DENDRITIC CELLS INDUCE T LYMPHOCYTES TO RELEASE B CELL-STIMULATING FACTORS BY AN INTERLEUKIN 2-DEPENDENT MECHANISM

BY KAYO INABA,* ANGELA GRANELLI-PIPERNO,‡ AND RALPH M. STEINMAN§

From The Rockefeller University, New York 10021

Dendritic cells (DC)[1] are essential accessory cells for thymus-dependent antibody responses (1). Culture systems that contain DC and T cells generate a lymphoproliferative response termed the syngeneic mixed leukocyte reaction (syn MLR) (2). There is evidence that syn MLR-conditioned media contain interleukin 2 (IL-2) (T cell growth factor) (3–5) and helper factors for human B cell responses (6, 7). In this paper, we have used anti-IL-2 antibodies and purified IL-2 to describe how DC/T cell-conditioned medium stimulates purified mouse B cells to develop into specific antibody-secreting cells. A three-step mechanism has been elucidated. The DC first induce T cells to release and become responsive to IL-2 (5). The IL-2 then acts as the trigger for the production of helper factors. These factors finally act in concert with antigen to induce the antibody response.

Materials and Methods

Mice. 6–10-wk-old mice of both sexes were purchased from the Trudeau Institute, Saranac Lake, NY. The principal strains used, with similar results, were (DBA/2 × BALB/c) F_1 (H-$2^{d\times d}$), B6.H-2k (H-2^k), and Swiss ICR (H-2^s, most likely, according to cytotoxicity studies [unpublished] with a panel of anti-H-2 K, I, and D alloantisera).

Antibodies. Monoclonal antibodies 33D1 anti-DC (8, 9), B21-2 anti-I-$A^{b,d,s}$ (10), TIB-99 anti-Thy-1.2 (11), and anti-Lyt-1.2 (12) were used as culture supernatants to selectively eliminate DC, Ia^+ cells, and T cells, respectively, with rabbit complement (C*), as described (9). An Ig fraction of B21-2 was also added to cultures at 10 μg/ml to block the production of helper factors. Anti-Ia^k alloantibodies (A.TH anti-A.TL) were obtained from Accurate Chemical & Scientific Corp., Hicksville, NY, while anti-Ia^d (B10.LG × A.TFR anti-B10.D2), anti-K^k (A.TL × 129 anti-A.AL), and anti-D^k [B10.A(2R) × C3H.SW anti-C3H] antibodies were provided by Dr. John Ray, Research Resources Branch of the NIH. A rabbit anti-IL-2 antiserum was prepared by one of us (A. G.-P.) as detailed below and added as a purified Ig fraction at 25 and 75 μg/ml.

Interleukin 2. IL-2 was purified from the supernatants of concanavalin A (Con A)-stimulated spleen cells as described (13). In a bioassay with Con A-stimulated spleen cells

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* Visiting Investigator from the Department of Zoology, Kyoto University, Kyoto, Japan 606.

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§ Established Investigator of the American Heart Association.

[1] *Abbreviations used in this paper:* C*, complement derived from fresh rabbit serum; Con A, concanavalin A; DC, dendritic cell; FCS, fetal calf serum; IL-2, interleukin 2; PFC, plaque-forming cell; syn MLR, syngeneic mixed leukocyte reaction.

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as responders (13), the purified IL-2 was active at 10^{-11} M and saturated at 10^{-9} M. In this paper, the IL-2 was used at 1.5×10^{-9} M. The IL-2 preparation (fraction VI; 13) contained a single 23 kD polypeptide on sodium dodecyl sulfate-polyacrylamide gels, and had no detectable interferon (A. G.-P, manuscript in preparation) or B cell-stimulating activity (see Results). Anti-IL-2 antibodies were prepared by injecting three rabbits intradermally with 1–10 μg of purified IL-2 mixed 1:1 with AluGel, every 4 wk. An Ig fraction was obtained from protein A-Sepharose columns (Pharmacia, Inc., Piscataway, NJ). The antibody progressively neutralized IL-2 activity over a dose range of 2–80 μg/ml (A. G.-P., manuscript in preparation). When coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia, Inc.), the anti-IL-2 totally removed IL-2 activity from T cell-conditioned media. A major 23 kD band could be eluted from the anti-IL-2 column with glycine at pH 2.8. The antibody did not react with immune interferon, IL-1, IL-3, cytolytic factor (manuscript in preparation), or B cell-stimulating factors (this paper).

Cells. DC were low density spleen adherent cells depleted of macrophages by a readherence method (2). DC-depleted B/T lymphocyte mixtures were Sephadex G10-nonadherent spleen cells (14). B cells were G10-nonadherent spleen cells treated with anti-Thy-1.2 and with anti-Lyt-1.2 and C*. T cells were largely eliminated, since the purified B cells did not proliferate to the T cell mitogen Con A and did not form antibody-secreting cells in response to DC (not shown). T cells were nylon wool-nonadherent spleen and lymph node cells (2). In many cases, high density spleen cells (80–90% of spleen), rather than whole spleen, were used to prepare B and T lymphocytes.

