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Immunology

Properties of memory T lymphocytes isolated from the mixed leukocyte reaction

(dendritic cells/antigen-presenting cells/helper lymphocytes/macrophages)

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ABSTRACT **During the primary mixed leukocyte reaction, T lymphocytes of the lyt-2⁻ helper subclass proliferate in response to transplantation antigens on allogeneic dendritic cells. We have isolated populations of antigen-specific proliferating lymphoblasts and recultured them in fresh medium. Within 2 days, the blasts become smaller in size, lose responsiveness to T-cell growth factor or interleukin 2, but retain vigorous reactivity to the original alloantigen. Two new biologic properties of these "memory" lymphocytes have been noted. First, they primarily respond to alloantigen on dendritic cells, whereas freshly sensitized lymphoblasts react to allogeneic dendritic cells, macrophages, and B lymphocytes. Second, the memory lymphocytes quickly aggregate with dendritic cells that are either syngeneic or allogeneic, but not with B cells. The aggregates that form with syngeneic dendritic cells disassemble within hours and do not release interleukin 2 or proliferate. The aggregates that form with allogeneic dendritic cells remain intact, release large amounts of interleukin 2 on the first day of culture, and synthesize DNA on the second day. Therefore, dendritic cells actively cluster memory lymphocytes by an antigen-independent mechanism, and this may underlie the heightened functional activity of each cell type.**

During the rejection of foreign tissue, the T lymphocytes of one individual respond to alloantigen presented by cells of another individual, who differs at genes of the major histocompatibility complex. A similar T-cell response occurs *in vitro* in the mixed leukocyte reaction (MLR). Since 1978, evidence has been accumulating that a particular class of leukocytes, termed dendritic cells (DC), are the principal stimulator of the MLR (1). When alloantigens encoded in the major histocompatibility complex (H-2 and Ia in the mouse) are presented by DC, a strong MLR occurs, whereas alloantigens on other leukocytes are less stimulatory or inactive (2–6). Likewise, selective removal of DC markedly reduces stimulatory function (3), whereas removal of other cell types has little or no effect (2, 6). Analogous findings have been made for the induction of certain allograft responses *in situ* (7–9) and other T-cell responses *in vitro* (4, 10–15).

An early manifestation of DC function in culture is the formation of helper T lymphoblasts that can release and respond to T-cell growth factor or interleukin 2 (IL-2; refs. 14–16). We have previously isolated these lyt-2⁻ lymphoblasts from the MLR and have shown that they can interact with and respond to other types of Ia⁺ allogeneic leukocyte, such as macrophages and B lymphocytes (16). Therefore, the weak MLR stimulatory capacity of the latter cells is not due to a failure of antigen presentation. Rather, the outcome of presentation seems to be influenced by the type of presenting cell and the physiologic state of the T cell. Here we extend our findings to secondary or "memory" helper lymphocytes, which we define as lymphoblasts that revert to smaller IL-2 unresponsive cells when the antigenic stimulus is removed. We will describe a method for isolating memory cells from the MLR and report several distinctive features in their response to antigenic challenge.

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MATERIALS AND METHODS

Cells. DC were derived from low-density spleen-adherent populations (10, 17). B cells were Sephadex G-10 nonadherent spleen cells that were treated with α-lyt-1 and α-thy-1 and complement to remove T lymphocytes (13). Macrophages were adherent resident peritoneal cells cultured 24 hr with interferon-γ (10 units/ml). T cells were nylon wool-nonadherent spleen and lymph node suspensions that were treated with α-Ia and α-lyt-2 monoclonal antibodies and complement to deplete accessory and cytotoxic cells, respectively (16). Allospecific T lymphoblasts were generated in primary MLRs using B6.H-2k DC and D2 × C F1 (H-2d) T cells (16). By surface immunofluorescence (16), the blast populations were >98% positive for thy-1, lyt-1, and L3T4 antigens and lacked cells reactive with α-Ia, α-Lyt-2, α-B cell, and α-macrophage monoclonal antibodies.

T-Cell Proliferative Assays. MLRs were induced in H-2d lyt-2⁻ unprimed lymphocytes, freshly sensitized lymphoblasts, or primed memory cells (see *Results*). Stimulator cells were irradiated with 900 rads of ^{137}Cs (1 rad = 1.0×10^{-2} gray) and added in graded doses. The culture medium was RPMI 1640 supplemented with 10% fetal calf serum/50 μM 2-mercaptoethanol/20 μg of gentamicin per ml.

