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Resting and sensitized T lymphocytes exhibit distinct stimulatory (antigen presenting cell) requirements for growth and lymphokine release

Kayo Inaba

Ralph M. Steinman

RESTING AND SENSITIZED T LYMPHOCYTES EXHIBIT DISTINCT STIMULATORY (ANTIGEN-PRESENTING CELL) REQUIREMENTS FOR GROWTH AND LYMPHOKINE RELEASE

BY KAYO INABA AND RALPH M. STEINMAN

From The Rockefeller University and The Irvington House Institute, New York

Resting T lymphocytes, in many instances, require dendritic cells (DC)[1] to initiate growth and differentiation. The models that have been studied in this regard include the mixed leukocyte reaction (MLR), T-dependent antibody responses, and oxidative mitogenesis in vitro, and graft rejection and contact sensitivity in situ (reviewed in 1). Little is known about the stimulatory requirements for T cells that have just been sensitized in concert with DC. For example, does the T cell that has differentiated into a lymphoblast interact effectively with other leukocytes, such as B cells and macrophages? It would seem essential that a clone of cells that has been stimulated by DC also be able to interact with other "antigen-presenting cells" to continue the immune response. An example would be T-dependent antibody formation. These responses require DC (2–4), as well as an interaction between helper T lymphocytes and antibody-forming precursors (5, 6).

In this paper we have prepared enriched populations of freshly sensitized, allospecific T lymphoblasts. To do so, we took advantage of the fact that DC and responding lymphocytes efficiently aggregate with one another during in vitro responses (3, 4, 7). Using the primary MLR as a model, we have isolated aggregates of DC and alloreactive T cells and shown that these clusters generate large numbers of allospecific Lyt-2^+ and Lyt-2^- blasts. Four features of the blasts will be outlined. First, the T cells rapidly aggregate allogeneic cells in an antigen-specific fashion. Second, sensitized cells require restimulation with antigen or IL-2 to grow and release B cell helper factors. Third, aldehyde-fixed allogeneic cells can restimulate sensitized T cells. Fourth, restimulation of growth and lymphokine release can be accomplished by many cell types expressing the appropriate alloantigen, including B cells and macrophages, whereas resting T cells (the primary MLR) are most effectively stimulated by DC.

K. Inaba is a Visiting Investigator from the Department of Zoology, Kyoto University, Kyoto, Japan 606. R. Steinman is an Established Investigator of the American Heart Association. This work was supported by grant AI13013 from the National Institutes of Health.

[1] *Abbreviations used in this paper:* Con A, concanavalin A; DC, dendritic cell; FCS, fetal calf serum; IL-2, interleukin 2; LPS, lipopolysaccharide; mAb, monoclonal antibody; MHC, major histocompatibility complex; MLR, mixed leukocyte reaction; PFC, plaque-forming cell; SRBC, sheep red blood cell; TNP-KLH, trinitrophenylated keyhole limpet hemocyanin.

Materials and Methods

Mice. Mice of both sexes were used at 7–12 wk of age. C × D2 F_1, C57BL/6, B6.H-2k, C3H, and ICR mice were from the Trudeau Institute, Saranac Lake, NY; B10.T(6R) and B10.MBR were from The Mayo Clinic, Rochester, MN.

Antibodies. Several monoclonal antibodies (mAb) were used for cell depletion (with rabbit complement) and staining (indirect immunofluorescence) as described elsewhere (2–4). The mAb included: TIB 99 and B5-3, α-Thy-1.2; TIB 150, α-Lyt-2.2; C3PO, α-Lyt-1.2; B21-2, α-I-$A^{b,d}$; 10-2.16, α-I-A^k; TIB 146, α-B cell.

Mixed Leukocyte Reactions. The stimulators were different populations of leukocytes prepared as described below and irradiated with 750–1,500 rad. Responders were nylon wool–nonadherent splenic T cells that had been treated with α-Ia and complement to deplete contaminating B cells and DC. 3–5 × 10^6 T cells were maintained in macroculture (16-mm wells, 1 ml culture medium) and 2–4 × 10^5 in microculture (6-mm wells, 0.2 ml medium). For restimulation of alloreactive blasts, the T cell doses were 2–3 × 10^5 cells in macroculture and 1–3 × 10^4 in microculture. The medium was 10% fetal calf serum (FCS) (Sterile Systems, Inc., Logan, UT) in RPMI 1640 (Gibco Laboratories, Grand Island, NY) with 20 μg/ml gentamycin sulfate and 5 × 10^{-5} M 2-mercaptoethanol. [^{3}H]thymidine (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, NY; 6.7 Ci/mM, 4 μCi/ml) was added at 24–38 h for restimulated blasts and 96–112 h for primary MLRs.

Cells. DC were macrophage-depleted, low density, spleen adherent cells (7). B cells were Sephadex G10 nonadherent spleen cells that had been treated with α-Thy-1 and α-Lyt-1 and complement (2–4). B blasts were 2–3-d cultures of splenic B cells stimulated with 10 μg/ml lipopolysaccharide (LPS) (Difco Laboratories, Inc., Detroit, MI) in medium containing 1% FCS. T blasts were prepared by a multistep procedure as follows: a primary MLR was set up with 5 × 10^4 DC and 5 × 10^6 allogeneic T cells per macroculture. At 36–40 h, the cell aggregates that had developed were isolated by velocity sedimentation on Percoll gradients as described (3, 4). Clusters sedimented to the bottom of the tube. Nonclustered cells, including T blasts and dendritic cells, were found 5 cm higher in the columns. Yields were typically 2–3 × 10^5 clustered cells and 3–4 × 10^6 nonclustered cells per culture. The clusters were maintained at 2–3 × 10^5 cells per macroculture for 48 h more, whereupon the released cells (almost entirely T blasts; see Results) were separated from residual clusters by velocity sedimentation. To obtain Lyt-2^- blasts, T cells were treated with TIB 150 α-Lyt-2.2 at the onset of the primary MLR and before restimulation of the blasts.

Interleukin 2. T cell growth factor was monitored by stimulation of [^{3}H]thymidine uptake in concanavalin A (Con A)–induced T blasts. A sample of recombinant human IL-2 (sp act, 8.5 × 10^5 U/μg) was kindly provided by Biogen, Cambridge, MA.

B Cell Stimulation. B cell helper factors in conditioned media were assayed on 2.5 × 10^6 splenic B cells to which sheep red blood cells (SRBC) were added as antigen (3). Plaque-forming cells (PFC) were measured at day 4. Helper activity for antibody responses (day 4 PFC) were also measured by coculturing B cells with DC–depleted helper T blasts, using SRBC or trinitrophenylated keyhole limpet hemocyanin (TNP-KLH) (5 μg/ml) as antigens. B cell proliferation in response to irradiated (3,000 rad) T blasts was monitored by culturing 2 × 10^5 B cells with graded doses of blasts in microculture, and measuring [^{3}H]thymidine uptake at 48–60 h or cell size by light scatter analysis at 48 and 60 h (see Results).

Indirect Immunofluorescence. A number of antigens were visualized using mAb (see above) and test cells that had been attached or spun onto slides (4). The visualizing system was mouse anti-rat Ig followed by fluorescent goat anti-mouse Ig (provided by Dr. Fritsch, Innsbruck, Austria).

Results

We will first describe how dendritic cells and alloreactive T cells cluster with one another during the primary MLR, and how these clusters can be used to

generate large numbers of allospecific T lymphoblasts. The functional properties of the Lyt-2$^-$ subpopulations will then be outlined. We will stress that sensitized T cells grow and release lymphokines when cultured with many different types of allogeneic leukocytes, and not just DC.

