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# DENDRITIC CELLS ARE THE PRINCIPAL STIMULATORS OF THE PRIMARY MIXED LEUKOCYTE REACTION IN MICE\*

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Until now, the study of dendritic cells (DC)<sup>1</sup> has required positive selection techniques. Populations enriched in DC have been shown to be powerful stimulators first of the mixed leukocyte reaction (MLR) (1) and subsequently of a number of other responses in vitro (2–11) and in situ (12, 13).

Negative selection experiments have yet to be performed. Specific depletion protocols would help establish if DC are the principal cells required for MLR stimulation and other accessory functions. Recently we obtained a stable hybridoma, 33D1, that secretes a specific anti-DC antibody (14). The 33D1 antigen appears to be found only on DC, at a frequency of  $1\text{--}1.5 \times 10^4$  antibody binding sites per cell. The antigen has yet to be identified biochemically but most likely, it is not an immune response associated or Ia antigen. In the presence of rabbit complement, 33D1 efficiently kills DC, but no other cells in spleen, node, thymus, marrow, blood, and peritoneal cavity. This monoclonal should provide a way to selectively eliminate DC and to measure the functional consequences that ensue.

Here we show that selective removal of DC dramatically reduces the MLR stimulatory capacity of both unfractionated spleen and spleen adherent cells. Function is reconstituted with small numbers of DC purified by a technique that is independent of the 33D1 antibody. We propose that antigen, in combination with DC, represents the physiologic pathway for inducing the MLR and other immune responses.

## Materials and Methods

**Mice.** 6–12-wk-old mice of both sexes were obtained from the Trudeau Institute, Saranac Lake, NY (BALB/c; C57B1/6; BALB/c  $\times$  C57B1/6 F<sub>1</sub>; DBA/2  $\times$  BALB/c F<sub>1</sub>; B6.H2k), and from the Mayo Clinic, Rochester, MN, courtesy of Dr. C. David (B10.TL; B10.S (7R)).

**Antibodies.** 33D1 (14) is a hybrid between the (P3 $\times$ 63-Ag8-U1) mouse myeloma and a rat spleen cell that secretes a DC-specific antibody (Ab). The original hybrid was cloned on soft agar and all seven clones evaluated were specifically cytotoxic for DC. One clone, 33D1-4, was injected into irradiated (600 rad), pristane primed (0.5 ml; Aldrich Chemical, Milwaukee, WI), D<sub>2</sub>  $\times$  C F<sub>1</sub> mice to provide ascites. Immunoglobulin (Ig) was precipitated with two cycles of 45% vol/vol saturated ammonium sulfate and further purified by passage over diethylaminoethyl cellulose (DE-52; Whatman, Ltd., Maidstone, England) in 0.0175 M phosphate buffer,

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<sup>1</sup> *Abbreviations used in this paper:* Ab, antibody; C', complement; CTL, cytotoxic T lymphocyte; DC, dendritic cells; EA, sheep erythrocytes opsonized with anti-sheep cell serum; FCS, fetal calf serum; MHC, major histocompatibility complex; MLR, mixed leukocyte reaction; M $\phi$ , macrophages; SDS, sodium dodecyl sulfate.

pH 6.3. 33D1 Ig was stored at 1–3 mg/ml phosphate-buffered saline at 4°C. On one-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, the 33D1 Ig had a single heavy chain band and two sharp light chain bands (see Fig. 1 and ref. 14). The heavy chain had the mobility of a  $\gamma$  chain, but the subclass has not been typed. In addition to purified Ig, we used 33D1-4 culture supernatants grown in Dulbecco's modified medium (Grand Island Biological Co., Grand Island, NY) supplemented with NCTC 109 medium (6%; M.A. Bioproducts, Walkersville, MD), horse serum (10%; Flow Laboratories, Rockville, MD) and fetal calf serum (FCS) (5%, HiClone; Sterile Systems, Logan, UT).

F4/80 is a rat-mouse hybridoma that is macrophage (M $\phi$ ) restricted (15) and does not bind to DC (16). It was kindly provided by Dr. J. Austyn and Dr. S. Gordon, and used as a culture supernatant to stain M $\phi$  (see below).

Two monoclonal anti-Ia reagents were used to stain and kill Ia-bearing cells. B21-2 is a rat anti-mouse I-A<sup>b,d</sup> (17). Purified Ig was obtained from ascites and used in cytotoxicity studies, while an F(ab')<sub>2</sub> fragment was used for indirect immunofluorescence. To prepare the F(ab')<sub>2</sub>, an Ig fraction of B21-2 at 1.5 mg/ml 0.1M acetate buffer, pH 4.0, was digested for 4 h at 37°C with 2% pepsin (Sigma Chemical Co., St. Louis, MO). The fragment, which lacked intact heavy chain on one-dimensional SDS polyacrylamide gel electrophoresis, was stored at 1.1 mg/ml in phosphate-buffered saline. 10-2.16 is a mouse anti-I-A<sup>k</sup> reagent characterized by Oi et al. (18) and provided by The Salk Institute, La Jolla, CA. Culture supernatant was used to eliminate I-A<sup>k</sup>-bearing cells in the presence of rabbit complement (C').

A mouse anti-rat Ig was produced for use in indirect immunofluorescence. Swiss mice were immunized with 100  $\mu$ g rat Ig (Sigma Chemical Co.) in complete Freund's adjuvant (Difco Laboratories, Detroit, MI). The injection was repeated after 1 mo. Subsequent boosts were with Ig in incomplete Freund's adjuvant. 4 d after boosting, tail blood was obtained, and an Ig fraction was precipitated with saturated ammonium sulfate (45% vol/vol). The Ig was digested at 4.3 mg/ml 0.1M acetate buffer, pH 4.3, using 2% pepsin for 18 h at 37°C. F(ab')<sub>2</sub> fragments were retrieved on a column of rat Ig bound to cyanogen bromide-activated sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ). The Ig was eluted in 3 M sodium thiocyanate and shown to contain no residual heavy chain on polyacrylamide gel electrophoresis run under reducing conditions. The F(ab')<sub>2</sub> at 1 mg/ml was dialyzed into 0.1 M sodium borate, pH 8.0. To 0.5 ml of F(ab')<sub>2</sub>, we added 50  $\mu$ l of dimethylsulfoxide with biotin hydroxysuccinimide ester at 1 mg/ml (a gift from Dr. S. Mojsos, The Rockefeller University) for 10 min at 4°C. The reaction was stopped with 50  $\mu$ l 1 M ethanolamine, and the biotinylated F(ab')<sub>2</sub> stored at 4°C.

**Cells.** Mouse spleen suspensions were obtained by teasing with fine forceps in RPMI-1640 (Grand Island Biological Co.). The suspension was mixed with an equal volume of 1.66% ammonium chloride, layered on a 30-ml column of 10% FCS-RPMI 1640, spun at 150 g for 20 min at room temperature, and the cell pellet was washed once more. This procedure removes most erythrocytes and cell debris, which are capable of fixing complement in rabbit serum.

