CONTRIBUTION OF DENDRITIC CELLS TO STIMULATION OF THE MURINE SYNGENEIC MIXED LEUKOCYTE REACTION*

By MICHEL C. NUSSENZWEIG and RALPH M. STEINMAN‡

From the Rockefeller University, New York 10021

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Dendritic cells (DC)[1] are a morphologically distinct subpopulation of lymphoid cells that can adhere to tissue culture surfaces (1-5). DC lack typical markers of T cells, B cells, and macrophages (Mϕ) (2), but do express Ia alloantigens linked to the I-A and I-E subregions (4). The recent finding that DC are at least 100 times more potent than other murine cells in stimulating the primary, allogeneic, mixed leukocyte reaction (MLR) demonstrates that DC are a functionally distinct cell type (6).

There is considerable evidence that Ia^+ adherent cells function as accessory cells in several immune responses (7-9). Whether or not DC were involved in these reactions became a feasible question to ask when we learned to purify DC free of Mϕ (4), the cell type most often considered to mediate accessory cell activity. The first system tested was antigen-induced, T cell proliferation. This led to the unexpected finding that DC stimulated extensive proliferation of syngeneic T cells in the absence of antigen. In this communication we demonstrate that the DC is a potent and possibly unique stimulator of the syngeneic MLR (SMLR). We present evidence that the SMLR is occurring in all cultures of unfractionated spleen and lymph node cells. This reaction need not involve modification of DC by antigen (Ag) or heterologous serum components. During the SMLR, DC and responding T cells associate with one another and formed discrete cell clusters that were isolated and characterized.

Materials and Methods

Mice. The experiments reported here have been performed on male and female C57BL/6, DBA/2, $B6D2F_1$ (The Trudeau Institute, Saranac Lake, N. Y.), BALB/c, and $CD2F_1$ (Flow Laboratories, Rockville, Md.) mice. In some of the allogeneic MLR experiments, C3H and CBA mice (The Jackson Laboratory, Bar Harbor, Maine) were used as responders.

Antisera. Cell surface markers were detected primarily with one-stage cytotoxicity assays with guinea pig complement (Cordis Laboratories Inc., Miami, Fla.) performed as previously described (4). Antisera obtained from outside sources were: sheep anti-mouse Ig (N. L. Cappel Laboratories Inc., Cochraneville, Pa.); rabbit anti-mouse brain (Accurate Chemical & Scientific Corp., Hicksville, N. Y.); anti-Ia-8+ $(A \times B10A)F_1$ anti-B10A(5R) and anti Ia-7 $(B10 \times HTI)F_1$ anti-B10A(5R) (Research Resources Branch, National Institutes of Health, Bethesda, Md.).

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[1] *Abbreviations used in this paper:* Ag, antigen(s); BCG, Bacille Calmette-Guérin; BPA, bovine plasma albumin; CFA, complete Freund's adjuvant; DC, dendritic cell(s); EA, erythrocyte(s) opsinized with antibody; EM, electron microscopy(ies); FCS, fetal calf serum; LODAC, low-density adherent cell(s); MHC, major histocompatibility complex; MLR, mixed leukocyte reaction(s); Mϕ, macrophage(s); s, surface; SAC, spleen-adherent cell(s); SMLR, syngeneic MLR; TdR, thymidine.

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We also used two monoclonal rat anti-mouse reagents prepared by immunizing rats with clusters of BALB/c T cells and DC (see Isolation of Cell Clusters from SMLR Cultures). Rats were immunized on days 0 and 30 with 5×10^6 cells i.v. and hybrids were obtained by fusing immune spleen cells and P3U cells on day 33 according to Kohler and Milstein (10). Clone B5-3 secretes a typical anti-Thy-1 antibody. By immunofluorescence it stains 95% of thymocytes and nylon wool-purified T cells. It does not stain either pure B cells, DC, or Mϕ. Immunoprecipitation from radiolabeled spleen cells with B5-3 yields a single polypeptide with an apparent molecular weight of 25,000 on sodium dodecyl sulfate polyacrylamide gels. Clone B21-3 is an anti-I-$A^{b,d}$ reagent. The specificity of this reagent was determined by cytotoxicity assays on appropriate recombinant mice. In sequential immunoprecipitation studies, B21-3 competes with anti-Ia-8 alloantiserum.

Cell Preparation. Spleen, mesenteric lymph node, and thymus were teased with forceps and further disrupted on stainless-steel sieves. Resident peritoneal cells were obtained by lavage with phosphate-buffered saline. T cell-enriched populations were obtained in two ways. Lymphoid cells were passed over nylon wool according to Julius et al. (11); for spleen, 15–20% of total applied cells and 30–50% of applied T cells were recovered in the nonadherent fraction, with >90% being T cells, i.e., reactive to rabbit anti-mouse brain serum and clone B5-3. Alternatively, enriched populations of T and B cells were obtained by panning on bacteriologic Petri dishes (No. 8-757-12, Fisher Scientific Co., Pittsburgh, Pa.) that were coated with 6–10 μg/ml affinity-purified sheep anti-mouse Ig exactly as described by Wysocki and Sato (12). The B cells were dislodged with Pasteur pipettes, or trypsinized (200 μg/ml trypsin, TRL3, Worthington Biochemical Corp., Freehold, N. J.) for 15 min at 37°C in the presence of DNAse (30 μg/ml, type I, Sigma Chemical Co., St. Louis, Mo.).

Various populations of adherent cells were studied shortly after harvest, or more frequently, after overnight culture. Resident peritoneal cells were maintained in 16-mm plastic tissue culture wells (No. 3524, Rochester Scientific Co., Rochester, N. Y.). Whole spleen-adherent cells (SAC) were prepared by adhering spleen cells for 2 h on 100-mm plastic Petri dishes (No. 3003, Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) or on 35-mm glass coverslips. Low-density adherent cells (LODAC) were obtained as previously described (4). After an overnight culture, most of the SAC or LODAC floated off or could be easily dislodged from the culture surface (4). LODAC or SAC suspensions were then separated into DC- and Mϕ-rich components by one of two techniques. Highly purified DC (usually <2–5% lymphocytes and Mϕ) were obtained by removing cells that were rosetting with erythrocytes opsonized with antibody (EA) (4). Alternatively, the cell suspension was applied to glass or plastic for 1–2 h, and the nonadherent cells dislodged by gentle pipetting. When the starting mixture contained 30% or more Mϕ, this readherence method provided a Mϕ-rich (>90%) adherent fraction and a DC-rich (50–90%) nonadherent fraction. The two cell types were enumerated on the basis of morphologic and EA-rosetting criteria (4), reviewed in Results.