Syngeneic Mixed Leukocyte Reactions. Syn MLR were generated by culturing $2–3 \times 10^4$ DC and 5×10^6 T cells in 1–1.5 ml culture medium (RPMI 1640 supplemented with 10% fetal calf serum (FCS), 20 μg/ml gentamycin sulfate, 5×10^{-5} M 2-mercaptoethanol) in 16-mm diam culture wells. Medium was collected, as a source of lymphokines, generally from day 0–4 or 2–4 cultures. Previous studies (2) have shown that most of the DC and responding T cells in syn MLR cultures cluster together to form discrete aggregates. In this paper, we purified the aggregates by sedimentation at 100 *g* at 4°C for 10 min on 10-ml Percoll gradients generated with a mixture of 4 parts Isoperc (Pharmacia, Inc.), 3 parts FCS, and 5.7 parts phosphate-buffered saline. Cells from 6–12, day 2 syn MLR cultures were sedimented on each 10-ml column. Clusters and nonclusters were separated by >5 ml on the column and were harvested with Pasteur pipettes. The fractions were washed twice in medium and maintained in culture at doses of $1.5–3 \times 10^5$ clusters and 3×10^6 nonclusters per 16-mm diam well, which were doses that provided optimal levels of helper factor per plated cell. In some cases, the cell fractions were processed for transmission electron microscopy as described (2).

Plaque Assay. Antibody-forming cells were enumerated with a direct plaque assay in agarose as described (14).

Assay for B Cell Helper Factors. $2–3 \times 10^6$ purified B cells were cultured for 4 d in 16-mm diam wells in 1 ml of 0, 12.5, 25, or 50% vol/vol, syn MLR-conditioned medium, as a source of helper factors, with or without 3×10^6 erythrocytes as antigen (sheep or horse; Colorado Serum Co., Denver, CO). In the presence of both antigen and helper factors, direct plaque-forming cells (PFC) began to appear at day 2–3 and peaked at day 4.

Proliferative Assays. For the syn MLR, 50 μl of a 1-ml culture was exposed in quadruplicate for 4–18 h to [^{3}H]thymidine (6.0 Ci/mM; Schwartz-Mann Div., Becton, Dickinson & Co., Orangeburg, NY) at 4 μCi/ml in 0.1 ml total volume. For the IL-2 bioassay, 2×10^4 Con A-stimulated spleen cells (13) were exposed to graded doses of test media (6–50% vol/vol) in 0.1 ml and pulsed with [^{3}H]thymidine at 14 μCi/ml at 18–22 h. IL-2 activity was recorded as cpm uptake above background (no IL-2) under conditions where uptake was directly proportional to the dose of test medium.

Results

The development of B lymphocytes into antibody-secreting cells or PFC requires DC and T cells (1). It is known that small numbers of DC (1/200 T

TABLE I

Helper Factors for Antibody Responses Are Produced by DC-T Cell Co-cultures

Exp.	Source of helper factor (concentration)	Antibody response (PFC) to:					
		No RBC		SRBC		HRBC	
		Anti-SRBC	Anti-HRBC	Anti-SRBC	Anti-HRBC	Anti-SRBC	Anti-HRBC
1	None	0	8	0	0	0	8
	Medium (50% vol/vol) from 3×10^4 DC	0	0	0	0	0	4
	Medium (50% vol/vol) from 5×10^6 T cells	0	4	4	0	0	0
	DC + T co-culture medium (50% vol/vol)	4	12	1,184	8	12	1,180
2	None	0	0	0	0	0	0
	DC + T co-culture medium						
	(50% vol/vol)	40	0	6,117	17	60	5,867
	(25% vol/vol)	60	3	4,153	0	10	1,890

Culture supernatants from DC, T, or DC/T cell co-cultures were collected and added to 2×10^6 purified B cells with or without erythocytes (RBC) as antigen. Direct PFC were measured 4 d later using sheep (SRBC) or horse (HRBC) erythrocytes in the plaque assay.

cells) induce a syn MLR (2). We will first present data indicating that syn MLR media are an active source of helper factor for specific antibody responses, bypassing the need for intact DC and T cells. Then we will characterize some of the requirements for the production and activity of helper factors, particularly the contribution of IL-2.

DC-T Cell Co-cultures Release B Cell Helper Factors. Syn MLR media (1 DC/200 T cells) reproducibly stimulated purified B cells to generate PFC to heterologous erythrocytes (Table I). The responses were antigen dependent and specific. DC alone or T cells alone produced little or no factor. Under our culture conditions, the syn MLR media were most active when taken from day 2–4 of the MLR culture and were weak or inactive when taken from day 0–2 or 4–6 (not shown). The helper factors were not genetically restricted, since factors produced by cells of one H-2 (H-2 k, s, or d) were active on B cells from syngeneic or allogeneic strains (Table II).

The essential role of DC in inducing the release of B cell-stimulating factors was evaluated by selective depletion experiments with the 33D1 anti-DC antibody and C*. DC-depleted spleen cells or spleen-adherent cells would not trigger the syn MLR, as monitored by T cell proliferation and release of IL-2 and helper factors (Table III). We conclude that DC/T cell co-cultures release soluble B cell-stimulating factors that induce differentiation to PFC in the presence of antigen.

Need for DC-T Cell Aggregates and Ia Antigens in Helper Factor Release. Previous studies (2), using cytology and [^{3}H]thymidine labeling, indicated that DC and responding T cells aggregated during the syn MLR to form distinctive clusters that could be separated by velocity sedimentation. Here we isolated cluster and noncluster fractions from day 2 syn MLR cultures. Recovery was 50–70% of the

TABLE II

Syngeneic MLR Helper Factors Stimulate Syngeneic or Allogeneic B Cells

Strain (H-2)		PFC response to doses of supernatant		
DC/T cell supernatant	B cells	None	25%	50%
(DBA/2 × BALB/c) F_1 (H-2 d × d)	(DBA/2 × BALB/c) F_1	0	4,153	6,117
		0	136	1,184
		0	816	4,520
		0	1,120*	—
	Swiss (H-2^s)	0	—	5,164
		0	—	3,166
	B6.H-2k (H-2^k)	0	1,760	4,870
Swiss (H-2^s)	Swiss	0	958	—
	B6.H2k	0	500*	1,644
		27	341	—
	(DBA/2 × BALB/c) F_1	0	2,864	2,860
		0	2,304	3,008
		0	147*	1,070
		0	2,320	4,192

Syn MLR supernatants were obtained from 4-d DC/T cell co-cultures and added at 0, 25, or 50% vol/vol to purified B cells in the presence of SRBC. PFC were measured 4 d later. In the absence of SRBC, PFC yields were 0–5% of those shown.

