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MONOCLONAL ANTIBODIES TO LFA-1 AND TO CD4 INHIBIT THE MIXED LEUKOCYTE REACTION AFTER THE ANTIGEN-DEPENDENT CLUSTERING OF DENDRITIC CELLS AND T LYMPHOCYTES

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Several lymphocyte responses occur in discrete clusters of dendritic cells (DC)¹ and T cells (1–8). The capacity to form these aggregates is not simply the result of antigen presentation and recognition. Macrophages and B cells, which present antigen to primed T cells (3–5), do not cluster resting lymphocytes (1–6). It has been proposed that DC first bind to T cells by an antigen-independent mechanism (5). Antigen-specific lymphocytes are then selected and activated within the DC–T cell cluster.

Lymphocyte function-associated antigen (LFA-1) and CD4 are found on the surface of most helper T lymphocytes and may be accessory molecules for the binding of T lymphocytes to APC, particularly low-avidity T cells (9–11). It is clear that mAb to these molecules inhibit T cell responses (12–16), yet there has been little direct analysis of the idea that anti-LFA-1 or CD4 block APC–T binding in primary systems. Given the prior work identifying DC–T cell interactions in vitro, it is feasible to pursue the mechanism whereby anti-LFA-1 and CD4 inhibit mitogenesis and to determine whether these molecules are required for DC–T clustering.

We find that mAb to LFA-1 and CD4 do not alter the initial clustering of DC with small and large CD4⁺ lymphocytes in the MLR. Both mAb block the function of clusters subsequently. Anti-LFA-1 interferes with the stability of DC–T clusters, whereas anti-CD4 blocks IL-2 release without altering the stability of DC–T conjugates.

Materials and Methods

T Cells. Lymphocytes were prepared from spleen suspensions, or from mixtures of spleen and mesenteric lymph node, from (C × D2)F₁ (H-2 d × d), (B6 × D2)F₁ (H-2 b × d), and B6.H-2k mice (The Trudeau Institute, Saranac Lake, NY). Mice of both sexes, 6–12 wk of age, were used. Unprimed CD4⁺ T cells were nylon wool nonadherent populations that were treated with anti-CD8 (HO 2.2 anti-Lyt-2.2, American Type Culture Collection [ATCC] Rockville, MD) and anti-Ia mAb (B21-2, TIB 229; 10-2.16, TIB 93; or M5/114, TIB 120; ATCC) and fresh rabbit serum (2). Antigen-sensitized T blasts and

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¹ *Abbreviations used in this paper:* DC, dendritic cell; LFA-1, lymphocyte function-associated antigen; Ti, T cell receptor for antigen.

memory cells were obtained from the primary MLR as described (3, 17). The primed T cells were alloantigen-specific in assays that measured APC-T cell binding (5, and see below) and the restimulation of IL-2 release (3, 5, 17).

DC-T Cell Interactions in Primary Responses. Unless stated in Results, MLRs were initiated by culturing 5×10^4 splenic DC with 5×10^6 allogeneic CD4⁺, Ia⁻ T cells in 1 ml of medium in 16-mm-diam tissue culture wells. The medium was 10% FCS-RPMI 1640 supplemented with 5×10^{-5} M 2-ME and 20 μ g/ml gentamicin. mAb were added at the doses and times indicated in Results. At 20–40 h (see Results), the cultures were applied to shallow Percoll gradients to separate cluster and noncluster fractions (2, 3). Isolated clusters were cultured in 6-mm microtest wells (3×10^4 cells in 200 μ l) or in 16-mm macrowells (3×10^5 cells in 1 ml). T cell proliferation was monitored by adding [³H]-TdR (4 μ Ci/ml) for 8–12 h. T cell growth factor in the medium (here termed IL-2 because growth factor activity was inhibited by an anti-IL-2 mAb, unpublished observations) was measured by adding a limiting dose of medium (6–50%, vol/vol) to 3×10^4 Con A-induced blasts in 100 μ l of total volume, and by measuring [³H]TdR uptake at 18–24 h (3, 17). All results are means of triplicates. Standard deviations were 5–20% and are not shown.

To monitor entry of APC into clusters, bulk spleen adherent cells (macrophages and DC) or enriched DC (2) were labeled with a fluorescent, stable, nontoxic carbocyanine dye (dil perchlorate, Molecular Probes, Inc., Junction City, OR) and added to the T cells (18). Spleen adherent cells were obtained by an improved technique developed by Dr. A. Nusrat (The Rockefeller Univ.). Spleen cells ($40\text{--}80 \times 10^7$ nucleated cells) were suspended in 15-ml conical polypropylene tubes (Sarstedt, Inc., Piscataway, NJ) in 5.5 ml of 70% Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) diluted with 3% FCS and PBS. The column was overlaid with 1.5 ml of culture medium and spun to equilibrium (18 min at 1,800 g at 4°C in an RC3b centrifuge [DuPont Sorvall, Newtown, CT]). 30–50% of the white cells floated, and this low-density fraction was harvested, washed twice, and adhered at 10^8 cells/100-mm petri dish. Nonadherent cells were dislodged by gently pipetting over the entire adherent layer. Adherent cells were cultured overnight, whereupon most of the DC were nonadherent, and most macrophages could be dislodged by pipetting. 60–80% of the spleen adherent cells and all of the DC (30% of the total adherent preparation) were stained with the B21-2 mAb to class II MHC antigens (1) and a polyclonal FITC mouse anti-rat Ig (605-540; Boehringer Mannheim Biochemicals, Indianapolis, IN). The adherent cells were labeled with dil, 20 μ g/ml in medium for 1 h at 37°C in 15-ml polypropylene tubes. The cells were reapplied to 16-mm-diam wells for 1 h and separated into readherent (macrophages) and nonadherent (>90% DC) fractions (1). Allogeneic T cells were added and the cultures were followed at daily intervals. The presence of the dye permitted enumeration of APC in cluster and noncluster fractions on a hemocytometer (18), as well as visualization of APC-T cell interactions in live cultures with an inverted microscope equipped for epifluorescence (Diaphot; Nikon, Inc., Garden City, NY).