SMLR. 4–5×10^6 responder cells were cultured 3–4 d with varying numbers of stimulators in 16-mm-Diam, flat-bottom, tissue culture wells. The culture medium (1 ml) was RPMI-1640 (Grand Island Biological Co., Grand Island, N. Y.) that was supplemented with penicillin, 5×10^{-5} M 2-mercaptoethanol, and heat-inactivated (56°C, 0.5 h) 5% fetal calf serum (FCS) (Flow Laboratories) or 2.5% heat-inactivated, isologous mouse serum, taken by cardiac puncture. Stimulator cells were treated with 25 μg/ml mitomycin-C (Sigma Chemical Co.) for 30 min at 37°C, washed three times in RPMI-1640, and added in suspension or as adherent cells on 15-mm glass coverslips.

To measure cell proliferation, the cultures were harvested with Pasteur pipettes, centrifuged, and replated in 6-mm microtest wells (No. 3396, Rochester Scientific Co.) in fresh culture medium that was supplemented with 2% dialyzed FCS. Proliferating cells did not remain adherent to the original culture vessel. 0.2 ml of cells (1/5 of the original culture) were pulsed with 50 μl of [^{3}H]thymidine (TdR) (8 μCi/ml, 6 Ci/mM sp act, Schwartz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.) for 2 h at 37°C and then harvested on a multisample harvestor. Data are expressed as counts per minute per culture, which is five times the value for individual microtest wells. In some instances, proliferating cells were enumerated by autoradiography. Cultures were labeled with 1 μCi/ml [^{3}H]TdR for 1 h and washed in RPMI-1640 that contained 200 μM nonradioactive TdR (Sigma Chemical Co.). Aliquots were spun onto

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12-mm coverslips, dried with a hair dryer, fixed in methanol, dipped in Ilford L-4 emulsion (Ilford Ltd., Basildon, Essex, England), exposed for 1-3 d, and developed in Kodak D-19 developer (Eastman Kodak Co., Rochester, N. Y.).

Cell-mediated Lympholysis. Varying doses of effector cells were tested on ^{51}Cr (New England Nuclear, Boston, Mass.) -labeled tumor cell lines, as described elsewhere (13). Effector and target cells were incubated together for 4 h at 36°C, and the results are expressed as the mean percent specific ^{51}Cr release for replicates of three wells. The formula used to calculate percent specific ^{51}Cr release was SR = ([TR − SR/A1 − SR] × 100), where TR is test release, SR is spontaneous release, and A1 is 1% acetic acid lysis.

Isolation of Cell Clusters from SMLR Cultures. Cell aggregates develop during the SMLR (Results) and can be isolated by velocity sedimentation. The cultures are layered on a 20-ml linear gradient bovine plasma albumin (BPA) (ρ = 1.031-1.008) in cellulose nitrate tubes (No. 302236, Beckman Instruments, Inc., Fullerton, Calif.). After centrifugation at 50 *g* for 5 min at 4°C, the clusters are found in the pellet, whereas the nonclustered cells remain in the upper part (1-5 cm) of the column. Under these conditions free lymphoblasts and DC do not pellet. Clusters were fixed and examined by both transmission and scanning electron microscopy (EM) as previously described (4).

Removal of Ia$^+$ Cells. 10^7 cells (usually surface [s]Ig$^-$ spleen or lymph node cells) were treated with monoclonal anti-Ia (final concentration of protein, 3 μg/ml) and guinea pig complement (Heme Lo, Accurate Chemical & Scientific Co.; final concentration, 1:10) for 45 min at 37°C, in culture medium supplemented with 30 μg/ml DNAse. The cells were then washed three times before being put into culture.

Priming to Tuberculin Ag. Mice were sensitized to tuberculin Ag administered in complete Freund's adjuvant (CFA) (0.5 ml i.p.) 2 wk before sacrifice, or live Bacille Calmette-Guérin (BCG) (10^6 i.v. Pasteur strain, The Trudeau Institute) 1-2 wk before sacrifice.

Results

DC Induce Syngeneic T Cells to Proliferate. Nylon wool-nonadherent spleen cells from normal animals (>90% T cells) were cultured for varying lengths of time in the presence or absence of small numbers of syngeneic DC. In five kinetic experiments, addition of DC resulted in a progressive increase in proliferative activity over a 3- to 5-d culture period (Table I). Either 2.5% isologous serum or 5% FCS could be used to supplement the culture medium, but responses were generally higher in FCS. Though 10^4 DC could induce detectable proliferative responses (see below), the 1-d culture medium of up to 5×10^5 DC did not. DC and T cells had to be cocultured because culturing the two cell types in adjacent wells that were separated by a patent 1- × 5-mm pore did not result in T cell proliferation.

More than 90% of the proliferating cells in cocultures of DC and T cells were killed by rabbit anti-mouse brain serum, or monoclonal rat anti-mouse Thy-1, plus complement. Mitomycin-C-treated and untreated DC induced similar responses. The expansion in proliferative activity measured by [^{3}H]TdR uptake was paralleled by a large expansion in lymphoblast numbers detected by autoradiography. With the latter technique, we noted that <1% of the cells could be pulse-labeled with [^{3}H]TdR in 1-d cultures. By day 3, 8-10% of the cells in DC-T cocultures were labeled blasts.

The proliferative response of cells from other lymphoid organs was then studied (Table II). In seven experiments, unfractionated spleen and mesenteric lymph node developed considerable background proliferative activity during a 3-d culture period. This could be further boosted one- to threefold by the addition of 10^5 syngeneic DC. However, the nylon wool-nonadherent fraction from either node or spleen showed little background proliferation and gave large absolute responses to added DC. Background proliferation and response to DC in thymus were <10% of that seen in spleen and node.

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TABLE I
Kinetics of T Cell Response to DC in FCS- or Mouse Serum-supplemented Cultures

Day of assay	Response in 2.5% isologous serum		Response in 5% FCS	
	T cells	T cells + DC	T cells	T cells + DC
		[^{3}H]TdR uptake/culture		
0	3,715	3,715	3,715	3,715
2	475	11,315	2,865	56,925
3	315	14,135	4,570	114,300
4	295	30,125	16,390	94,530
5		46,280		79,730

5×10^6 C57BL/6 nylon wool-nonadherent spleen cells were cultured with or without 10^5, mitomycin-C-treated, purified DC. The culture medium was supplemented with either 2.5% isologous or 5% FCS. Day 1 TdR uptake data, not done in this experiment, were always intermediate between day-0 and day-2 values.