* In these three experiments, supernatant at a dose of 25% vol/vol was also tested on 5×10^6 B/T lymphocyte mixtures (DC-depleted, Sephadex G10 nonadherent spleen) in addition to B cells. In the order of their appearance in table, their PFC were 1,010, 704, and 773.

applied cells, and the aggregates represented 5–10% of total recovered cells. By Giemsa staining and transmission electron microscopy, >90% of the clustered cells were lymphoblasts as compared with 5% of the nonclusters. >80–90% of the clustered cells could be killed with anti-Thy-1 or anti-Lyt-1 and C*.

When tested for the production of B cell helper factors, the cluster fraction was exclusively active even though 10 times as many nonclustered cells were evaluated (Table IV). The medium conditioned by clusters was 50–100% as active as the medium from cluster-noncluster mixtures. The clusters were also the principal source of T cell growth factor or IL-2 (Table IV), which is known to be released in the syn MLR (3–5).

The addition of specific anti-Ia antibodies, but not mixtures of anti-H-2K or D reagents, blocked the release of helper factors from preformed DC/T cell clusters (Table V). Anti-Ia also blocked T cell proliferation and IL-2 release (Table V). The anti-Ia antibodies probably interfered with T cell recognition of DC Ia antigens, since most T cells in the syn MLR are I-restricted (15) and since >90% of the clustered cells were neither stained (indirect immunofluorescence) nor killed (C*) with anti-Ia antibodies (not shown).

The addition of anti-IL-2 antibodies to the cluster cultures also ablated the helper activity of the conditioned medium (Table VI). Unlike the experiments

TABLE III

DC Requirement for Stimulating T Cell Proliferation and Lymphokine Production in the syn MLR

Stimulators		T cell growth	Activity in supernatant	
Treated cells	Anti-DC MAb/C*		IL-2	Helper factor
		cpm	*cpm*	*PFC*
Whole spleen	−/−	4,123	12,607	264
	+/−	5,300	13,328	260
	−/+	4,147	14,851	280
	+/+	1,765	1,665	0
Adherent spleen cells	−/−	7,198	5,310	296
	+/−	6,619	10,929	316
	−/+	7,570	17,320	252
	+/+	2,081	2,349	0
None	−/−	1,270	2,828	8

Spleen cells (2.5×10^6) or spleen-adherent cells (5×10^4) from ($D_2 \times C$) F_1 mice were irradiated and treated with or without (+/−) 33D1 anti-DC monoclonal antibody (MAb) and rabbit complement (C*) as described (9). The treated cells were washed and used as stimulators of 4×10^6 responder T cells in 1.5 ml medium. At day 4, 50-μl aliquots of the cell suspensions were taken for measurements of T cell growth (cpm [^{3}H]thymidine uptake). The culture media were also assayed in graded doses (50% vol/vol is shown) for IL-2 (cpm [^{3}H]thymidine uptake; background, 1,000 cpm) or helper factor (PFC response by purified B cells in the presence of sheep erythrocytes). Stimulator cells cultured alone did not release IL-2 or helper factor (not shown).

TABLE IV

DC/T Cell Clusters Produce B Cell Helper Factors and IL-2

Exp.	Cells	Helper factor (PFC)			IL-2
		50%	25%	12.5%	
					cpm
1	syn MLR clusters, 3×10^5	1,328	616	212	16,809
	syn MLR nonclusters, 3×10^6	0	0	0	173
	Mix nonclusters + 3×10^5 clusters	1,984	796	176	19,939
	Mix nonclusters + 1.5×10^5 clusters	868	216	12	17,847
2	syn MLR clusters, 3×10^5	4,112	3,416	2,816	158,242
	syn MLR nonclusters, 3×10^6	0	0	0	11,014
	syn MLR, unseparated 2×10^6	3,008	2,304	1,552	182,147
	Cultured T cells only 2×10^6	424	20	12	Not done

2.5×10^4 DC and 5×10^6 T cells were cultured for 2 d and separated into cluster and noncluster fractions. Each fraction was cultured an additional 2 d to provide conditioned media that were tested for B cell helper activity (PFC response to sheep erythrocytes with varying doses of medium) and IL-2 or T cell growth factor (growth on Con A-stimulated spleen cells; only the 50% dose is shown). The distinction between cluster and noncluster fractions that is shown here was repeated in six of six additional experiments described in subsequent tables.

TABLE V
Anti-Ia Antibodies Inhibit Lymphokine Release from DC/T Cell Clusters

Cells	Alloantibody	Helper factor (PFC)			Growth	IL-2
		50%	25%	12.5%		
					cpm	*cpm*
H-2k						
T cells only	—	0	0	0	1,357	2,338
Clusters	—	964	511	180	22,606	226,706
	Anti-Ia^k, 1%	36	44	4	7,220	8,211
	Anti-Ia^k, 0.5%	11	36	12	14,046	10,334
	Anti-K,D^k, 1% each	984	640	132	23,058	194,841
	Anti-K,D^k, 0.5% each	1,064	676	168	18,618	178,915
Nonclusters	—	24	8	0	1,549	13,886
	Anti-Ia^k, 1%	16	12	4	1,413	11,379
H-2s						
Clusters	—	1,136	696	220	11,315	211,713
	Anti-Ia^k, 1%	1,464	1,052	220	11,243	221,585
	Anti-Ia^k, 0.5%	1,332	620	184	11,788	211,836
	Anti-K,D^k, 1% each	1,028	644	204	14,423	91,598
	Anti-K,D^k, 0.5% each	1,512	644	184	14,664	169,973
Nonclusters	—	44	20	12	1,369	11,031
	Anti-Ia^k, 1%	24	20	4	1,579	10,421