Adherent cells were prepared by plating 3–4  $\times 10^6$  spleen cells in 16-mm diam wells (Costar 3524; Rochester Scientific, Rochester, NY) or 150  $\times 10^6$  cells in 100-mm diam plastic petri dishes (3003; Falcon Labware, Oxnard, CA). Nonadherent cells were dislodged with pasteur pipettes. The firmly adherent population corresponded to 1–3% of the total cells plated and were enumerated by direct counts under an inverted phase contrast microscope. For some experiments adherent cells were cultured overnight (16–24 h), after which most cells could be dislodged into suspension with pasteur pipettes.

DC were obtained from cultured low density spleen-adherent cells as described (2). Contaminating M $\phi$  were depleted by reculturing the suspension in fresh medium on plastic petri dishes. Most M $\phi$  adhered firmly while the DC-rich suspension (80–95% pure) could be dislodged by gentle pipetting.

Enriched spleen and node T cells were obtained by passage over nylon-wool columns as described (2). Generally, 50–80  $\times 10^7$  spleen and node were passed through 1.5 gm nylon-wool columns with a yield of 15% of total applied cells.

**Cytotoxicity Assays.** Equal volumes of cells, Ab, and C' were mixed at 37°C for 1 h in the presence of deoxyribonuclease (DNAase I, Sigma Chemical Co.) at 30  $\mu$ g/ml. All reagents were diluted in cytotoxicity medium, which contained RPMI 1640 medium with 0.3% bovine albumin and 25 mM Hepes buffer. Spleen cells were treated at a concentration of 10<sup>7</sup>/ml, and

spleen adherent cells and DC were used at  $6 \times 10^6$ /ml. The antibodies were: 33D1, either culture supernatant or purified Ig at 100  $\mu$ g/ml; B21-2 at 10  $\mu$ g/ml; or 10-2.16 culture supernatant. The function of M $\phi$ , B, and T cells was unimpaired after exposure of spleen to 33D1 and C'. Specifically, M $\phi$  expressed Fc receptors and phagocytosed (14), while B and T cells proliferated normally to lipopolysaccharide and alloantigens, respectively (unpublished results). C' was fresh rabbit serum obtained from any of three rabbits. Rabbit blood was allowed to clot for 1 h at room temperature; the serum was retrieved and stored at  $-70^\circ\text{C}$  in a lyophilized state. Cells were exposed to Ab and C' in plastic test tubes (2059 and 2054; Falcon Labware) or during adherence to 16-mm diam tissue culture wells. All cells were washed twice in RPMI 1640 before use.

**Immunofluorescence.** Staining was carried out on cells that were naturally adherent to glass coverslips (12-mm diam, CGS; Propper Manufacturing, Long Island City, NY) or attached to coverslips coated with 50  $\mu$ g/ml poly-L-lysine (type VII, Sigma Chemical Co.) in phosphate-buffered saline. Reagents were applied for 30–60 min at  $4^\circ\text{C}$  in the presence of 5% newborn calf serum (North American Biologicals, Miami, FL) and 0.02% sodium azide. Coverslips were exposed successively to primary Ab (the monoclonal reagents 33D1, B21-2, and F4/80); 10  $\mu$ g/ml of biotin-labeled, affinity purified F(ab')<sub>2</sub> mouse anti-rat Ig; and rhodamine-avidin (Vector Laboratories, Burlingame, CA) at 5  $\mu$ g/ml. Because 33D1 exhibits some Fc-mediated binding to IgG<sub>2A</sub> receptors (19), we mixed 33D1 with a blocking IgG<sub>2A</sub> myeloma, LPC-1, at 100  $\mu$ g/ml. LPC-1 was not reactive with our mouse anti-rat Ig.

After staining, the coverslips were rosetted on ice with antibody coated sheep erythrocytes (EA) prepared by opsonizing sheep erythrocytes with a subhemagglutinating dose of hyperimmune antiserum. The EA were spun onto the coverslips and allowed to rosette for 15 min; nonrosetted EA were removed by washing. The coverslips were rapidly dried with a hair dryer, fixed in absolute methanol for 5–10 min, and mounted in Elvanol for examination in a Zeiss Photomicroscope II equipped for epifluorescence (Carl Zeiss Inc., New York). Photomicrographs were prepared using a constant exposure time of 60 s and Tri-X film (Eastman Kodak Co., Rochester, NY).

**MLR.** Primary MLR were set up in flat-bottomed macro- (16-mm diam, Costar 3524) or micro- (6-mm diam, Costar 3596, Costar, Data Packaging, Cambridge, MA) wells. All stimulator populations were irradiated with 1,500 rad (<sup>137</sup>Cs, Gammacell 1000; Atomic Energy of Canada, Ottawa, Canada). Responder cells were T cells added at a dose of  $4 \times 10^6$ /macroculture; and  $3\text{--}4 \times 10^6$ /microculture. Whole spleen was never used as responders because DC syngeneic to the responding T cells could promote MLR to alloantigens on Ia negative stimulators (see for example ref. 19). The culture medium used was RPMI 1640 supplemented with 20  $\mu$ g/ml gentamycin sulfate (Schering Corp., Kenilworth, NJ), 1 mM glutamine (Grand Island Biological Co.),  $5 \times 10^{-5}$  M 2-mercaptoethanol (Eastman Kodak Co.), 5% vol/vol FCS (HiClone; Sterile Systems) and 1  $\mu$ g/ml indomethacin (Sigma Chemical Co.) to block the potential immunosuppressive effects of macrophage prostaglandins (5). In macrocultures, two doses of stimulators were used while in microcultures, four serial two dilutions were used [<sup>3</sup>H]thymidine (6.0 Ci/mM, 2.0  $\mu$ Ci/ml final concentration; Schwartz Mann, Orangeburg, NY) was added for 14–20 h beginning at 68 or 92 h of culture. Often the earlier time point was required to obtain [<sup>3</sup>H]thymidine uptakes that varied linearly with the dose of stimulator cells (see Results). Syngeneic MLR were small (<5–10% of the allogeneic response) and were not included in the data.

To monitor the development of cytotoxic T lymphocytes (CTL), we used H-2<sup>d</sup> stimulators and <sup>51</sup>Cr-labeled P815 (a DBA/2, H-2<sup>d</sup> tumor) as targets as described (5). The CTL assay used 0.1 ml of effector cells obtained from MLR cultures in microtest wells (usually at  $1\text{--}3 \times 10^6$  viable cells/ml) and  $1 \times 10^4$  <sup>51</sup>Cr-labeled targets. Percent specific <sup>51</sup>Cr-release was measured as experimental – spontaneous release/total – spontaneous release. Total cpm was measured as release in 0.1 N acetic acid; spontaneous release was 10% of total.

