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DENDRITIC CELLS INITIATE A TWO-STAGE MECHANISM FOR T LYMPHOCYTE PROLIFERATION*

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Previous studies of dendritic cells (DC)[1] (reviewed in 1–3) have revealed the potent immunostimulatory capacities of this class of leukocyte. However, relatively little is known of their mechanism of action. We have used a polyclonal lymphocyte mitogenesis system to examine this question. Klinkert et al. (4) and Phillips et al. (5) showed that T cells, modified with sodium periodate, proliferate quickly and extensively in the presence of DC. This assay has been used to identify two stages in oxidative mitogenesis. In the first stage, viable DC induce both the release of T cell growth factor, or interleukin 2 (IL-2) (reviewed in 6–8), and T lymphocyte responsiveness to IL-2. In the second stage, which can occur in the absence of DC, IL-2 alone mediates proliferation of these responsive T cells.

Materials and Methods

Animals. Mice were of either sex and 4–14 wk old. Swiss mice were from Taconic Farms, Germantown, NY, and the Laboratory Animal Research Center of The Rockefeller University; C3H/HeJ were from The Jackson Laboratory, Bar Harbor, ME; all other inbred strains were from The Trudeau Institute, Saranac Lake, NY.

Antibodies. 33D1 (9) and B21-2 (10) are rat monoclonal antibodies specific for mouse DC and $IA^{b,d}$ antigens, respectively. The latter also binds to Swiss mouse cells. Both antibodies were purified by M. Witmer, The Rockefeller University, by diethylaminoethyl cellulose fractionation of ascites.

Primary cells. Mice were killed in chloroform vapor or by cervical dislocation. Spleen cell suspensions were prepared by teasing with fine forceps and pressing through a stainless steel mesh in RPMI 1640 (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY). For unfractionated spleen cells, the suspension was treated with ammonium chloride (0.88%: Mallinckrodt Inc., Science Products, Div., St. Louis, MO) to lyse erythrocytes. Spleen adherent cells, representing 1–2% of the total were obtained by culture in medium (see below) on 100-mm petri dishes (3002; Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, CA) for 2–3 h at 37°C. Nonadherent cells were then removed by rigorous washing; the adherent cells remaining consisted primarily of DC and macrophages in a ratio of ~1:3 (11). After overnight

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[1] *Abbreviations used in this paper:* Con A, concanavalin A; CTLL, cytotoxic T lymphocyte line; DC, dendritic cell; IL-1, IL-2, interleukins 1 and 2; MHC, major histocompatibility complex; MLR, mixed leukocyte reaction.

culture the cells were harvested by gently pipetting over the tissue culture surface. Enriched populations of DC were derived from low density adherent cells that consist predominantly of DC and macrophages. After overnight culture, macrophages were depleted by readherence to 60-mm petri dishes (3002; Falcon Labware).

T lymphocytes (>90% Thy-1 positive) were obtained from mixed spleen and mesenteric lymph node cell suspensions passed over nylon wool columns as described (12).

Medium for cell culture was RPMI 1640 supplemented with 2.5–10% heat-inactivated fetal bovine serum (KC Biologicals Inc., Lenexa, KS), 2-mercaptoethanol (5×10^{-5} M; Sigma Chemical Co., St. Louis, MO), gentamycin sulphate (20 μg/ml; Schering Corp., Kenilworth, NJ) and, for experiments in Table II, indomethacin (1 μg/ml; Sigma Chemical Co.). Cells were irradiated with 1,500 rad from a ^{137}Cs source (Gamma Cell 1000; Atomic Energy of Canada Ltd., Ottawa, Canada).

Oxidative Mitogenesis Assay. T cell mitogenesis was induced by modification of cells with periodate (13). In one experiment (see Fig. 1) stimulators and/or responders were treated, but in all others only responder T cells were modified. T lymphocytes were washed twice in ice-cold phosphate-buffered saline with calcium and magnesium (Gibco Laboratories) and resuspended in the same to 2×10^7/ml. An equal volume of a freshly prepared solution of sodium *m*-periodate (Sigma Chemical Co.) at 0.5 mg/ml was added to the cells and incubated on ice for 15 min. Control cells were treated with buffer alone. The cells were centrifuged, without further dilution, at 300 *g* for 10 min and washed once in medium (2.5% serum) before use.

Graded numbers of irradiated stimulators were added to $4–5 \times 10^5$ periodate-treated T cells in a total volume of 200 μl in flat-bottomed microtiter plates (3596; Costar, Data Packaging, Cambridge, MA). At 24–27 h, 0.5 μCi/well of [^{3}H]thymidine (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, NY) was added and cells were harvested 14–18 h later on a multisample harvester. Kinetic studies (not shown) demonstrated that irrespective of stimulator dose, the bulk of DNA synthesis occurred from ~24 h onwards. Unless otherwise stated, results are given as the means of triplicate cultures; standard deviations were generally <15% of the mean.

Primary Mixed Leukocyte Reaction. Graded doses of irradiated unfractionated spleen or spleen adherent cells were used to stimulate proliferation of allogeneic T lymphocytes in microtiter wells as described (11).

Cytotoxic Elimination of Accessory Cells in Oxidative Mitogenesis. Accessory cells were eliminated by treatment with monoclonal anti-Ia or anti-DC antibody and complement, in two different types of experiment. Medium for treatment contained 2.5% serum throughout.

TREATMENT OF STIMULATORS BEFORE ADDITION TO T CELLS (SEE TABLES I, II). DC were eliminated from fresh spleen adherent cells by treatment with antibody and complement during the adherence step as described (11). Cultured adherent and unfractionated spleen cells were resuspended at $5–10 \times 10^6$/ml. They were incubated on ice with an equal volume of 33D1 or B21-2 antibody for 5–45 min at final, saturating concentrations of 10 μg/ml. Another volume of complement was added and the mixture was incubated at 37°C for 45–55 min. The complement source was rabbit serum screened for low background toxicity, lyophilized, and stored at −70°C before use at a final dilution of 1:16. After treatment the cells were washed two to three times in ice-cold RPMI, irradiated, and used as stimulators for T cell oxidative mitogenesis.