B6.H2k and Swiss (H-2s) DC and T cells (1:200) were co-cultured for 2 d and separated into cluster (10% of total cells) and noncluster fractions by velocity sedimentation. The fractions were cultured an additional 2 d with or without alloantibodies that react with $H\text{-}2^k$ (I^k, K^k, and D^k; see Materials and Methods) but not with $H\text{-}2^s$. Proliferative activity (growth) was measured as in Table III. B cell helper activity was tested on Swiss B cells, which did not react with the alloantibodies. IL-2 data are shown for the 50% vol/vol dose; background was 2,700 cpm. The capacity of anti-Ia^k or monoclonal B21-2 (anti-$Ia^{b,d,s}$) to block lymphokine release was repeated in five of five additional experiments, many of which are included in subsequent tables.

with anti-Ia above (Table V), we could not determine if the anti-IL-2 was blocking the activity of helper factors in the B cell assay as has been reported (16, 17) or the production of factors in the DC/T cell culture. Therefore, we did two types of experiments with the rabbit anti-IL-2 reagent to determine if IL-2 made a direct contribution to PFC development.

Evidence that IL-2 Is Not a B Cell Helper Factor. First, syn MLR media were passed over control (Sepharose preimmune Ig) and anti-IL-2 columns (Fig. 1). IL-2 was found entirely in the effluent fraction of the control column and in the acid eluate of the anti-IL-2 column, with 30–70% recovery of total activity from both columns. Yet the effluents of both columns were exclusively and similarly active as a source of helper factor (Fig. 1). Therefore, anti-IL-2 does not absorb B cell helper factors.

In the second approach, we made use of the fact that the anti-IL-2 antibody neutralized 90–100% of the IL-2 present in syn MLR (e.g., Table VI) and other T cell-conditioned (A. G.-P., manuscript in preparation) media. However, the anti-IL-2 did not neutralize B cell helper activity using purified B cells or DC-

TABLE VI

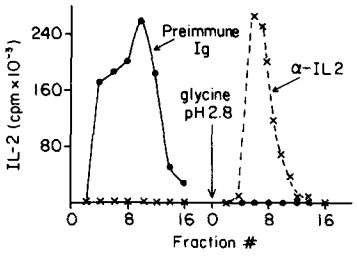
Anti-IL-2 Reduces Helper Activity in Medium Conditioned by DC/T Clusters

Exp.	Antibody added to DC-T cell culture	PFC response			IL-2
		50%	25%	12.5%	
					cpm
1	(D2 × C) F_1 ($H\text{-}2^d$)				
	None	1,816	804	224	
	Anti-IL-2, 80 μg/ml	0	0	0	Not
	Anti-IA, B21-2, 10 μg/ml	4	0	0	tested
2	(D2 × C) F_1 ($H\text{-}2^d$)				
	None	1,712	388	50	87,846
	Anti-IL-2, 75 μg/ml	272	20	8	2,811
	25 μg/ml	316	8	0	7,741
	Anti-IA, B21-2, 10 μg/ml	4	0	0	20,286
	Anti-I^d, 1% vol/vol	144	32	0	33,321
	0.5% vol/vol	276	8	4	47,150
3	C3H ($H\text{-}2^k$)				
	None	1,440	460	148	6,779
	Anti-IL-2, 75 μg/ml	0	0	4	235
	25 μg/ml	72	16	8	349
	Control Ig, 75 μg/ml	1,208	424	112	6,529
	25 μg/ml	1,388	468	120	6,479
	Anti-IA, B21-2, 10 μg/ml	1,276	472	116	9,404
	Anti-Ia^k, 1% vol/vol	108	28	4	1,066
4	(D2 × C) F_1 ($H\text{-}2^d$)				
	None	1,328	616	212	15,167
	Anti-IL-2, 75 μg/ml	0	0	0	100
	25 μg/ml	0	0	0	738
	Control Ig, 75 μg/ml	1,132	604	190	10,772
	25 μg/ml	1,676	620	200	10,675

2 d after the onset of the syn MLR (1 DC/200 T cells), aggregates of DC and responding T cells were isolated and cultured an additional 2 d with or without anti-IL-2 or anti-Ia antibodies (the B21-2 monoclonal reacts with $H\text{-}2^d$ as in experiments 1, 2, and 4 but not $H\text{-}2^k$, as in experiment 3). The supernatants were collected and tested for helper activity on B cells from a haplotype (H-2 k or s) that did not react with the anti-Ia antibody added to the DC/T cell cultures. IL-2 was assayed on Con A-stimulated spleen cells from BALB/c mice (backgrounds were 7,000, 400, and 500 cpm in experiments 2–4 and were subtracted; data are for media tested at 25% vol/vol).

depleted B/T lymphocyte mixtures as responders (Table VII) even at limiting doses of syn MLR medium (not shown). In contrast, the same anti-IL-2 reagent markedly reduced the yield of helper factor in DC/T cell cultures (Table VI) and the yield of PFC in cultures of DC, B, and T lymphocytes (Table VII). We conclude that factors distinct from IL-2 mediate B cell helper activity in syn MLR supernatants. Instead, IL-2 seems essential for factor production.