IL-2 Bioassay. ConA splenic lymphoblasts were incubated for 18 hr in 100 μl of medium with 50% (vol/vol) test supernatant, and then pulsed for 6 hr with 1.75 μCi (1 Ci = 37 GBq) of [^{3}H]thymidine in 0.025 ml. Data are mean cpm uptake in which the standard deviation was <15%. All uptakes were <50–75% maximum uptake as assessed with saturating levels of recombinant human IL-2 (rIL-2; 100 units/ml; Biogen, Cambridge, MA).

Clustering of T Cells with DC or B Lymphocytes. T cells (10^5) were mixed with 10^4–10^5 DC or B cells in 1 ml of medium in polypropylene tubes (Falcon, 2054) for 1–2 hr at 37°C. Aliquots were observed in a hemocytometer, or by phase-contrast microscopy after transfer into flat-bottomed wells containing 1% glutaraldehyde fixative.

RESULTS

Preparation of Alloreactive Memory T Cells. To prepare memory populations, we first isolated T lymphoblasts from

Abbreviations: MLR, mixed leukocyte reaction; IL-2, interleukin 2; rIL-2, recombinant IL-2; DC, dendritic cells.

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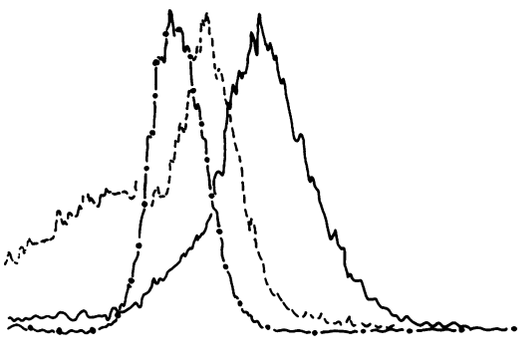

FIG. 1. Relative cell size of the populations used in this study. Abscissa, light scatter; ordinate, particle number for unprimed T lymphocytes (●–●), freshly sensitized blasts (—), and primed memory cells obtained by culturing the blasts for 2 days (----). The latter profile has a shoulder of "dead cells."

the primary MLR as described (16). H-2k DC and allogeneic H-2d lyt-2$^-$ T lymphocytes were cultured for 1–2 days. Cell aggregates, containing most of the DC and antigen-reactive T cells, developed and were separated by velocity sedimentation. During 2–3 more days of culture, many lymphoblasts were released but the DC remained within the aggregates. The released blasts were isolated from the clusters by a second velocity sedimentation. The blasts were antigen specific, as demonstrated by the capacity to cluster with Ia^k but not with Ia^b or Ia^d B cells, and were IL-2 responsive, as demonstrated by cell doubling within 14–18 hr of exposure to exogenous IL-2 (16). The isolated blasts stopped proliferating once the alloantigenic stimulus had been removed. Within 48 hr, most of the cells reverted to medium-sized lymphocytes (Fig. 1). The viability of the cultured cells at 48–72 hr was 50–90% and was improved by coculture with 1% syngeneic peritoneal macrophages (3–10 × 10^3 per 3 × 10^5 blasts in 16-mm wells). On a per cell basis, T cells that had been rested in the presence or absence of macrophages functioned similarly when restimulated (see below).

The medium-sized lymphocytes that were derived from the blasts were termed "memory" cells for two reasons. First, the populations responded rapidly and actively to DC from the priming strain. High levels of IL-2 were released on the first day after restimulation, and proliferation began on the second day (Table 1). Unprimed T cells at day 3 had just begun to respond. Second, the memory cells differed from freshly sensitized lymphoblasts in that there was no response to, or absorption of, exogenous IL-2 (Table 1).

Kinetics and Specificity of Responses by *in Vitro* Primed Memory Cells. The proliferation of H-2d lyt-2$^-$ T cells, which were either unprimed or primed to H-2k (blasts and memory cells), were then compared in more detail. Primed T blasts gave large and rapid (18–24 hr) responses to human rIL-2 and to H-2k DC (Fig. 2 *Upper Left*), and 10^5 cpm of [^{3}H]thymidine uptake corresponded to a doubling of all cultured cells within 18 hr (16). The early IL-2 response of memory cells (blasts that had been rested for 3–7 days) was <1% of that observed with lymphoblasts (Fig. 2 *Upper Left*). Later responses to IL-2 were evident (3–5 days) in memory and unprimed T-cell cultures. These responses could be due to the growth of a few IL-2 responsive "contaminants" and/or the induction of IL-2 responsiveness by factors in culture.