TREATMENT OF MIXED POPULATIONS TO EXAMINE DC-MEDIATED T CELL ACTIVATION (SEE TABLES III–V AND FIGS. 2, 3). Unirradiated DC were mixed with periodate-modified T cells at ratios of 1:100 to 1:30 as indicated. The mixture was either treated immediately (t = 0) or distributed into 16-mm wells (3524; Costar) at 10^7/well in 1 ml of medium. At subsequent times, cells were harvested and the culture supernatant was saved. Treatment with antibody and complement was as described above except that the initial cell density was $10–12 \times 10^7$/ml and the cells were not irradiated. After washing, the mixture was distributed into microculture plates at $2–5 \times 10^5$/well and 200 μl total volume. The cultures were maintained in fresh medium, culture supernatant at 50%, crude rat IL-2 at 2.5–5%, purified mouse IL-2 at ~10^{-9} M, or supplemented with DC at 1×10^4/well.

Interleukin Assays. Two assays (modifications of those in refs. 14 and 15, respectively) were used to test for the presence of interleukins:

THYMOCYTE MITOGENESIS. C3H/HeJ thymocytes were plated at 4–5 × 10^5/well in flat-bottomed microtiter wells in a total volume of 100 μl. Medium contained 2.5% serum, 0.8 μg/ml phytohemagglutinin (Burroughs Wellcome, Greenville, NC), a concentration determined to be suboptimal to induce mitogenesis alone, and 5 × 10^{-5} M 2-mercaptoethanol, which is essential for optimal responses to IL-1. Proliferation was assessed in a 14–18 h [^{3}H]thymidine pulse from day 2–3.

PROLIFERATION OF AN IL-2-DEPENDENT CELL LINE. A cytotoxic T lymphocyte line (CTLL) was used which proliferates in response to IL-2 but not to IL-1 (16). Two clones, CTLL-A2 and CTLL-A11, were used interchangeably. The line was maintained in minimal essential medium, Eagle (Gibco Laboratories) supplemented with 10% serum, 5 × 10^{-5} M 2-mercaptoethanol, 5 μg/ml glutamine, minimal essential medium amino acids (Gibco Laboratories), and 4–5% polyclone (Collaborative Research Inc., Lexington, MA) as a source of IL-2. It was passaged after harvesting with 0.5% EDTA (Mallinckrodt Inc.). On the day of assay, growth medium was replaced with IL-2-free medium at least twice over 4–5 h. After harvesting and washing to remove EDTA, cells were plated at 5–10 × 10^3/well in flat-bottomed wells. Serial dilutions of sample were added to a total volume of 100 μl. Proliferation was assessed in a [^{3}H]thymidine pulse from day 1–2.

IL-2. IL-2 was obtained from three sources. Supernatants from oxidative mitogenesis cultures that were found to contain IL-2 (see Results) were stored at −20°C before use. A standard source of crude rat IL-2 was prepared and generously provided by W. van Voorhis, The Rockefeller University. This was derived from Sephadex G150 fractionation of a serum-free, concanavalin A (Con A)-stimulated spleen cell supernatant; fractions in the 14,000–28,000 mol wt range, which supported proliferation of Con A blasts, were pooled. Purified mouse IL-2 was prepared as described (17). Activity migrated as a single band of 23,000 mol wt by polyacrylamide gel electrophoresis and a single peak of isoelectric point (pI) 4.0–4.1 by isoelectric focusing. The concentration of IL-2 used in studies on T cell activation was saturating in the CTLL assay.

Results

The DC is the Critical Accessory Cell for T Lymphocyte Proliferation in Oxidative Mitogenesis. To study the mechanism of action of DC, we chose a polyclonal mitogenesis system in which T cells proliferate after treatment with sodium periodate. Initial studies confirmed the work of Klinkert et al. (4) showing that DC were potent stimulators of oxidative mitogenesis (Fig. 1). Periodate treatment of DC, T cells, or both, lead to large T cell proliferative responses that were 10–100 times greater than those of unmodified cells. Irradiated (1,500 rad) and unirradiated DC had similar effects (not shown). On a per cell basis, enriched DC (>90% pure) were at least 100 times more active as accessory cells than unfractionated spleen cells, which contained <1% DC (Fig. 1).

To document the contribution of DC further, selective depletion experiments were performed using the specific anti-DC monoclonal antibody, 33D1. Treatment with 33D1 and complement removed 90% or more of the T cell stimulatory capacity of spleen adherent cells. These were tested immediately after isolation from the animal (Table I) or following overnight culture (Table II). Anti-DC antibody and complement also removed 50–75% of the stimulatory capacity of freshly prepared, unfractionated spleen cells (Table II). The effects of 33D1 and complement were similar to those produced by specific anti-Ia antibody and complement (e.g., Table I, experiment 2). Selective elimination of DC had similar effects on accessory function in oxidative mitogenesis and the primary mixed leukocyte reaction (MLR) (Table II and ref. 11). Treatment with 33D1 antibody alone, antibody, and heat-inactivated complement (Tables I and II), or the continuous presence of antibody at 20 μg/ml (not shown) did