TABLE II
Proliferative Response of Mesenteric Lymph Node and Thymus Cells to Syngeneic DC

Responders	Response after stimulation with			
	Experiment 1		Experiment 2	
	No DC	10^5 LODAC	No DC	10^5 DC
		[^{3}H]TdR uptake/culture		
Unfractionated mesenteric lymph noae	30,735	54,000	40,240	94,845
Nylon wool-nonadherent node	4,265	67,075	8,475	102,450
Unfractionated thymus	275	2,315	1,160	7,435
Nylon wool-nonadherent thymus	200	2,220	255	11,680

5×10^6 responder cells from BALB/c mice (experiment 1) and C57BL/6 mice (experiment 2) were cultured 3 d in medium supplemented with 5% FCS. In experiment 1, 10^5 LODAC, a mixture of adherent macrophages and DC, were used as stimulators, whereas in experiment 2 purified DC were used.

Comparison of DC with Other Cell Types as SMLR Stimulators. Dose-response curves were obtained to compare the capacity of various cell populations to stimulate proliferative responses in syngeneic nylon wool-nonadherent spleen cells. Suitable quantitative data were obta ned by harvesting the cells at day 3, before the peak of the responses, and by exposing the cells to brief pulses of [^{3}H]TdR in fresh culture medium.

In all experiments lymphocyte preparations were <100 times as active as pure DC in stimulating the SMLR (Fig. 1). B cells prepared by selection on anti-Ig-coated plates (12), and nylon wool-passed T cells, were even less active as stimulators than unfractionated spleen. In contrast, the sIg$^-$ fraction, which is both T and DC enriched (Table III), was enriched in stimulatory capacity. Thus, SMLR stimulatory capacity correlated with DC content rather than T or B cell content.

Next we studied SMLR stimulation by cultured LODAC, which are a mixture of DC and Mϕ (4). LODAC were fractionated into DC- and Mϕ-rich components either by EA rosetting (4) or by readherence to plastic (5). The EA$^-$ fraction, which is highly enriched in DC, was at least 10-fold more potent than the Mϕ-rich EA$^+$ fraction (Table IV). The small numbers of nonrosetted DC (<10%) that contaminate the EA$^+$ cells accounted for their stimulatory capacity. These results were confirmed with a second separation technique in which LODAC were divided into a Mϕ-rich readher-

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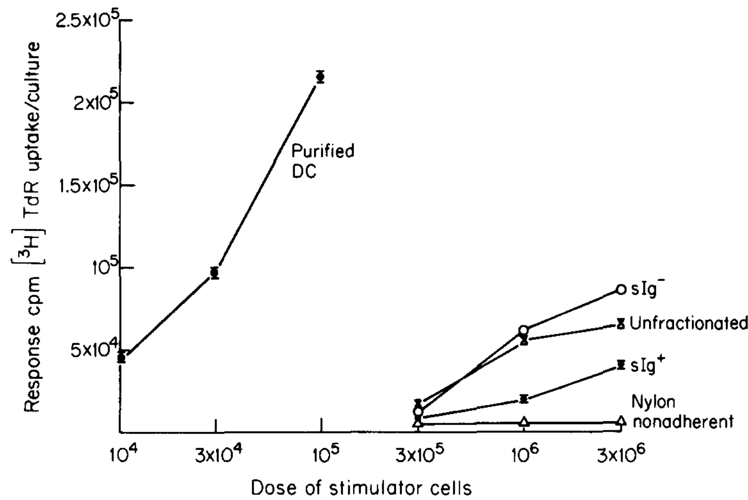

FIG. 1. The relative potencies of purified DC and different populations of lymphocytes in stimulating syngeneic T cell proliferation. 5×10^6 C57BL/6 nylon wool-nonadherent cells were cultured for 3 d with varying doses of mitomycin-C-treated stimulators in 5% FCS-supplemented medium. Uptake in unstimulated cultures was 5,780 cpm.

TABLE III

Proliferative Response of Subpopulations of Spleen Cells to Syngeneic DC

Splenic responder population	Percent cells bearing				Proliferative response	
	Ia	Brain	Ig	DC	Without DC	With DC
			%		$[^3H]TdR$ *uptake/culture*	
Experiment 1						
Unfractionated	50–60	25–35	45–55	0.3–0.5	42,510	65,435
Ig^+	<5	>90	<5	0.8–1	109,460	106,175
Nylon wool nonadherent	<5	>90	5–10	<0.1	6,210	98,875
Ig^+	92	<5	ND	<0.1	33,065	32,455
Experiment 2						
sIg^-	<5	75–80	<5	0.8–1	42,905	46,910
sIg^- nylon wool nonadherent	<5	>95	<5	<0.1	2,035	70,440

Experiment 1: 5×10^6 C57BL/6 spleen cells were cultured 3 d in the presence of 5% FCS, with or without 3×10^4 purified DC. Cytotoxicity assays were used to enumerate those cells in the responding populations that were reactive to monoclonal rat anti-mouse Ia, rabbit anti-brain, and sheep anti-mouse light chain (Ig). DC were enumerated after adherence and spreading on glass coverslips (5). ND, not done. Experiment 2: 5×10^6 BALB/c spleen cells were cultured for 3 d in 2.5% isologous mouse serum with or without 10^5 LODAC added as a source of DC. sIg^- cells were compared with sIg^- cells passed over nylon wool.

ent population and a DC-rich nonadherent fraction (Fig. 2). Mϕ obtained by readherence contained fewer DC than the EA^+ fraction and exhibited less SMLR-stimulating activity. In contrast, the DC-enriched nonadherent cells were similar to the DC prepared by EA rosetting in stimulatory capacity (Table IV).

