vol/vol in the assay. Supernatants from unstimulated T cells induced incorporation of 2,887 cpm. The response to anti-Ig in the absence of supernatants was 11,103 cpm. The reponse to supernatants alone, in the absence of anti-Ig, ranged from 1,900 to 7,000. The maximum response with murine rIL-4 (100 U/ml) + anti-Ig was 80,878 cpm, and with murine rIL-4 alone was 7,860 cpm.

BCGF Assay.   Supernatants were tested at 25% vol/vol for BCGF activity on anti-Ig pre-activated blasts. [^{3}H]TdR incorporation of anti-Ig blasts recultured in the absence of supernatants was 3,850 cpm. The maximum response with EL-4-conditioned medium was 35,556 cpm.

that anti-Ig blasts could also form clusters with allogeneic T cells in the primary MLR, while small B cells could not form these clusters, as described previously (7, 29). We therefore designed experiments to test if the differences between the efficiency of dendritic cells and anti-Ig blasts as stimulators for a primary MLR related to their abilities to cluster T cells or, alternatively, to activate the T cells once contact was made. The stimulator dendritic cells and anti-Ig blasts were labeled with a fluorescent, lipophilic carbocyanine dye. Dye labeling, as described (21), did not interfere with stimulatory activity or undergo transfer to the T cells, so that the number of APC in the clusters could be enumerated on a hemocytometer. Clusters were isolated at 48 h of the MLR. After enumerating total and dye-labeled cells (which was used to calculate the number of responder T cells in the clusters), the APC:T aggregates were returned to culture to monitor T cell proliferation. Although the clusters were dissociated during their isolation, the aggregates quickly reformed in culture (data not shown).

In Table II we have tabulated the results from a typical primary MLR involving MHC but not Mls stimuli (B6.H-2^k stimulating CxD2). The unseparated cultures demonstrated a much higher MLR (30-fold when stimulator dose-response curves were compared) with dendritic cells as compared to anti-Ig blasts (2nd line). In parallel macrowell cultures, anti-Ig blasts were much less efficient at clustering T cells; 20-fold fewer T cells were aggregated in the first 48 h of the MLR as compared with comparable numbers of dendritic cells (3rd line). In addition, a much lower percentage of the added anti-Ig blasts were found in clusters with nearly all being

TABLE II

Comparison of Dendritic Cells and Anti-Ig Blasts in the Initial Clustering of Antigen-reactive T Cells

	Dendritic cells	Anti-Ig blasts
Initial APC:T cell ratio	1:30	1:30
Proliferation of bulk cultures (cpm × 10^{-3})	248	4.5
Percent of recovered T cells in clusters at 48 h	10.1	0.4
Percent of recovered APC in clusters at 48 h	58.8	2.3
Proliferation of clusters (cpm × 10^{-3})	24.9	11.0

5×10^6 or 5×10^5 CxD2 bulk T cells were cultured in 24- or 96-well flat-bottomed plates, respectively. Specified doses of irradiated, diI-labeled, B6.H-2K spleen dendritic cells or anti-Ig blasts were added. At 48 h, the macrowell cultures (5 wells per dose of APC) were gently harvested and layered on Percoll velocity gradients. Cluster and noncluster bands were harvested separately and washed twice. Aliquots of each sample were dissociated by pipetting and counted on a hemocytometer under bright field and green fluorescence optics in order to enumerate the total and dye-labeled cell recoveries. The difference between the two numbers was calculated, in each case, to represent the number of T cells recovered. The ratio of dendritic cells to T cells in the cluster was 1:4; anti-Ig blasts/T cells, 1:3. In all cases the total recovery of cells was <50%, primarily due to losses on the velocity gradients. Based on the above calculations, equivalent numbers of clustered T cells (6×10^3/well) were returned to culture. All cultures were pulsed from 91 to 99 h.

found in the noncluster fraction. In contrast, the majority of dendritic cells were recovered in the cluster fraction (4th line).

When equivalent numbers of clustered T cells were recultured, comparable levels of proliferation were noted (5th line). These studies suggest that anti-Ig blasts, once they have bound to T cells, can induce a proliferative response just like dendritic cells, but that the ability of anti-Ig blasts to form clusters is relatively weak.