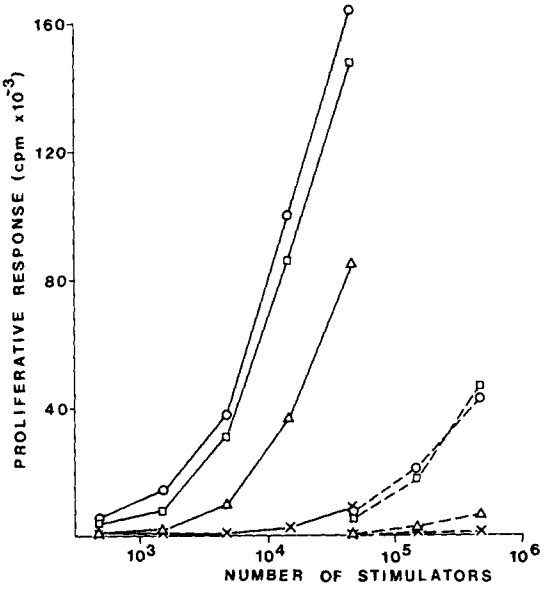

FIG. 1. The relative potency of DC compared with unfractionated spleen cells as stimulators in oxidative mitogenesis. Graded numbers of irradiated Swiss mouse DC (——) or unfractionated spleen cells (- - -) were added to 5×10^5 T cells in medium containing 5% serum, and proliferation was assessed. Periodation was carried out before mixing, on both stimulators and responders (○), responders only (□), stimulators only (△), or neither (×).

not alter DC function. We conclude that the DC is the critical cell required to stimulate the proliferation of periodate-modified T cells.

Release of Interleukins during Oxidative Mitogenesis. Interleukins, notably IL-1 (lymphocyte-activating factor, reviewed in 19) and IL-2 (T cell growth factor, reviewed in 6–8), can act as effector molecules for lymphocyte mitogenesis. We therefore tested supernatants from DC, T cells, and DC-T cell co-cultures for interleukin activity in two standard bioassays. These were costimulation with phytohemagglutinin of thymocyte mitogenesis, which detects both IL-1 and IL-2 (18), and proliferation of an IL-2-dependent T cell line, which detects only IL-2.

The supernatants from DC-T cell co-cultures were active in stimulating both thymocyte mitogenesis and proliferation of the IL-2-dependent CTLL line (Fig. 2). The interleukin activity in both bioassays was parallel. Interleukins were not detected in cultures of DC alone (up to 10^6/ml; not shown). Low levels of IL-2 activity were found in supernatants of T cells cultured in the absence of DC, but these were always <10% of that of the DC-T cell mixture. During oxidative mitogenesis (DC plus periodate-modified T cells), IL-2 activity was detected at the first time point studied (3 h), peaked by 20 h, and then fell rapidly in the 2nd d of culture (Fig. 2). Similar kinetics were observed in four consecutive experiments. If the T cells were not periodate-modified (the syngeneic MLR), the rate of IL-2 release was slower, but proceeded continuously over 4 d in culture (Fig. 2). We conclude that DC stimulate the release of IL-2 during DC-T cell co-culture. The bioassays did not allow us to determine whether IL-1 was also present.

The Dendritic Cell Induces Periodate-Modified T Cells to Respond to Soluble Factors Released into the Culture Medium. Although the culture medium from oxidative mitogenesis

TABLE I

Selective Elimination of DC Reduces the Accessory Activity of Freshly Prepared Spleen Adherent Cells

Experiment	Strain	Treatment	Proliferation
			cpm
1	C × B6 F_1	O	66,123
		O/C*	53,199
		33D1	62,177
		33D1/C*	7,802
2	C × B6 F_1	O/heat	56,484
		33D1/heat	52,856
		B21-2/heat	51,140
		O/C*	44,024
		33D1/C*	6,339
		B21-2/C*	1,486
3	D2 × C F_1	O/C*	47,067
		33D1/C*	4,535
4	D2 × C F_1	O/C*	33,226
		33D1/C*	1,288
5	B10.TL	O/C*	52,206
		33D1/C*	4,059

Freshly harvested, unfractionated spleen cells from the mouse strains indicated were irradiated and cultured at 3–4 × 10^6 per 16-mm well in the presence (33D1; B21-2) or absence (O) of antibody and complement (C*, fresh; heat, heated at 56°C for 30 min; no symbol, medium only at this stage), as indicated for 60 min at 37°C. Nonadherent cells were removed by washing, thus providing adherent spleen cells at doses estimated to be 5–10 × 10^4 per well. 20–30% of the adherent cells were DC. Periodate-modified T cells were added as responders in 2.5% serum. Proliferation was measured on the second day of culture and the responses of T cells only were subtracted.

cultures contained IL-2 activity, these supernatants alone would not induce proliferation of periodate-modified T cells (see below for examples). We therefore designed experiments to test if T cells became responsive to growth factors after co-culture with DC. DC and T cells were cultured for varying lengths of time (0–20 h), after which the DC were killed with anti-DC or anti-Ia monoclonal antibody and complement. Removal of DC with either of the antibodies reduced the subsequent proliferative response of the treated cells, recultured in fresh medium. However, anti-Ia was more effective than anti-DC at later times (10–20 h, Table III). Proliferation of DC-depleted cultures was restored by adding back small numbers (1–3%) of DC, showing that treatment with antibody and complement had not altered the T cells (Table III).

The striking finding was that T cells that had been cultured with DC became progressively responsive to soluble factors (Table III and Fig. 3). The latter were obtained from the DC-T cell-conditioned medium, or from a standard interleukin source (crude rat IL-2 derived from Con A-stimulated rat spleen cells). Responsiveness to soluble factors increased progressively with time (Table III and Fig. 3), and with increasing numbers of DC (Fig. 3), but T cells cultured in the absence of DC were not responsive (Fig. 3). We conclude that at least two events occur in the 1st d of an oxidative mitogenesis culture: exposure to DC causes the T cells to become responsive

TABLE II

Selective Elimination of DC Reduces Accessory Cell Function of Cultured Spleen Adherent Cells and Fresh Unfractionated Spleen Cells