Mϕ from two other sources–whole spleen and peritoneal cavity–were also tested. The use of SAC rather than LODAC did not increase the yield of adherent cells, and there was significant contamination by lymphocytes and granulocytes. Nevertheless, the Mϕ-rich component of SAC was weak stimulators (Table IV), whereas the DC-

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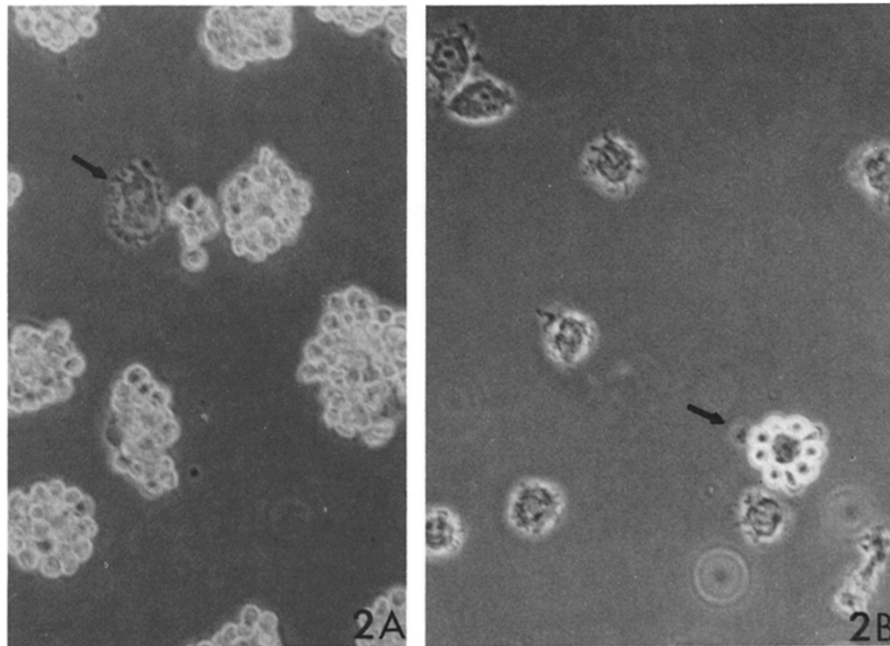

FIG. 2. Phase contrast micrographs of subpopulations of splenic LODAC. The LODAC were dislodged from glass coverslips after overnight culture and separated into adherent and nonadherent fractions by readherence to glass. The readherent cells (A) were >90% typical Mϕ in appearance, phagocytosis of immune complexes (5), and, as shown here, heavy rosetting of opsonized EA. 5–10% of the cells in this fraction look like DC (arrow) and fail to rosette EA. The nonadherent cells (B) are largely nonphagocytic (5), EA-rosette$^-$ cells, with the irregular shape and other typical cytologic features of DC. EA-rosette$^+$ cells (arrow) comprise a variable percentage of this fraction, usually <20%, and they have been further identified as Mϕ by morphology and phagocytosis of immune complexes. Of the nonadherent cells, <10% were small lymphocytes. × 640.

enriched, nonadherent component stimulated according to its DC content (Fig. 3; Table IV). Peritoneal Mϕ were also weak or inactive as stimulators (Table IV).

Not only were Mϕ-rich preparations weak stimulators at all doses, but levels of 2% or more Mϕ in our cultures inhibited the effects of added DC. The degree of inhibition varied in different experiments, but the source of this variability was not pursued.

Comparison of the Allogeneic and SMLR Induced by DC. The potency of DC relative to other cell types in inducing syngeneic responses was reminiscent of data generated for the stimulation of allogeneic MLR (6). We therefore compared the responses of syngeneic and allogeneic T cells to purified DC. Dose-response curves generated in either 2.5% responder serum (Fig. 4) or FCS (not shown) revealed that DC were 10-fold more potent in stimulating allogeneic than syngeneic responses. Similar results were obtained if a single batch of T cells was stimulated with syngeneic and allogeneic DC (not shown). The slopes of the dose-response curves (Fig. 4), as well as the response kinetics, were similar for the two kinds of MLR. Mitomycin-C- and nonmitomycin-treated DC stimulated similarly in both systems.

Large cytotoxic T cell responses specific for the stimulating DC haplotype were detectable by day 4 in allogeneic cultures, induced by as few as 1 DC/150 responders. However, cytotoxic activity was not detectable in the syngeneic system (Table V).

Occurrence of SMLR in Cultured Spleen and Lymph Node. Because cultured spleen and

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TABLE IV

Stimulatory Capacity of Various DC- or Mϕ-Enriched Preparations

Stimulator dose	Response to Mϕ-rich populations				Response to DC-rich populations		
	Adherent SAC	Adherent LODAC	EA-rosette$^+$ LODAC	Adherent peritoneal	Nonadherent SAC	Nonadherent LODAC	EA-Rosette$^-$ LODAC
	[^{3}H]TdR uptake/culture						
3×10^5		1,300	50,025				
2×10^5			29,460	550			
1.8×10^5					33,520	76,000	
10^5	550	500	10,190				99,290
6×10^4				600	23,605	62,110	
3×10^4	500	425	13,595				46,010
2×10^4				750	6,990	13,710	
10^4	475						19,225
0	550	550	550	550	550	550	550

5×10^6 BALB/c nylon wool-nonadherent spleen cells were cultured for 3 d in 2.5% isologous serum with or without mitomycin-C-treated stimulators. The Mϕ and DC content of the stimulator populations was determined by morphology and EA rosetting (Fig. 2). Adherent peritoneal cells, SAC, and LODAC were >95% Mϕ. EA-rosette$^+$ LODAC were 90% Mϕ and 5–10% DC. Nonadherent SAC were 25% DC, 71% Mϕ, and 3% unidentified cells. Nonadherent LODAC were 40% Mϕ, 55% DC, and 5% unidentified cells. EA-rosette$^-$ LODAC were 90% DC and 10% Mϕ. The incidence of Mϕ in the nonadherent SAC and LODAC was atypically high in this experiment, but this is the only experiment in which all subpopulations of adherent cells were tested simultaneously; generally these populations are >70–90% DC.

lymph node exhibit extensive background proliferation (Table II), we designed experiments to test if part of this proliferative activity represented a response of cultured T cells to endogenous DC. We separated cell suspensions into sIg-bearing (sIg$^+$, >95% B cells) and sIg$^-$ (>90% T cells) populations on anti-Ig-coated plates (Table III). DC were enriched in the sIg$^-$ and depleted from the sIg$^+$ fractions, which was as expected because DC lack sIg (2). Cultured sIg$^-$ cells behaved like unfractionated spleen, i.e., there was high background proliferative activity and little response to added DC (Table III). Passage of these sIg$^-$ cells over nylon wool reduced background proliferation and rendered the cells responsive to DC (Table III, experiment 2). sIg$^+$ cells proliferated to a lesser extent than sIg$^-$, and did not respond to added DC (Table III), even at very high doses (5). Finally, unfractionated spleen behaved as a composite of its sIg$^+$ and sIg$^-$ fractions. We inferred that the differences in background proliferative activity and responses to DC in the two kinds of enriched T cell preparations could be ascribed to the presence of DC in the sIg$^-$ fraction and their depletion by passage over nylon wool.