Clustering of Dendritic Cells and Allospecific T Lymphocytes. An early indicator that an MLR was occurring in DC/T cell cocultures was the development of discrete cell clusters after 20–40 h of culture. As in previous studies of the syngeneic MLR and T-dependent antibody responses (3, 4), the clusters could be isolated by velocity sedimentation and shown to consist of DC and lymphocytes. By α-Ia staining (compare Fig. 1, *A* and *C*) most DC were in the cluster fraction, compared with 5–10% of total T cells. If the isolated clusters were cultured for an additional 48 h, cell yields increased two- to threefold, and T lymphoblasts were the predominant cell within and around each aggregate. The released cells could be separated from residual clusters by a second velocity sedimentation. The released cells were almost entirely Thy-1$^+$ T lymphoblasts and lacked allogeneic Ia antigens, either on DC or adsorbed to the T cells (Fig. 1, *E* and *F*). The residual clusters consisted of T blasts clustered about large Ia-rich DC (Fig. 1, *G* and *H*).

Both Lyt-2$^+$ cytolytic and Lyt-2$^-$ noncytolytic populations were generated by the clusters (Table I). The Lyt-2$^-$ blasts all stained strongly with α-Lyt-1 mAb and were used in most of the subsequent studies. When cluster-derived T blasts were rechallenged with DC from various mouse strains, responses were directed primarily to the original stimulating or "specific" DC. Proliferation to other allogeneic or "third-party" strains was reduced some 10-fold, and little or no response to syngeneic DC was observed (Fig. 2, *left*). Nonclustered T cells exhibited primary rather than accelerated MLR responses. These were directed to third-party rather than specific DC (Fig. 2, compare *middle* and *right*). We conclude that DC efficiently aggregate allospecific T cells.

Homogeneity of Cluster-derived T Lymphoblasts. Unlike resting T cells, which require 1–2 d to begin DNA synthesis, most of the cluster-derived blasts quickly resumed growth (generation time of 14–18 h) for 2–3 d when stimulated with DC (Fig. 3). Cell densities of 2–3 $\times$ 10^6 blasts/ml were reached. Optimal initial DC/T cell ratios were 3 $\times$ 10^4 DC/1–3 $\times$ 10^5 blasts in macroculture, and 0.3–3 $\times$ 10^3 DC/2 $\times$ 10^4 blasts in microculture. If the blasts were restimulated with recombinant IL-2 (10–100 U/ml), cell growth occurred, but only for 20–24 h (Fig. 3, *right*); probably because the exogenous IL-2 was rapidly depleted from the culture medium (not shown). When placed in fresh medium, the T blasts died in 2–3 d (Fig. 3).

To test whether most T blasts specifically recognized alloantigens, we did binding assays in which [^{3}H]thymidine-labeled blasts were mixed with small B lymphocytes from different strains. At limiting T cell doses (1:20–1:60), >80% of the blasts quickly (within 2 h) clustered with specific B cells, even at 4°C (Table II). The clusters had T/B cell ratios of 1:5–1:10, and had a rosette appearance with a central large blast and several surrounding small B cells (not shown). Little or no clustering with syngeneic and third-party B cells was evident. A limited strain analysis indicated that the blasts were restricted to the I region of the allogeneic major histocompatibility complex (MHC) (Table II, Exp. 3).

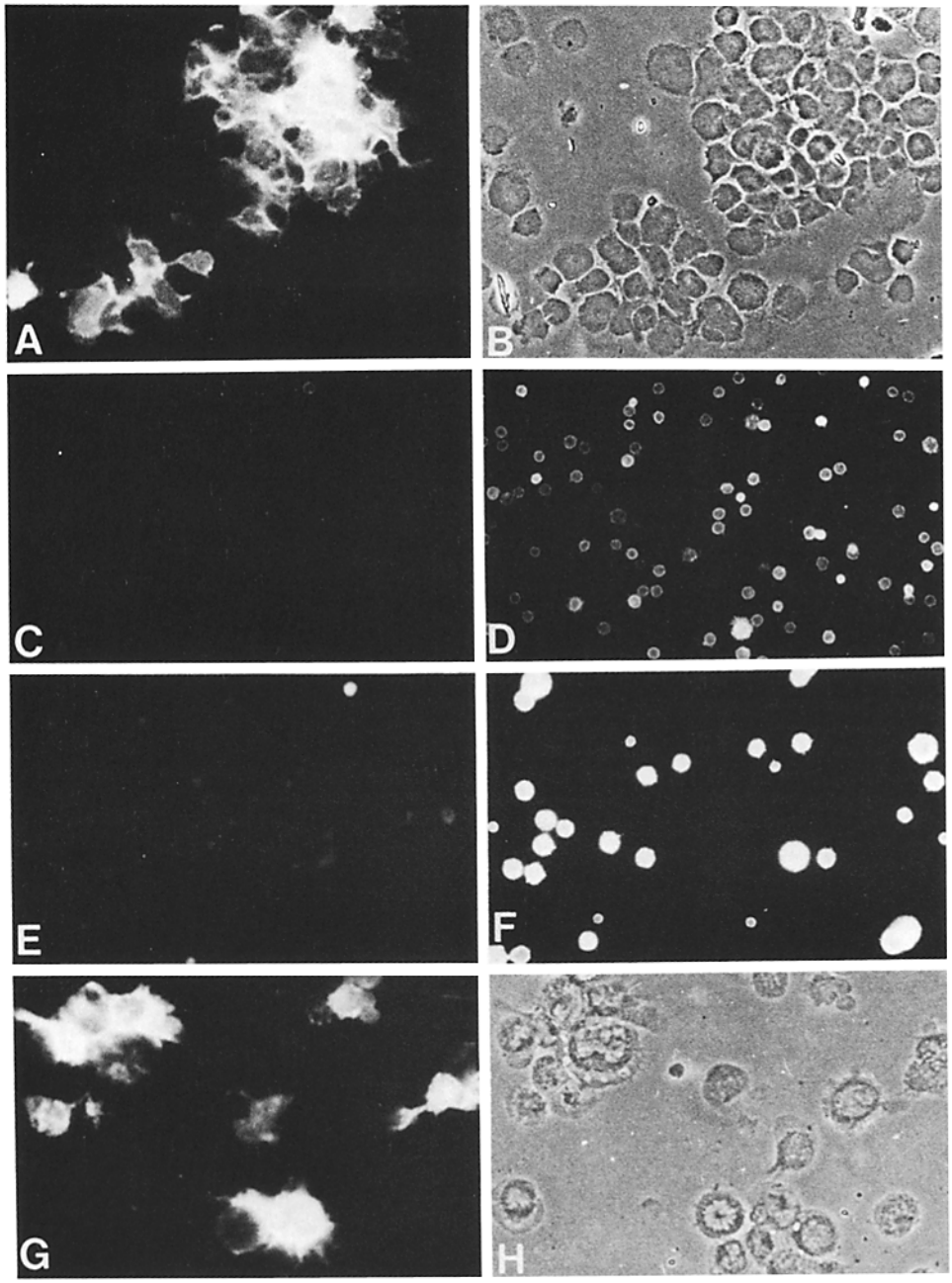

FIGURE 1. During a primary MLR, dendritic cells cluster with T cells and generate T lymphoblasts. Micrographs are shown of MLRs between 5×10^4 H-2d DC and 5×10^6 H-2k T cells. Cluster and noncluster fractions were spun onto coverslips and stained with α-I-A^d (B21-2) and α-Thy-1 (B5-3) mAb. The clusters exhibited abundant Ia staining (*A*, fluorescence; *B*, phase contrast) due to the presence of one or more DC. The nonclusters lacked Ia^+ DC (*C*) and were almost entirely small Thy-1^+ cells (*D*). Additional aliquots of the fractions (not shown) were stained with other mAb (F4/80, α-macrophage; TIB 146, α-B cell; and 2.4G2, α-Fc receptor) to prove that stimulator and responder populations lacked macrophages and B cells. The clusters were cultured an additional 2 d to provide two fractions. The released cell fraction was >80% blasts that lacked the stimulating B21-2 alloantigens (*E*), but stained strongly for Thy-1 (*F*). >90% of the blasts also lacked endogenous Ia (not shown). The residual clusters (corresponding to day 4 of primary MLR) contained large Ia^+ DC (*G*), (*H*) and many blasts. × 480.