Stimulators	Treatment	Proliferative response of 5×10^5 responders to stimulators at a dose of ($\times 10^{-3}$)			
		cpm			
Oxidative mitogenesis		24	12	6	3
Spleen adherent cells	O	49,010	28,710	15,050	7,150
	O/C*	40,765	23,752	18,238	7,505
	33D1	38,689	30,324	18,887	8,903
	33D1/C*	8,765	4,685	1,782	664
		320	160	80	40
Unfractionated spleen cells	O	11,147	9,269	5,220	3,186
	O/C*	19,461	10,280	5,871	3,657
	33D1	25,046	13,864	7,728	3,796
	33D1/C*	8,071	3,194	1,687	713
Allogeneic MLR		24	12	6	3
Spleen adherent cells	O	87,524	61,697	44,347	24,724
	O/C*	76,766	60,498	45,453	19,375
	33D1	75,995	53,513	31,249	12,686
	33D1/C*	2,707	1,150	790	523
		320	160	80	40
Unfractionated spleen cells	O	60,739	30,880	12,763	2,079
	O/C*	37,464	20,264	6,211	2,052
	33D1	63,943	41,976	17,254	6,544
	33D1/C*	14,874	3,064	655	355

C × B6 F_1 stimulator cells were treated in the presence or absence of antibody and complement (designated as in Table I). The stimulators were irradiated and added to C × B6 F_1 periodate-treated T cells (oxidative mitogenesis) or untreated BALB/c T cells (allogeneic MLR). Oxidative mitogenesis was assayed from day 1–2: control responses with unmodified T cells were <700 cpm. The allogeneic MLR was assayed from day 4–5: syngeneic MLR responses were <2,250 cpm. Cultures were a 2.5% serum. The proportion of DC, macrophages and Ia-bearing (Ia^+) cells was determined by indirect immunofluorescence using specific monoclonal antibodies 33D1, F4/80 (38), and B21-2, respectively. Spleen adherent cells were the same population characterized previously (ref. 11). They contained a two- to threefold excess of Ia^+ macrophages to Ia^+ DC. Unfractionated spleen cells contained <1% DC, 3% macrophages, and 65% Ia^+ cells. Killing with 33D1 and complement was ~25% in spleen adherent cells but was not detectable in unfractionated spleen cells.

to soluble factors, and DC induce release of the required factors into the culture medium.

Purified IL-2 Drives Proliferation of Responsive T Cells. To pinpoint the molecule responsible for mitogenesis, we tested a purified preparation of mouse IL-2, prepared as previously described from the supernatants of Con A-stimulated spleen cells (17). This IL-2 had an apparent molecular weight of 23,000, migrated as a single band on isoelectric focusing with a pI of 4.0–4.1, and saturated the IL-2 bioassay at 10^{-9} M.

After varying times of DC-T cell co-culture, DC were removed with anti-Ia antibody and complement. Then the T cells were recultured in fresh medium, the original DC-T cell culture supernatant, or purified IL-2 (Table IV). The purified IL-

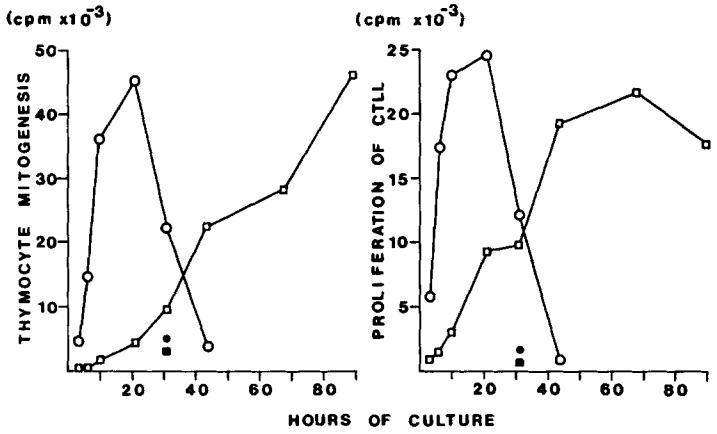

FIG. 2. Kinetics of interleukin production during DC-induced T lymphocyte proliferation. The responses examined were oxidative mitogenesis (○) and the syngeneic MLR (□). Swiss mouse DC were mixed with periodate-modified (●, ○) or untreated (■, □) T cells at a ratio of 1:50 in medium containing 2.5% serum. Closed symbols indicate T cells cultured in the absence of DC. The mixture was distributed at 8×10^6 per well in 1 ml into 16-mm wells. Cells from individual wells were harvested at the times shown. The supernatant was assayed for interleukin activity by determining the induction of thymocyte mitogenesis (left) and proliferation of an IL-2-dependent T cell line (right).

2, tested at 10^{-9} M, had similar activity to the DC-T cell conditioned medium in promoting proliferation (Table IV), which was likewise restored by crude rat IL-2 or small numbers of DC (not shown). Fresh T cells, or T cells cultured in the absence of DC, did not respond to IL-2. Unmodified T cells (1 d syngeneic MLR) also became responsive to IL-2 when cultured with DC, but the responsiveness was <20% of that seen in oxidative mitogenesis (Table IV, experiment 2). We conclude that IL-2 itself mediates the proliferation of DC-stimulated T cells.

IL-2 and Viable DC Do Not Act Synergistically. IL-2 replaces DC after an initial period (6–20 h) of DC-T cell co-culture. We asked whether IL-2 could enhance the function of live DC when both were present continuously from the onset of culture. Purified IL-2, used at a concentration that saturated the IL-2 bioassay, and mediated the proliferation of activated T cells, did not enhance oxidative mitogenesis induced by a wide range of DC doses (Table V). This is in agreement with the results of experiments in Table IV where purified IL-2 did not enhance proliferation of cells treated with complement alone. We conclude that IL-2 mediates proliferation of DC-activated T cells but does not act synergistically with live DC in the activation step. As a corollary, the restoration of proliferation in activated T cells is not due to a synergistic effect of IL-2 with very small numbers of residual DC.