We pursued this hypothesis by removing DC from sIg$^-$ cells with anti-Ia and complement treatment. Although <10% of the sIg$^-$ cells were killed, background proliferative activity fell 80–95% in node (five experiments) and 50–60% in spleen (four experiments [Table VI]). Reconstitution with small numbers of mitomycin-C-treated DC restored background proliferative activity (Table VI). The number of DC needed to fully reconstitute, i.e., 1 DC/100 anti-Ia + complement-treated cells, corresponds to the number of DC present in the untreated sIg$^-$ fraction (Table III). We conclude that T cell proliferation in response to small numbers of endogenous DC is occurring in mouse lymphoid cell cultures.

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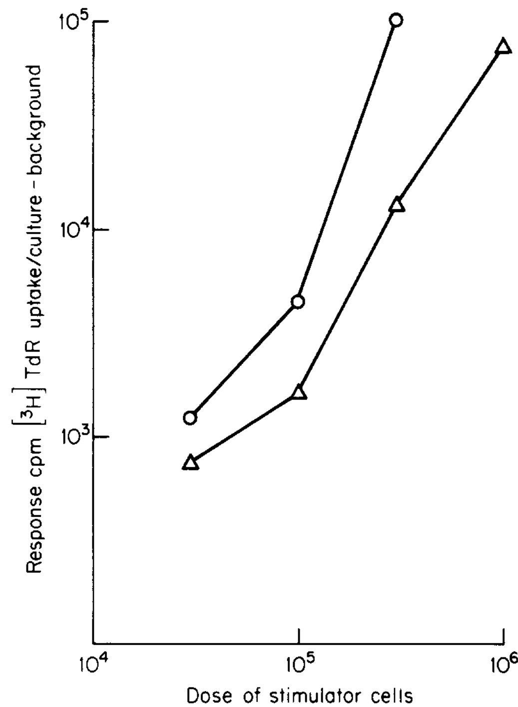

FIG. 3. Relative stimulatory capacity of whole SAC (Δ) vs. EA-rosette⁻, purified DC (○). 5×10^6 C57BL/6 nylon wool-nonadherent responder spleen cells were cultured for 3 d in 2.5% isologous mouse serum with varying doses of mitomycin-C-treated stimulators. SAC were prepared entirely in isologous mouse serum and contain 15% DC by cytologic and EA-rosetting criteria. [^{3}H]TdR uptake in unstimulated cultures was 300 cpm.

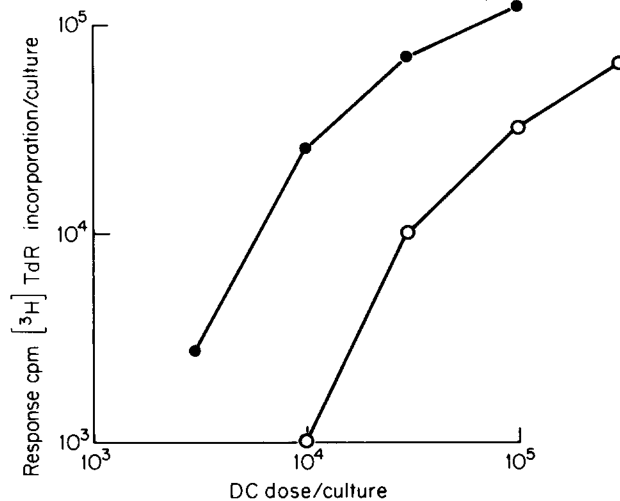

FIG. 4. Varying doses of C57BL/6 DC were used to stimulate 5×10^6 allogeneic (DBA/2) (●) and syngeneic (C57BL/6) (○), nylon wool-nonadherent spleen cells. Cultures were maintained for 3 d in 2.5% responder serum. Response in the absence of stimulators was 14,195 cpm for the DBA/2 and 4,350 cpm for the C57BL/6 T cells.

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TABLE V

Generation of Cytotoxic Cells in Allogeneic vs. SMLR

Stimulator	Responder	Effector:target	Percent ^{51}Cr specific release from	
			P3U H-2^d	EL4 H-2^b
			%	
10^5 C57BL/6 DC	5 × 10^6 C57BL/6 T cells	50:1	0	0
		15:1	0	0
		5:1	0	0
		1.5:1	0	0
3 × 10^4 C57BL/6 DC	5 × 10^6 BALB/c T cells	50:1	0	47
		15:1	0	28
		5:1	0	13
		1.5:1	0	6

Syngeneic (top) and allogeneic (bottom) mixtures of nylon wool-nonadherent responder spleen cells and purified DC were maintained 4 d in medium supplemented with 2.5% isologous serum. Cytotoxicity assays were performed on ^{51}Cr-labeled P3U 51R, H-2d (spontaneous release of 11%) and EL4-BU, H-2b (spontaneous release of 8%) tumor targets.

TABLE VI

α-Ia + C′ Depletion of Endogenous Proliferative Activity in sIg⁻ Spleen Cells

Treatment	Response to			
	No DC	3 × 10^4 DC	10^5 DC	3 × 10^5 DC
		[^3H]TdR incorporated/culture		
C′	78,790	135,300	168,950	ND
Ab + C′	14,310	99,915	156,890	185,375

5 × 10^6 DBA/2 Ig^- spleen cells were cultured 3 d in medium supplemented with 5% FCS after treatment with complement (C′) alone +/− clone 21-3 antibody (Ab) (a monoclonal rat anti-mouse I-A). Mitomycin-C-treated DBA/2 DC were added in the doses indicated above. ND, not done.

Studies on the Mechanism of DC-induced SMLR. To determine if carryover of endogenous Ag influenced the SMLR, we compared the responses of unprimed mice and animals primed with either CFA or 10^6 live BCG, to DC from the same mice (Table VII). Ag priming did not affect either the magnitude of the T cell-proliferative response or the stimulatory capacity of DC. In control experiments, we noted that Ag (purified tuberculin protein derivative) induced the primed T cells to proliferate.