IL-2 Triggers the Production of B Cell-stimulating Factors. We next evaluated several T cell populations to document directly that T cells could release helper factor upon the addition of purified IL-2 (see Materials and Methods). DC/T cell clusters were a logical starting point but proved unsuitable, because the high constitutive release of helper factor (Tables IV–VI) could not be reduced >80%



Fraction	Helper activity (PFC)		
	50%	25%	12.5%
Unseparated medium	2,524	1,208	380
Effluent: control Ig	2,252	652	220
Eluate: control Ig	0	0	0
Effluent: anti-IL-2	1,696	494	120
Eluate: anti-IL-2	4	0	0
Purified IL-2	8	8	4

FIGURE 1. 10 ml of DC/T cell-conditioned medium were passed over Sepharose columns conjugated with preimmune Ig (●) or anti-IL-2 Ig (×). 1-ml fractions were collected and 25-μl aliquots assayed for IL-2 (cpm [^{3}H]thymidine uptake). Then the columns were eluted with glycine buffer, pH 2.8 (arrow). The eluate fractions were immediately neutralized with Tris buffer and 5-μl aliquots were assayed for IL-2.

by treatment with anti-Ia antibodies and C* (not shown). The nonclustered fraction, in contrast, released little or no helper factor unless IL-2 was added to the culture (Table VIII). The purified IL-2 itself did not stimulate PFC development from B cells and did not induce helper factor release from T cells that had been cultured in the absence of DC (Table VII and VIII). T cells were the likely source of helper factor in the IL-2-stimulated nonclustered fraction, since factor production was not reduced after the elimination of trace DC and B cells with anti-Ia and C* (Table VIII).

The IL-2-responsive cells in the noncluster fraction presumably arose as progeny of lymphocytes that were proliferating in DC/T cell clusters. Indeed, when clusters were isolated and returned to culture, nests of lymphoblasts were released over a 2-d interval. These released blasts formed little IL-2 or helper factor, indicating that the population was effectively depleted of DC (Table IX). When exposed to purified IL-2, the lymphoblasts proliferated and released large amounts of helper activity in spite of the small numbers of cells tested (1×10^5 per 16-mm diam well in Table IX vs. 3×10^6 bulk nonclustered cells in Table VIII). We conclude that DC/T cell aggregates generate lymphoblasts that in turn release helper factors upon the addition of IL-2.

Although anti-Ia antibodies blocked the production of helper factor from DC/T cell clusters (Table V), anti-Ia did not reduce IL-2-initiated factor production significantly (Table X). We interpret these findings to mean that recognition of Ia is needed to trigger the release of IL-2, but then the IL-2 acts directly on T cells to release helper factor.

Evidence that IL-2 Is the Immediate Stimulus for Helper Factor Release. Two observations were made which indicated that IL-2 was directly stimulating helper

TABLE VII

Effects of Anti-IL-2 on PFC Responses Mediated by DC or Helper Factors

Components of culture during the generation of PFC responses	No. of exp'ts	PFC (percent of control)
2×10^6 B cells + syn MLR medium (control)	6	100
Plus anti-IL-2, 75 μg/ml	6	86 ± 9
Plus anti-IL-2, 25 μg/ml	4	93 ± 20
Plus pre-Ig, 75 μg/ml	6	101 ± 16
Plus pre-Ig, 25 μg/ml	4	109 ± 28
2×10^6 B cells only	6	0
2×10^6 B cells + pure IL-2	3	0
5×10^6 B/T cells + syn MLR medium (control)	3	100
Plus anti-IL-2, 75 μg/ml	3	115 ± 15
Plus anti-IL-2, 25 μg/ml	1	104
Plus pre-Ig, 75 μg/ml	3	101 ± 27
Plus pre-Ig, 25 μg/ml	1	117
5×10^6 B/T cells only	3	0
5×10^6 B/T cells + pure IL-2	3	1
5×10^6 B/T cells + 2.5×10^4 DC (control)	3	100
Plus anti-IL-2, 75 μg/ml	3	24 ± 17
Plus anti-IL-2, 25 μg/ml	2	50 ± 21
Plus pre-Ig, 75 μg/ml	3	97 ± 8
Plus pre-Ig, 25 μg/ml	2	102 ± 7

4-d anti-SRBC responses were generated by adding syn MLR supernatants to purified B cells or B/T cell mixtures, or by adding DC to B/T cell mixtures (Sephadex G10-nonadherent spleen). The contribution of IL-2 to the PFC responses was evaluated by adding purified IL-2 (1.5×10^{-9} M) or either a neutralizing rabbit anti-IL-2 or control preimmune Ig (pre-Ig) at 75 or 25 μg/ml. PFC responses are given as the mean ± SD relative to the control (100%), which was 2,816 (B cells + syn MLR medium), 976 (B/T cells + syn MLR medium), and 1,081 (DC + B/T cells). Responses in the absence of SRBC were 0–2% of the responses in the presence of SRBC. In two of the above experiments, antibody neutralization of IL-2 was confirmed with a bioassay and shown to be 95–100%.

factor production, rather than inducing T cell growth that was followed by release of helper factor to some other stimulus. First, irradiated (1,500 rad) T cells released normal and often elevated amounts of helper factor in response to IL-2 (Table XI). Second, the release of helper factor was rapid with high levels detectable in 3 h and peak levels in 6–12 h. The kinetics were similar using either normal or irradiated T cells as IL-2 responders (Table XI).