Role of LFA-1 in Cluster Formation in the Dendritic and Anti-Ig Blast MLRs.   In view of the difference in efficiency with which dendritic cells and anti-Ig blasts formed aggregates with T cells, we investigated whether the two types of cells might use different cell–cell binding mechanisms. We evaluated the effects of anti-LFA-1 mAb, which is known to reduce leukocyte cell–cell adhesions in several systems (30).

In keeping with prior observations (31), dendritic-T cell clustering appeared similar in the presence or absence of anti-LFA-1 mAb over the first 24–48 h of the primary MLR (Fig. 6, *top*). At later time points, anti-LFA-1 appeared to weaken the clusters, which began to fall apart. In contrast, mAb to LFA-1 totally blocked the clustering of anti-Ig blasts and T cells from the onset of the primary MLR (Fig. 6, *bottom*). Other mAbs (to C3biR, FcR, common leukocyte antigen, or Thy-1) did not block clustering with either dendritic cells or anti-Ig blasts as stimulators (data not shown).
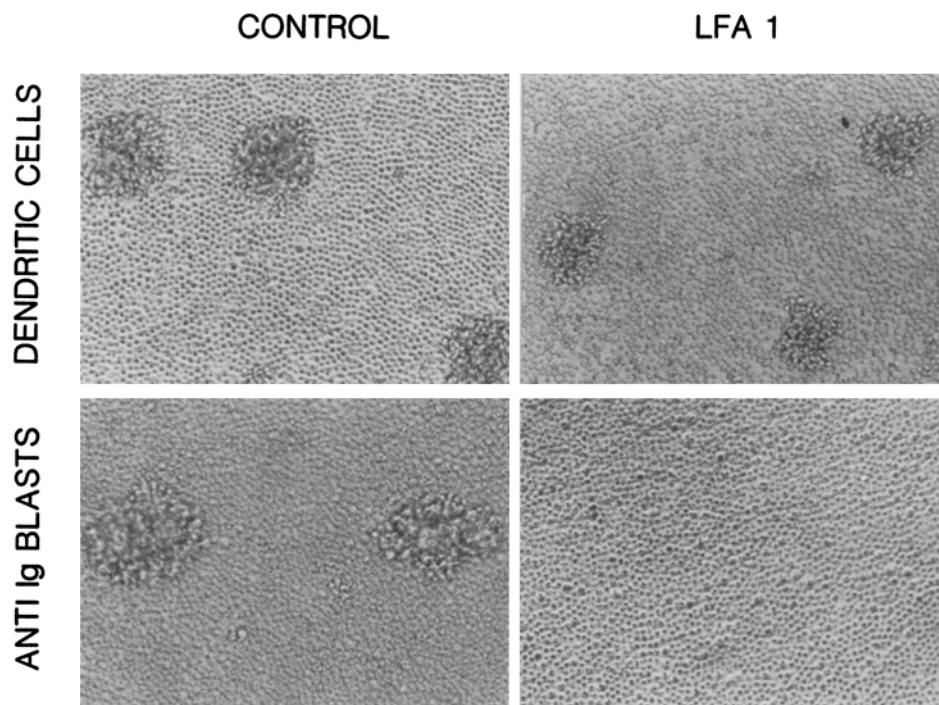

FIGURE 6.   Contrasting effects of anti-LFA-1 on the capacity of dendritic cells and anti-Ig blasts to cluster responding T cells. Micrographs of MLRs between 5×10^5 CD4$^+$ B6.H-2K T cells and 1.5×10^5 CxD2 dendritic cells (*top*) or anti-Ig blasts (*bottom*) in the presence (*right*) or absence (*left*) of 25% (vol/vol) anti-LFA-1 hybridoma supernatant (FD441.8 ATCC TIB 213). Cultures were photographed from 96-well flat-bottomed plates at 24 h of the MLR.

In addition, co-addition of 2.4G2 anti-FcR mAb with anti-LFA-1 did not inhibit the blocking effect of the anti-LFA-1 mAb, suggesting that the blocking effect was not mediated by a negative signal delivered through binding to the anti-Ig blast FcR. Furthermore, anti-LFA-1 caused only a minimal block of clustering and proliferation in the secondary MLR that was restimulated by either dendritic cells or anti-Ig blasts (data not shown).