We then considered the possibility that the various steps involved in DC purification altered their stimulatory capacity. By using whole spleen-adherent cells rather than LODAC, we were able to obtain DC-enriched populations entirely in isologous serum. SAC stimulated syngeneic T cell proliferation according to DC content (Fig. 3; Table IV). The potency of DC was unaltered when the cells were used immediately after removal from the animal (LODAC) or if used after 3 d of culture (not shown). Thus, we could find no evidence that heterologous serum (FCS), BPA columns, or tissue culture altered the stimulatory capacity of DC.

DC and Responding T Cells Are Physically Associated during the SMLR. Cell aggregates appear in cultures of sIg^- spleen cells and in cocultures of purified DC and nylon wool-passed T cells. After 1 d, the clusters were 3–10 cells in diameter, and by 3 d, the

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TABLE VII
Effect of Ag Priming on SMLR

Responder	Stimulator	Response stimulated by			
		No DC	2×10^4 DC	6×10^4 DC	2×10^5 DC
		[^{3}H]TdR incorporation/culture			
Experiment 1					
Unprimed T cells	Unprimed DC	5,150	40,140	139,430	283,955
Unprimed T cells	CFA DC	5,150	62,285	146,600	282,950
CFA-primed T cells	Unprimed DC	10,385	37,090	70,520	148,010
CFA-primed T cells	CFA DC	10,385	34,610	81,285	163,805
		No DC	10^4 DC	3×10^4 DC	10^5 DC
Experiment 2					
BCG-primed T cells	Unprimed DC	12,900	15,185	20,495	85,555
BCG-primed T cells	BCG DC	12,900	16,485	32,020	86,910

In experiment 1 the same shipment of C57BL/6 mice was divided into two groups. One was primed with 0.2 ml of CFA i.p. 2 wk before the experiment, and the other group was left unprimed. In experiment 2 $B6D2F_1$ mice were divided into two groups. One was primed with 10^6 live BCG i.v. 2 wk before the experiment, and the other group was left unprimed. Cultures of nylon wool-nonadherent spleen cells and varying numbers of purified DC were maintained in 5% FCS for 3 d before TdR uptake assays.

clusters enlarged and fused with one another (Fig. 5A–C). The aggregates could be purified by velocity sedimentation and studied directly.

Morphologic examination indicated that the cluster fraction contained both lymphocytes and irregularly shaped DC, whether isolated after 1 or 3 d of culture. In scanning EM (Fig. 6A), the presumptive DC had large, smooth, leaflike extensions, whereas the lymphocytes were 8- to 10-μm spherical cells with variable numbers of microvilli. By transmission EM, the cells that gave rise to the large cytoplasmic extensions had the cytologic features of typical DC (Fig. 6B). The nuclei were large and irregularly shaped, and the cytoplasm contained many mitochondria but relatively few other organelles; e.g., there were few free or membrane-bound ribosomes and the Golgi region contained multivesicular bodies but few vesicles and granules. Close contacts between DC and lymphocytes were numerous, but specialized junctions were not detected.

To enumerate cluster-associated, proliferating T cells, we pulse-labeled 3-d DC-T cell cocultures with [^{3}H]TdR, separated clusters and nonclusters, and examined the two fractions by autoradiography. The cluster fraction had only 10–20% of the cells, but contained >90% of the labeled blasts (Table VIII). This corresponds to a 100-fold increase in specific proliferative activity relative to the nonclustered cells. DC were enumerated by phase contrast after centrifugation onto poly-L-lysine coverslips. DC comprised 17 and 1.5% of the cells in the clusters and nonclusters, respectively.

The percentage of T cells and DC in the clusters was verified by surface markers. 85% of the clustered cells were killed by Thy-1 and complement, and 15% were Ia^+. Sequential killing studies showed that the Ia^+ and Thy-1^+ cells were not overlapping populations. In addition, anti-Ia plus complement did not reduce TdR uptake in day 3 SMLR. We conclude that responding Ia^- T cells physically associate with Ia^+ DC during the SMLR (Table VIII).

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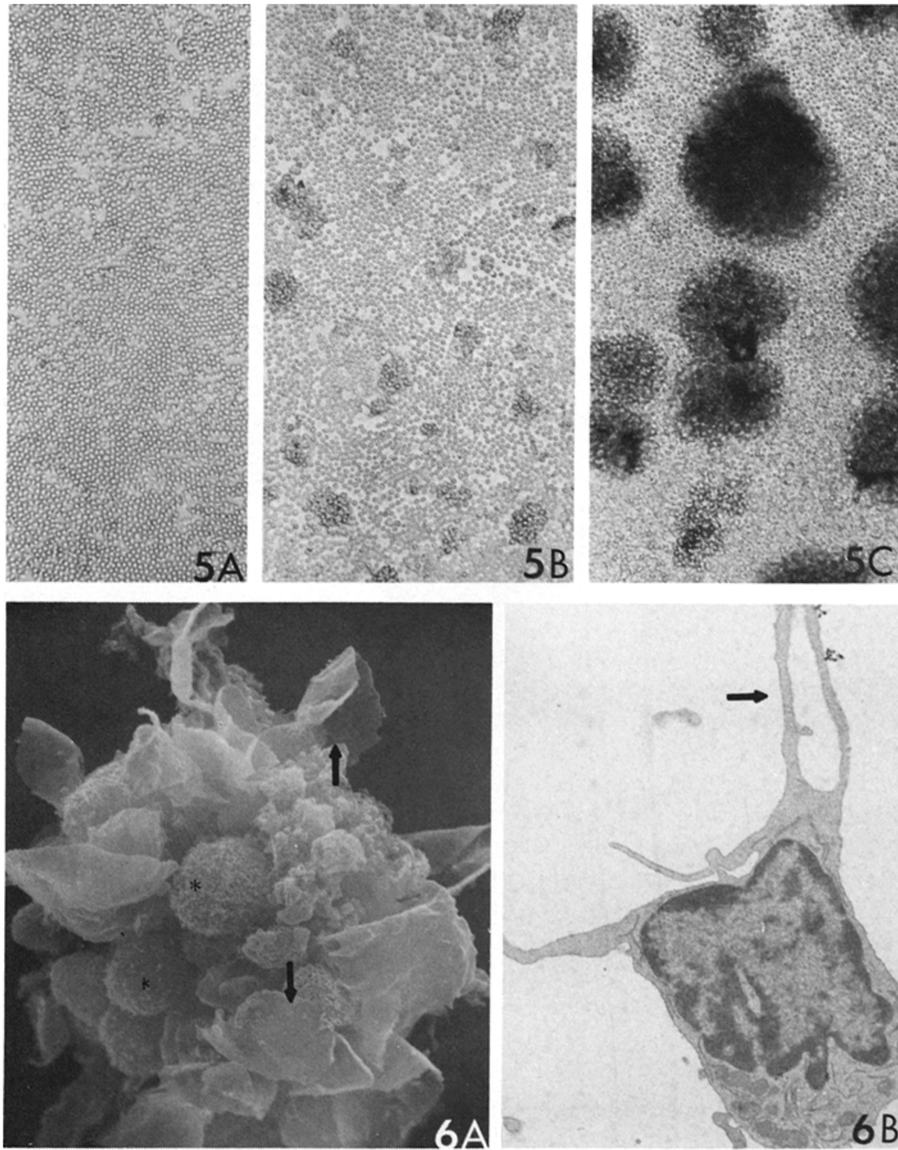