TABLE I

Cytolytic, Lyt-2$^+$, and Noncytolytic, Lyt-2$^-$ T Blasts Are Generated by Dendritic Allogeneic T Cell Clusters

Effector cells	Specific ^{51}Cr release at effector/target ratios of:			
	30:1	10:1	3:1	1:1
Total blasts	67	47	39	29
Lyt-2$^-$ blasts	11	7	9	14
Lyt-1$^-$ blasts	57	47	35	23

T lymphoblasts were derived from DC/T cell (H-2k/H-2d) clusters (equivalent to day 4 of the primary MLR) and treated with TIB 150 (α-Lyt-2.2) or C3PO (α-Lyt-1.2) mAb and complement before testing on ^{51}Cr-labeled Con A blasts (H-2k) targets. Percent specific ^{51}Cr release was: (experimental − spontaneous release [7%])/(total − spontaneous release [4 h assay]). The frequency of Lyt-2$^+$ cells varied considerably between experiments and mouse strains, ranging from 10 to 80%.

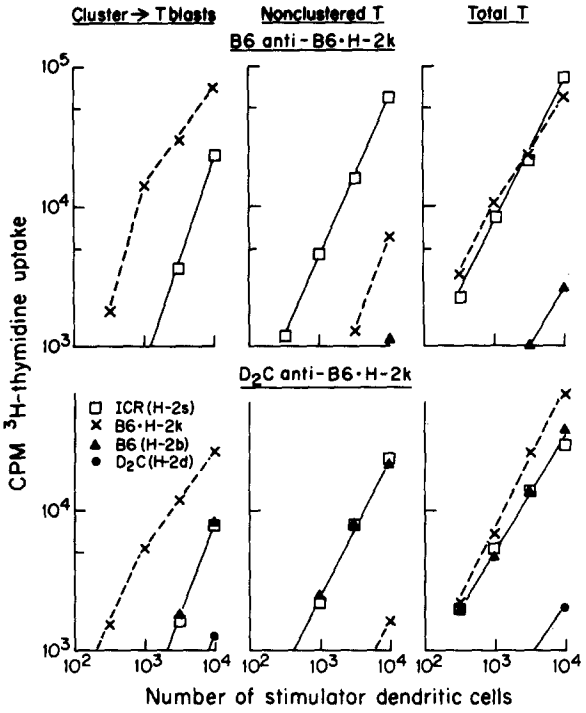

FIGURE 2. Alloreactivity of clustered and nonclustered T cells. Two experiments are shown in which DC from several strains (see legend) were used to stimulate proliferation in cluster-derived T blasts (*left*; [^{3}H]thymidine uptake at 26–42 h) as well as nonclustered (*middle*) and unprimed T cells (*right*) ([^{3}H]thymidine uptake at 96–112 h). Note that the clustered cells responded actively to the strain used to initiate the MLR but exhibited depleted reactivity to third party. Nonclustered cells preferentially responded to third party.

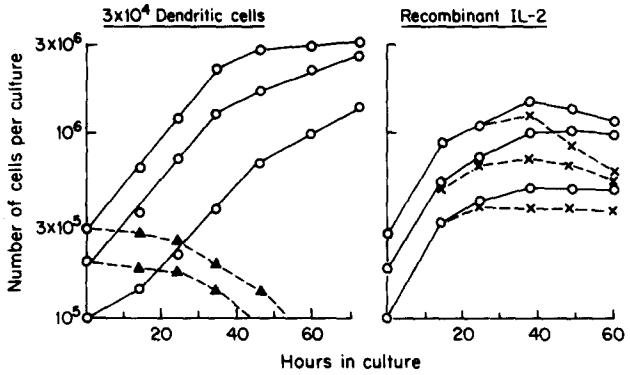

FIGURE 3. Growth curves of T blasts restimulated with DC or IL-2. 1, 2, and 3 × 10^5 Lyt-2^- T blasts were placed in 16-mm tissue culture wells with 1 ml culture medium and restimulated with 3 × 10^4 irradiated (900 rad) DC. Data with 10^5 DC were identical and are not shown. Cell counts were made at varying time intervals to show that all cells resumed exponential growth (*left*). T blasts died if not restimulated (▲). Blasts also responded to recombinant IL-2 at 100 (○) and 30 (×) U/ml (*right*).

When resting T cells (Lyt-2^- or total nylon-nonadherent cells) were mixed with allogeneic B cells, no clustering occurred even over a 5 d period in culture. Therefore, most of the T blasts we isolated had acquired an enhanced capacity to bind specific antigen (allogeneic B cells).

B Lymphocytes as Stimulators for Resting and Sensitized T Cells. DC induced strong proliferative responses in allogeneic resting T cells, but fresh splenic B cells were inactive even at 300 times the dose needed to detect stimulation by DC (Fig. 4). Irradiated (750–1,500 rad) and mitomycin-treated (50 μg/ml), 30 min) B cells were equally inactive. Populations of LPS-induced B blasts stimulated the MLR but were only 1–2% as active as DC (Fig. 4). The B populations were >90% blasts and expressed Ia at half the level of DC (8). Minor contamination with DC was not excluded. B cells and B blasts were weak MLR stimulators whether total or Lyt-2^- T cells were used as responders (see Figs. 6 and 8 below).

Freshly sensitized T blasts, however, proliferated when cultured with allogeneic B lymphocytes (Fig. 5). Stimulatory activity, as assessed by dose response curves, was 2–3- and 5–20-fold less effective with B blasts and fresh spleen B cells than with DC. Therefore, stimulatory capacity seemed directly related to the levels of class II MHC products on the allogeneic populations, as determined previously by quantitative binding studies with ^{125}I-anti-Ia antibodies (8).

T blasts that had been depleted of Lyt-2^+ cells showed enhanced responses to allogeneic DC and B cells (Fig. 6), probably because the Lyt-2^+ component contained cytolytic cells capable of eliminating the stimulators (Table I). Even 30 DC induced detectable proliferation at 28–42 h in 2 × 10^4 blasts.

The response to allogeneic B cells was MHC-restricted, as measured by T cell proliferation and IL-2 release (Fig. 7). As in the T/B clustering studies (Table II), the restriction of Lyt-2^- blasts was primarily to the I region or MHC class II products (Fig. 7). The MHC restriction was tighter when IL-2 release was measured. Presumably, some IL-2 would be released from true cross-reactive

TABLE II

Aggregation of Alloreactive T Blasts with B Lymphocytes

Exp.	[^{3}H]thymidine C × D2 α-B6.H-2k T blasts added to B cells			B cells (6 × 10^6) used	T blasts in:	
	Number of cells	Time	Temp.		Clusters	Nonclusters
		h	*°C*		*cpm*	
1	1 × 10^5	1	37	B6.H-2k	44,486	4,363
				C × D2 F_1	17,661	30,352
				None	ND	51,402
		2	37	B6.H-2k	48,391	3,763
				C × D2 F_1	14,959	33,341
				None	ND	47,815
		3	37	B6.H-2k	41,723	4,027
				C × D2 F_1	7,542	37,765
				None	ND	46,974
		5	37	B6.H-2k	36,864	7,280
				C × D2 F_1	4,842	38,478
				None	ND	46,417
2	1.5 × 10^5	2	37	B6.H-2k	36,946	3,944
				C × D2 F_1	4,754	38,956
				None	ND	44,963
		5	37	B6.H-2k	36,086	4,882
				C × D2 F_1	3,064	37,760
				None	1,175	37,912
	3 × 10^5	2	37	B6.H-2k	85,924	8,524
				C × D2 F_1	10,995	84,268
				None	ND	91,577
		5	37	B6.H-2k	79,953	8,129
				C × D2 F_1	3,212	87,092
				None	3,934	86,908
3	1.5 × 10^5	2	37	C3H	44,114	4,050
				B10.MBR	44,011	4,360
				B6	4,942	41,384
				C × D2 F_1	3,212	43,332
				None	3,512	41,375
		3	4	C3H	41,477	5,106
				B10.MBR	44,885	5,167
				B6	15,554	38,874
				C × D2 F_1	8,550	40,242
				None	5,585	41,497