DC-T Cell Interactions with Antigen-sensitized T Cells. Rapid (10–20 min) binding assays were set up with graded doses of DC and a single dose (usually 10^5) T blasts or memory cells. The latter were derived from the MLR and were tagged with fluorescein diacetate as described (5). DC and T cells were sedimented at 4°C and then kept on ice or at 37°C. 10–60 min later (the results were similar over this time period), the cell pellets were resuspended twice with a 100- μ l Eppendorf pipette. The number of nonclustered T cells were counted, because the DC-T clusters were typically multicellular and difficult to count. DC-T binding was antigen-dependent on ice, because >80% of the blasts bound to allogeneic but not syngeneic or third-party DC. In contrast, binding was antigen-independent at 37°C.

mAb. A large panel of hybridoma culture supernatants (ATCC) were tested for blocking activity in the MLR (see Tables I and V, Results). The culture supernatants contained 50–150 μ g/ml of rat Ig as titered in an ELISA. None of the mAb agglutinated T cells or T lymphoblasts in short- (<1 h) or long- (4 d) term culture. Reactivity of the mAb with DC and with T cells was monitored by indirect immunofluorescence again

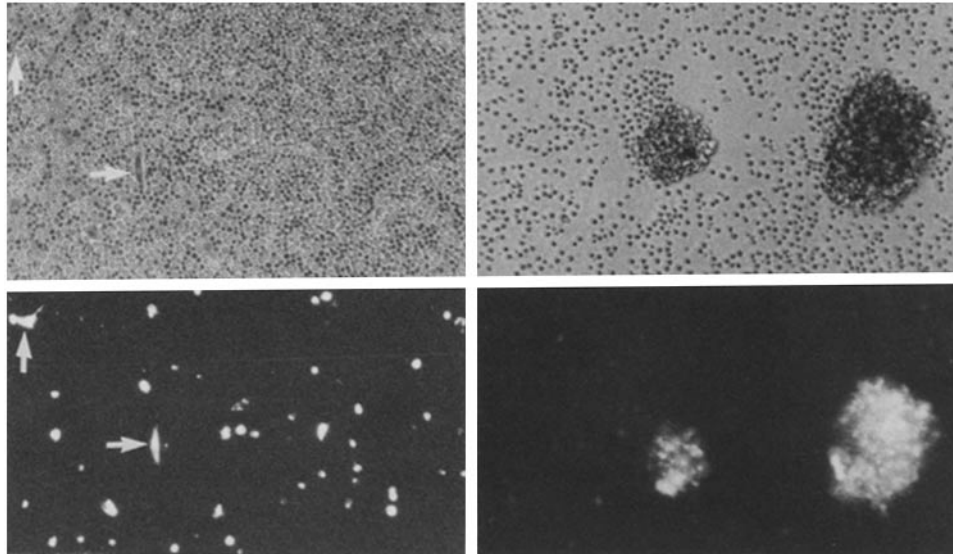


FIGURE 1. Relative capacity of macrophages and DC to aggregate T cells. Macrophages (*left*) and DC (*right*) were labeled with a fluorescent carbocyanine dye and mixed with allogeneic CD4⁺ T lymphocytes. At 1–4 d (day 2 is shown here), the cultures were examined with an inverted microscope by phase-contrast (*top*) and fluorescence (*bottom*) microscopy. Note that the fluorescent macrophages, some of which can be detected by phase contrast (*arrows*), do not form clusters whereas the DC do. ~5% of the clustered cells are DC (see reference 18 and Table I). $\times 100$.

using a polyclonal FITC mouse anti-rat Ig. The mAb to CD4 and to LFA-1 produced optimal staining at ~3%, vol/vol, and stained most small and large lymphocytes in the DC-Lyt-2⁻ MLR. The mAb produced a uniform ring pattern of fluorescence without capping even after 24-h in culture. DC stained strongly with anti-Ia, weakly with anti-LFA-1, but not at all with anti-CD4.

Results

Clustering of CD4⁺ T Cells with DC but Not Macrophages in the MLR. The availability of carbocyanine dyes as stable nontoxic fluorescent labels has made it possible to monitor the formation of APC-T cell clusters more directly than in prior experiments (1–4). When DC were cultured with allogeneic T cells, most of the dye-labeled DC were in DC-T aggregates within 24 h (Fig. 1, *right*) and large numbers of lymphoblasts were produced (3, 5). When allogeneic macrophages were tested, no aggregates were seen during 5 d of culture (Fig. 1, *left*) and no T cell proliferation occurred (3). However, polyclonal mitogens, such as anti-CD3 and Con A, mediated the formation of T cell clusters around most of the macrophages (not shown).