## Results

*33D1 Reacts Specifically with the Dendritic Cell Component of Mouse Spleen.* Previous work on the specificity of 33D1 used DC-enriched populations as a positive control

(14). Additional protocols were needed to monitor that 33D1 only reacted with DC in heterogeneous cell mixtures. A biotin-avidin immunofluorescence technique was developed and first tested on spleen adherent cells. Adherent DC could be distinguished from adherent macrophages ( $M\phi$ ), because DC lacked Fc receptors and would not rosette EA (2, 20, 21). 33D1 only stained cells that were  $EA^-$  (Fig. 1), while the  $M\phi$  restricted monoclonal, F4/80, primarily stained  $EA^+$  cells (Table I). The B21-2 anti-Ia monoclonal stained similar numbers of  $Fc^-$  cells as 33D1, and variable numbers of  $Fc^+$  cells (Table I). In unfractionated spleen suspensions, 33D1 failed to stain above the background (no primary antibody) of 0.5–1%; F4/80 stained 2–4% of spleen cells.

The specificity of 33D1 for DC was also evident in Ab-mediated cytotoxicity. Here DC were identified by cytologic criteria (20–23). That is, DC were flattened, well-spread cells with mitochondria-filled processes;  $M\phi$  were primarily round cells, and when spread, exhibited clear ruffles, lysosomes, and/or vesicles. When spleen adherent cells were prepared in the presence of 33D1 Ab and  $C'$ , only viable cells adhered and 90% of cells with the appearance of DC were eliminated. Total adherent cell numbers were reduced 10–40% relative to controls (No Ab or  $C'$ ; Ab or  $C'$  only; Ab and heated  $C'$ ). 1-d cultures of spleen adherent cells, which contained many  $M\phi$  and some DC (Fig. 2, left), were also treated with 33D1 and  $C'$ . 95% of the cells with the bulbous

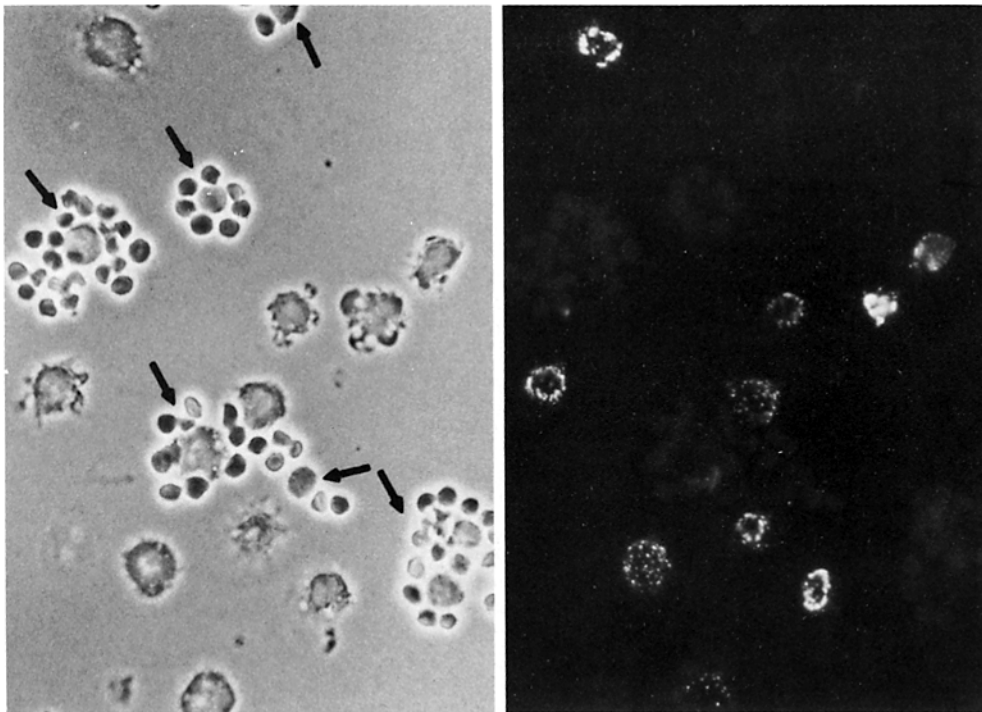


FIG. 1. Indirect immunofluorescence with biotin-avidin (see Materials and Methods) to visualize 33D1 positive spleen adherent cells. After staining, the preparations were rosetted with EA, which distinguishes  $EA^+$   $M\phi$  from  $EA^-$  DC (phase contrasts, left). Useful cytologic detail has been lost, because the cells were stained on ice for 3 h, dried, and fixed in methanol. 33D1 produced a speckled stain (immunofluorescence, right) on many  $EA^-$  cells, but did not stain  $EA^+$  cells. The reciprocal pattern was seen with F4/80 (see ref. 19).  $\times 800$ .

TABLE I  
33D1 Stains Adherent Spleen Dendritic Cells, but not Macrophages

Mouse strain	Spleen adherent cells		Percent cells stained with					
	Time in culture	Percent EA <sup>+</sup> cells	33D1 ( $\alpha$ -DC)		F4/80 ( $\alpha$ -M $\phi$ )		B21-2 ( $\alpha$ I-A <sup>b,d</sup> )	
			EA <sup>+</sup>	EA <sup>-</sup>	EA <sup>+</sup>	EA <sup>-</sup>	EA <sup>+</sup>	EA <sup>-</sup>
	<i>h</i>							
C $\times$ B6	1	74	2	45	78	3	55	40
	20	70	0	52	74	1	68	53
BALB/c	1	76	1	48	70	8	23	43
	20	61	0	71	77	1	29	65

Spleen adherent cells from C  $\times$  B6 F<sub>1</sub> and BALB/c mice were studied after 1 or 20 h in culture. Cells on coverslips were exposed successively at 4°C to: primary antibody; biotin-labeled, affinity-purified, F(ab')<sub>2</sub> mouse anti-rat Ig; and rhodamine-avidin. EA were then spun onto the monolayers to identify Fc-bearing cells. Finally, the coverslips were dried rapidly and fixed in absolute methanol. At least 100 EA<sup>+</sup> and EA<sup>-</sup> cells were evaluated for expression of three different surface antigens, as illustrated in Fig. 1.

pseudopods typical of cultured DC were killed (Fig. 2, compare middle and right). No killing was detectable in fresh or cultured whole spleen. Together with previous data (14), we conclude that 33D1 reacts with the trace DC component of mouse spleen. Specificity can be monitored by cytotoxicity and immunofluorescence assays on adherent spleen cells.

*Effects of the 33D1 Ab on the Function of Enriched Spleen DC.* When enriched populations of DC were exposed for 1 h to both 33D1 Ab and C', the cells were killed and MLR stimulatory capacity was ablated (Table IIA). Killing of DC and elimination of function did not occur with Ab only, C' only, or Ab plus heated C'. 33D1 Ab and C' also eliminated function when lysis was performed in the presence of large numbers (30-fold excess) of "filler" or nonstimulatory lymphocytes (Table IIA).