DC/T cell clusters were isolated from a C × D2 anti-B6.H-2k MLR and cultured an additional 2 d to generate populations of alloreactive, Lyt-2$^-$ T blasts. The latter were [^{3}H]thymidine-labeled with 3.3 μCi/ml for 16 h, washed, and mixed with 6 × 10^6 fresh splenic B cells for 1–5 h in tubes (Falcon No. 2057) on ice or at 37°C. The suspensions were then layered onto ice-cold Percoll gradients to separate clusters from nonclusters. Each fraction was then counted in the hemocytometer (the T blasts were 2–3 times the diameter of the B cells) or in a scintillation counter (10% aliquots). For some time points, separate gradients were run from tubes containing T blasts without B cells, since small numbers of T cells aggregated in the absence of B cells (ND, not determined). The haplotypes of the mice used in Exp. 3 were: C3H, $K^kI^kD^k$; B10.MBR, $K^bI^kD^q$; B6, $K^bI^bD^b$; and C × D2, $K^dI^dD^d$.

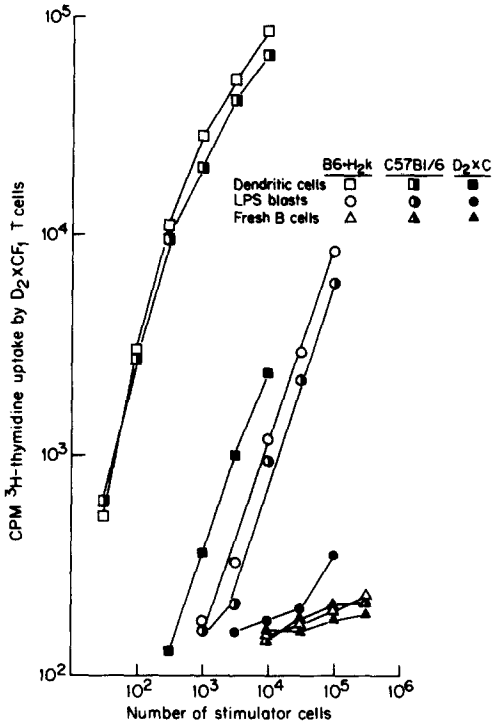

FIGURE 4. The relative capacity of DC (rectangles), LPS-induced B blasts (circles), and fresh splenic B cells (triangles) to stimulate a primary MLR. Responders were 4×10^5 C × D2 F_1 Ia^- T cells. Stimulators were irradiated with 900 rad and added in microcultures at varying doses for 96 h before 15 h exposure to 1 μCi of [^{3}H]thymidine. Note that the responses to both strains of allogeneic DC (B6.H-2k and B6) were similar and that the syngeneic response (D2 × C F_1) was 100-fold less active.

clones (clones recognizing both specific and third-party cells, e.g., ref. 9), and this would drive the proliferation of specific T blasts (Fig. 7, left).

Whereas resting T cells did not produce B cell helper factors in the absence of DC (3), T blasts released these factors when stimulated with either DC or B cells (Table III). IL-2 also induced the production of helper factors from T blasts (Table III), as has been documented in more detail previously with stimulated T cells derived from the syngeneic MLR (3).

Macrophages as Stimulators of T Blasts. More complex dose response curves were obtained when macrophages were evaluated as stimulator cells. The test populations were either fresh resident peritoneal cells (not shown) or adherent macrophages cultured for 1 d in immune interferon to provide Ia-rich and DC-depleted populations (10). Low doses of macrophages actively stimulated the T blasts, while higher doses did not (Fig. 8). The mechanism underlying the high dose inhibition is under study. Macrophages were weak or inactive in stimulating a primary MLR at all doses (Fig. 8), as previously reported (10–12).

Interaction of Aldehyde-fixed Stimulators with Resting and Stimulated T Cells. Previous studies indicated that aldehyde-fixed cells could present antigens to T-T hybrids and T cell clones (13, 14). To further compare the requirements

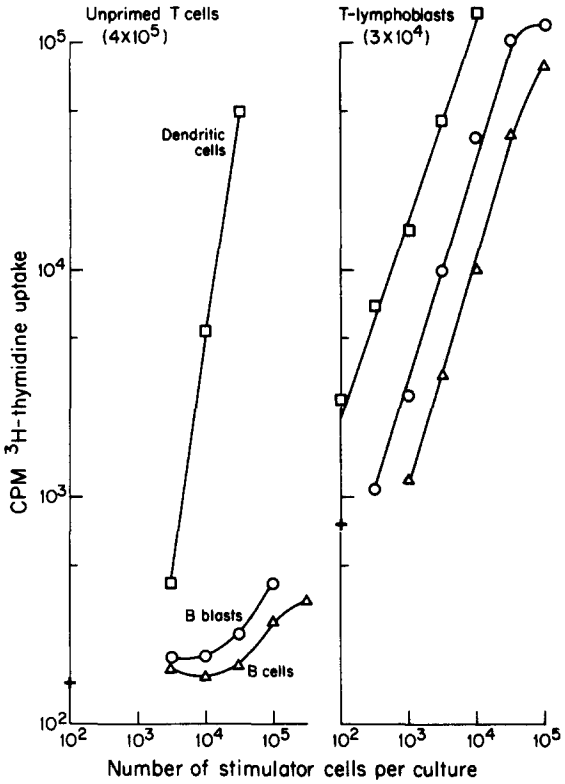

FIGURE 5. Stimulator cell requirements for resting and sensitized T cells. The same stimulator cell populations were used to induce proliferation in unprimed T cells (*left*; cpm [^{3}H] thymidine at 96–111 h) and cluster-derived T lymphoblasts (*right*; cpm [^{3}H]thymidine at 24–38 h). The stimulators were DC (□), B blasts (○), and fresh splenic B cells (△). Background proliferation (+) is shown.

of resting and sensitized T cells, we tested DC and macrophages that had been fixed. Glutaraldehyde and formaldehyde impaired the stimulation of the primary MLR to a much greater extent than stimulation of T blasts (Table IV). This experiment provides additional evidence that sensitized T cells respond to alloantigen per se, whereas unprimed T cells respond to viable DC.

B Lymphocyte Responses to Sensitized T Cells. In the absence of DC, unprimed T cells did not exhibit helper function for syngeneic or allogeneic B lymphocytes (e.g., Table X of ref. 4). In contrast, we noted that Lyt-2$^-$ T blasts actively induced B cell proliferation and differentiation. After 2 d of coculture, most of the B cells had enlarged, with ~50% recovery of starting B cell numbers (Fig. 9). Only B cells of the appropriate MHC underwent the increase in cell volume (Fig. 9) and incorporated [^{3}H]thymidine (Table V). The Lyt-1$^-$, 2$^+$ blasts quickly (~6 h) killed the stimulators and, accordingly, did not induce B cell growth (Table V).