Quantitative studies confirmed that most of the DC in the culture had aggregated with the CD4⁺ T cells within 24 h (Table I, legend). Two observations suggested that the majority of antigen-responsive cells also had aggregated. Mixing clusters and nonclusters did not increase the proliferative response beyond that seen with clusters alone (Table I), and the nonclusters responded normally to rechallenge by a third party but had <10% of the response to the original stimulating DC (see Fig. 2 in Inaba et al. [2]).

TABLE I
Clustering of Dendritic and Antigen-responsive CD4⁺ Cells Early in the MLR

Time of proliferation measurement (h)	Proliferative response (cpm $\times 10^{-5}$)		
	Clusters	Nonclusters	Mix
36-48	40.0	0.8	50.2
60-72	88.4	0.7	69.9
84-96	144.8	0.5	161.1
108-120	59.8	0.7	66.9
132-144	13.5	1.0	22.0

(C \times D2)F₁ DC, labeled with a carbocyanine dye (18), and B6.H-2k Lyt-2⁻ T cells were cultured 24 h in 16-mm wells. The cultures were separated on Percoll velocity gradients into cluster and noncluster fractions representing 9 and 91% of the viable cells per culture. Dye-labeled DC corresponded to 5% of the clustered cells and <0.1% of the nonclusters. 3×10^4 clustered cells, 2×10^5 nonclusters, or mixtures of clusters and nonclusters were cultured in microtest wells at the indicated cell doses. Proliferative activity was measured at daily intervals after a pulse of 1 μ Ci [³H]TdR in 0.2 ml. The first time (36-48 h) is 12-24 h after separating the clusters. The experiment was repeated with similar results in the reciprocal strain combination.

Anti-LFA-1 and CD4 Block Proliferation but Not Cluster Formation in the MLR. A large panel of previously defined mAb were tested as inhibitors of lymphocyte proliferation and cluster development in the MLR. Only mAb to LFA-1, CD4, and class II MHC products inhibited proliferation and IL-2 production (Table II). Higher doses of DC partially overcame the block (Table II). The addition of mAb to LFA-1 and CD4 did not alter the number of clustered DC or T lymphocytes early in the MLR (Fig. 2, *top* and Table III). Therefore it seemed that LFA-1 and CD4 were somehow involved in the MLR, but not in the early stages of DC-T clustering.

Effects of mAb on the Function of DC-T Clusters. To measure the clustering of antigen-specific cells, we first examined the proliferative response of aggregates that had formed in the presence of mAb during the first day of the MLR. If clusters had formed for 24 h in the presence of anti-LFA-1 and were then washed, a normal proliferative response was observed (Table III). Some reduction in proliferative activity was observed if clusters were isolated after 40 h of culture with anti-LFA-1. Therefore anti-LFA-1 did not block the efficient clustering of antigen-specific cells with DC at the onset of the MLR.

When anti-CD4 was added for 24 h and then the cells were washed, clusters that had formed in the presence of mAb exhibited 25-50% of the proliferative activity of controls (Table III). We suspect that anti-CD4 (remaining on the T cells after washing) was acting after, rather than during clustering, because additional experiments to be described below indicated that anti-CD4 did not interfere with the binding of DC to antigen-specific T cells.

Although anti-LFA-1 did not block DC-T cell clustering, aggregates that had formed in the presence of mAb were easily disassembled by gentle pipetting (Fig. 2, *bottom*). The aggregates did not reform upon further culture. Clusters that

TABLE II
Inhibition of T Cell Proliferation and IL-2 Production by mAb Added to the Primary MLR

mAb (antigen/ hybridoma)	Dose (%vol/vol)	Response to graded doses dendritic cells							
		Growth (cpm $\times 10^{-3}$ at 88-96 h)				IL-2 (cpm $\times 10^{-3}$ at 48 h)			
		3×10^4	10^4	3×10^5	10^5	3×10^4	10^4	3×10^5	10^5
Exp. 1: C \times D2 (H-2d) anti-B6.H-2k									
None		60.0	133.1	47.3	14.2	29.5	25.4	2.8	0.5
Anti-CD4 (GK1.5)	40	38.0	37.0	5.9	0.6	1.9	0.6	0.2	0.2
	4	39.5	41.6	6.0	0.8	11.0	2.0	0.2	0.1
Anti-LFA-1 (F441.8)	40	190.5	16.3	1.1	0.5	5.4	0.5	0.2	0.2
	4	244.7	50.0	3.0	1.7	22.0	3.0	0.4	0.2
Anti-I-E (M5/114)	40	58.1	39.0	1.4	0.5	1.6	0.5	0.2	0.1
	4	65.6	49.0	3.6	0.7	5.0	1.0	0.3	0.1
Anti-H-2K (M1/42)	40	74.7	128.4	56.5	15.1	45.2	27.6	2.5	0.2
Exp. 2: B.6 H-2k anti-(C \times D2) _i (H-2d)									
None		298.7	240.7	110.8	19.9	55.9	38.8	9.3	1.1
Anti-CD4 (GK1.5)	25	56.7	20.4	6.1	2.2	2.3	0.5	0.3	0.2
	2.5	101.8	35.3	8.3	3.1	6.6	1.1	0.2	0.2
Anti-LFA-1 (F441.8)	25	51.7	25.1	3.9	1.0	5.0	1.0	0.2	0.1
	2.5	63.9	42.6	12.2	4.0	21.7	10.2	0.3	0.2
Anti-I-A,E (M5/114)	25	18.3	11.0	2.7	0.8	0.3	0.2	0.1	0.1
	2.5	41.9	16.0	4.3	0.6	0.8	0.3	0.1	0.2
Anti-H-2K (M1/42)	25	281.9	245.5	131.6	23.3	35.1	29.4	15.3	0.6
	2.5	289.8	256.4	108.8	27.2	48.4	24.8	7.4	0.5