Discussion

The DC is the Principal Accessory Cell in Oxidative Mitogenesis. Novogrodsky and Katchalski (20) discovered that lymphocytes proliferated after treatment with sodium periodate, or with neuraminidase and galactose oxidase. Greineder and Rosenthal (21) noted that oxidative mitogenesis in the guinea pig, like other T cell responses, required accessory cells. Either the accessory cells, assumed to be macrophages, or the T cells needed to be oxidized for mitogenesis to ensue. Klinkert et al. (4) demonstrated

TABLE IIIA

DC Progressively Activate T Cells to Respond to Soluble Factors Released into the Culture Supernatant

Co-culture of DC with periodate T cells		Proliferative response of 3×10^5 treated cells after reconstitution with:			
Time	Treatment	Medium	DC	Supernatant	Crude rat IL-2
h			*cpm*		
0	O	32,107	46,864		30,598
	O/C*	18,978	69,200		22,239
	33D1/C*	407	48,663		1,761
	B21-2/C*	46	37,835		393
3	O	28,983	48,633	28,420	38,194
	O/C*	24,365	68,779	24,114	31,159
	33D1/C*	698	45,334	1,706	7,669
	B21-2/C*	106	33,683	349	3,424
6	O	46,800	66,809	57,801	55,107
	O/C*	40,103	79,038	58,125	62,245
	33D1/C*	4,117	73,804	28,903	37,864
	B21-2/C*	340	64,094	12,879	23,041
10	O	68,411	80,622	63,337	81,827
	O/C*	57,165	94,790	76,165	78,137
	33D1/C*	15,128	77,545	53,536	60,292
	B21-2/C*	1,487	83,974	47,032	58,895
20	O	59,939	113,962	98,781	92,952
	O/C*	50,422	84,596	91,731	66,807
	33D1/C*	25,493	56,655	70,082	46,724
	B21-2/C*	16,858	63,742	90,504	61,654

At successive times, mixtures of Swiss mouse DC and periodate-modified T cells at a ratio of 1:100 were treated with antibody and complement as indicated. They were then recultured in fresh medium containing 2.5% serum, fresh DC at 3% of the total, a saturating (2.5% vol/vol) concentration of crude rat IL-2 (used to obtain an estimate of the maximum response attainable at each time after removal of DC), or 50% of the culture supernatant collected at each time point. This contained progressively increased concentrations of IL-2 in a CTLL assay as shown below. Rat IL-2 was diluted to a 10% starting concentration and the background was 1,122 cpm.

IIIB

Time of supernatant collection	Proliferative response of CTLL cells with supernatant at concentration of:			
	50%	25%	12%	6%
h		*cpm*		
3	6,424	3,708	2,673	1,536
6	39,283	28,340	17,739	9,668
10	47,859	40,881	33,536	18,831
20	42,128	38,433	38,799	26,470
rat IL-2	48,244	44,058	37,103	ND*

* Not determined.

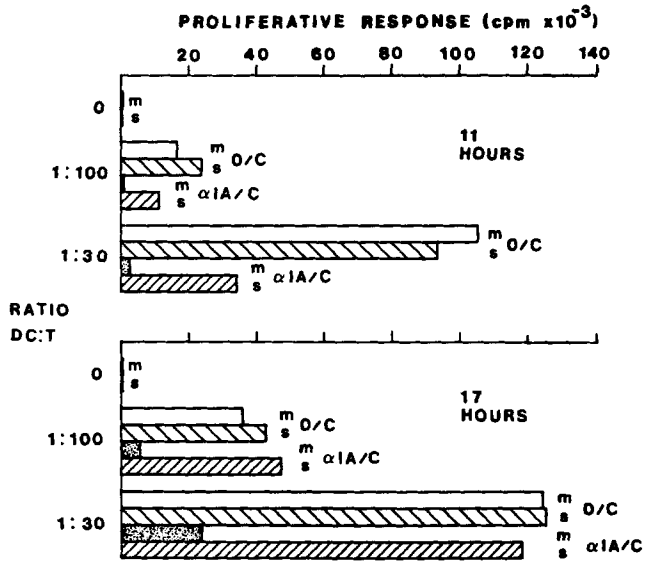

FIG. 3. DC activate T lymphocytes to become responsive to soluble factors. The experiment illustrated is similar to that in Table III, except that 11- and 17-h points were examined, two ratios of DC:T were used, and the medium contained 10% serum. Briefly, at the times indicated, the cells were treated with B21-2 and complement (αIa/C) or complement alone (O/C). They were recultured in fresh medium (m), or 50% supernatant (s) from the original 11- or 17-h cultures. Cultures in medium with 2.5% serum were also tested with qualitatively similar results. However the degree of proliferation was reduced when the cells were cultured in 2.5% serum before treatment (the activation step) but the serum concentration had no effect after activation (results not shown).

that enriched populations of DC were active accessory cells in the rat, and that macrophages and lymphocytes were in fact weak or inactive. Philips et al. (5) and van Voorhis et al. (22) found similarly in mouse and humans, respectively. We confirmed in this paper that highly enriched (>90% pure) mouse spleen DC were potent stimulators of oxidative mitogenesis (Fig. 1). Furthermore, selective elimination of DC with the specific 33D1 monoclonal antibody and complement, markedly reduced the accessory function of unfractionated spleen and spleen adherent cells (Tables I and II). This specific, negative selection approach, which was applied to fresh or cultured cell suspensions, extends previous evidence that the DC is the principal leukocyte mediating oxidative mitogenesis. 33D1 is the first cell-specific antibody to so strikingly diminish accessory function.