Since all the above studies on the release of helper factor were performed with DC/T cell co-cultures, we verified that similar events occurred in the presence of B cells. Cultures of DC and B/T cell mixtures (G10-nonadherent spleen) formed aggregates that contained all three cell types (manuscript in preparation). These clusters were an active source of lymphokines (IL-2 and helper factor; Table XII). The nonclustered fraction, which lacked DC, contained radioresistant cells that released helper factor in response to purified IL-2 (Table XII). The production of factor by both cluster or noncluster fractions was ablated by treatment with either anti-Thy-1 and C* (manuscript in preparation). Therefore,

TABLE VIII

IL-2 Induces the Nonclustered Cells in the syn MLR to Release Helper Factor

Cells and supplements	Growth	IL-2	Helper factor (PFC)		
			Exp. 1	Exp. 2	Exp. 3
	cpm	*cpm*			
syn MLR clusters, 3×10^5	7,913	109,974	1,712	1,816	1,368
syn MLR nonclusters, 3×10^6	987	21,121	0	4	0
Plus IL-2	8,656	61,657	300	796	540
Plus IL-2 plus anti-IL-2	3,027	2,168	44	—	—
Ia-negative nonclusters	402	166	0	0	0
Plus IL-2	3,364	59,509	452	2,364	408
Plus IL-2 plus anti-IL-2	984	3,843	116	—	156
Plus IL-2 plus pre-Ig	2,949	47,041	436	—	—
Cultured T cells only	—	—	—	24	4
Plus IL-2	—	—	—	64	92
IL-2 only	—	—	24	—	20

2-d syn MLR cultures were separated into cluster and noncluster components and each was cultured an additional 2 d with or without IL-2 or anti-IL-2. Aliquots of the nonclustered cells were also treated with anti-Ia and complement before culture. The culture supernatants were then tested for helper activity. In experiment 1, cell growth and IL-2 (50% vol/vol; 6,000 cpm background) were measured as well.

TABLE IX

IL-2-responsive T Blasts Are Released by DC-T Cell Clusters

Cells in culture	Duration	Growth	IL-2	Helper factor (PFC)		
				50%	25%	12.5%
	h	*cpm*	*cpm*			
T blasts only	9	7,798	2,207	4	0	0
	25	3,620	1,793	40	24	0
T blasts + IL-2	9	11,658	8,677	2,744	1,600	580
	25	17,671	5,467	1,609	676	136
T blasts, 1,500 rad	9	1,748	8	0	0	4
	25	1,026	0	12	8	4
T blasts, 1,500 rad + IL-2	9	3,308	10,545	3,320	2,356	892
	25	2,042	10,597	2,776	1,500	384
IL-2 only	25	—	10,211	36	28	12

DC/T cell aggregates were isolated at day 2 of the syn MLR and then cultured an additional 2 d. Nests of lymphoblasts developed around the clusters. The lymphoblasts and residual clusters were separated by velocity sedimentation. 10^5 lymphoblasts were recultured for 9 or 25 h with or without purified IL-2 in 1 ml medium. In some cases, the cells were irradiated with 1,500 rad immediately before culture. At each time, aliquots of the cells were assayed for [^{3}H]thymidine uptake (cpm uptake at 9–15 or 25–31 h) and the supernatants were assayed for IL-2 (25% vol/vol; background of 600 cpm) and helper factors (PFC).

TABLE X

Effects of Anti-Ia Antibodies on the Release of Helper Factor by DC/T Cell Clusters or by IL-2-stimulated Nonclusters

Exp.	Cells and supplements	Helper factor (PFC)		
		50% vol/vol	25% vol/vol	12.5% vol/vol
1	B6.H-2k			
	Clusters, 3×10^5	580	384	292
	Plus anti-Ia^k, 1%	32	20	8
	0.5%	100	80	68
	0.25%	244	132	56
	Nonclusters, 3×10^6	12	8	4
	Plus IL-2	1,004	432	228
	Plus IL-2 plus anti-Ia^k, 1%	936	436	168
	0.5%	900	424	132
	IL-2 only	24	8	4
2	(D2 × C) F_1 ($H\text{-}2^d$)			
	Clusters, 3×10^5	1,368	736	252
	Plus B21-2, 10 μg/ml	4	0	0
	Nonclusters, 3×10^6	0	0	0
	Plus IL-2	540	276	32
	Plus IL-2 + B21-2	360	170	1
	IL-2 only	20	8	4

2-d syn MLR cultures were separated into cluster and noncluster fractions and each was cultured an additional 2 d as a source of B cell helper factor. As described above (Tables IV, V, VIII) clusters make helper factor "constitutively," while nonclusters require stimulation with IL-2. The test B cells in experiments 1 and 2 were from Swiss (H-2s) and B6.H-2k mice, respectively, and did not react with the anti-Ia antibodies added during the production of helper factors.

in the presence or absence of B cells, DC stimulate the formation of T cells that will release helper factor in response to IL-2.

Discussion

This paper describes one pathway whereby DC stimulate the development of antibody-secreting cells or PFC. Small numbers of purified DC trigger blastogenesis in a subpopulation of syngeneic T lymphocytes (the syn MLR) (2). IL-2 is released and the T cells become IL-2 responsive, as shown previously by Austyn et al. (5). The IL-2 then stimulates the production of B cell helper factors, which act independently of DC, T cells, and IL-2 to induce a PFC response. Three new points emerged from this study:

The Syn MLR Is an Active Source of Helper Factors for Antigen-specific Antibody Responses. Previous studies in man have shown that syn MLR media enhance responses to pokeweed mitogen (6) and induce some B cells to differentiate into plasma cells (7). In this paper, DC/T cell-conditioned media helped murine B cells develop primary, antigen-specific responses. Few PFC formed in the absence

TABLE XI

IL-2-induced Release of Helper Factor by Activated T Cells: Kinetics and Resistance to Ionizing Irradiation