Purified 33D1 (30  $\mu$ g/ml; saturation is at 10–15  $\mu$ g/ml [14]) was next added continuously to cocultures of T cells responding to small numbers of irradiated, enriched DC. No inhibition of the MLR was noted (Table IIB). Purified DC populations lacked M $\phi$ , and it was conceivable that 33D1 could inhibit function by opsonizing DC in the presence of Fc receptor-bearing cells. We therefore included experiments in which adherent peritoneal cells (primarily M $\phi$ ) were added to the DC-T cell co-cultures. The M $\phi$  were not themselves active as MLR stimulators and had little effect on DC function, even in the continuous presence of 33D1 (Table IIB). We conclude that 33D1 does not itself interfere with MLR stimulation, but that killing DC with Ab and C' abolishes function.

*Effects of Selective Dendritic Cell Depletion on the MLR Stimulating Capacity of Unfractionated Spleen.* Treatment of spleen cells with 33D1 Ab and C' reduced MLR stimulatory capacity 75–90% (Table III). Stimulation by both fresh and cultured (24 h) spleen cells was reduced similarly. In general, the inhibition was similar to that produced by anti-Ia Ab and C'. However, 33D1 killed <1% of spleen cells, whereas anti-Ia routinely killed 60–70%. In most experiments, 33D1 Ab alone did not diminish stimulatory capacity; specific anti-Ia Ab alone usually reduced stimulation by 50% or more, particularly at high stimulator/responder ratios (Table III).

Treatment with 33D1 and C' also reduced stimulation of the CTL response typical of the primary MLR (Table IV). Small numbers of purified DC (0.5% of the total

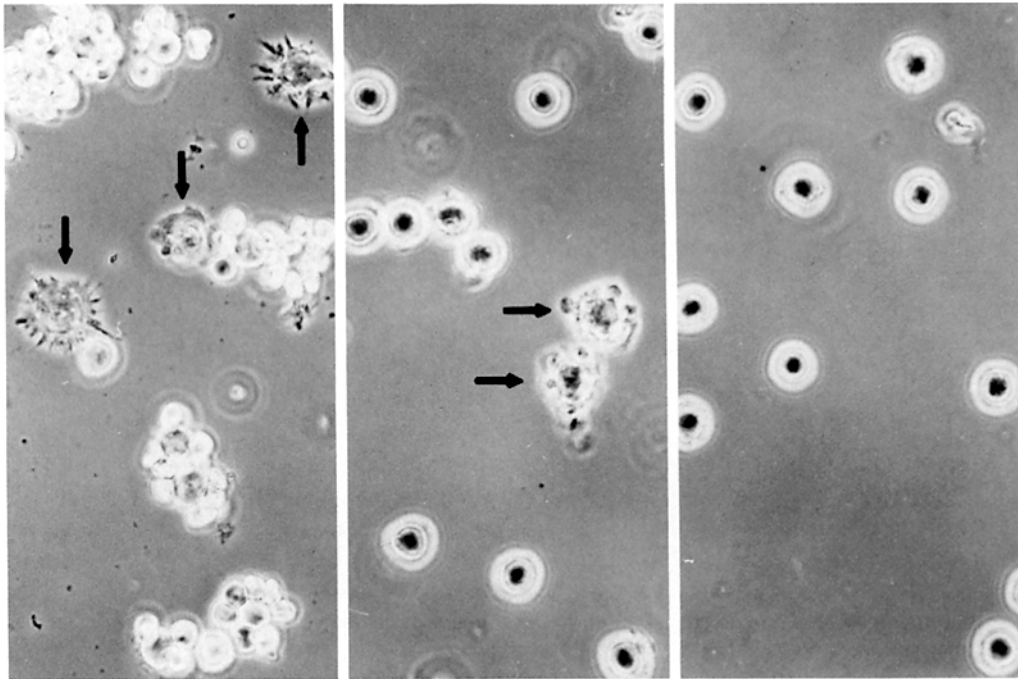


FIG. 2. (Left) Identification of the DC and M $\phi$  components of cultured spleen adherent cells. Adherent spleen cells were cultured 24 h and dislodged from the tissue culture dish.  $2-3 \times 10^4$  cells were attached to poly-L-lysine-coated coverslips for 30 min at 4°C, and then EA were spun onto the coverslips for 20 min at 4°C to mark Fc $^+$  cells. Nonrosetted EA were removed by washing, and the coverslips were fixed in 1.25% glutaraldehyde. This technique provides concentrated viable populations in which most of the cells strongly rosette EA. The latter are M $\phi$  defined by phagocytic activity and expression of the F4/80 M $\phi$  restricted antigen (e.g., Table I). The EA $^-$  cells include spiny DC (arrows) and some round cells.  $\times 800$ . (Middle) Cultured spleen adherent cells that were exposed to rabbit C' for 1 h at 37°C, before attachment to poly-L-lysine coated coverslips at 4°C. Most of the cells were round, but DC covered with cell processes (arrows) could be identified.  $\times 800$ . (Right) Same as middle, but the adherent cells were treated with both 33D1 anti-DC Ab and C'. Only viable cells attached to the coverslips. The irregularly shaped DC were eliminated.  $\times 800$ .

stimulator dose) restored function, whether DC had been removed with 33D1 or anti-Ia Ab and C' (experiment B, Table IV). We conclude that small numbers of DC are essential for primary MLR stimulation by mouse spleen.

*Effect of Selective Dendritic Cell Depletion on MLR Stimulation by Spleen Adherent and Nonadherent Cells.* To compare MLR stimulation by spleen DC and M $\phi$  directly, we studied the effects of 33D1 and C' on isolated spleen adherent cells. The latter were prepared under conditions in which most lymphocytes were removed by pipetting over the tissue culture surface. Adherent cells represented 1–3% of the cultured inoculum.

Fresh spleen suspensions were first adhered to plastic in the presence of 33D1 Ab and C', or suitable controls (Table VA). Stimulation by adherent cells was reduced >90% if DC had been eliminated. Nonadherent spleen was much less active as stimulators on a per cell basis, but stimulation was also sensitive to 33D1 and C'. Small numbers of enriched DC ( $2 \times 10^4$ /macroculture) reversed the inhibition induced by 33D1 and C' (Table VA).