When proliferative responses were monitored, anti-LFA-1 substantially, but incompletely, reduced the dendritic cell–induced MLR. In contrast, the anti-Ig blast MLR was totally blocked by anti-LFA-1 (Fig. 7, *A* and *B*). Even in MLRs involving MHC and Mls stimulation, where the anti-Ig blasts were nearly equipotent with dendritic cells as stimulators, the anti-LFA-1 mAb still incompletely blocked dendritic cell stimulatory activity but completely blocked anti-Ig blast stimulatory activity (Fig. 7 *A*). These data suggest that LFA-1 plays a critical role in anti-Ig blast–T cell binding, but that binding and some proliferation can occur when anti-LFA-1 is added to dendritic cell–stimulated responses.

Stimulating Activity of LPS-induced B Blasts.   We studied B cells that were activated with LPS to see whether these blasts were similar to anti-Ig–activated B cells. Indeed, LPS blasts also formed clusters with T cells in a primary MLR and this led to T cell blastogenesis. The efficiency of this process was similar to that seen with anti-Ig blasts. Furthermore, the formation of LPS blast–T cell clusters was completely inhibited by mAb to LFA-1 as was any subsequent T cell proliferation (Fig. 7 *C*). Therefore, at this point in our studies, we are not aware of any differences between anti-Ig blasts and LPS blasts as stimulators of the MLR.
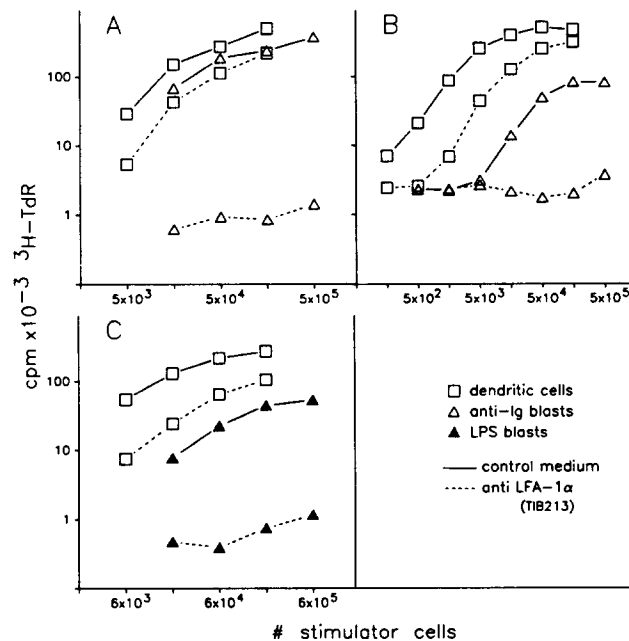

Figure 7.   Role of LFA-1 in dendritic cell vs. anti-Ig blast–stimulated primary MLR. (*A*) CxD2 spleen dendritic cells and anti-Ig blasts were added in graded doses to 5×10^5 CD4$^+$ B6.H-2K T cells. At the start of the reaction, 25% (vol/vol) anti-LFA-1 hybridoma supernatant (FD441.8 ATCC TIB 213) or control medium was added. Cultures were pulsed from 88 to 96 h of the MLR. (*B*) B6.H-2^k spleen dendritic cells and anti-Ig blasts were added in graded doses to 5×10^5 CD4$^+$ CxD2 T cells. Anti-LFA-1 mAb was added as above and cultures were pulsed from 73 to 88 h. (*C*) B6.H-2K spleen dendritic cells and LPS blasts were added in graded doses to 5×10^5 CD4$^+$ CxD2 T cells. Anti-LFA-1 mAb was added as above and cultures were pulsed from 72 to 90 h.