Exp.	Cells and supplements	Helper factor (PFC) present at (h)					IL-2	Growth
		3	6	12	24	48		
							cpm	*cpm*
1	Nonclusters	0	0	0	0	—	1,222	
	Plus IL-2	1,112	2,032	2,328	2,400	—	23,950	
	1,500 rad	0	0	3	3	—	0	Not tested
	1,500 rad + IL-2	2,092	2,576	2,972	3,032	—	6,052	
	IL-2 only	—	—		52	—	14,392	
2	Nonclusters	—	—	—	—	0	2,969	3,752
	Plus IL-2	—	344	536	1,032	280	11,532	36,819
	1,500 rad	—	—	—	—	0	505	395
	1,500 rad + IL-2	—	744	776	844	1,268	4,527	1,419
	IL-2 only	—	—	—	—	56	32,804	—
3	Nonclusters	—	0	0	24	404		
	Plus IL-2	—	1,488	2,012	2,116	1,824		
	1,500 rad	—	0	12	12	28	Not tested	
	1,500 rad + IL-2	—	2,276	2,812	2,728	2,636		
	IL-2 only	—	—	—	—	68		

The noncluster component of 2-d syn MLR cultures was isolated and cultured with or without IL-2 and 1,500 rad irradiation for 3–48 h. Aliquots of the culture medium were tested for helper activity at the times indicated. Cell growth and IL-2 (25% vol/vol; background of 1,000 cpm in all experiments) were also monitored in some experiments; data are shown for the last time points.

TABLE XII

Production of Helper Factors from Mixtures of DC, B, and T Cells

Cells used to prepare factor	Helper activity (PFC)			IL-2 (cpm)		
	50%	25%	12.5%	50%	25%	12.5%
G10-nonadherent spleen	16	4	0	13,332	8,415	3,900
DC only	0	0	0	0	0	0
G10 + DC						
Clusters,	1,996	1,024	372	292,492	155,147	77,100
Nonclusters	68	12	0	32,381	15,099	9,004
Nonclusters + IL-2	1,000	488	120	92,616	45,164	20,319
Nonclusters, 1,500 rad, + IL-2	2,276	642	212	86,472	40,981	26,071
IL-2 only	4	4	0	162,402	81,568	37,903

2.5×10^4 DC were cultured with 5×10^6 Sephadex G10-nonadherent spleen (B/T lymphocyte mixtures) for 2 d and the cultures were separated into cluster and noncluster fractions. The fractions were maintained another 2 d in vitro with or without purified IL-2. The supernatants were removed and tested at 12.5, 25, and 50% vol/vol for B cell helper function and IL-2 (backgrounds were 0 PFC and 2,000 cpm).

of antigen (e.g., Table I). Thus, the murine syn MLR helper factors should not be considered as true polyclonal activators. Instead, the factors probably act on B cells that have been selected and/or activated in vitro by the added antigen.

The precise stimulus which triggers T cell growth and lymphokine release in the syn MLR is not known. The T cells are I restricted (15), as is typical of helper cells, but it is not clear if the T cells recognize Ia or Ia plus some undefined "antigen."

Syn MLR helper factors likely are similar to the major histocompatibility complex-nonrestricted factors that have been recovered previously from cultures stimulated with lectin, alloantigen, and specific antigen (18–23, reviewed in 24) and which include such moieties as B cell growth and B cell differentiation factors (25–29). In our experience, media collected between day 2 and 4 of the syn MLR are similar in activity to media from T cells stimulated with lectin and alloantigen for 24–48 h. Since DC and T cells are necessary components of any T-dependent PFC culture system, syn MLR helper factors would be present in the absence of additional stimulation with antigen or mitogen. It remains to be determined if syn MLR-conditioned medium contains a representative sample of all the factors which can activate B cells (reviewed in 24). For example, production of antigen-specific helper factors (e.g., 30, 31) might require the addition of the appropriate erythrocyte to the syn MLR culture.

DC as the Principal Stimulator of the Syn MLR. Removal of DC with a specific antibody and C* severely inhibited cell proliferation and lymphokine release in the syn MLR (Table III). This selective depletion approach complemented previous experiments in which DC were shown to be active stimulators of the syn MLR, in contrast to macrophages, B cells, and T cells (2).

The specialized stimulatory capacity of DC was also manifested by the development of DC/T cell aggregates that contained the bulk of the T cells involved in helper factor release (Tables IV, V). Clusters did not form if T cells were cultured with splenic macrophages or B cells (not shown). Most syn MLR-reactive T cells formed clusters during 2 d of co-culture with DC at a DC/T cell ratio of 1:100; i.e., the addition of DC to the noncluster fraction resulted in little additional cell aggregation. Previous studies have shown that primed T cells can bind to monolayers enriched in macrophages (32–36), leading to a reduction of antigen-responsive cells in the nonattached fraction. However, the data obtained with DC/T cell clusters seem decidedly different from the data on macrophage/lymphocyte binding. First, the DC/T cell clusters were formed at a ratio of 1 DC per 200 unprimed T cells, while macrophage/lymphocyte binding has usually been done at a 1:1 ratio. Second, few small lymphocytes were noted in the syn MLR clusters even though the clusters were bathed in a 10–40-fold excess of small cells. Macrophage monolayers have been reported to bind a majority of small, nonspecific cells accounting for 50% or more of the added inoculum (35, 36). Third, the DC/T cell aggregates exhibited most of the functional activity (proliferation, production of IL-2 and helper factors) in the cultures and, on a per cell basis, were enriched some 10–20-fold relative to the unfractionated culture (Table IV). Macrophage monolayers have been used to deplete antigen-primed T cells, but it has been difficult to show that the bound lymphocytes are enriched in functional activity relative to the starting inoculum (e.g., 34, 35). We

suspect this is in part due to nonspecific sticking of small lymphocytes and in part due to the inability of macrophages to independently sustain T cell proliferation.

IL-2 as an Essential Stimulus for the Release of B Cell Helper Factors. A priori, the IL-2 that is present in syn MLR-conditioned medium could influence antibody responses in three ways: (*a*) IL-2 could act directly on the B cell as has been proposed in some studies (16, 17) but not in others (28, 29); (*b*) IL-2 could promote T cell growth, and then the growing T cells would release helper factor in response to antigen or other stimuli, as is usually conceived; (*c*) IL-2, rather than antigen, Ia, or accessory cells, could trigger helper factor production. The latter proved to be the case.