Polyclonal antibody responses were induced by alloreactive T blasts as well, and were measured as α-SRBC or α-TNP PFC. The α-TNP response almost certainly required direct B-T interaction. Thus MHC–appropriate B cells (B6.H-

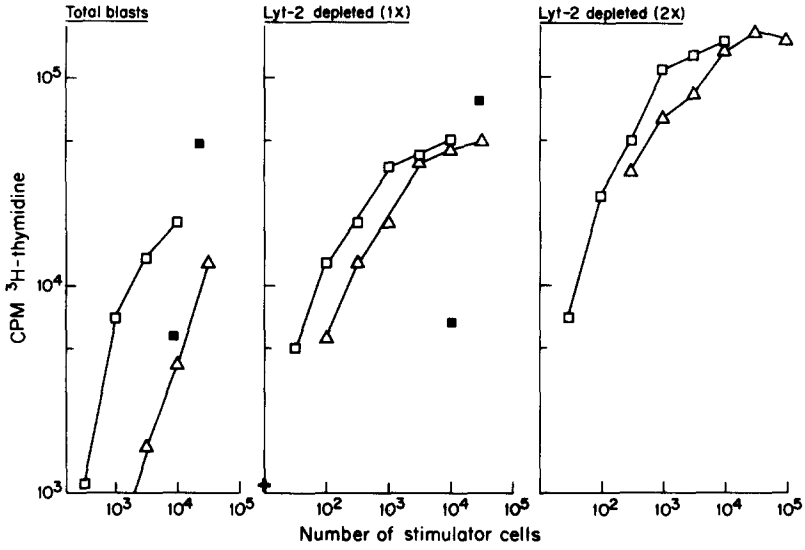

FIGURE 6. B cells stimulate Lyt-2$^-$ T blasts. DC (□) and fresh splenic B cells (△) were compared as stimulators for unfractionated, cluster-derived T blasts (80% Lyt-2$^+$; *left*); blasts derived from MLRs between DC and Lyt-2$^-$ T cells (20% Lyt-2$^+$ blasts; *middle*); and blasts that were treated with α-Lyt-2 at the start of the MLR and prior to restimulation (2× Lyt-2$^-$; *right*). Primary MLRs with unprimed total and Lyt-2$^-$ T cells were studied as well, but only DC (■) gave significant responses.

2k in Table VI) formed α-TNP PFC, whereas inappropriate B cells (H-2d) did not, even if the cultures were supplemented with H-2k DC (Table VI). Also, α-TNP responses did not occur if the B cells were exposed to antigen and the helper factors found in syngeneic MLR supernatants (group 5, Table VI).

Polyclonal PFC responses to alloreactive T blasts were enhanced by the inclusion of specific antigen in the culture, i.e., 10-fold fewer blasts were required to induce equivalent levels of α-SRBC and α-TNP PFC (Table VI). We conclude that unprimed T cells are inactive as helper cells until sensitized by DC; then direct and effective T-B interaction can occur.

Discussion

Isolation of Freshly Sensitized T Lymphoblasts. There is relatively little direct information on freshly sensitized, antigen-specific T lymphoblasts, primarily because these cells have not been available for study. Alloreactive T blasts can be isolated in substantial yield and purity by using DC as a "cellular immunoadsorbent." Virtually all the DC in primary MLR cultures formed clusters with T cells (Fig. 1). ~5–10% of total T cells aggregated, which is in keeping with data on the frequency of T cells specific for allogeneic MHC (e.g., ref. 15). The nonclustered cells were depleted of specific alloreactivity (Fig. 2) even though a dose of only 1% DC was used to induce clustering. Isolated clusters generated large numbers of T blasts that were restricted to the original stimulating MHC and showed little response to third-party cells (Figs. 2 and 7). Growth of the blasts upon antigen restimulation was efficient, such that cultures expanded with a generation time of 14–18 h (Fig. 3). The population seemed homogeneous in

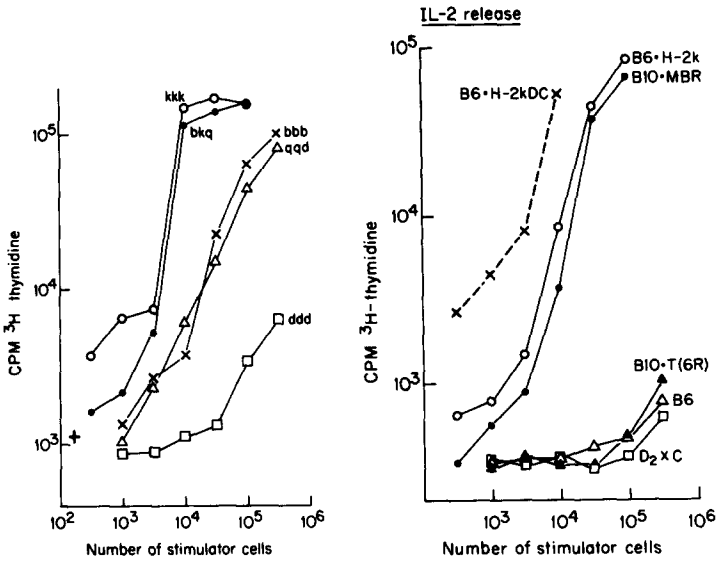

FIGURE 7. Specificity of the response of alloreactive T blasts. C × D2 F_1 α-B6.H-2k T blasts were cultured with irradiated B6.H-2k DC (×), or fresh splenic B cells from various strains, as shown. The haplotype of the MHC K, I, D regions are given on the left. T cell proliferation was measured at 26–41 h (*left*); IL-2 activity in the culture medium at 26 h was measured in a bioassay (*right*). Since the T blasts showed little reactivity to third-party stimulators, and since B6.H-2k and B10.MBR stimulate with similar efficacy, the responsiveness of the T blasts was directed primarily to I^k.

its capacity to recognize antigen, since most of the T cells quickly aggregated with lymphocytes from the appropriate MHC (Table II). We concentrated on the Lyt-2^- helper subpopulation, but the cytolytic Lyt-2^+ subset also entered the clusters (Table I).

Comparable populations of specific lymphoblasts might have been retrieved by using bulk 4-d primary MLR cultures. For example, the blasts could have been floated on dense albumin columns (16); copurified DC could then have been removed with α-Ia antibody and complement. However, we focused on the cluster approach, to help establish that direct interaction between DC and T cells led to the production of MHC-restricted T blasts, and to avoid potential contamination by nonspecific cells responding to the IL-2 released from specific DC/T cell clusters. It is probable that T cells responding to antigen and self MHC products would also cluster, but in this instance, the blasts would be contaminated with many cells stimulated in the syngeneic MLR (3).

The retrieval of T cell clones by chronic restimulation with antigen and lymphokines provides sensitized populations that are highly specific, rather than oligoclonal, as were the cells we studied. However, long periods of growth are required to obtain sufficient numbers of cells. Clones represent a small sample of the stimulated population, and the cloning procedure could apply considerable selection pressure on the very parameters one wishes to study, such as the efficacy of antigen recognition and the capacity to release lymphokines. Therefore, there

TABLE III

Alloreactive T Blasts Release B Cell Helper Factors After Restimulation with Allogeneic Cells or with IL-2

Allogeneic cells as stimulators:

Cell dose	Helper factor (PFC) from T blasts stimulated by:		
	Dendritic cells	B cells	LPS-B blasts
3×10^6	—	740	—
1×10^6	—	336	1,176
3×10^5	—	188	988
1×10^5	1,480	4	1,084
3×10^4	528	4	176
1×10^4	156	—	0
None		0	

IL-2 as stimulator:

IL-2 dose	IL-2 only	Il-2 + DC/T supernatant	IL-2 + supernatant from T blasts	T blasts treated with IL-2
1,000	5	2,150	4	—
300	5	1,970	4	—
100	0	2,150	0	920
30	0	1,970	—	84
10	—	1,940	—	8
0	—	1,955	0	0

2×10^5 Lyt-2^- C × D2 F_1 α-B6.H-2k T blasts were stimulated with different populations of allogeneic, irradiated (750 rad) stimulator cells or with recombinant IL-2. The conditioned medium was collected at 24 h and assayed for helper activity in the anti-SRBC PFC response of purified B cells. The data shown are for 40% vol/vol doses of conditioned medium, but 20% vol/vol doses gave qualitatively similar results. Stimulator cells alone did not release helper factors, and irradiated (3,000 rad) T blasts released higher (50–200%) levels of factor (neither shown). In the second part, we tested media containing IL-2 only; IL-2 mixed with helper factors released by DC/T cell allo-MLR clusters; IL-2 mixed with the conditioned medium from T blasts cultured in the absence of stimulator cells; and conditioned medium from T blasts cultured with graded doses of IL-2.

are potential drawbacks in the use of chronically restimulated clones as models for T blasts that are freshly sensitized during cell-mediated immune responses.