Discussion

Previously, it was reported that freshly isolated B cells failed to activate resting T cells in a primary MLR, but the same B cells effectively restimulated T lymphoblasts that had been initially activated by dendritic cells (7, 8). Since preactivation of the T cells with antigen permitted T-B interactions, we considered whether preactivation of the B cells with anti-Ig might lead to effective T-B interactions. Prior studies had shown that LPS-induced B blasts were more potent than small B cells as MLR stimulators (7, 14). In this paper, we found that both dendritic cells and anti-Ig–activated B cells expressed similar levels of class II MHC products, and that both cell types could elicit strong proliferation and lymphokine production from allogeneic, $CD4^+$ resting T cells, in an antigen-dependent fashion. In addition, both stimulators formed stable aggregates, or clusters, with the responding T cells that could be isolated during the first 48 h of the primary MLR. However, there were marked differences in the potency of allogeneic stimulation and apparent differences in the mechanisms whereby dendritic cells and anti-Ig blasts bound antigen-reactive T cells.

Variability in the Potency of Anti-Ig Blast Stimulatory Activity.   Although anti-Ig blasts could independently stimulate a primary MLR, in many strain combinations tested, their potency as stimulators, on a per cell basis, ranged from 3- to 30-fold less than dendritic cells. One source of this variability pertained to the distinct roles of MHC and Mls antigens in an MLR. If only an MHC mismatch was present, dendritic cells were 20–30-fold more potent than anti-Ig blasts, whereas if Mls was the stimulatory locus, anti-Ig blasts were at least 10-fold more potent than dendritic cells (Fig. 4). It was not clear whether dendritic cells carried Mls antigens at all, since the amount of proliferation with Mls disparate combinations (B6.H-2^k stimulated by CBA) was equivalent to that observed with fully syngeneic dendritic and T cells (Fig. 4). In the presence of both Mls and MHC stimuli, i.e., CxD2 stimulating B6.H-2^k, both dendritic cells and anti-Ig blasts were potent stimulators of primary T cells (Fig. 7 A).

Further interpretation of these results is difficult due to the limited information currently available on the nature of allogeneic MHC or Mls antigens. Recent data on the structure of MHC molecules and their capacity to handle peptide antigens suggest a role for more than just the MHC itself in allogeneic MHC stimulation; it is likely that the complete transplantation antigen includes a peptide bound to the MHC product (32). It is possible that anti-Ig blasts and dendritic cells, while carrying equivalent levels of the MHC determinants recognized by anti-Ia mAbs, differ significantly in the array of peptides that they are presenting during the MLR. Anti-Ig blasts increase their surface levels of Ia antigens by 10-fold during the 48 h of preparative culture, presumably by complexing newly synthesized class II molecules with the limited array of foreign proteins in FCS. Dendritic cells, in contrast, have high levels of Ia antigens upon isolation, so it follows that these class II molecules are assembled in situ and may be charged with the complex array of foreign and self proteins encountered in lymphoid and nonlymphoid tissues. This hypothesized difference between the source of the peptides presented on the class II molecules of these two APC is one possible explanation for the observed difference in their MLR stimulatory abilities. Still it is unlikely that B cells are totally devoid of these complete transplantation antigens since even small, freshly isolated B cells can restimulate alloreactive T cells in an antigen-dependent manner.

The nature of the Mls antigen(s) is even more obscure. Mls reactive T cell clones

show little MHC restriction (26), yet a limited range of TCR Vβ genes, which typically see antigen in association with MHC, seem to be used in Mls responses (33, 34). The latter has suggested that the Mls antigen is a peptide that is presented on MHC molecules to the conventional TCR (33). If so, strong stimulation by anti-Ig blasts in an Mls response could be explained if this locus encoded a protein/peptide that was specifically expressed by B cells, in association with MHC molecules, and that reacted with a large percentage of peripheral T cell receptors.

Differences in T Cell Binding Mechanisms. In the absence of a strong Mls antigen, dendritic cells were always more efficient MLR stimulators than anti-Ig blasts. This increased efficiency was reflected in an increased capacity to find and aggregate alloreactive T cells. A much higher percentage of dendritic cells formed clusters with T cells, and per input dendritic cell, many more T cells aggregated (Table II). Once a dendritic cell or anti-Ig blast had clustered with a T cell, then the level of the proliferative response of the clustered T cells was similar. This suggests that these two types of APC, once bound to the T cells, can exert whatever accessory functions are required to make the T cell grow and secrete lymphokines.