With respect to the possibility that IL-2 is itself a helper factor, we found that an anti-IL-2 antibody neither neutralized nor absorbed helper activity (Table VII, Fig. 1), and that purified IL-2 did not stimulate B cells or DC-depleted B/T cell mixtures (Tables VII–XII). The reported effects of IL-2 on B cells could represent contaminating factors in the IL-2 preparation, or possibly IL-2 triggered helper factor production by contaminating T cells.

Since our probes (anti-IL-2 and purified IL-2) did not act directly on the B cell helper assay, we could evaluate the contribution of IL-2 to the generation of helper activity from DC/T co-cultures. The anti-IL-2 markedly blocked the production of helper factor (Table VI), suggesting that most factor release was IL-2 dependent. Likewise, purified IL-2 stimulated factor release from T cells that had been rendered IL-2 responsive by co-culture with DC (Tables VIII–XII). The best source of these T cells was the population of lymphoblasts derived from the DC/T cell aggregates (Table IX). 10^5 blasts released as much helper factor in 9 h as did 5×10^6 T cells in a 4-d syn MLR or 5×10^6 Con A- or alloantigen-stimulated spleen cells in 24–48 h (not shown). T cell growth was not required, since IL-2 induced a rapid release of helper factor (most was released in 3–6 h) even if the T cells were irradiated to inhibit growth. Most likely the IL-2 was acting on the T cell directly, because the responding population was effectively depleted of DC and because anti-Ia antibodies did not block the IL-2 effect (Table X).

Therefore IL-2 is more than a T cell growth factor. It is the trigger needed by the activated helper T cell to express its functional potential. It is intriguing that even if T cells are allowed to grow, IL-2 does not induce the release of more helper factor than is produced by irradiated T cells (Table XI). Presumably, the lymphoblast has to encounter DC again before it can respond to IL-2 and release lymphokine a second time. Studies to test this hypothesis are underway. We would predict that the functional potential of helper T cells is limited once the cell leaves the DC-rich environment that exists in lymphoid tissues or in DC/T cell clusters. Since DC are scarce in certain exudates (37), an immigrating helper T blast may only be able to exhibit a single round of IL-2-mediated lymphokine release and division after an encounter with antigen in the inflammatory site.

Previous studies have shown that IL-2 triggers the release of another lymphokine, immune interferon (38, 39). In lepromatous leprosy, there exists a defect in immune interferon release that can be reversed by the combination of M. Leprae antigen and IL-2 (40). These studies have not determined if accessory cells or T cell proliferation are needed for IL-2-induced interferon release.

However, it is possible that there are helper T cells which produce interferon in two stages similar to that seen for B cell helper factors, i.e., DC-mediated T cell activation followed by IL-2-mediated lymphokine release.

The studies of Austyn et al. (5) first demonstrated the importance of DC in inducing T cell responsiveness to IL-2. DC were added to T cells and then removed at defined time intervals before the onset of DNA synthesis. The data indicated that after co-culture with DC, T cells could synthesize DNA in response to the IL-2 that had been released into the culture medium. The DC so tightly controlled IL-2 release and responsiveness that the addition of exogenous growth factor had no effect unless the DC were first eliminated with antibody and C*. Future experiments should clarify if IL-2 induces the release of lymphokines required for the development of other effector cells, e.g., interferon for activated macrophages and distinct differentiation factors for cytolytic T cells. However the data presented in the work of Austyn et al. (5) and this paper begin to explain the capacity of DC to induce such complex multicellular responses as cytolytic T cell formation (2, 41), contact sensitivity (42), antibody formation (1), and graft rejection (43). In each system, helper T cells can respond to antigen and mediate the development of other effector cells. We reason that DC are the physiologic stimulators of IL-2 responsiveness and that the latter underlies the subsequent release of helper lymphokines and the formation of effector cells. Thus, the strategy of a helper T cell response is not simply antigen-induced clonal expansion. Rather, antigen and DC induce IL-2 release and responsiveness, which then mediates the production of helper factors and effector cells in the absence of additional T cell growth.

Summary

Dendritic cells (DC) are essential accessory cells for T-dependent antibody responses in culture (1). We have outlined a three-stage mechanism to explain the capacity of DC to stimulate primary antibody responses to heterologous erythrocytes. First, DC induced T cells to produce and to become responsive to interleukin 2 (IL-2). This stage corresponded to the syngeneic mixed leukocyte reaction (2) and involved the clustering of DC and T cells into discrete aggregates. Isolated clusters, representing 5–10% of the culture, were critical for IL-2 release and the production of IL-2-responsive T blasts. In the second stage, IL-2 directly triggered the responsive T cells to release B cell helper factors. This role for IL-2 was documented with a rabbit anti-IL-2 reagent, purified IL-2, and T cells that had been rendered IL-2 responsive by an initial co-culture with DC. T cell growth was not required for IL-2-mediated helper factor release, since irradiated and untreated responders produced similar levels of factor and did so within 3 h of the addition of IL-2. In the final stage, helper factors stimulated the development of antibody-secreting cells from purified B lymphocytes. The helper factors were not H-2 restricted, but for both sheep and horse erythrocytes, the response to factors was antigen dependent and specific. The IL-2 that was present in the DC/T cell-conditioned medium did not act on B cells, since helper activity was neither neutralized nor absorbed by our anti-IL-2 reagent. We conclude that the ability of the DC to induce IL-2 release and responsiveness underlies its capacity to trigger both T and B lymphocyte reactions.

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