TABLE II  
Effect of Monoclonal Anti-DC Ab on MLR stimulation by Enriched Populations of DC

(A) Initial Exposure to 33D1 Ab with or without C'

Stimulators	Treatment (A/C')	Proliferative response in $3 \times 10^5$ B6 T cells (cpm [ $^3$ H]-thymidine uptake) induced by D Cells at a dose of:			
		$2 \times 10^4$	$1 \times 10^4$	$5 \times 10^3$	$2.5 \times 10^3$
DC-C $\times$ B <sub>6</sub> F <sub>1</sub>	None/heat	58,887	46,237	26,930	12,730
DC-C $\times$ B <sub>6</sub> F <sub>1</sub>	None/fresh	56,813	38,033	20,960	11,172
DC-C $\times$ B <sub>6</sub> F <sub>1</sub>	33D1/heat	46,532	32,826	17,691	8,655
DC-C $\times$ B <sub>6</sub> F <sub>1</sub>	33D1/fresh	3,305	1,727	1,343	Not done
DC + B6 T cells	None/fresh	43,209	24,280	11,834	6,229
DC + B <sub>6</sub> T cells	33D1/fresh	2,500	1,283	1,001	1,159
B <sub>6</sub> T cells only	None/fresh	548	957	1,210	1,265
B <sub>6</sub> T cells only	33D1/fresh	692	901	1,012	1,235

DC were prepared from BALB/c  $\times$  C57BL/6 F<sub>1</sub> low density spleen adherent cells. 40  $\mu$ l of cells at  $5 \times 10^6$ /ml were treated with equal volumes of medium or 33D1 antibody (100  $\mu$ g/ml) and rabbit serum (heated 56°C for 30 min or reconstituted fresh from lyophilized stocks). After 1 h, the cells were washed, irradiated, and used to stimulate allogeneic C57BL/6, nylon wool-nonadherent spleen and lymph node cells (T cells). In the lower half of the experiment,  $6 \times 10^6$  C57BL/6 T cells were treated in conjunction with DC, to test whether filler cells interfered with 33D1-mediated killing. The T cells were themselves insensitive to 33D1, and were used in the cultures at doses of 4, 2, 1, and  $0.5 \times 10^5$ /well. Proliferative responses were monitored at 67–72 h; the background without stimulators was 1,345 cpm and was not subtracted.

(B) Continuous Exposure to 33D1 Ab

Supplement		Proliferative response in $4 \times 10^5$ BALB/c T cells (cpm [ $^3$ H]thymidine uptake) induced by DC at a dose of:			
33D1 Ab (30 $\mu$ g/ml)	$2 \times 10^4$ C $\times$ B <sub>6</sub> F <sub>1</sub> macrophages	$8 \times 10^3$	$4 \times 10^3$	$2 \times 10^3$	$1 \times 10^3$
—	—	58,420	22,840	8,122	4,692
+	—	59,975	26,872	8,389	3,996
—	+	60,929	32,443	18,211	10,753
+	+	40,161	23,488	13,018	8,840

Primary MLR were generated by adding irradiated DC to allogeneic T cells. Some of the cultures were supplemented with purified 33D1 antibody at a final concentration of 30  $\mu$ g/ml, and/or with irradiated, 1-d cultures of peritoneal macrophages ( $2 \times 10^4$  adherent peritoneal cells, which are >95% macrophages and have few detectable DC). Thymidine pulse was at 76–92 h. Maximum response of syngeneic T cells was 4,000–5,000 cpm for all supplements. All “no stimulator” backgrounds were 900–1,200 cpm and were not subtracted. Responses to allogeneic peritoneal cells alone were 4,050 and 4,176 cpm  $\pm$  33D1 antibody, respectively.

This type of experiment was performed seven consecutive times using strain combinations that differed in the entire major histocompatibility complex (MHC) (e.g., B6.H<sub>2</sub>k stimulating D<sub>2</sub>  $\times$  C F<sub>1</sub> responders) or in specific MHC subregions [B10.S(7R) = K<sup>s</sup>I<sup>s</sup>D<sup>d</sup> stimulating B10.TL = K<sup>s</sup>I<sup>k</sup>D<sup>d</sup> responders and B6.H<sub>2</sub>k = K<sup>k</sup>I<sup>k</sup>D<sup>k</sup> stimulating B10.TL = K<sup>s</sup>I<sup>k</sup>D<sup>d</sup> responders]. In all cases, a similar phenomenon was observed (Table VB). The bulk of the stimulating capacity ( $62 \pm 8\%$ ) was in the adherent population and was ablated when DC were removed. A small fraction of MLR-stimulating activity was induced by nonadherent cells; stimulation was reduced by 33D1 and C', but not to the same extent as in the adherent fraction. A number of approaches were tested to obtain complete depletion of stimulating capacity, but

TABLE III  
*Treatment With Monoclonal Anti-DC Antibody (33D1) and C' Inhibits The MLR Stimulating Capacity of Fresh and Cultured Mouse Spleen Cells*

Treatment of stimulators (Ab/C')	Proliferative response (cpm [ <sup>3</sup> H]Thymidine uptake) induced by stimulators at a dose of			
	4 × 10 <sup>5</sup>	2 × 10 <sup>5</sup>	1 × 10 <sup>5</sup>	5 × 10 <sup>4</sup>
Experiment A				
None/heat	29,992	16,694	6,436	3,129
None/fresh	24,712	13,257	9,422	6,321
α-DC/heat	22,825	17,575	9,941	7,494
α-DC/fresh	6,998	2,076	1,301	950
α-I-A <sup>k</sup> /heat	13,557	11,762	7,932	3,590
α-I-A <sup>k</sup> /fresh	2,750	1,565	1,075	860
Experiment B	2 × 10 <sup>5</sup>	1 × 10 <sup>5</sup>	5 × 10 <sup>4</sup>	2.5 × 10 <sup>4</sup>
None/heat	59,497	46,839	21,981	10,144
None/fresh	60,726	35,868	19,664	8,283
α-DC/heat	78,753	46,619	25,117	10,409
α-DC/fresh	17,988	4,370	2,165	1,749
α-I-A <sup>k</sup> /heat	31,876	23,938	15,384	6,821
α-I-A <sup>k</sup> /fresh	13,726	9,546	5,020	3,277
Experiment C				
None/heat	27,680	15,916	7,227	3,475
None/fresh	30,063	22,584	10,616	4,231
α-DC/heat	29,241	16,913	6,275	4,070
α-DC/fresh	6,191	1,299	990	881
α-I-A <sup>k</sup> /heat	15,012	7,164	4,315	2,503
α-I-A <sup>k</sup> /fresh	10,279	7,163	3,678	2,469

Fresh or cultured (24 h) spleen cells were exposed to Ab and C' (heat inactivated for 30 min at 56°C, or freshly reconstituted from lyophilized stocks) for 1 h at 37°C in polypropylene tubes. The cells were washed twice and were irradiated (1,500 rad). Serial two dilutions of cells were pipetted into microtest wells and cultured with nylon wool nonadherent, spleen and lymph node cells (T cells). The "no stimulator cell" background cpm were not subtracted from the data. (A) Fresh B6.H2k spleen (H-2<sup>k</sup>) stimulating D2 × C F<sub>1</sub> (H-2<sup>d</sup> × H-2<sup>b</sup>) T cells. [<sup>3</sup>H]thymidine pulse at 72–90 h. No stimulator cultures incorporated 998 cpm. (B) Cultured B6.H2k spleen stimulating D2 × C F<sub>1</sub> T cells. [<sup>3</sup>H]thymidine pulse at 48–64 h. No stimulator cultures incorporated 1,775 cpm. (C) Cultured B6.H2k spleen stimulating C × B6 F<sub>1</sub> T cells (H-2<sup>d</sup> × H-2<sup>b</sup>). [<sup>3</sup>H]thymidine pulse at 48–64 h. No stimulator cultures incorporated 602 cpm.

without success. These approaches (not shown) included: testing different preparations of 33D1 purified Ab and culture supernatants; use of 20 different nontoxic rabbit sera as C' sources; varying the dose of cells, Ab, and C' during the lytic steps; double treatments with 33D1 and C'; and elimination of DC in the responding T cells with 33D1 or anti-Ia Ab and C'.