FIG. 5. Micrographs of SMLR generated by 5×10^6 C57BL/6 T cells cocultured with 10^5 C57BL/6 DC. (A) At the time of initiation of the SMLR, T cells and DC are evenly distributed in the culture vessel. (B) The same culture 12 h later contains discrete cell aggregates. (C) After 3 d at culture the aggregates have fused, enlarged, and are concentrated in the center of the culture vessel. These aggregates do not form if DC are omitted from the culture. × 210.

FIG. 6. Clusters were isolated from 24-h cocultures of 5×10^6 DBA/2 T cells and 10^5 DC by centrifugation on linear gradients of BPA. (A) Scanning EM of a single cluster that shows lymphocytes (*) and DC (arrows). × 2,500. (B) Transmission EM showing that the flaps of cytoplasm (arrow) in the clusters arise from cells with the typical cytologic features of DC. × 6,400.

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TABLE VIII

T-Blast and DC Content of Cluster and Noncluster Fractions

	Unfractionated	Cluster	Noncluster
Number cells per culture	1.5×10^6	0.3×10^6	1.1×10^6
Labeled blasts per culture*	0.15×10^6 (100%)	0.14×10^6 (93%)	0.004×10^6 (3%)
DC per culture*	0.075×10^6 (100%)	0.051×10^6 (68%)	0.016×10^6 (21%)
Percent killed by			
α-Thy-1 + C′	—	75%	—
α-Ia^{bd} + C′	—	25%	—
α-Thy-1 + C′ → α-Ia^{bd} + C′‡	—	100%	—

5×10^6 DBA/2 nylon wool-nonadherent spleen cells were cultured for 3 d with 10^5 DBA/2 DC in the presence of 5% FCS. Clusters were separated from nonclustered cells after a 1-h pulse of 1 μCi/ml [^{3}H]-TdR. Labeled lymphoblasts were enumerated after autoradiography. DC were identified by phase contrast. 5-3 and 21-3 monoclonal antibodies, α-Thy-1 and α-Ia^{bd}, respectively, were used at a final concentration of 3 μg/ml. C′, complement.

* Numbers in brackets represent percentage of total blasts or DC in a given fraction.

‡ Sequential killing with 21-3 followed by 5-3, or vice versa, gave identical results. This experiment was repeated with similar results with C57BL/6 cells.

Discussion

The term autologous or SMLR refers to the expansion of T cell-proliferative activity in vitro, after the addition of syngeneic stimulator cells. This phenomenon has been studied primarily in man (14–20) and to a lesser extent in mice (21–23). We have documented four new features of the mouse SMLR: (*a*) the DC is a uniquely potent stimulator cell; (*b*) DC-dependent T cell proliferation occurs in all cultures of mouse spleen and lymph node; (*c*) DC and responding T cells efficiently associate with one another in cell aggregates; and (*d*) neither T cell responsiveness nor DC stimulatory capacity are modified by immunization with tuberculin Ag.

The singular potency of DC in stimulating syngeneic T cells was documented by a combination of two approaches. First, we devised conditions in which the proliferative response was related to the dose of stimulating cells. As noted in previous studies of the allogeneic MLR (6), this involves the use of short proliferative assays on washed lymphoid cells before the peak in the response. Second, we monitored and manipulated DC numbers in both stimulator and responder populations with a series of morphologic and cell-surface markers that have been previously described (4). This enabled us to assess the potential contribution of DC to the responses we measured.

Studies of the autologous MLR in man have emphasized the role of B cells as stimulators. This was based on the findings that B lymphocyte lines (14), E rosette-depleted leukocytes (15), and anti-Ig-selected leukocytes (20) functioned as stimulators. However, there is evidence that B cells do not represent the major stimulators in primary leukocyte populations (17, 20). DC have yet to be identified in man, so their contribution has not been analyzed directly. In the mouse, we found that stimulating capacity in heterogeneous populations correlates with DC content. Thus, sIg^- spleen cells, which are enriched in DC, were better stimulators than whole spleen. In contrast, nylon wool-purified T cells and sIg^+ B cells, which are depleted of DC, were less potent than unfractionated spleen. In all instances, lymphocyte-rich populations were at least 100 times less active than pure DC (Fig. 1).

Mφ prepared by several different techniques were also weak stimulating cells.

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Proliferation of T cells did occur in response to some of our Mϕ-rich preparations, but, again, this could be attributed to contaminating DC. For example in Table IV, the Mϕ-rich EA-rosette fraction from LODAC has ~10% of the stimulatory capacity of EA^- DC and contains 10% contaminating DC. The readherent LODAC fraction from the same preparation had few identifiable DC and was two orders of magnitude less potent than DC purified by two independent procedures. We conclude that mouse T cells, B cells, and Mϕ are poor SMLR stimulators and that conceivably contaminating DC account for any stimulation that is observed.

In rodents, SMLR have been noted in most studies where Mϕ-enriched accessory cell preparations were mixed with nylon wool-nonadherent cells (24, 25). Our data indicate that Mϕ are weak or inactive as stimulators. Therefore, it is possible that significant numbers of DC have been present in the accessory cell preparations used in previous studies. We are currently studying proliferative responses of primed T cells to evaluate the relative contributions of DC and Mϕ as accessory cells.