Graded doses of DC were added to 3×10^5 , Ia⁻ Lyt-2⁻ T cells in the presence of the indicated mAb (hybridoma culture supernatants added at two doses). At 48 h (Exp. 1) or 96 h (Exp. 2), 50 μ l of medium was removed and added to 50 μ l of Con A lymphoblasts to measure growth factor (IL-2) activity. None of the mAb inhibited the effect of IL-2 in the bioassay. At 88-96 h, 1 μ Ci of [³H]TdR was added to monitor the MLR. Maximal response in the IL-2 bioassay (to 10 U/ml human rIL-2) was 92×10^3 cpm. The T cell only responses were <200 cpm. The following mAb (ATCC) were tested but did not reduce or enhance proliferation or IL-2 release: 53-7.1 (CD5); 53-6.7 (CD8); KJ 16 (Ti); 33D1 (DC); M1/70 (C3biR); M1/9.3 (leukocyte); B5-3 (Thy-1).

had formed in the presence of anti-CD4 were not disaggregated by pipetting (Fig. 2, *bottom*).

In the next experiments, clusters were generated in the absence of mAb, and then mAb were added at varying times. Anti-LFA-1 had to be added shortly after cluster formation to achieve a profound block; the block was much reduced if the addition of mAb was delayed by 7 h (Table IV) or 5 h in another experiment (not shown). In each case, the anti-LFA-1 mAb stained the clustered lymphocytes as evaluated by indirect immunofluorescence. In contrast to LFA-1, mAb to CD4 and Ia were markedly inhibitory when added either at 0 or 7 h after cluster formation (Table IV). Whenever the mAb blocked proliferation, there was a marked reduction of IL-2 activity in the medium (not shown).

When isolated clusters were examined in an inverted microscope, many of the DC-T clusters slowly disassembled in the presence of anti-LFA-1. Yet many of the disaggregated cells were large blasts (Fig. 3). Anti-CD4 did not disassemble the clusters, but few lymphoblasts were produced in or around the cluster (Fig. 3).

We conclude that anti-LFA-1 primarily interferes with the stability rather than

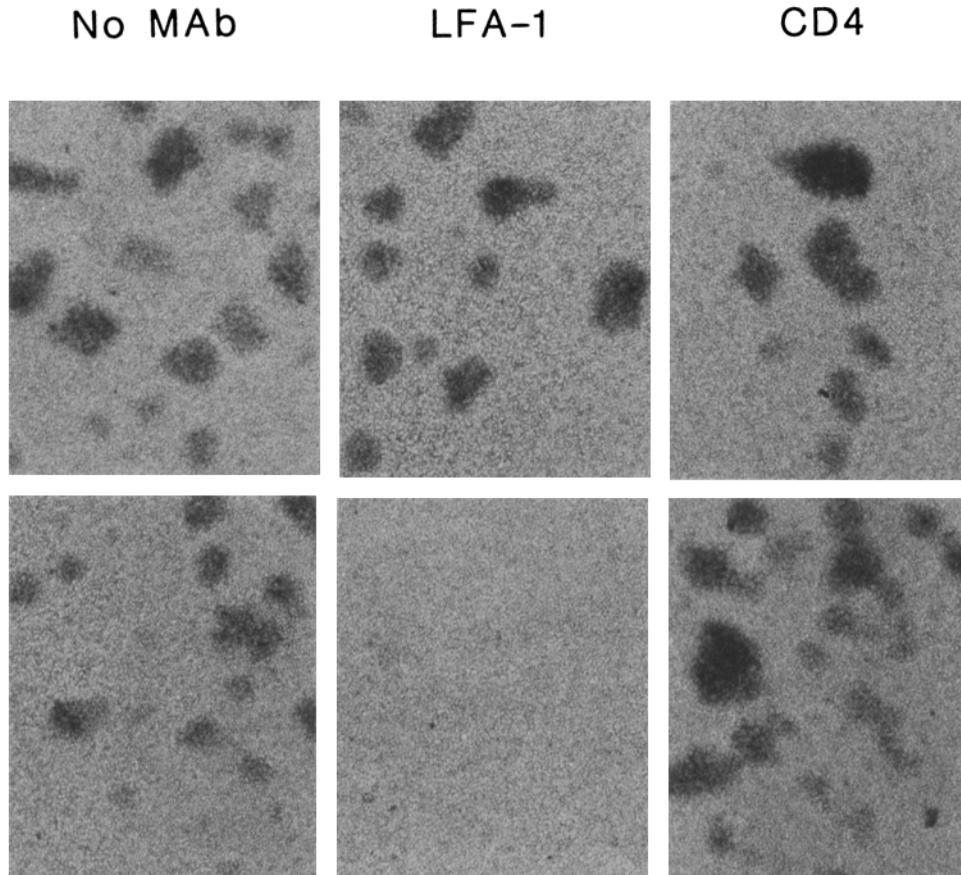


FIGURE 2. Formation of DC-CD4 clusters in the presence of mAb. The top row contains micrographs of the primary MLR at 24 h and shows the normal development of cell clusters in the presence of anti-LFA-1 and -CD4 mAb. Quantitative data on the number of DC and T cells in the cluster fraction are given in Table III. On the bottom is the appearance of the same cultures 1 h after the cells were gently resuspended by aspirating and discharging half of the culture in an Eppendorf pipette three times. The clusters which formed in the presence of anti-LFA-1 were easily dissociated and did not reassemble. $\times 75$.