The memory cells were clearly enriched in alloreactivity. Memory cells (3 × 10^4) gave larger and faster responses than unprimed lyt-2$^-$ T cells (3 × 10^5), especially at lower doses of DC (Fig. 2 *Upper*, compare △ and □). The reactivity of memory cells was primarily directed to H-2k and not to self (H-2d) or third party (H-2b), whereas unprimed H-2d T cells responded similarly to H-2k and H-2b DC (Fig. 2 *Lower*).

The relationship between stimulator dose and the proliferative response of primed cells was striking. For blasts, 30–100 DC induced a half-maximal response in 30,000 T cells (56,000 cpm vs. a background without DC of 2280 cpm). For memory cells, 300–1000 DC induced a half-maximal response. Therefore, allorestricted blasts and memory cells can be recovered from the MLR, and both populations are stimulated by small doses of allospecific DC.

Kinetics and Specificity of IL-2 Production by Memory Cells. The level of IL-2 was also monitored in the media of the cultures described above by using a standard bioassay and subsaturating volumes of conditioned medium. Exogenous IL-2 was rapidly consumed by T blasts, but not by unprimed or memory populations (Fig. 3 *Upper Left*; Table 1). When H-2k DC were added to α-H-2k primed memory cells, IL-2 was released into the medium within 3 hr (not shown) and reached high levels at 18 hr prior to the onset of DNA synthesis (Fig. 3; Table 1). Much higher levels of IL-2 were detected in memory cultures relative to lymphoblasts, probably because the former did not bind and consume the IL-2.

Antigen-Presenting Cell Requirements of Memory Cells. The stimulatory capacity of DC was compared to allogeneic peritoneal macrophages and B lymphocytes (Table 2). DC were active stimulators of the primary MLR, while the other

Table 1. Kinetics of T-cell growth and IL-2 release in unprimed and memory T lymphocytes: Comparison of DC and IL-2 as stimuli

T cell and stimuli	[^{3}H]Thymidine uptake, cpm × 10^{-3}					
	T-cell growth			IL-2 in medium		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Unprimed and H-2k DC	0.4	1.3	2.8	0.6	2.6	8.5
Unprimed and H-2d DC	0.2	0.2	0.3	0.6	0.7	0.8
Memory and H-2k DC	0.8	50.2	174.7	44.6	48.1	52.0
Memory and H-2d DC	0.3	0.4	0.8	0.6	0.6	0.9
Unprimed (3 × 10^5)	0.2	0.2	0.2	0.5	0.6	0.6
Unprimed (3 × 10^5) and IL-2 (500 units/ml)	0.3	1.0	1.6	48.5	51.0	51.9
Unprimed (3 × 10^5) and IL-2 (100 units/ml)	0.2	0.6	0.5	14.1	14.9	15.0
Memory (3 × 10^4)	0.3	0.2	0.2	0.6	0.7	0.8
Memory (3 × 10^4) and IL-2 (500 units/ml)	0.3	8.4	13.5	44.8	52.5	53.1
Memory (3 × 10^4) and IL-2 (100 units/ml)	0.2	3.4	6.7	14.9	15.8	13.7
No T cells, IL-2 (500 units/ml)						48.6
No T cells, IL-2 (100 units/ml)						12.5

Growth and IL-2 release of H-2d memory (3 × 10^4) and unprimed (3 × 10^5) T cells cultured in flat-bottomed microtest wells. The stimuli were syngeneic (H-2d) and allogeneic (H-2k) DC or rIL-2. Responses were monitored by proliferation (1 μCi of [^{3}H]thymidine per ml for 16 hr at 18, 42, and 66 hr) and by release of IL-2 into the medium.

leukocytes were weak, as described (2–6). All cell types stimulated the primed lymphoblasts, as reported (16). DC were more potent, but this could be attributed to the higher levels of Ia antigens on DC (18, 19). Memory lymphocytes behaved much like unprimed responders, because DC were the principal stimulating cell.