In the previous section, we suggested one possible explanation for these observed differences in clustering efficiency; dendritic cells may have more of the appropriate alloantigens that participate in the binding of antigen-specific T cells. An alternative, or additional, possibility is that the two types of APC use different accessory molecules to bind T cells. A variety of adhesion molecules have been implicated in leukocyte cell–cell binding events. One of these, LFA-1, does not appear to be critical for the initial binding of murine dendritic cells to T cells (31). In contrast, we find that the anti-Ig blast binding to T cells is completely inhibited by anti-LFA-1 mAb.

There are several possible explanations for this difference in sensitivity to anti-LFA-1. (*a*) The dendritic cell may initially bind T cells via a ligand that differs from that recognized by LFA-1; (*b*) relative to anti-Ig blasts, the dendritic cell might have very high levels of a ligand for LFA-1, so that it is difficult to block function with a mAb; and (*c*) a T cell subpopulation may be responding to the anti-Ig blasts, and this subpopulation is uniquely dependent on accessory cell binding via an LFA-1 mechanism. Recently, it has been suggested that in the human system, memory T cells are distinguished from virgin T cells by displaying higher levels of several molecules, including LFA-1 (35). Though a similar subpopulation has yet to be identified in the mouse, they may be the cells that are specifically stimulated by MHC-mismatched anti-Ig blasts. Any of the above explanations could also account for the observed efficiency displayed by dendritic cells in binding T cells, compared with anti-Ig blasts.

Pathways for B-T Interaction during T-dependent Antibody Responses. The capacity of B cells to interact directly with T cells in situ is likely given the observations that many T-dependent antibody responses are restricted to the MHC of the B cell, and both MHC and TCR molecules are transmembrane glycoproteins. However, the studies of the MLR and antibody responses in culture have indicated that either the T cell or B cell must first be stimulated before an MHC-restricted cell–cell interaction occurs.

T lymphoblasts can be restimulated by small B cells in an MHC-restricted fashion, and these blasts can bind B cells in rapid binding assays (7, 36). However, dendritic cells are required to generate these blasts from resting precursors. It was therefore

proposed that one pathway for generating an antibody response would be for the dendritic cell to induce T lymphoblasts and then the latter could interact with the B cell (7, 11).

The data on B cells, in this and other studies, are that B lymphoblasts can initiate a primary MLR whereas small B cells are weak stimulators. In fact, when high density B cells were isolated, we were unable to detect any primary MLR stimulatory activity, suggesting that the weak activity of freshly isolated small B cells was due to their contamination with lower density B blasts generated in situ. To further assess the physiological impact of our findings, it will be important to determine if T-dependent antigens can induce a B blast that is comparable to the anti-Ig and LPS blasts that have been studied. It also will be of interest to compare B blasts and dendritic cells as stimulators of allogeneic T cells in vivo to further assess their capacity to initiate cell-mediated immune responses.

Summary

Highly enriched populations of B lymphoblasts have been isolated after culture with anti-Ig-Sepharose and compared with dendritic cells as stimulators of CD4$^+$ T cells in the murine MLR. The two populations clearly differed in phenotype; anti-Ig blasts were FcR$^+$, B220$^+$, 33D1$^-$, while dendritic cells were FcR$^-$, B220$^-$, 33D1$^+$. However, as MLR stimulators, they shared many common features. Both cells (a) expressed comparable levels of class II MHC products; (b) independently stimulated the primary MLR and the production of several T derived lymphokines including IL-2 and IL-4; and (c) were comparable in stimulating freshly sensitized T cells. However, the relative potencies of dendritic cells and anti-Ig blasts as primary MLR stimulators varied in a strain-dependent fashion. Only anti-Ig blasts could stimulate across an Mls barrier, being at least 100 times more active in stimulating Mls-mismatched, MHC-matched T cells, relative to syngeneic T cells. In contrast, dendritic cells were 10–30 times more potent than anti-Ig blasts when stimulating across an MHC barrier and were likewise more effective in binding MHC-disparate T cells to form the clusters in which the MLR was generated. Dendritic cell–T cell clustering was resistant to anti-LFA-1 mAb, while B blast–T cell clustering was totally blocked. Thus, anti-Ig B lymphoblasts and dendritic cells, two cell types which differ markedly in phenotype, also differ in efficiency and mechanism for initiating responses in allogeneic T cells.

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