Sensitized Helper T Blasts Must be Restimulated by Antigen to Function. An important feature of sensitized T cells is their lack of function in the absence of antigen. Isolated Lyt-2^- blasts placed in fresh medium neither grew nor released lymphokine. Once challenged, however, the blasts rapidly aggregated with antigen-specific cells (Table II). Growth resumed immediately (Fig. 3), and IL-2 and B cell helper factor release was apparent in 6 h (not shown). Blasts generated in the syngeneic MLR (3) and with lectins (17) also required restimulation to trigger lymphokine release. In one sense the helper cell is analogous to the cytolytic T cell, in that it exhibits little function or "help" constitutively, but instead must reencounter antigen. The cytolytic T cell probably stores its lysin in preformed granules (18), whereas there seems to be no storage of preformed lymphokines. This has been studied recently by Piperno, et al., who found that

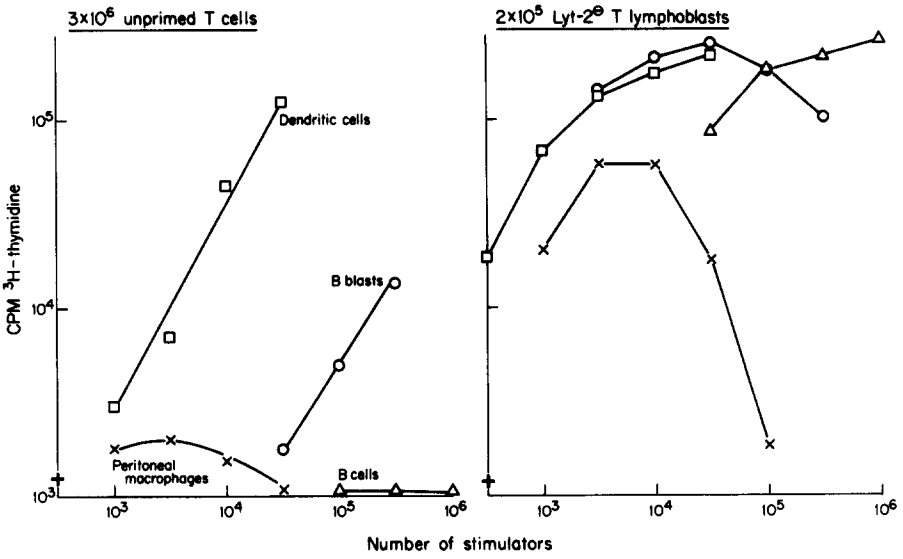

FIGURE 8. Macrophages stimulate Lyt-2⁻ T blasts. Adherent peritoneal macrophages (×), which had been cultured for 1 d in immune interferon (100 U/ml), were compared with DC (□) and fresh splenic B cells (△) as stimulators of Lyt-2⁻ T blasts. [³H]thymidine uptake was measured at 28–42 h. Background proliferation is shown (+).

the release of lymphokines from mitogen-induced blasts requires new lymphokine mRNA and protein synthesis (17).

Antigen-presenting Cell Requirements of Resting and Sensitized T Cells. In the primary MLR, resting or unprimed T cells respond vigorously to DC but not to other leukocytes (reviewed in 12, 19). A striking property of sensitized T cells is that restimulation occurs with many kinds of allogeneic leukocytes, including B cells, B blasts, and macrophages (Figs. 5–8). DC are still the most potent stimulators of the T blasts. It is known that DC express 2 and 10–20 times as much Ia as B blasts and fresh spleen B cells, respectively (8), and this relationship persists in terms of stimulating capacity for allogeneic helper T blasts (Figs. 5–8).

As a consequence of its interaction with B cells, the T blast can initiate antibody responses and release B cell helper factors (Table III, V, and VI). Similar events have been observed in long-term alloreactive T cell lines (20, 21). The capacity of T blasts to interact directly with B cells helps explain recent data on dendritic-T-B clustering during T-dependent antibody responses. Antigen-specific B cells of the appropriate MHC entered dendritic–T cell aggregates, became responsive to lymphokines released therein, and developed into PFC (4). We observed that PFC development was abolished if DC were removed from the clusters on day 1, but not on day 2. We would reason that, by day 2, the clusters contained sensitized, MHC-restricted T cells capable of directly stimulating B cells to become PFC.

The distinct stimulatory requirements of resting and sensitized T cells are important when comparing the functional properties of DC with other leukocytes. If the responding population includes primed cells, then there may be no

TABLE IV

Aldehyde-fixed Cells More Actively Stimulate Alloreactive T Blasts Than Resting T Cells

Exp.	Stimulators*	MLR‡	Fixative§	Proliferation with stimulators fixed in:				
				0.3%	0.1%	0.03%	0.01%	None
						cpm		
1	LODAC	1°	F	706	9,641	18,422	48,996	79,696
		2°	F	50,762	67,485	123,973	—	139,116
		1°	G	182	155	157	373	
		2°	G	2,218	3,412	16,032	83,391	
2	Macrophages + DC	1°	F	316	447	573	6,757	38,720
		2°	F	6,592	8,697	16,206	54,149	67,436
		1°	G	318	317	307	317	
		2°	G	—	6,353	17,515	38,223	
	Macrophages	1°	F	318	310	356	717	5,919
		2°	F	4,259	6,452	10,551	25,256	34,284
		1°	G	261	321	308	323	
		2°	G	—	4,579	9,592	19,524	

* In Exp. 1, the stimulators were 10^4 B10.BR ($K^kI^kD^k$) low density adherent spleen cells (LODAC); this dose is well above the saturating dose (e.g., Figs. 5–8). In Exp. 2, the stimulators were 10^5 B10.MBR ($K^bI^kD^q$) spleen adherent cells that had been prepared in the presence of complement only (~80% macrophages and ~20% DC) or in 33D1 anti-DC antibody and complement (macrophages primarily, ref. 12).

‡ Primary (1°) MLR (with 2×10^5 unprimed C × D2 F_1 T cells) were pulsed at 96–112 h, and secondary (2°) MLR (with 1×10^4 Lyt-2^- C × D2 α-B6.H-2k T blasts) at 28–42 h. Nylon T cells had cpm of only 258 and 336 in the 1° MLRs and 1,702 and 1,552 in the blast cultures.

§ Cells that were adherent to round-bottomed microtest wells were fixed for 10 min at room temperature with graded doses of formaldehyde (*F*) (made from formalin solution in Exp. 1 and paraformaldehyde in Exp. 2) or glutaraldehyde (*G*) (Polysciences, Inc.) and washed twice each in phosphate-buffered saline and RPMI 1640.