the formation of the DC-T cluster, whereas anti-CD4 primarily retards blastogenesis and IL-2 release of clustered T cells.

Effects of mAb on the Binding of DC to Sensitized, Antigen-specific T Cells. To study antigen-specific T cells more directly, we isolated blasts from MLR clusters at day 4 and cultured in the absence of IL-2 and APC to provide memory cells (3, 17). Prior work had shown that blasts and memory T cells were >80% antigen-specific in rapid binding assays (5). In these assays, the T cells were tagged with a fluorescent dye and sedimented with graded doses of DC. After an additional 10 min, nonclustered fluorescent cells were counted. mAb were added to T blasts and memory cells for 1 h (either at 4 or 37°C) before mixing with DC, or during the binding assays. The results were similar regardless of the method of antibody addition.

mAb to class II MHC products (reactivity with both I-A and I-E was necessary)

TABLE III
Anti-LFA-1 and Anti-CD4 mAb Do Not Inhibit the Formation of DC-T Clusters Early in the MLR

Stimulating antibody	Total cells $\times 10^{-6}$ (%DC in parentheses)		T cell growth (cpm [^3H]TdR)		
	Clusters	Noncluster	No anti-body	LFA-1	CD4
mAb given at 24-96 h:					
At 0-24 h:					
None	0.60 (4.0)	13.2 (0)	69.7	10.8	4.0
LFA-1	0.52 (5.3)	12.8 (0)	65.1	4.1	2.8
CD4	0.53 (4.3)	13.1 (0)	27.3	3.1	2.7
mAb given at 40-96 h:					
At 0-40 h:					
None	0.68	11.5	125.1	112.2	31.4
LFA-1	0.43	12.2	72.0	6.1	15.6
CD4	0.44	10.9	32.5	3.1	3.1

5×10^4 B6.H-2k DC (labeled with a fluorescent carbocyanine dye, [18]) were used to stimulate 5×10^6 Ia⁻, Lyt-2⁻ H-2d T cells for 24 or 40 h with and without mAb. Cluster and noncluster fractions from a pool of four cultures were isolated by velocity sedimentation. The number of cells (DC + T) and dye-labeled DC were measured in a hemocytometer. 3×10^4 clustered cells were then cultured in microtest wells with and without mAb at 40% (vol/vol). [^3H]TdR uptake was measured at 88-96 h. The experiment was repeated with similar results in the reciprocal strain combination.

TABLE IV
Anti-LFA-1 Has to Be Added Shortly after Cluster Formation to Block T Cell Proliferation

mAb added	[^3H]TdR uptake	
	68-80 h	92-104 h
<i>cpm</i>		
At 20 h:		
None	33.0	78.8
CD4	3.9	6.5
LFA-1	5.1	7.1
Ia	3.3	4.4
At 27 h:		
None	33.0	78.8
CD4	4.1	9.3
LFA-1	22.4	65.2
Ia	7.2	8.5

5×10^4 H-2d DC were used to stimulate 5×10^6 Ia⁻ Lyt-2⁻ H-2k T cells for 20 h. Clusters were isolated and cultured with and without mAb. mAb were added at 20 h (immediately after the isolation of clusters) or 27 h. [^3H]TdR uptake was measured at the indicated times. Several mAb were used as controls (anti-Lyt-2, Thy-1, Mac-1, leukocyte, and H-2K) and had no blocking effects.

profoundly blocked antigen-dependent binding at 4°C (Table V). The anti-Ti (T cell receptor for antigen) reagent KJ16 blocked to a lesser extent (Table V), presumably because only 20% of T cells expressed the KJ16 antigen (19). None