Clustering of Dendritic Cells and T Lymphocytes. To corroborate the antigen specificity of the memory cells, we performed 1-hr aggregation assays in which T cells were mixed with allogeneic B cells or DC. As noted (16), H-2d α-H-k T blasts did not spontaneously aggregate but did form clusters with H-2k, and not H-2d, B cells. In contrast, both T blasts and memory cells aggregated with either syngeneic or allogeneic DC (Fig. 4). At a DC/T-cell ratio of 1:1, >75% of the T cells and >90% of the DC clustered; at a ratio of 1:100, clustering was clearly detectable. Memory cells did not aggregate with either allogeneic or syngeneic B cells (not shown). DC also aggregated unprimed syngeneic or allogeneic lymphocytes, but the clusters accounted for <20% of the added T cells at a DC/T ratio of 1:10.

Clusters that had been formed between H-2d α-H-2k T cells and syngeneic (H-2d) or allogeneic DC were returned to culture. The allospecific clusters remained intact and exhibited active IL-2 release and proliferation (Table 3). The syngeneic clusters disassembled within hours and no IL-2 release or growth occurred. Clustering and stimulation by DC were unaffected by exposure to 1000 rads of ^{137}Cs. Therefore, DC can cluster T cells by an antigen-nonspecific pathway, but antigen is required for cluster stability and function.

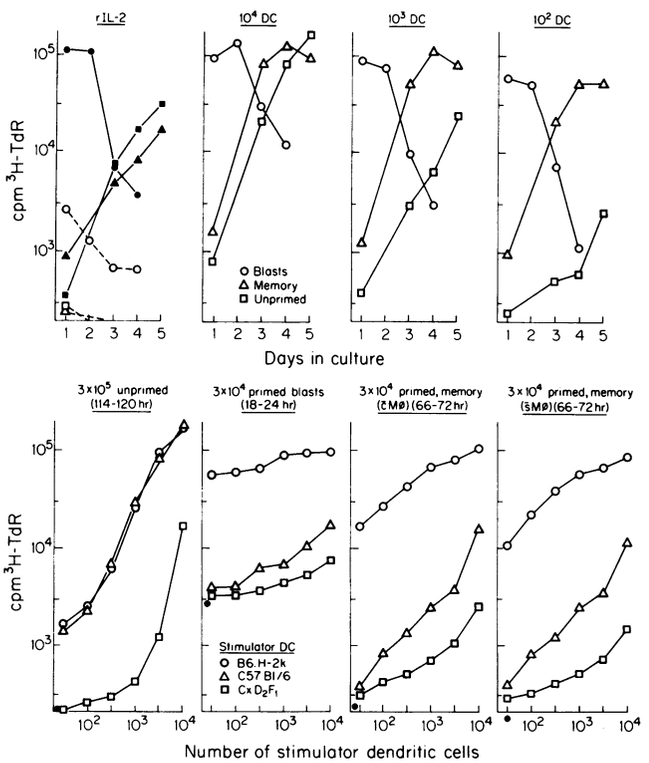

FIG. 2. (*Upper*) Kinetics of cell proliferation in three different C × D2 F1 H-2d, lyt-2⁻ T-cell populations in response to human rIL-2 (100 units/ml) or to different doses of H-2k DC. Closed symbols, rIL-2 responses; open symbols, activities in the absence of IL-2. All data are mean cpm of triplicates given [^{3}H]thymidine (5 μCi/ml) for 6 hr at the end of each day. (*Lower*) Antigenic specificity is plotted for the same T cells as described above, except that an additional panel is added (*Right*) for memory cells that were rested in the absence of feeder macrophages for 2 days. The time at which T-cell proliferation was measured is at the top of each panel. DC from three haplotypes were tested. Background responses in the absence of DC are shown (●).

DISCUSSION

Enriched populations of memory T lymphocytes have been isolated from the MLR. The key steps for enrichment were the isolation of clusters of interacting DC and helper T cells, which provides populations of antigen-specific lymphoblasts (16), and the removal of these blasts from the original DC, whereupon the IL-2 responsive blasts rapidly become IL-2 unresponsive medium-sized lymphocytes.