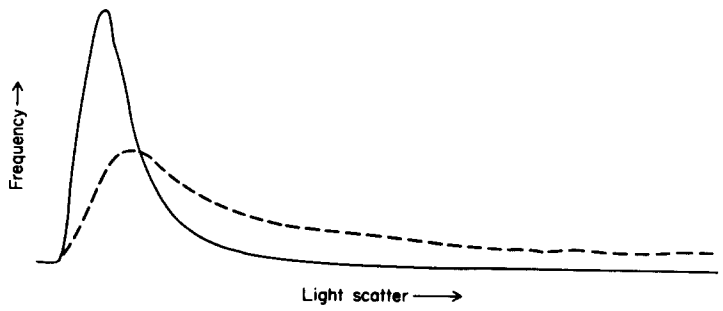

FIGURE 9. B cell responses to T blasts. Macrocultures were set up with 3×10^6 B cells from various strains with or without 10^5 C × D2 F_1 α-B6.H-2k, irradiated (3,000 rad) T blasts. At 48 h, cell volumes were measured by light scattering using an Orthocytofluorograph IV. Viable cell yields were $0.5–1.5 \times 10^6$ cells per culture and 30,000 cells were counted. (——) Profile shown by B6.H-2k B cells; similar profiles were obtained with B cells from H-2d and H-2b mice, even when cultured with 10^5 T blasts. (– – –) Profile of B6.H-2k B cells cultured with10^5 alloreactive blasts; similar results were obtained with B10.MBR B cells and T blasts.

TABLE V
Alloreactive Lyt-2⁻ T Blasts Induce B Cell Proliferation

H-2d α-H-2k T blasts		Strain of B cells responding to T blasts			
Type	Number	C × D2 (ddd)	B6.H-2k (kkk)	B10.MBR (bkq)	C57Bl/6 (bbb)
Lyt-2⁻T	3×10^4	5,408	66,828	75,824	20,158
	10^4	2,266	73,517	50,995	8,855
	3×10^3	1,498	36,054	22,231	1,718
	10^3	1,366	11,112	8,213	1,211
	3×10^2	1,388	5,192	2,940	1,211
Lyt-1⁻T	3×10^4	1,109	429	1,309	1,045
	10^4	1,292	533	1,192	978
	3×10^3	1,248	823	1,034	924
	10^3	1,302	893	917	957
	3×10^2	1,283	1,027	955	863
None		1,205	783	886	934

2×10^5 B cells were cultured with graded doses of irradiated (3,000 rad) C × D2 F_1 α-B6.H-2k T blasts that had been treated with α-Lyt-1.2 or 2.2 mAb and complement. Proliferation was measured 48–60 h with a 4 μCi/ml pulse of [^{3}H]thymidine.

requirement for DC, except that stimulators bearing more of the relevant MHC products might be expected to function more effectively. If the response involves resting T cells—as in the primary MLR (11, 12, 22), graft rejection in situ (23, 24), oxidative mitogenesis (22, 25, 26), contact sensitivity (27), or "memory" lymphocytes (22)—then there is a marked catalytic effect exerted by DC. If a primary response is followed for a long period, a mixture of phenomena could occur. DC could initiate a response, then other leukocytes could trigger the sensitized T cell. This hypothesis would explain recent data that macrophages do not stimulate the MLR but can synergize with small numbers of DC (28).

The literature frequently groups DC with other "antigen-presenting" Ia$^+$ cells. Indeed, many cell types express the needed MHC molecules that are recognized by T cell receptors for allo-MHC, or for MHC plus nominal antigen. In the latter case, recognition of antigen plus cell-associated MHC products may not require any active role by the presenting cell, i.e., the cell bearing the MHC products. The presentation event even occurs with fixed cells (13, 14, and Table IV). The biologic significance of DC may be obscured by the use of a single term—antigen-presenting cell—to encompass all the events required for immune stimulation. The capacity of the DC to initiate responses in helper cells seems to be its most distinctive role. Once sensitized, T blasts can interact with other leukocytes to propagate the various effector components of cell-mediated immunity.

The T-B binding studies which we have performed (Table II) provide a clue to a major difference between the resting and sensitized T cell. We have never observed clustering of unprimed T with allogeneic B cells. Yet the T blast quickly aggregated small B cells in an antigen-specific manner and at 4°C. The simplest explanation for this finding is that unprimed T cells express few functional and/or suitably disposed receptors for antigen. Perhaps DC have a unique capacity

TABLE VI

Alloreactive T Blasts Induce Polyclonal B Cell Responses

Group	B cells (2×10^6)	α-B6.H-2k Lyt-2⁻ T blasts	C × D2 fresh T	Syngeneic supernatant, or 10^4 DC	α-TNP-PFC +/− TNP-KLH	α-Sheep PFC +/− SRBC
1	B6.H-2k	—	—	—	25/50	0/0
2		—	10^6	—	35/60	0/0
3		—	10^6	H-2k DC	1,035/60	1,340/30
4		—	10^6	H-2d DC	390/75	300/5
5		—	—	40% supernatant	0/0	1,560/0
6		10^5	—	—	2,755/480	3,080/470
7		3×10^4	—	—	4,735/1,800	4,600/780
8		10^4	—	—	4,105/1,530	1,630/190
9		3×10^3	—	—	1,905/740	385/45
1	C × D2	—	—	—	55/45	0/0
2		—	10^6	—	75/55	0/0
3		—	10^6	H-2k DC	400/125	1,175/5
4		—	10^6	H-2d DC	1,535/95	1,695/0
5		—	—	40% supernatant	45/35	1,365/0
6		10^5	—	—	270/145	0/0
7		3×10^4	—	—	116/55	0/0
8		10^4	—	—	125/70	5/5
9		3×10^3	—	—	35/40	5/0
10		10^5	—	H-2k DC	380/220	1,150/35
11		10^5	—	H-2d DC	275/135	15/5
1	B10.T(6R)	—	—	—	25/5	0/0
5		—	—	40% supernatant	15/10	785/5
6		10^5	—	—	1,320/450	655/130
7		3×10^4	—	—	865/160	421/25
8		10^4	—	—	105/5	20/0
9		3×10^3	—	—	5/0	0/0
10		10^5	—	H-2k DC	3,180/955	935/225
11		10^5	—	H-2d DC	1,560/525	615/220

Allospecific C × D2 F_1 α-B6.H-2k Lyt-2⁻, irradiated (3,000 rad) T blasts induce polyclonal antibody responses. 2×10^6 B cells from B6.H-2k, C × D2, and third-party B10.T(6R) were placed in macroculture with, or without (+/−) antigen (5 μg/ml TNP-KLH or 2×10^6 SRBC). α-TNP and α-SRBC PFC were measured on day 4. Groups 1–5 describe the capacity of B cells to generate primary antibody responses under typical conditions while groups 6–11 describe responses to T blasts. The purified B cells, as expected, made no response to antigen alone (group 1), or antigen and 10^6 unprimed T cells (group 2). Responses were obtained when unprimed T cells and 10^4 DC were present (compare groups 3 and 4). Addition of helper factors derived from DC/T-conditioned medium (syngeneic MLR supernatants, group 5) enhanced the α-sheep but not the α-TNP PFC responses as described previously (4). When C × D2 α-B6.H-2k T blasts were added to B cells (groups 6–9), large antibody responses were obtained with B6.H-2k B cells but not with C × D2 B cells. B10.T(6R) third-party B cells were <1/50th as responsive to T blasts as B6.H-2k B cells. The PFC responses in B6.H-2k B cells were polyclonal, since antigen was not required. However, antigen enhanced the efficacy of the T blasts ~10-fold (compare groups 6–9). The α-TNP response in B6.H-2k was probably initiated by a direct interaction between T blast and B cell, rather than by soluble factors, since stimulation of the T blasts with H-2k DC (group 10) helped H-2d cells make α-sheep but not α-TNP responses.

to induce resting T cells to express functioning clonotypic or antigen receptors, in concert with the capacity to induce responsiveness to IL-2 (25).