Stimulation by cultured spleen adherent cells was also studied. Because cultured cells could be dislodged from the plastic surface into a viable suspension, more concentrated populations than fresh adherent cells could be tested. Selective removal of DC with 33D1 and C' again eliminated the stimulating activity of cultured adherent cells, even though Ia<sup>+</sup> Mφ outnumbered DC several-fold (Table VI). Therefore, DC and not Ia<sup>+</sup> Mφ are responsible for MLR stimulation in spleen adherent cells.

TABLE IV  
Dendritic Cells are Required to Stimulate Both Proliferative and Cytotoxic Responses in the Primary MLR

Stimulators (cell/treatment)	T cell response to graded doses of allogeneic stimulators: cpm [ <sup>3</sup> H]thymidine uptake (percent <sup>51</sup> Cr Release)			
	4 × 10 <sup>5</sup>	2 × 10 <sup>5</sup>	1 × 10 <sup>5</sup>	0.5 × 10 <sup>5</sup>
Experiment A				
Spleen, no Ab/C'	43,041 (83)	26,743 (64)	14,515 (37)	7,604 (11)
Spleen, α DC/C'	10,911 (14)	6,094 (5)	1,937 (0)	1,249 (0)
Spleen, α I-A <sup>k</sup> /C'	35,289 (77)	22,144 (39)	10,881 (18)	5,325 (9)
Spleen, α I-A <sup>b,d</sup> /C'	1,980 (0)	1,547 (0)	1,192 (0)	1,109 (0)
Experiment B				
Spleen, no Ab/C'	29,479 (45)	14,822 (25)	9,101 (19)	5,840 (7)
Spleen, α DC/C'	5,499 (4)	3,326 (2)	1,602 (0)	1,249 (0)
Spleen, α I-A <sup>b,d</sup> /C'	4,565 (5)	3,712 (4)	1,761 (0)	Not done
DC only	33,759 (26)	10,514 (20)	4,035 (7)	2,536 (3)
DC + spleen, α DC/C'	37,138 (51)	20,605 (45)	5,925 (27)	2,200 (3)
DC + spleen, α I-A <sup>b,d</sup> /C'	23,814 (55)	16,400 (40)	7,018 (22)	3,033 (9)

Spleen cells were treated with monoclonal Ab and fresh rabbit C' for 1 h at 37°C, washed, irradiated (1,500 rad), and added in varying doses to 3 × 10<sup>5</sup> nylon wool-nonadherent spleen cells. One plate was used to monitor the proliferative response (cpm [<sup>3</sup>H]thymidine uptake at 70–90 h). A replicate plate was used to quantitate lytic activity (percent <sup>51</sup>Cr release from P815 targets assayed at 116–120 h at effector/target ratios of 10–30:1; spontaneous <sup>51</sup>Cr release of 10%). In experiment A, BALB/C (H-2<sup>d</sup>) stimulated B6.H2k (H-2<sup>k</sup>) responders. [<sup>3</sup>H]thymidine uptake in the no-stimulator background was 1,438 cpm (not subtracted). In experiment B, C × B6F<sub>1</sub> (H-2<sup>d</sup> × H-2<sup>b</sup>) stimulated C57Bl/6 (H-2<sup>b</sup>) responders. [<sup>3</sup>H]thymidine uptake in the no-stimulator background was 1,493 cpm (not subtracted). DC were obtained from low density spleen adherent cells as described (2). DC were used as stimulators at a top dose of 1.2 × 10<sup>3</sup> either alone, or in conjunction with spleen cells treated with αDC or αI-A<sup>b,d</sup> Ab and C'. DC accounted for 0.5% of stimulators at all doses.

### Discussion

*DC as Unique Stimulators of the MLR.* MLR-stimulating capacity is reduced by 75–90% when the number of DC in whole spleen or spleen adherent cells is diminished with specific Ab (clone 33D1) and C'. Function can be restored by adding back DC in numbers comparable to those found in the spleen (0.5% of total cells) and prepared by a technique that is independent of 33D1 Ab. These observations provide the best evidence for the unique functional properties of DC. Previous experiments with positive selection have led to a similar conclusion. Specifically, populations enriched in DC are potent stimulators, whereas lymphocytes and macrophages are not (1, 2, 9, 11, 17). Current positive selection procedures have some drawbacks, however. No cell type is obtained in >25–50% yield; considerable manipulation, e.g., adherence and tissue culture, is required for purification; and it is difficult to rigorously correlate functional activity with numbers of DC, since it is difficult to count DC in any cell population that has not undergone some sort of enrichment procedure. Negative selection provides a minimum estimate of the amount of activity that can be attributed to DC, whether the cell acts by direct stimulation of responding T cells or by enhancing stimulation to antigens on other cells. Furthermore, negative selection can be performed on fresh spleen suspensions.

Cell-specific reagents have seen little use in the identification of MLR stimulators. It has been shown that B and T cell elimination (with anti-Ig or anti-Thy-1 plus C') does not alter stimulatory capacity (1). Specific anti-mouse Mφ reagents have yet to

TABLE V  
*The Effect of Selective DC Depletion on the MLR Stimulating Capacity of Spleen Adherent and Nonadherent Cells*  
 Part A