Oxidative Mitogenesis as a Model for a DC-mediated T Cell Response. Oxidative mitogenesis is a particularly useful model to study the mechanism of action of DC. First the response is large, comparable in magnitude to lectin-stimulated proliferation. Second, unlike lectin models, it is easy to make T cells accessory cell dependent: a single passage of spleen or lymph node cells over nylon wool reproducibly yields T cells that are dependent upon small numbers of DC. Third, responses are rapid so that within 2 d it is possible to monitor early events in moving the T cell from a resting or unprimed state into its first cell cycle. Responses to specific antigens, such as alloantigens in the primary MLR, or to soluble protein antigens, require long assay times and/or primed T cells. Fourth, oxidative mitogenesis is blocked by anti-Ia antibodies (23 and J. M. Austyn, unpublished observations) administered continu-

TABLE IV

Purified IL-2 Mediates the Proliferation of T Lymphocytes after Their Activation by DC

Experiment/Assay	Co-culture		Proliferative response of 3×10^5 treated cells recultured in:		
	Time	Treatment	Medium	Supernatant	Pure IL-2
	h			*cpm*	
Experiment 1					
Oxidative mitogenesis	0	O/C*	59,452	53,678	47,556
		B21-2/C*	65	1,097	507
	6	O/C*	129,158	136,812	105,801
		B21-2/C*	719	18,395	12,450
	10	O/C*	81,667	84,366	82,395
		B21-2/C*	3,426	46,407	29,968
	20	O/C*	136,451	136,715	133,023
		B21-2/C*	46,446	127,608	102,386
Experiment 2					
Oxidative mitogenesis	0	O/C*	99,707	83,846	79,049
		B21-2/C*	86	232	617
	15	O/C*	120,906	154,594	125,406
		B21-2/C*	32,982	116,633	112,995
1-d syngeneic MLR	0	O/C*	3,463	3,659	3,139
		B21-2	67	577	289
	15	O/C*	17,308	17,067	16,975
		B21-2/C*	4,832	12,521	11,751
Periodate T cells only	0	O/C*	141	145	105
		B21-2/C*	83	711	129
	15	O/C*	905	3,618	1,500
		B21-2/C*	149	871	528

As in Table III and Fig. 3, cells were cultured for varying lengths of time before treatment with or without anti-Ia antibody and complement. They were then recultured in either fresh medium containing 2.5% or 10% serum (experiments 1 and 2, respectively) or the same supplemented with a saturating concentration of purified mouse IL-2, or DC-T cell culture supernatant. In experiment 1, the supernatant added at t = 0 was from a 20-h culture of DC and periodate-modified T cells; those at later times were from the original cultures. In experiment 2, the supernatant used throughout was from a similar 18-h oxidative mitogenesis culture. Addition of small numbers of purified DC restored the proliferation of B21-2/C*-treated cultures and the effect of crude rat IL-2 was similar to that of pure mouse IL-2 (results not shown). In experiment 2, where pure IL-2 was added at 15 h, T cells were exposed to IL-2 for 12 h before the thymidine pulse. In other experiments, incubation was continued for 28 h before pulsing. The results were similar to those shown here. Cultures of DC with unmodified T cells are termed 1-d syngeneic MLR.

ously, indicating that polyclonal activation with periodate requires major histocompatibility complex (MHC) antigens as do many antigen-induced proliferative responses.

The precise mechanism whereby periodate enhances T cell mitogenesis is currently unknown. Possibly periodate simply enhances the so-called syngeneic MLR that occurs when DC and T cells are mixed (24). All the events that occur when DC are added to periodate-modified T cells are quantitatively increased relative to events occurring in the syngeneic MLR (Table IV, experiment 2; and Fig. 2).

Two Stages in T Cell Mitogenesis. Experiments with periodate-modified T cells have

TABLE V

Purified IL-2 Does Not Enhance the Effect of DC in Oxidative Mitogenesis

Number of DC	Proliferative response of 4×10^5 periodate T cells in	
	Medium	IL-2
	cpm	
2×10^4	90,895	82,803
1×10^4	46,045	46,138
5×10^3	27,788	23,058
2.5×10^3	10,133	9,803
1.2×10^3	3,405	4,485
6×10^2	1,747	2,328
3×10^2	805	1,480
None	350	1,068
	Proliferative response of 4×10^5 unmodified T cells	
2×10^4	3,573	6,393
1×10^4	2,007	2,417
5×10^3	837	1,760
None	88	538

Graded numbers of irradiated Swiss mouse DC were added to periodate-treated or unmodified T cells. The proliferative response was assessed in medium containing 10% serum or the same supplemented with a saturating concentration of pure mouse IL-2 (as used for experiments in Table IV).

provided a two-stage model of DC-initiated mitogenesis. In the first stage, viable DC induce both the release of IL-2 and T lymphocyte responsiveness to this factor. In the second, which does not require viable DC, T cells proliferate in response to the IL-2 that is generated in the culture medium.

Release of IL-2 activity into the culture medium, and acquisition of responsiveness to IL-2, occurred over a similar time course (compare Fig. 2 and Table III). Both events were induced by DC and were required for mitogenesis. It is likely that the IL-2 was derived from T cells, rather than DC, since T cell lines are the only homogeneous populations known to release this factor (e.g., 25, 26). We do not know if the subpopulation of T cells that responds to IL-2 is different from, or overlaps, that which releases IL-2. IL-2 itself, in the form of supernatant from DC-T cell cocultures, or as crude or pure preparations from Con A-stimulated spleen cells, had no effect on T cells that were not first activated by DC.

Previous work indicated that T cell growth factor, or IL-2, does not trigger the growth of resting T cells (27, 28). Rather, IL-2 acts in conjunction with other signals, or these cells must first be "activated" to respond to IL-2, a process which includes expression of functional receptors (28–30). The distinctive findings in this paper, which were verified with a highly purified preparation of IL-2, were: (*a*) responsiveness to IL-2 was acquired progressively (6–20 h) during exposure to small numbers of DC, before the onset of the first cell cycle; (*b*) sufficient IL-2 was generated in DC-T cell co-cultures to account for the proliferative response; and (*c*) IL-2 seems to be the only mediator required by the activated T cell to ensure its first cell division.