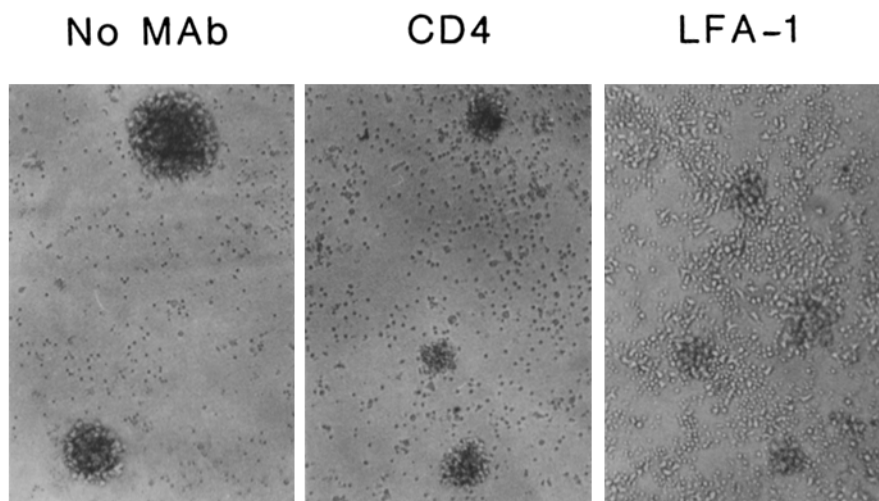


FIGURE 3. Effect of mAb on DC-T aggregates. At 20 h of a control MLR, DC-T clusters were isolated by velocity sedimentation and cultured 20 h more in the presence of mAb. The no mAb controls (or several noninhibitory mAb, see Table I legend) contain spherical aggregates composed of many large lymphoblasts. The latter are distinguished as large clear profiles around the rim of the clusters. In the presence of anti-CD4, the clusters remain intact but there is no blastogenesis. In the presence of anti-LFA-1, the clusters appear smaller and flatter (DC in the cluster probably do not disassemble). Note the numerous single lymphoblasts that are released by LFA-1. $\times 75$.

of the other mAb that reacted with T cells or with DC blocked clustering, including anti-LFA-1 and anti-CD4.

To assess the capacity of DC to bind in an antigen-independent fashion, we mixed alloreactive blasts with syngeneic DC at 37°C (5). None of the antibodies, including anti-I-A and -I-E, blocked antigen-independent binding (Table V). Conjugates that were formed with T cells that had been precoated with anti-LFA-1 were readily dissociated by gentle pipetting; conjugates formed with anti-CD4-coated T cells were as stable as controls (not shown). Therefore, the results from these rapid binding assays suggest that anti-LFA-1 and -CD4 were not acting on the initial steps whereby DC bound to T cells.

Effects of mAb on IL-2 Release and Responsiveness of Sensitized T Cells. T blasts were coated with mAb, allowed to bind to DC at 37°C (Table V), and returned to culture. Microscopic examination of the live cultures revealed that lymphoblasts were emerging from the aggregate within 5 h in control and anti-LFA-1-treated cultures. In the presence of anti-CD4 or anti-Ia, the clusters remained intact but the blasts began to revert to smaller lymphocytes. IL-2 release was detectable within 2 h in control cultures (not shown) and increased progressively over 5–18 h (Tables VI and VII). Anti-LFA-1-treated conjugates made normal levels of IL-2 (unless the cultures were studied at intervals longer than 1 d, not shown) whereas anti-CD4 and anti-Ia-coated conjugates made little or no IL-2 (Tables VI and VII).

The IL-2-responsive, alloreactive lymphoblasts were challenged with graded doses of exogenous human rIL-2 (0.5–100 U/ml). The proliferative response

TABLE V
Effects of mAb on DC-T Clustering

mAb		Percent alloreactive T cells that cluster at stated DC-T ratios							
		Freshly sensitized T blasts				Rested T memory			
		Allogeneic DC, 4°C			Syngeneic DC, 37°C	Allogeneic DC, 4°C			Syngeneic DC, 37°C
Clone	Determinant	1:2	1:10	1:50	1:10	1:2	1:10	1:50	1:10
None	None	68	42	15	73	79	51	13	82
14-4-4S +10.216	I-E +I-A	34	16	4	73	38	0.4	0.7	83
KJ-16	Ti	50	33	11	74	62	26	5	81
M1/42	H-2K	67	41	14	73	79	50	14	80
53-6.7	Lyt-2	69	40	13	74	76	52	13	81
53-7.1	Lyt-1	71	41	16	73	78	52	12	82
GK1.5	L3T4	73	42	12	73	78	55	12	87
B5-3	Thy-1	69	42	16	73	80	51	13	82
FD441.8	LFA-1	72	44	18	73	77	55	14	89
M1/70	C3biR	69	42	16	73	85	51	13	81

Antigen-specific T blasts and memory cells were isolated from the primary MLR (3, 17). Antigen-dependent DC-T binding occurred when alloreactive (H-2d anti-H-2k) T cells were mixed with allogeneic (H-2k) DC at 4°C (top line of the tabular data). There was no binding of syngeneic cells at 4°C (not shown). At 37°C, DC-T binding was antigen-independent because allogeneic (H-2k) and syngeneic (H-2d) DC bound similarly to T cells (5). When a variety of mAb were added to the binding assay (this experiment), or to the T cells for 1 h before the assay (other experiments), the only block that was observed was for mAb to Ti (KJ-16) and to Ia (a mixture of anti-I-E and anti-I-A mouse mAb). The top four mAb are rat IgG2a and the bottom rat IgG2b.

was not blocked by any of the mAb we tested, including antibodies to Ia, LFA-1, and CD4 (Table VII).

Discussion

It has been observed that DC efficiently cluster T cells during many immune responses (1-8; Table I, Fig. 1). We have used mAb to LFA-1 and CD4 to see whether these molecules are involved in the DC-T aggregation mechanism, and if not, why do they inhibit T cell proliferation so profoundly (Table II)? Our data indicate that mAb to LFA-1 and CD4 do not block the early steps whereby DC bind to unprimed or sensitized CD4⁺ cells but inhibit the function of DC-CD4 conjugates. LFA-1 and CD4 do not seem to be accessory molecules for binding. Instead they play a subsequent and independent role that influences most CD4⁺ cells in the primary response.