The memory populations exhibited several noteworthy properties. The first was that memory cells were qualitatively better responders than their unprimed precursors (Figs. 2 and 3; Tables 1 and 2); 3×10^4 cells gave more rapid and/or larger responses, and to smaller doses of DC, than unprimed lymphocytes (3×10^5), which should contain some 10% allospecific precursors (20). This qualitative change in memory cells was appreciated long ago by Wilson and Nowell (21). They noted that populations that had been primed to alloantigens *in situ* exhibited more rapid secondary MLRs *in vitro*. Yet, the frequency of responding units in the primed population had not increased. A second and new finding was that DC were the principal cell type that reactivated the secondary MLR (Table 2). Macrophages and B cells were weak stimulators. This result could not be ascribed to a lack of alloantigen, since the same populations of macrophages and B cells triggered freshly sensitized lymphoblasts across a broad dose range (Table 2; ref. 16). This distinction could be important in interpreting accessory cell requirements, which traditionally have been studied in cells that have been primed *in situ* or chronically stimulated *in vitro* and that may

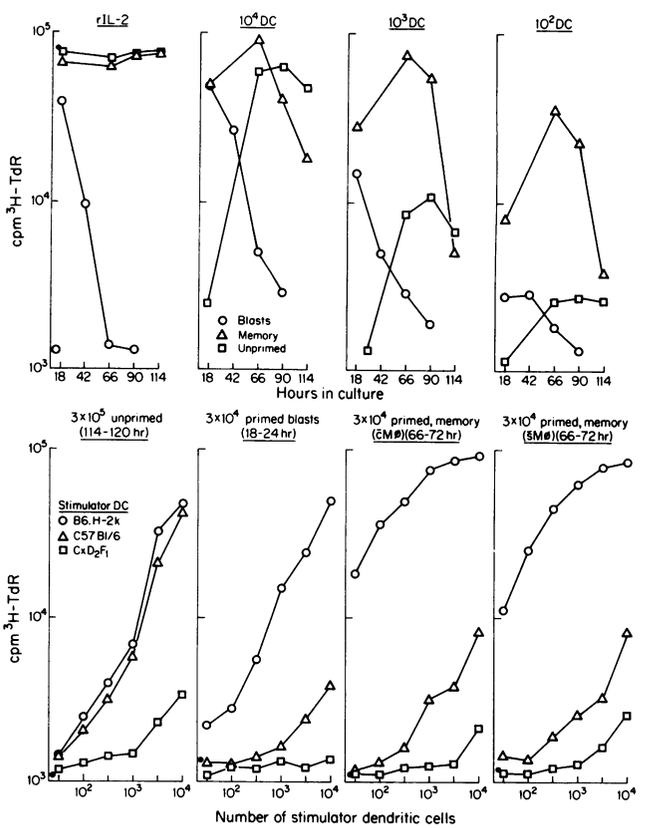

FIG. 3. (*Upper*) Kinetics of IL-2 accumulation in the primary and secondary MLR. This is the same experiment as in Fig. 2, except that samples of the culture medium were applied to an IL-2 bioassay of 2×10^4 ConA lymphoblasts (14) and the IL-2 response is recorded here. Note that T blasts rapidly absorb IL-2 whereas memory and unprimed populations do not. (*Lower*) The antigen specificity of IL-2 release. H-2k DC are the principal stimulators for IL-2 release in primed cells, and only small numbers are required.

Table 2. Antigen-presenting cell requirements of unprimed, freshly sensitized, and memory T cells