The above conclusions were derived from experiments (Tables III and IV, Fig. 3)

in which T cells were cultured with DC for varying lengths of time. The DC were then killed with anti-Ia or anti-DC antibody and complement. Such treatment drastically inhibited subsequent proliferation in fresh medium. The greater efficacy of anti-Ia antibody, compared with 33D1 at later times was presumably due to more efficient killing of DC by the former: the 33D1 antigen may be modulated, become inaccessible, or other factors could be involved. Anti-Ia also became less efficient at the later times studied (15–20 h). Possibly the T cells become committed to replication in the absence of DC and exogenous IL-2. Finally, in these experiments, mitogenesis was triggered by soluble factors, including a highly purified preparation of IL-2. The latter does not seem to enhance the function of DC (Tables III, IV, and V, and Fig. 3). Rather, the DC triggers the T cell to release and to respond to IL-2: the number of DC in the culture tightly controls the extent of lymphocyte activation, and endogenous IL-2 release and action.

It is important to relate oxidative mitogenesis to other T lymphocyte responses. Primed cytolytic T cells may be stimulated by antigen acting in concert with soluble factors, and may not require interaction with intact accessory cells (31). Some lectins and other stimuli (27, 32–35) induce IL-2 responsiveness, again in the apparent absence of accessory cells. For example, Larsson and co-workers (34, 35) studied T cells depleted of Ia-bearing cells. Culture with Con A altered the T cell in a few hours so that it then responded to exogenous factors from Con A-stimulated spleen cell supernatants. In contrast, DC-depleted, periodate-modified T lymphocytes proliferate little if at all in the presence of IL-2. Ia-bearing cells are required for Con A mitogenesis (36, 37), and it has been suggested that such cells function solely to induce IL-2 release. In oxidative mitogenesis, the Ia-bearing DC itself alters the T cell to respond to IL-2, in addition to inducing IL-2 release. Conceivably Con A mimics the action of DC in inducing T cell responsiveness. Because the first stage in oxidative mitogenesis demands cellular interaction with live DC, and because sufficient IL-2 is produced to drive the second, proliferative stage, this model may approximate more closely to the physiological triggering of T cells. We are currently examining the extension of this model to antigen-driven T cell responses.

Summary

T cells oxidized with sodium periodate proliferate polyclonally in response to accessory cells. We confirmed previous work showing that DC are potent stimulators of this response. In addition, the accessory function of unfractionated mouse spleen and spleen adherent cells was markedly reduced after elimination of DC with a specific monoclonal antibody and complement. Therefore oxidative mitogenesis was used as a model to study the mechanism by which DC stimulate T cell proliferative responses.

A two-stage mechanism was identified. The first stage occurred during the first 20 h of culture, required live DC, and involved the progressive release of interleukin 2 (IL-2) into the medium and acquisition of responsiveness to this growth factor. The second stage occurred between 20 and 40 h, did not require live DC, and involved DNA synthesis in response to IL-2. Similar events occurred during culture of DC with unmodified T cells (syngeneic MLR) but were quantitatively reduced. The experimental approach was to co-culture DC and T cells for up to 20 h and then kill the DC with specific antibody, or anti-Ia antibody, and complement. Subsequent prolif-

eration was inhibited if the T cells were cultured in fresh medium. However, proliferation was restored when the lymphocytes were cultured in the original DC-T cell medium, or with a crude or a purified preparation of IL-2. IL-2 did not induce the proliferation of T cells that had been cultured in the absence of DC, and did not synergize with viable DC. We conclude that DC induce proliferation by tightly coordinating the release of, and responsiveness to, T cell growth factor or IL-2.

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References

1. Steinman, R. M., and M. C. Nussenzweig. 1980. Dendritic cells: features and functions. *Immunol. Rev.* **53:**127.
2. Steinman, R. M., M. D. Witmer, M. C. Nussenzweig, B. Gutchinov, and J. M. Austyn. 1983. Studies with a monoclonal antibody to mouse dendritic cells. *Transplantation (Baltimore)*. In press.
3. Steinman, R. M. 1981. Dendritic cells. *Transplantation (Baltimore)*. **31:**151.
4. Klinkert, W. E. F., J. H. Labadie, J. P. O'Brien, C. F. Beyer, and W. E. Bowers. 1980. Rat dendritic cells function as accessory cells and control the production of a soluble factor required for mitogenic responses of T lymphocytes. *Proc. Natl. Acad. Sci. USA.* **77:**5414.
5. Phillips, M. L., J. A. Parker, J. A. Frelinger, and R. L. O'Brien. 1980. Characterization of responding cells in oxidative mitogen stimulation. II. Identification of an Ia-bearing adherent accessory cell. *J. Immunol.* **124:**2700.
6. Smith, K. A. 1980. T-cell growth factor. *Immunol. Rev.* **51:**337.
7. Watson, J., and D. Mochizuki. 1980. Interleukin 2: a class of T cell growth factors. *Immunol. Rev.* **51:**257.
8. Watson, J., D. Mochizuki, and S. Gillis. 1980. T-cell growth factors: interleukin 2. *Immunol. Today (Amst.)*. **1:**113.
9. Nussenzweig, M. C., R. M. Steinman, M. D. Witmer, and B. Gutchinov. 1982. A monoclonal antibody specific for mouse dendritic cells. *Proc. Natl. Acad. Sci. USA.* **79:**161.
10. Steinman, R. M., N. Nogueira, M. D. Witmer, J. D. Tydings, and I. S. Mellman. 1980. Lymphokine enhances the expression and synthesis of Ia antigens on cultured mouse peritoneal macrophages. *J. Exp. Med.* **152:**1248.
11. Steinman, R. M., B. Gutchinov, M. D. Witmer, and M. C. Nussenzweig. 1983. Dendritic cells are the principal stimulators of the primary mixed leukocyte reaction in mice. *J. Exp. Med.* **157:**613.
12. Julius, M. H., E. Simpson, and L. A. Herzenberg. 1973. A rapid method for the isolation of functional thymus derived murine lymphoyctes. *Eur. J. Immunol.* **3:**645.
13. Beyer, C. F., and W. E .Bowers. 1978. Lymphocyte transformation induced by chemical modification of membrane components. II. Effect of neuraminidase treatment of responder cells on proliferation and cytotoxicity in indirect stimulation. *J. Immunol.* **121:**1790.
14. Mizel, S. B., J. J. Oppenheim, and D. L. Rosenstreich. 1978. Characterization of lymphocyte activating factor (LAF) produced by a macrophage cell line P388D_1. II. Biochemical characterization of LAF induced by activated T cells and LPS. *J. Immunol.* **120:**1504.
15. Gillis, S., M. M. Ferm, W. Ou, and K. A. Smith. 1978. T cell growth factor: parameters of production and a quantitative microassay for activity. *J. Immunol.* **120:**2027.
16. Palladino, M. A., Y. Obata, E. Stockert, and H. F. Oettgen. 1983. Characterization of IL2-dependent cytotoxic T cell clones. I. Specificity, cell surface phenotype and susceptibity to blocking by Lyt antisera. *Cancer Res.* **43:**572.