The fact that anti-LFA-1 and -CD4 do not block the initial clustering in the MLR perhaps is not surprising. The available evidence is that clustering with unprimed T cells is DC-specific and not observed with other APC (1-6, Fig. 1). Yet the presumptive ligands for LFA-1 and CD4 are likely to be widely distributed, in that mAb to these molecules block the binding of CD4⁺ cytolytic cells to a variety of targets (14-16).

There seem to be at least four interactions that contribute to the onset of a primary T-dependent immune response in DC-CD4⁺ lymphocyte aggregates.

Antigen Recognition. Antigen specificity is a major component of cluster

TABLE VI
Anti-CD4 mAb Blocks IL-2 Release from DC-T Clusters

Exp. 1	IL-2 released (cpm $\times 10^{-3}$)				
	mAb	T blasts: DC: DC-T ratio:	H-2d anti-H-2k		
			H-2k		H-2bxd
		1:2	1:10	1:10	
None		26.8	8.2	2.2	
I-A ^k + I-E ^{d,k}		3.6	1.0	0.8	
LFA-1 α		28.7	7.0	0.6	
CD4		0.4	0.4	0.3	

Exp. 2	T blasts: DC: DC-T ratio:	H-2d anti-H-2k		H-2d anti-H-2bxd
		H-2k		H-2bxd
		1:3		1:3
None		128.1		87.0
I-A ^k + I-E ^{d,k}		61.3		80.3
LFA-1 α		117.3		89.2
CD4		22.7		6.5

3×10^5 alloreactive T blasts were exposed to mAb (30%, vol/vol) for 1 h. DC of the indicated MHC were added. The cells were sedimented (50 g, 5 min), and DC-T clusters were allowed to form as in Table V. The cells were resuspended once and cultured in 1 ml of medium for 5 h at 37°C. Graded aliquots of the medium were assayed for growth factor activity on Con A T blasts. Data are cpm $\times 10^{-3}$ at 50% (vol/vol). Half-maximal activities in the bioassay were $70-55 \times 10^3$, respectively. IL-2 activity without DC was <500 cpm.

TABLE VII
Effects of mAb on T Lymphoblasts: IL-2 Release and Responsiveness

mAb	Response to half-maximal dose of human rIL-2	IL-2 activity released (cpm $\times 10^{-3}$) by T blasts in response to graded doses of DC		
		10^4	3×10^3	10^3
None	23.2	57.1	21.5	5.3
CD4	23.8	18.4	3.6	0.7
LFA-1	24.4	54.8	25.9	3.0
I-A, I-E	24.7	21.2	3.8	0.8
CD8	24.5	57.9	32.4	4.9

H-2d anti-H-2k Lyt-2⁻ T blasts were isolated from the primary MLR and 3×10^4 cells were challenged with human rIL-2. Proliferation is shown only for the half-maximal dose of IL-2. Other aliquots of the blasts (3×10^4 cells/well) were challenged with graded doses of H-2k DC. IL-2 activity in the medium is shown. Data are cpm $\times 10^{-3}$ at 18 h.

function. Antigen-specific blasts are produced in the cluster (2-4), whereas the noncluster fraction (representing $>90\%$ of the cells in the culture) are substantially depleted of antigen reactivity (3). Antigen specificity likely involves the interaction between Ti molecules on the lymphocyte and MHC products on the APC (20, 21). At this time, the most direct assay for the Ti-MHC interaction is the rapid binding assay between sensitized T cells and DC at 4°C. This assay is blocked by antibodies to Ti and MHC but not by other mAb to DC or T cells

including anti-LFA-1 and anti-CD4 (Table V). However, the Ti-MHC interaction may not be the first one between DC and unprimed T cells. Macrophages and B lymphocytes express the MHC products that are recognized by T cells but do not cluster as do DC (1-6). Therefore other molecules on DC, or additional features of DC MHC products, seem necessary for early clustering (see below).

CD4 Function. The expression of CD4 molecules on peripheral T cells is tightly associated with the recognition of Ia glycoproteins (class II MHC products), whereas expression of CD8 is linked to the recognition of class I (22). For this reason it has been proposed that CD4 interacts with Ia and contributes to APC-T cell binding. Nevertheless, there is data that anti-CD4 blocks mitogenesis in the apparent absence of Ia molecules suggesting a signaling rather than Ia-binding function (23, 24). The large (45-amino-acid) cytoplasmic domain of CD4 exhibits marked (78%) similarity in rodents and humans (25, 26) consistent with a conserved intracellular role.

Our data (Tables III and V; Figs. 2 and 3) indicate that most antigen-responsive T cells in the MLR bind to DC in the presence of blocking levels of anti-CD4 mAb. However, the effect of anti-CD4 on the ensuing function of DC-T conjugates is dramatic. IL-2 production immediately ceases (Table VI), and the blasts revert to smaller memory cells within 3-5 h (not shown). These findings imply a cell signaling vs. binding function for CD4, in which Ia could be the physiologic trigger. The putative interaction of Ia (or Ia-associated molecule) with CD4 may only occur after polymorphic Ia determinants have been engaged by the Ti/T3 receptor complex.

It has recently been shown that the administration of anti-CD4 mAb in situ results in antigen-specific tolerance (27, 28). If CD4⁺ T cells see antigen to be tolerized, then these in situ results support our in vitro findings that APC can efficiently bind to specific T cells in the presence of anti-CD4 (Table V).