T-cell responder	Stimulator Cells	H-2	[^{3}H]Thymidine uptake to graded doses of stimulators, cpm × 10^{-3} 3×10^5	10^5	3×10^4	10^4	3×10^3	10^3	3×10^2	10^2	3×10^1
Unprimed T cell (120–128 hr)	DC	k				280	75.2	13.1	1.8	0.8	0.3
		b				260	77.9	10.7	0.8	0.3	0.3
		d				16.1	1.0	0.3	0.2	0.2	0.2
	Peritoneal macrophage	k			1.0	0.8	0.6	0.4			
		b			0.8	0.6	0.3	0.2			
		d			0.7	0.6	0.3	0.3			
	B lymphocyte	k	0.6	0.4	0.3	0.3					
		b	0.3	0.3	0.2	0.2					
		d	0.3	0.3	0.3	0.3					
Primed T blast (26–34 hr)	DC	k				60.8	52.0	39.6	18.3	14.8	12.3
		b				16.3	4.3	2.2	1.1	1.0	1.0
		d				1.6	1.3	1.0	0.9	1.0	1.0
	Peritoneal macrophage	k			5.0	36.0	31.0	28.0			
		b			8.2	16.2	4.5	1.6			
		d			1.4	1.1	1.1	1.1			
	B lymphocyte	k	45.2	47.4	39.3	31.7	21.3	12.9			
		b	21.9	16.1	8.7	3.2	1.4	1.4			
		d	2.1	1.8	1.1	1.1					
Primed or memory T cell (55–60 hr)	DC	k				95.9	67.3	53.2	23.9	10.7	3.9
		b				12.9	7.9	2.7	1.1	0.6	0.2
		d				2.0	0.8	0.4	0.3	0.2	0.2
	Peritoneal macrophage	k			13.6	4.6	1.6	0.5			
		b			1.3	0.5	0.2	0.2			
		d			0.4	0.2	0.2	0.3			
	B lymphocyte	k	4.5	2.8	1.3	0.7					
		b	0.3	0.3	0.2	0.2					
		d	0.3	0.2	0.2	0.2					

Antigen-presenting cell requirements for unprimed (top), freshly sensitized (middle), and memory (bottom) lyt-2$^-$ T cells. Unprimed (3×10^5) and primed (3×10^4) H-2d anti-H-2k cells were cultured in microtest wells with the indicated doses of irradiated (900 rads) stimulator cells. The latter were from B6.H-2k (specific), C × D2F1 (syngeneic), or C57BL/6 (H-2b, third party) mice (The Trudeau Institute, Saranac Lake, NY). Background counts in the absence of stimulators were 215, 1151, and 227 cpm for unprimed, blast, and memory T cells, respectively. In additional experiments, C3H/He (H-2k) were found to have the same activity as B6.H-2k stimulators.

therefore be mixtures of blasts and memory cells. In effect, DC seem to be needed for the stimulation of IL-2-unresponsive helper T cells—such as unprimed or memory populations—whereas other leukocytes can stimulate IL-2-respon-