17. Granelli-Piperno, A., J.-D. Vassalli, and E. Reich. 1981. Purification of murine T cell growth factor. A lymphocyte mitogen with helper activity. *J. Exp. Med.* **154:**422.
18. Smith, K. A., L. B. Lachman, J. J. Oppenheim, and M. F. Favata. 1980. The functional relationship of the interleukins. *J. Exp. Med.* **151:**1551.
19. Oppenheim, J. J., B. M. Stadler, R. P. Siraganian, M. Mage, and B. Mathieson. 1982. Lymphokines: their role in lymphocyte responses. Properties of interleukin 1. *Fed. Proc.* **41:**257.
20. Novogrodsky, A., and E. Katchalski. 1971. Induction of lymphocyte transformation by periodate. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **12:**297.
21. Grieneder, D. K., and A. S. Rosenthal. 1975. The requirement for macrophage-lymphocyte interaction in T lymphocyte proliferation induced by generation of aldehydes on cell membranes. *J. Immunol.* **115:**932.
22. van Voorhis, W. C., L. S. Hair, R. M. Steinman, and G. Kaplan. 1982. Human dendritic cells. Enrichment and characterization from peripheral blood. *J. Exp. Med.* **155:**1172.
23. Phillips, M. L., P. J. Wettstein, R. L. O'Brien, J. A. Parker, and J. A. Frelinger. 1980. Characterization of responding cells in oxidative mitogen stimulation. I. Ia^+ cells and Ly 1^+2^+ cells are required for proliferative responses. *J. Immunol.* **124:**2693.
24. Nussenzweig, M. C., and R. M. Steinman. 1980. Contribution of dendritic cells to stimulation of the murine syngeneic mixed leukocyte reaction. *J. Exp. Med.* **151:**1196.
25. Gillis, S., M. Scheid, and J. Watson. 1980. Biochemical and biologic characterization of lymphocyte regulatory molecules. III. The isolation and phenotypic characterization of interleukin-2 producing T cell lymphomas. *J. Immunol.* **125:**2570.
26. Gillis, S., and J. Watson. 1980. Biochemical and biological characterization of lymphocyte regulatory molecules. V. Identification of interleukin 2-producing human leukemia cell line. *J. Exp. Med.* **152:**1709.
27. Larsson, E.-L., J. Andersson, and A. Coutinho. 1978. Functional consequences of sheep red blood cell rosetting for human T cells: gain of reactivity to mitogenic factors. *Eur. J. Immunol.* **9:**587.
28. Coutinho, A., E.-L. Larsson, K.-O. Gronvick, and J. Andersson. 1979. Studies on T lymphocyte activation. II. The target cells for Con A-induced growth factors. *Eur. J. Immunol.* **9:**587.
29. Robb, R. J., A. Munck, and K. A. Smith. 1981. T cell growth factors. Quantitation, specificity and biological relevance. *J. Exp. Med.* **154:**1455.
30. Bonnard, G. D., K. Yasaka, and D. Jacobson. 1979. Ligand-activated T cell growth factor-induced proliferation: absorption of T cell growth factor by activated T cells. *J. Immunol.* **123:**2704.
31. Weinberger, O., S. Herrmann, M. F. Mescher, B. Benacerraf, and S. J. Burakoff. 1981. Cellular interactions in the generation of cytolytic T lymphocyte responses. Analysis of the helper T cell pathway. *Eur. J. Immunol.* **11:**405.
32. Larsson, E.-L., and A. Coutinho. 1980. Mechanism of T cell activation. I. A screening of "step one" ligands. *Eur. J. Immunol.* **10:**93.
33. Konaka, Y., M. A. Norcross, V. C. Maino, and R. T. Smith. 1981. Anti-Thy-1-mediated T cell activation. Role of soluble factors and expression of interleukin 2 receptors on T cells. *Eur. J. Immunol.* **11:**445.
34. Larsson, E.-L., N. N. Iscove, and A. Coutinho. 1980. Two distinct factors are required for induction of T-cell growth. *Nature (Lond.).* **283:**664.
35. Larsson, E.-L., and A. Coutinho. 1979. The role of mitogenic lectins in T-cell triggering. *Nature (Lond.).* **280:**239.
36. Andersson, J., K.-O. Gronvick, E.-L. Larsson, and A. Coutinho. 1979. Studies on T lymphocyte activation. I. Requirements for the mitogen-dependent production of T cell growth factors. *Eur. J. Immunol.* **9:**581.

37. Habu, S., and M. C. Raff. 1977. Accessory cell dependence of lectin-induced proliferation of mouse T lymphocytes. *Eur. J. Immunol.* **7**:451.
38. Austyn, J. M., and S. Gordon. 1981. F4/80: a specific monoclonal anti-macrophage antibody. *Eur. J. Immunol.* **11**:805.