Mechanism of SMLR Stimulation. The SMLR may be mediated by Ag that are present in the animal before sacrifice and carried over into tissue culture. If endogenous Ag were needed for stimulation, DC would be functioning as powerful accessory or Ag-presenting cells. Work in progress in this laboratory indicates that DC do act as accessory cells for Ag-dependent T cell responses in vitro. Nevertheless, we could not alter the magnitude of the SMLR by deliberately immunizing mice with tuberculin Ag in living mycobacteria or CFA (Table VIII). Furthermore, the SMLR was not reduced if we maintained DC and/or T cells for 2 d in vitro before coculture. Therefore, we doubt that carryover of Ag that may be present in the animal before sacrifice mediates the SMLR.

We also considered the possibility that antigenic determinants could be introduced by experimental manipulations. We prepared DC entirely in isologous serum, thus avoiding exposure to BPA or FCS, but this did not change DC stimulatory capacity. Although we cannot rigorously exclude the possibility that DC-associated Ag mediate the SMLR, our data suggest that T cells or some subpopulation of T cells recognize and respond to DC directly.

Both Ag and I-region determinants can influence proliferative responses in primed T cells (26, 27). There is evidence in man that $F(ab)_2'$ fragments of anti-Ia antibodies block the autologous MLR (20). Thus, major histocompatibility complex (MHC) recognition seems to occur in both the autologous and Ag-driven proliferative responses. If the SMLR in the mouse is indeed independent of Ag, then I-region determinants on DC may be the primary stimulants for T cell proliferation. Following this line of reasoning, the function of Ag would be to select T cells that will respond to DC.

A subpopulation of T cells appears to respond in the SMLR, because increasing the dose of stimulators beyond 10^5 DC/5 × 10^6 T cells does not significantly increase the proliferative response. It needs to be determined if the responder T cells bear a specific Ly-1,2 phenotype, if they are restricted to recognize self MHC products, or if they have previously been activated by antigen *in situ*. Our studies agree with work in the human system that showed that self cytotoxic cells do not develop during the SMLR (16). Thus the cytotoxic T cell subpopulation is not directly involved in the SMLR.

Association of DC and Responding T Cells. One specific property of the responding T

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cells is the ability to aggregate with stimulator DC. Aggregation of the two cell types is remarkably efficient because they each represent only a few percentages of the cells in the culture; yet DC and T blasts associate to the exclusion of most of the nonproliferating cells. Our current studies have revealed that if the aggregates are disrupted, they reform within 1–2 h, and, again, the clusters include most of the dividing T cells in the culture. Clusters isolated after 24 h of coculture contain few lymphoblasts, will remain intact, and are highly enriched in T cells that will subsequently proliferate. The ability of responding T cells and DC to form stable aggregates could play an important role in T cell responses influenced by DC. That is, any stimulatory determinant that is associated with the DC would remain closely apposed to the T cell for long periods of time, thereby promoting induction of a response. Alternatively, any stimulant associated with the T cell may be able to act in concert with DC determinants such as Ia Ag.

The clustering capacity of DC may explain what is a striking phenomenon, i.e., the same cell type that is such a potent stimulator of the allogeneic MLR also contributes to the SMLR. We had previously reasoned that MHC-linked alloantigens on DC may not in themselves be responsible for the capacity of DC to stimulate the allogeneic MLR, because other cell types bearing alloantigens are weak stimulators (5, 6). Clustering of DC and responding T cells occurs in the allogeneic system and this may provide an efficient and stable form of alloantigen presentation.

Significance of the SMLR. The SMLR is the first system in which DC have been demonstrated to influence the behavior of syngeneic cells. The small numbers of DC required to induce the SMLR is striking; as few as 1 DC/50–100 responders leads to optimal proliferation (Fig. 4). By using sIg^- cells, we have shown that SMLR are probably a component of all murine spleen and lymph node cell cultures. This is relevant because studies in man have shown that autologous reactions contribute to the development of cytotoxic and helper activities (8, 16, 19).

An obvious question raised by our findings is whether recognition of self DC in the SMLR is a component of self MHC recognition during Ag and thymic-dependent T cell development. In Ag-induced T cell development, it is thought that a complex of Ag and MHC product is recognized on a nonlymphocytic, accessory cell. However, in the thymus, MHC restrictions can be imposed presumably in the absence of Ag (28). We now know that typical DC are present in thymus and that DC act as accessory cells in Ag-dependent, peripheral T cell responses. We would propose that self-recognition in certain systems involves recognition and proliferation to self DC by a process equivalent to a DC-mediated SMLR.

Summary

We have studied the proliferative response of unprimed T cells to syngeneic dendritic cells (DC) (syngeneic mixed leukocyte reaction [SMLR]) in cultures of mouse spleen and lymph node. T cells purified by passage over nylon wool contain few DC and exhibit little proliferative activity during several days of culture. Addition of small numbers of purified syngeneic DC induces substantial, dose-dependent, T cell-proliferative responses that peak at day 4–5. B cells purified on anti-Ig-coated plates do not respond to DC at all doses tested. DC culture medium does not induce proliferation, and coculture of DC and T cells is required. Purified mouse B and T lymphocytes stimulate SMLR weakly if at all. Likewise, peritoneal and spleen

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macrophages are weak or inactive. Therefore, DC are potent and possibly unique primary cells for stimulating the SMLR in mice.

sIg^- spleen and lymph node cells show extensive background proliferative responses in vitro, and fail to respond to small numbers of purified DC. If the sIg^- cells are treated with anti-Ia and complement, or passed over nylon wool, DC are removed and proliferative activity falls. Proliferative activity is restored by adding back DC at levels similar to those present in sIg^- cells (1–2%). Thus, DC-dependent, T cell proliferation probably occurs in all spleen and lymph node cultures.

As expected from previous work (6), DC are also potent inducers of allogeneic MLR. On a per DC basis, the syngeneic response is 10 times weaker than the allogeneic MLR, and it is not accompanied by the development of cytotoxic lymphocytes.

The magnitude of the SMLR was not altered by antigen priming, and DC maintained in isologous rather than fetal calf serum were active stimulators. Therefore, syngeneic stimulation appears to be an intrinsic property of DC, and modification by exogenous agents does not seem to be required.

Coculture of DC and T cells results in the development of cell clusters that can be isolated and characterized directly. The clusters account for 10–20% of the viable cells in the culture, but contain >80% of the responding T cells and stimulating DC by morphologic and surface-marker criteria. The efficient physical association of DC and responding T cells implies specific cell-cell recognition.

We conclude that the SMLR reflects the ability of T cells, or some subpopulation of T cells, to interact with and proliferate in response to small numbers of DC.

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