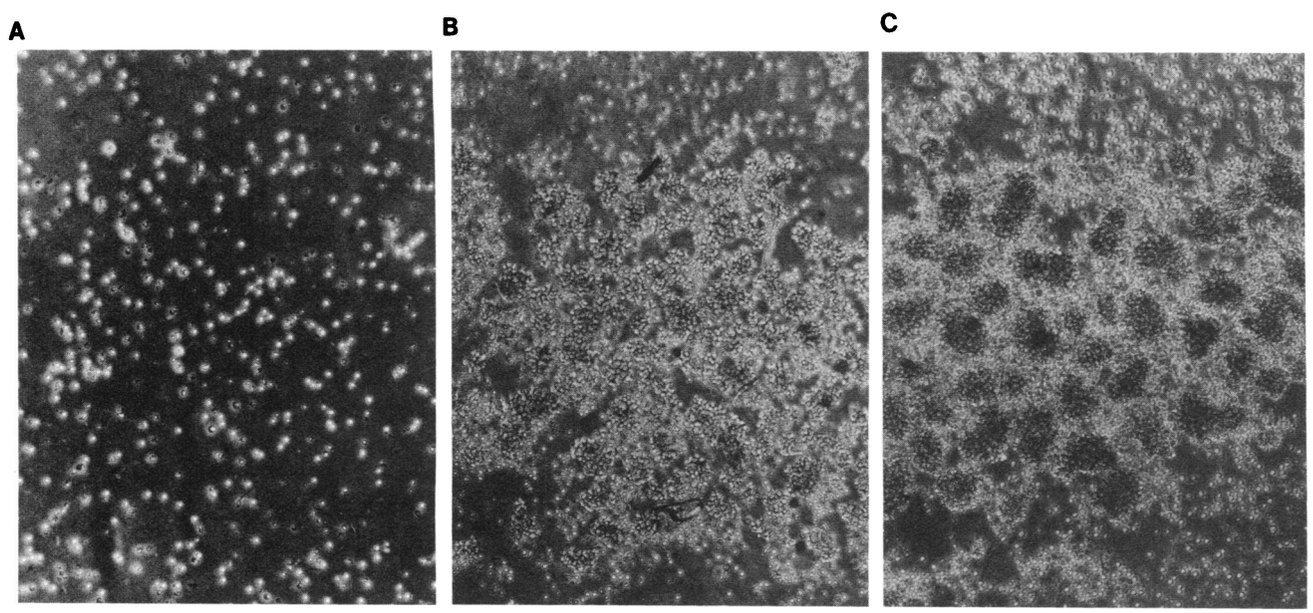

FIG. 4. Micrographs obtained with an inverted phase-contrast microscope showing the antigen-independent T-cell clustering capacity of DC. The latter were mixed with memory T cells (1 and 3×10^5 cells per ml, respectively) in 12 × 50 mm round-bottomed polypropylene tubes for 2 hr at 37°C. The mixtures were resuspended by gently tapping the tube and were transferred to fixative in 16-mm flat-bottomed wells for photography. With both syngeneic and allogeneic DC, the numbers of clusters decreased progressively as the DC/T ratio was lowered to 1:300 (not shown). (*A*) T cells (H-2d anti-H-2k) only. The cells do not cluster and are distributed over the entire culture surface. (*B*) T cells and H-2k DC. Most cells cluster and the clusters quickly move to the center of the culture well, so that the cell density appears large relative to *A*. (*C*) T cells and H-2d DC. Syngeneic DC also cluster the H-2k-specific T cells. (×45.)

Table 3. Kinetics of cell proliferation and IL-2 release by primed (blasts and memory) H-2d anti-H-2k T lymphocytes after clustering with DC

Days in culture	DC		[^{3}H]Thymidine uptake, cpm × 10^{-3}			
			T lymphoblasts (10^3/10^4 DC)		Memory T cells (10^3/10^4 DC)	
	H-2	Irradiated	Growth	IL-2 release	Growth	IL-2 release
1	H-2k	−	47.6/77.0	2.3/25.8	0.5/1.1	25.5/32.5
		+	42.3/65.0	1.2/20.8	0.5/0.6	27.4/47.4
	H-2d	−	4.3/11.5	0.3/1.0	0.2/0.2	0.3/0.7
		+	2.7/10.4	0.3/1.0	0.3/0.2	0.3/0.7
	None		1.3	0.3	0.3	0.3
2	H-2k	−	44.7/113.8	1.1/17.2	10.7/22.2	39.8/47.8
		+	34.3/83.3	1.5/14.8	9.0/21.5	35.0/49.2
	H-2d	−	1.3/10.5	0.5/1.0	0.2/1.3	0.4/0.8
		+	0.7/9.6	0.4/1.0	0.2/0.5	0.3/0.7
	None		0.3	0.3	0.2	0.3
3	H-2k	−	8.5/54.6	0.9/9.3	53.1/94.3	40.7/44.9
		+	5.3/43.6	0.7/8.5	43.4/98.0	35.3/44.8
	H-2d	−	1.4/13.1	0.5/1.1	0.2/1.5	0.3/0.3
		+	0.4/9.9	0.4/1.0	0.2/0.6	0.3/0.4
	None		0.2	0.3	0.2	0.3

Blasts or memory T cells (3×10^5) (H-2d α-H-2k) were allowed to aggregate with 10^5 or 10^4 H-2k or H-2d DC for 2 hr in tubes with 1 ml of medium. Aliquots (0.1 ml) containing many clusters (Fig. 4) were then removed and cultured in microtest wells with an additional 100 μl of medium. DC were exposed or not to 1000 rads of ^{137}Cs. T-cell growth and IL-2 in the medium (cpm of [^{3}H]thymidine uptake) were measured at 18, 42, and 66 hr as in Table 1.

sive blasts. The third and surprising feature was that alloreactive memory cells quickly clustered with both syngeneic and allogeneic DC (Fig. 4). Unlike allospecific DC–T-cell clusters, syngeneic aggregates disassembled quickly and did not release IL-2 or grow (Table 3). Therefore, DC could interact in a reversible antigen-independent fashion with T cells, and this interaction could be stabilized by an antigen/T-cell receptor event. Previous studies of the primary MLR (16) had indicated that clusters that had been isolated at day 2 were enriched in alloreactivity (vs. self and third party). However, a nonspecific interaction could have preceded and/or contributed to the antigen-specific aggregation. Additional studies will further quantitate and characterize the clustering phenomenon, but the capacity of primed lymphocytes to aggregate with DC in an antigen-independent fashion may underlie the accelerated nature of second-set MLRs and graft rejection.

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