Summary

Previous studies have shown that unprimed or resting T lymphocytes will grow and release lymphokines when stimulated by dendritic cells (DC). We now have

examined the stimulatory requirements for antigen-primed or blast-transformed T cells. The latter were derived from dendritic/T cell clusters that developed during the primary mixed leukocyte reaction (MLR). The specificity of the blasts was established by a binding assay in which most T cells aggregated small B lymphocytes of the appropriate haplotype within 2 h at 4 or 37°C. Since unprimed T cells did not aggregate allogeneic B cells, we suggest that DC induce T lymphocytes to express additional functioning receptors for antigen.

Lyt-2$^-$ T blasts did not grow or release interleukin 2 or B cell helper factors unless rechallenged with specific alloantigen, whereupon growth (generation time of 14–18 h) and lymphokine release rapidly resumed. The blasts could be stimulated by allogeneic macrophages, B cells, and B lymphoblasts, whereas the primary MLR was initiated primarily by DC. Responsiveness appeared restricted to the I region of the major histocompatibility complex, and varied directly with the level of Ia antigens on the stimulator cells.

The interaction of B cells and T blasts was bidirectional. The T blasts would grow and form B cell helper factors, while the B cells grew and secreted antibody. However, the efficacy of T cell–mediated antibody formation was enhanced some 10-fold by the addition of specific antigen.

Therefore, responses of resting helper T cells, then, are initiated by antigen plus DC. Once sensitized, T blasts interact independently with antigen presented by other leukocytes.

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References

1. Steinman, R. M., W. C. Van Voorhis, and D. M. Spalding. Dendritic cells. *In* Handbook of Experimental Immunology, D. M. Weir, L. Herzenberg, and L. Herzenberg, editors. Blackwell Scientific Publications, Oxford. Fourth ed. In press.
2. Inaba, K., R. M. Steinman, W. C. Van Voorhis, and S. Muramatsu. 1983. Dendritic cells are critical accessory cells for thymus-dependent antibody responses in mouse and in man. *Proc. Natl. Acad. Sci. USA.* 80:6041.
3. Inaba, K., A. Granelli-Piperno, and R. M. Steinman. 1983. Dendritic cells induce T lymphocytes to release B cell-stimulating factors by an interleukin 2-dependent mechanism. *J. Exp. Med.* 158:2040.
4. Inaba, K., M. D. Witmer, and R. M. Steinman. 1984. Clustering of dendritic cells, helper T lymphocytes, and histocompatible B cells during primary antibody responses in vitro. *J. Exp. Med.* 160:858.
5. Katz, D. H., T. Hamaoka, and B. Benacerraf. 1973. Cell interactions between histocompatible T and B lymphocytes. II. Failure of physiologic cooperative interactions between T and B lymphocytes from allogeneic donor strains in humoral response to hapten-protein conjugates. *J. Exp. Med.* 137:1405.
6. Sprent, J. 1978. Restricted helper function of F_1 hybrid T cells positively selected to heterologous erythrocytes in irradiated parental stain mice. II. Evidence for restrictions affecting helper cell induction and T-B collaboration, both mapping to the *K*-end of the *H-2* complex. *J. Exp. Med.* 147:1159.

7. 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.
8. Nussenzweig, M. C., R. M. Steinman, J. C. Unkeless, M. D. Witmer, B. Gutchinov, and Z. A. Cohn. 1981. Studies of the cell surface of mouse dendritic cells and other leukocytes. *J. Exp. Med.* 154:168.
9. Taswell, C., H. R. MacDonald, and J.-C. Cerottini. 1980. Clonal analysis of cytolytic T lymphocyte specificity. I. Phenotypically distinct sets of clones as the cellular basis of cross-reactivity to alloantigens. *J. Exp. Med.* 151:1372.
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., and M. D. Witmer. 1978. Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice. *Proc. Natl. Acad. Sci. USA.* 75:5132.
12. 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.
13. Shimonkevitz, R., J. Kappler, P. Marrack, and H. Grey. 1983. Antigen recognition by H-2 restricted T cells. I. Cell-free antigen processing. *J. Exp. Med.* 158:303.
14. Allen, P. M., and E. R. Unanue. 1984. Differential requirements for antigen processing by macrophages for lysozyme-specific T cell hybridomas. *J. Immunol.* 132:1077.
15. Ford, W. L., S. J. Simmonds, and R. C. Atkins. 1976. Early cellular events in a systemic graft-vs-host reaction. II. Autoradiographic estimates of the frequency of donor lymphocytes which respond to each Ag-B determined antigenic complex. *J. Exp. Med.*141:681.
16. Steinman, R. M., B. G. Machtinger, J. Fried, and Z. A. Cohn. 1978. Mouse spleen lymphoblasts generated in vitro: Recovery in high yield and purity following floatation in dense bovine plasma albumin solutions. *J. Exp. Med.* 147:279.
17. Granelli-Piperno, A., K. Inaba, and R. M. Steinman. 1984. Stimulation of lymphokine release from T lymphoblasts: Requirement for mRNA synthesis and inhibition by Cyclosporin A. *J. Exp. Med.* 160:1792.
18. Henkart, P. A., P. J. Millard, C. W. Reynolds, and M. P. Henkart. 1984. Cytolytic activity of purified cytoplasmic granules from cytotoxic rat large granular lymphocyte tumors. *J. Exp. Med.* 160:75.
19. 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. *Trans. Proc.* 15:299.
20. Augustin, A. A., and A. Coutinho. 1980. Specific T helper cells that activate B cells polyclonally. In vitro enrichment and cooperative function. *J. Exp. Med.* 151:587.
21. Waterfield, J. D., G. Dennert, S. L. Swain, and R. W. Dutton. 1979. Continuously proliferating allospecific T cells. I. Specificity of cooperation with allogeneic B cells in the humoral antibody response to sheep erythrocytes. *J. Exp. Med.* 149:808.
22. Van Voorhis, W. C., J. Valinsky, E. Hoffman, J. Luban, L. S. Hair, and R. M. Steinman. 1983. The relative efficacy of human monocytes and dendritic cells as accessory cells for T cell replication. *J. Exp. Med.* 158:174.
23. Lechler, R. I., and J. R. Batchelor. 1982. Restoration of immunogenicity to passenger cell-depleted kidney allografts by the addition of donor strain dendritic cells. *J. Exp. Med.* 155:31.
24. Faustman, D., R. M. Steinman, H. Gebel, V. Hauptfeld, J. Davie, and P. Lacy. 1984. Prevention of rejection of murine islet allografts by pretreatment with anti-dendritic cell antibody. *Proc. Natl. Acad. Sci. USA.* 81:3864.

25. Austyn, J. M., R. M. Steinman, D. E. Weinstein, A. Granelli-Piperno, and M. A. Palladino. 1983. Dendritic cells initiate a two-stage mechanism for T lymphocyte proliferation. *J. Exp. Med.* 157:1101.
26. Klinkert, W. E. F., J. H. LaBadie, and W. E. Bowers. 1982. Accessory and stimulating properties of dendritic cells and macrophages isolated from various rat tissues. *J. Exp. Med.* 156:1.
27. Britz, J. S., P. W. Askenase, W. Ptak, R. M. Steinman, and R. K. Gershon. 1982. Specialized antigen-presenting cells. Splenic dendritic cells, and peritoneal-exudate cells induced by mycobacteria activate effector T cells that are resistant to suppression. *J. Exp. Med.* 155:1344.
28. Naito, K., S. Komatsubara, J. Kawai, K. Mori, and S. Muramatsu. 1984. Role of macrophages as modulators but not as stimulators in primary mixed leukocyte reactions. *Cell. Immunol.* 88:361.