Stimulator cells*	Treatment (Ab/C')‡	T cell proliferative response (cpm [ <sup>3</sup> H]Thymidine uptake)§
None	None	1,205; 695
1.2 × 10 <sup>5</sup> Adherent spleen	None/heat	70,779; 68,035
1.2 × 10 <sup>5</sup> Adherent spleen	None/fresh	60,647; 55,997
0.3 × 10 <sup>5</sup> Adherent spleen	None/fresh	19,930; 20,500
1.2 × 10 <sup>5</sup> Adherent spleen	33D1/heat	65,307; 74,180
0.8 × 10 <sup>5</sup> Adherent spleen	33D1/fresh	2,197; 4,336
0.2 × 10 <sup>5</sup> Adherent spleen	33D1/fresh	965; 724
0.8 × 10 <sup>5</sup> Adherent spleen	33D1/fresh	76,654; 55,467
+ 0.2 × 10 <sup>5</sup> DC	None	
2.4 × 10 <sup>6</sup> Nonadherent spleen	None/heat	35,875; 32,627
2.4 × 10 <sup>6</sup> Nonadherent spleen	None/fresh	32,943; 33,468
0.6 × 10 <sup>6</sup> Nonadherent spleen	None/fresh	10,900; 8,517
2.4 × 10 <sup>6</sup> Nonadherent spleen	33D1/heat	31,747; 35,179
2.4 × 10 <sup>6</sup> Nonadherent spleen	33D1/fresh	5,875; 7,021
0.6 × 10 <sup>6</sup> Nonadherent spleen	33D1/fresh	1,946; 1,363
2.4 × 10 <sup>6</sup> Nonadherent spleen	33D1/fresh	62,108; 48,870
+ 0.2 × 10 <sup>5</sup> DC	None	
0.2 × 10 <sup>5</sup> DC only	None	32,461; 41,423

\* 3.3 × 10<sup>6</sup> B6.H2k spleen cells were irradiated (1,500 rad), and exposed to antibody and C' during adherence to 16-mm diam plastic tissue culture wells in duplicate. After 60–80 min at 37°C, the nonadherent cells were removed by gently pipetting over the surface of the well. The nonadherent cells, which represented >90% of the recovered cells, were washed and added to separate wells.

‡ Cells were exposed to medium (no antibody) or an Ig fraction of 33D1, anti-DC antibody at 100 µg/ml. Complement was rabbit serum, final dilution of 1:16, that had been heat inactivated (56°C, 30 min) or reconstituted "fresh" from lyophilized stocks.

§ 4 × 10<sup>6</sup> nylon wool-nonadherent spleen and lymph node cells from D<sub>2</sub> × C F<sub>1</sub> mice (T cells) were used as responders. Total culture volume was 2.0 ml. At 96 h, triplicate aliquots of 0.2 ml were transferred from the 16-mm wells to microtest plates (6-mm diam wells) and exposed to [<sup>3</sup>H]thymidine (2 µCi/ml final concentration) for 16 h.

|| DC were obtained from low density, spleen adherent monolayers; the latter were cultured overnight and depleted of macrophages by readherence to plastic as described (2).

be used because current monoclonal Ab are not cytotoxic. However, van Voorhis et al. (manuscript in preparation) have selectively eliminated human Ia<sup>+</sup> monocytes with a specific cytotoxic monoclonal Ab, and have found no reduction in MLR stimulation by blood leukocytes. Most investigators have used anti-Ia and C' to eliminate MLR stimulators. Ia is not a cell-specific marker and can be expressed on nonleukocytes as well as all classes of mononuclear leukocyte. Treatment with anti-Ia plus C' would remove the Ia-rich DC. We think it is by virtue of its DC specificity that 33D1 represents the first cell specific reagent to have such marked effects on MLR-stimulating activity.

## Part B

Primary MLR strain combination		Treatment of stimulator cells	Proliferative response (cpm [ $^3$ H]thymidine uptake) induced by	
Irradiated stimulator	T cell responder		Adherent cells	Nonadherent cells
B6.H <sub>2</sub> k	D <sub>2</sub> × CF <sub>1</sub>	No	57,372	32,256
		33D1	2,316	5,498
B6.H <sub>2</sub> k	D <sub>2</sub> × CF <sub>1</sub>	No Ab	37,264	10,685
		33D1	5,545	5,565
B6.H <sub>2</sub> k	D <sub>2</sub> × C F <sub>1</sub>	No Ab	31,040	20,057
		33D1	2,400	10,681
B10.S(7R)	B10.TL	No Ab	30,570	24,767
		33D1	1,628	7,657
B6.H <sub>2</sub> k	B10.TL	No Ab	41,738	23,462
		33D1	580	10,213
B6.H <sub>2</sub> k	D <sub>2</sub> × C F <sub>1</sub>	No Ab	40,907	32,046
		33D1	6,327	21,730
B6.H <sub>2</sub> k	C × B6 F <sub>1</sub>	No Ab	24,052	16,930
		33D1	1,970	3,807

As described above (A), adherent and nonadherent spleen cells were prepared during treatment with fresh C' ± 33D1 anti-DC antibody. Three different rabbits were used as C' donors. The data in the first experiment are taken from Table VA. The second experiment was performed in the same detail as above with similar results (heated complement controls; low and high doses of stimulators; reconstitution with low doses of enriched dendritic cells). In all experiments, adherent cells and nonadherent cells numbered  $\sim 1 \times 10^6$ /culture and  $2.5 \times 10^6$ /culture, respectively. Pulsing with [ $^3$ H]thymidine was performed by transferring aliquots to microtest wells before labeling (as Table VA); alternatively, [ $^3$ H]thymidine was added to the 16-mm wells followed by transfer to microtest wells at the time of harvest. [ $^3$ H]thymidine incorporated by T cells alone, which ranged from 800 to 2,000 cpm, has been subtracted from all entries.

If DC are unique MLR stimulators, why does treatment with 33D1 and C' not remove 100% of stimulating capacity in spleen? One possibility is that 33D1 does not kill all DC. The Ab efficiently eliminates DC enriched by adherence (Tables II, V, VI). However, there may be DC that are more difficult to kill, particularly in nonadherent spleen cells. Nonadherent DC could express less antigen, or the antigen may be occupied and inaccessible to Ab. Such differences in antigen expression may in turn reflect differences in DC maturation and/or activation. A second possibility relates to the contribution of soluble mediators (interleukins). For example, antigen on any cell plus soluble factors may effectively trigger development of CTL (e.g., 24, 25). Interleukins might be generated by very small numbers of DC resisting 33D1 plus C', or by spleen cells that were triggered by antigen in the animal before killing.