LFA-1. A third DC-T interaction mechanism involves LFA-1. A ligand for LFA-1 is not yet identified. Anti-LFA-1 does not appear to block the initial stages of the DC-T interaction (Tables III, V-VII) but significantly inhibits other aspects. If clusters of DC and either unprimed (Fig. 2) or primed (not shown) T cells are gently resuspended, the clusters dissociate and do not reform. When anti-LFA-1 is added to recently formed primary clusters, the mAb slowly (4-20 h) disassembles the aggregates and markedly reduces subsequent proliferation (Fig. 3; Table IV). Prior studies in which activated T cells have been mixed with APC have concluded that anti-LFA-1 has either a modest (9, 29) or large (30) effect on conjugate formation. A critical variable in these experiments may be the vigor with which the conjugates are resuspended before measurement. Another variable is that prior work has not used DC. It is possible that other APC are more dependent on LFA-1 for the initial APC-T binding step.

Although our observations indicate that LFA-1 contributes to the stability of DC-CD4 conjugates, the molecule may have other effects. The C3bi receptor, which is a member of the LFA-1 family, has both binding and signaling functions which are regulated independently (31). We are finding that DC-T aggregates contain many antigen-nonspecific cells early in the MLR (8-24 h) but exclude nonspecific cells later on (48-72 h; Freudenthal, manuscript in preparation). LFA-1, once engaged, may signal the DC or T cell to turn off the antigen-

nonspecific binding mechanism that brings DC and T cells together (see below). DC-T clusters once dissociated do not recluster in the presence of anti-LFA-1.

LFA-1 is expressed on resting T cells, but it only seems to influence binding after the T cell has interacted with DC. The adhesion function of LFA-1 therefore may be regulated independently of its levels of expression. A similar phenomenon has been noted in studies of the C3bi receptor, a member of the LFA-1 family that uses a common 90-kD β chain. The binding function of C3biR is reduced when monocytes are cultured in IFN- γ , without major changes in the expression of this molecule (31).

Our observations may explain why patients who lack LFA-1 do not exhibit a profound clinical T cell deficiency (32). Antigen recognition (Table V), blastogenesis (Fig. 3), and IL-2 release (Tables VI and VII) all may begin in the absence of LFA-1, but a prolonged DC-T interaction will not occur. Patients may therefore have sufficient T cell function to avoid symptoms.

Antigen-independent DC-T Cell Binding. A fourth interaction mechanism could be critical for cluster formation in the primary response. We suspect that DC use a unique adhesion molecule to reversibly sample or survey T cells. Binding would be stabilized when complementary MHC and Ti molecules on DC and T cells interact. There are three reasons for postulating a distinct, early DC-T cell interaction mechanism that is required for antigen-specific responses. First, the Ti-MHC interaction does not in itself seem to initiate cluster formation, since many types of APC express MHC molecules but do not form clusters. Second, clear-cut antigen-independent DC-T binding is evident in rapid binding assays, particularly with sensitized T cells where the antigen-specificity of the lymphocyte is known (5). APC that do not initiate primary responses—such as macrophages, B lymphocytes, and freshly isolated Langerhans cells—do not bind T cells in the absence of antigen (5, 33). Third, none of the existing mAb to DC or to T cells, including anti-LFA-1 and -CD4, block antigen-independent binding (Tables III and V). Recent studies in the human MLR (Freudenthal et al., manuscript in preparation) indicate that antigen-nonspecific T cells rapidly enter and leave the DC-T aggregate while simultaneously, specific lymphocytes are selected and induced to proliferate.

Summary

T cell proliferation in response to many stimuli is known to occur in discrete clusters of dendritic cells (DC) and CD4⁺ helper lymphocytes. The role of lymphocyte function-associated antigen (LFA-1) and CD4 in the formation and function of these clusters has been evaluated in the mixed leukocyte reaction (MLR).

By day 1 of the control MLR, most of the DC and responsive T cells are associated in discrete aggregates. Addition of anti-LFA-1 and CD4 reagents does not block DC-T aggregation but reduces the subsequent proliferative response by 80–90%. Anti-LFA-1 disassembles newly formed DC-T cell aggregates, whereas anti-CD4 inhibits blastogenesis without disrupting the cluster.

Binding of DC to sensitized, antigen-specific CD4⁺ cells has been studied using lymphoblasts isolated at day 4 of the MLR. It has been shown previously that >80% blasts rebind to DC in an antigen-specific fashion in rapid (10 min) binding

assays. Antigen-dependent DC-T binding is blocked by anti-Ia but not by mAb to LFA-1 or CD4. However, the bound anti-CD4-coated lymphocytes are unable to release IL-2. Anti-LFA-1-coated T cells release IL-2 but are easily disaggregated after binding to DC.

These findings lead to two conclusions. LFA-1 and CD4 are not involved in the initial steps whereby DC bind to T cells but exert an independent and subsequent role. LFA-1 acts to stabilize the DC-T cluster, while CD4 contributes to lymphocyte blastogenesis and IL-2 release. Because DC but not other presenting cells cluster unprimed lymphocytes, it seems likely that an antigen-independent mechanism distinct from LFA-1 and CD4 mediates aggregate formation at the onset of cell-mediated immunity.

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