*Relevance of the Primary MLR Data to Tissue Transplantation.* Allografts could contain small numbers of allogeneic DC that trigger first set rejection. Thus allograft rejection would be comparable to the primary MLR, in which trace numbers of DC induce the response. This hypothesis gains support from several recent studies in rats. Hart and Fabre (26) identified cells in tissue sections of most nonlymphoid organs that are similar to DC. Klinkert et al. (11) have isolated DC from liver and skin, and have shown that their accessory function is comparable to lymphoid DC. Cells similar to DC have been identified in rat afferent lymph (7, 8), so that DC could move from allograft to host lymphoid organ once lymphatic connections are established. Lechler and Batchelor (13) showed that small numbers of DC, obtained from rat lymph,

TABLE VI  
*Selective Depletion of DC Ablates the MLR Stimulating Capacity of Cultured Spleen Adherent Cells*

Treatment of stimulators (Ab/C')	Proliferative response (cpm [ <sup>3</sup> H]thymidine uptake) induced by stimulators at a dose of			
	3.2 × 10 <sup>4</sup>	1.6 × 10 <sup>4</sup>	0.8 × 10 <sup>4</sup>	0.4 × 10 <sup>4</sup>
Experiment A: C × B6 F <sub>1</sub> stimulators, B6 responders				
No Ab/No C'	38,582	21,420	6,511	2,582
No Ab/C'	23,940	10,404	3,518	710
αDC/No C'	26,210	16,405	8,293	2,436
αDC/C'	180	141	89	55
αI-A <sup>b,d</sup> /No C'	18,622	11,393	2,265	583
αI-A <sup>b,d</sup> /C'	NT*	NT	120	57
αI-A <sup>k</sup> /No C'	31,687	16,311	7,202	2,091
αI-A <sup>k</sup> /C'	31,396	18,035	7,655	1,622
Experiment B: B6.H <sub>2</sub> k stimulators, B6 responders				
No Ab/No C'	27,525	25,698	13,896	4,254
No Ab/C'	28,060	16,282	5,986	1,433
αDC/No C'	29,541	16,501	8,206	3,084
αDC/C'	72	80	162	166
αI-A <sup>b,d</sup> /No C'	36,117	25,923	13,263	6,701
αI-A <sup>b,d</sup> /C'	22,292	11,860	3,056	283
αI-A <sup>k</sup> /No C'	16,988	16,433	9,399	3,064
αI-A <sup>k</sup> /C'	NT	169	193	87

Spleen adherent cells were prepared from Balb/c × C57Bl/6 F<sub>1</sub> (I-A<sup>b,d</sup>, experiment A) or from B6 H<sub>2</sub>k (I-A<sup>k</sup>, experiment B) mice. Aliquots of cells in suspension were treated with Ab (none, 33D1 α-DC, B21-2 α-I-A<sup>b,d</sup>, or 10-216 α-I-A<sup>k</sup>) with or without C'. The cells were washed, irradiated, and added in graded doses to 4 × 10<sup>5</sup> nylon wool nonadherent spleen cells. [<sup>3</sup>H]thymidine uptake was measured at 72–90 h. Background (no stimulators) was 105 and 135 cpm and was not subtracted. In both experiments, 33D1 killed 10% of the adherent cells. Specific α-I-A killed 75% of the adherent cells in experiment A, and 50% in experiment B. Since cultured spleen adherent cells were not detectably killed with αIg or αthy-1, Ia<sup>+</sup> macrophages had to outnumber Ia<sup>+</sup> DC several-fold in both experiments.

\* Not tested.

triggered rejection of rat kidney allografts. Therefore, depletion of allogeneic DC might well explain the survival of allografts that have been cultured for prolonged periods (27, 28) or that have been treated with anti-Ia and C' (29).

A second possibility is that host DC, especially in lymphoid organs, act as accessory cells for responses to alloantigens on other cells. In support of this possibility, there is in vitro evidence that DC syngeneic to the responder, as well as third-party allogeneic DC, markedly enhance CTL responses to alloantigens on thymocytes and Ia-negative spleen cells (19). Nussenzweig et al. (5) have also demonstrated that DC enhance the CTL response to hapten-modified but otherwise nonimmunogenic T cells.

A final possibility is that certain grafts bypass the need for DC by themselves producing soluble mediators, termed costimulators by Lafferty and associates (30). Costimulators in turn may resemble those produced during DC-T cell interaction.

*The MLR as a Model for Accessory Cell Function.* The term "accessory cell" refers to a nonlymphocytic element required, in addition to antigen or other stimulus, to induce a lymphocyte response. Our interest in accessory cells began with a study of adherent spleen cells (22). At the time, adherent spleen was the principal source of accessory cells in mice, and function was evaluated by stimulation of antibody forming responses in vitro. It was quickly apparent that adherent populations contained a

distinct class of DC and were not simply a source of  $M\phi$  as is often assumed (reviewed in 23). Further analysis required that DC and  $M\phi$  be studied separately, either by positive or negative selection techniques. Both have now been accomplished for the primary MLR (1, 2, 9, 11, 17, 23). The MLR effectively illustrates the need for DC as accessory cells in the induction of immune responses. The antigens are almost certainly the known class I (H-2) and II (Ia) alloantigens of the major histocompatibility complex.  $M\phi$  and lymphocytes express antigen, yet they need not stimulate the MLR. Antigens, to be immunogens, require accessory cells. Further studies must explain how DC, which may be considered to be "nature's adjuvant," function in the MLR and other responses.

### Summary

Clone 33D1 is a mouse-rat hybridoma that secretes a specific anti-dendritic cell (DC) monoclonal antibody (14). Because the antibody kills DC in the presence of rabbit complement, it can be used to study the functional consequences of selective DC depletion. Previous data on the cell specificity of 33D1 were first extended. By cytotoxicity (rabbit complement) and indirect immunofluorescence (biotin-avidin technique), 33D1 reacted with DC but not with macrophages nor other splenocytes. In contrast, the monoclonal antibody, F4/80 (15), reacted with macrophages but not DC.

The functional assay evaluated in this paper was stimulation of the primary mixed leukocyte reaction (MLR). 33D1 antibody itself did not inhibit stimulation by enriched populations of DC. In the presence of complement, 33D1 killed DC and ablated stimulatory function.

The effect of 33D1 and complement on MLR stimulation by heterogeneous cell mixtures was then evaluated. Removal of DC from unfractionated spleen suspensions reduced stimulatory capacity 75–90%, comparable to that produced with specific anti-Ia antibody and complement. Stimulation of both proliferative and cytotoxic responses was reduced. DC depletion had similar effects on MLR generated across full strain differences, or across selected subregions (H2I, H-2K/D) of the major histocompatibility complex.

To further compare the functional properties of spleen DC and macrophages, MLR stimulation by adherent and nonadherent fractions of spleen were tested separately.  $62 \pm 8\%$  of the total stimulatory capacity of spleen was in the plastic adherent population. Activity was ablated >90% after elimination of DC. MLR stimulation by 24-h cultures of spleen adherent cells, which contained a three- to sixfold excess of  $Ia^+$  macrophages, was also ablated when DC were removed. Stimulation by nonadherent spleen was more resistant, but was reduced 50–75% by 33D1 and complement.

The function of spleen cells treated with 33D1 or anti-Ia antibody and complement was restored with a small inoculum of purified DC. The latter corresponded to 0.5% of total stimulator cells and were enriched by previously described techniques that did not require the 33D1 antibody.

We conclude that the DC, a trace component of mouse spleen, is the principal cell type required for stimulation of the primary MLR. Because other cells are not immunogenic, but do express Ia and H-2 alloantigens, DC likely represent the critical accessory cell required for the induction of lymphocyte responses.

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