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CLUSTERING OF DENDRITIC CELLS, HELPER T LYMPHOCYTES, AND HISTOCOMPATIBLE B CELLS DURING PRIMARY ANTIBODY RESPONSES IN VITRO

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Primary antibody responses can be generated from cell suspensions of mouse spleen after 4–5 d of culture with specific antigen. Lymphocyte aggregates develop in such cultures, but little is known about their composition and significance. We have now prepared cluster and noncluster fractions after 1–2 d of culture and studied their properties over an additional 2–3 d. The clusters prove to be the site in which most antibody-forming cells develop during the response to thymus-dependent antigens, like foreign red blood cells and hapten-carrier conjugates. We will show that the aggregates contain all three cell types that are required for antibody responses, i.e., dendritic cells (DC),[1] helper T cells, and antigen-specific B cells. B cells enter the aggregates after encountering antigen and appear to be recognized by the clustered T cells in a major histocompatibility complex (MHC)-restricted fashion. After entry into the cluster, antibody-forming cells can develop in response to soluble factors released during the interaction of DC and T cells therein.

Materials and Methods

Mice. Mice of either sex were used at 6–10 wk of age. DBA/2 × BALB/c F_1 (D2C F_1, H-2^d), C57BL/6 (B6, H-2^b), C57BL/6 × DBA/2 F_1 (B6D2 F_1, H-2^{bxd}), B6.H-2k, and ICR Swiss (H-2^s) were purchased from Trudeau Institute, Saranac Lake, NY. A/J (H-$2^{k/d}$) and A.SW (H-2^s) were obtained from The Jackson Laboratory, Bar Harbor, ME.

Antigens. Sheep and horse erythrocytes (RBC) were purchased from Colorado Serum Co., Denver, CO. RBC were stored in Alsever's solution at 4°C and used within 2 wk of shipment. Dinitrophenylated (DNP) keyhole limpet hemocyanin (KLH) (Calbiochem-Behring Corp., La Jolla, CA) was prepared as described previously at 12.7 mol DNP/100 kD KLH (1).

Antibodies. Monoclonal antibodies 33D1 α-DC, B21-2 α-I-$A^{b,d}$, TIB 99 α-Thy-1.2, TIB 150 α-Lyt-2.2, and C3PO α-Lyt-1.2 (see 2 for references) were used as culture supernatants to selectively eliminate DC, Ia-bearing cells, and T cells, respectively. Rabbit serum was the source of complement (C). Several rat α-mouse monoclonal antibodies (3, 4) were used as culture supernatants to identify leukocyte subsets by indirect immunofluorescence as described (5). The monoclonals have been summarized previously and include

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[1] *Abbreviations used in this paper:* C, complement; DC, dendritic cell; DNP, dinitrophenyl; IL-2, interleukin 2; KLH, keyhole limpet hemocyanin; MLR, mixed leukocyte reaction; MHC, major histocompatibility complex; PFC, plaque-forming cell; RBC, erythrocyte; syn MLR, syngeneic MLR; TNP, trinitrophenyl.

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B5-3 α-Thy 1.2, 53-7.3 α-Lyt-1, 53-6.7 α-Lyt-2, TIB 146 α-B cell, and TIB 145 α-B cell. α-Ia^k alloantibodies (A.TH α-A.TL) were purchased from Accurate Chemical & Scientific Corp., Westbury, NY α-Ia^d (B10.LG × A.TFR α-B10.D2), α-K^k (A.TL × 129 α-A.AL), α-D^k (B10.A(2R) × C3H.SW α-C3H), α-K^s (B10.A × A.AL α-A.TL), α-K^b (B10.D2 × A α-B10.A(5R)), α-D^b (B10.A(5R) × LP.RIII α-B10) alloantibodies were provided by Dr. John Ray, Research Resources Branch of the NIH. A purified Ig fraction of rabbit α-interleukin 2 (α-IL-2) antibodies was donated by Dr. A. Granelli-Piperno (2).

Cells. DC (macrophage-depleted, low density spleen-adherent cells), B/T lymphocyte mixtures (Sephadex G10-nonadherent spleen cells), T cells (nylon wool–nonadherent cells), and B cells (G10-nonadherent, T cell–depleted cells) were prepared as described previously (2, 6). In most experiments, T cells were treated with α-Ia and C to eliminate residual B cells and DC.

Culture Conditions and Isolation of Cell Clusters. 5×10^6 B/T lymphocytes, or mixtures of 2×10^6 T cells and 3×10^6 B cells, were cultured with $2–3 \times 10^4$ DC in 1.2 ml culture medium (RPMI 1640 from Gibco Laboratories, Grand Island, NY; 10% fetal calf serum from Sterile Systems, Inc., Logan, UT; 20 μg/ml gentamycin sulfate; 5×10^{-5} 2-mercaptoethanol) in 16-mm diam wells. Antigen doses were 5×10^6 RBC or 10 μg/ml DNP-KLH. After 1–2 d of DC-B-T cell co-culture, cell aggregates were separated on Percoll gradients as described (2). Cluster and noncluster preparations were cultured at densities of 2×10^5 and 2×10^6 per well, respectively, for an additional 2–3 d in the presence or absence of antigen. Cytologic features of the fractions were assessed after spinning aliquots of 10^5 cells onto 12-mm coverslips coated with 50 μg/ml poly-L-lysine (Sigma Chemical Co., St. Louis, MO). DC were identified after 20 min of spreading at 37°C (see Results). Cell surface phenotype was determined by indirect immunofluorescence, or by complement-mediated cytotoxicity, using monoclonal antibodies as summarized above.

B Cell Helper Factors. These were obtained from the conditioned medium (25–40% vol/vol) of DC/T syngeneic mixed leukocyte reactions (syn MLRs) (1 DC/200 T cells) as described (2).

Assay for Plaque-forming Cells (PFC). Antibody-forming cells were enumerated with a direct plaque assay in agarose. α-DNP PFC were measured using TNP-conjugated sheep RBC as indicator cells (7). The H-2 type of the PFC was determined by incubating the test cultures in a volume of 0.5 ml medium containing no antiserum or 1:100 concentrations of anti-H-2 alloantibodies (see above) and C for 1 h at 37°C. Recovered cells were suspended in fresh medium and assayed for PFC. All data are for single cultures in which the plaque assay was done in triplicate and found to have standard deviations of <10%.

Results

The antibody response to sheep RBC in culture requires DC, B, and T cells (6). It is known that DC and stimulated T cells aggregate with one another, and that the clusters release large amounts of B cell helper factors (4). Here we report that antibody-secreting cells (PFC) also develop within clusters. We will show that DC and T cells act together to bring MHC-compatible, antigen-specific B cells into the clusters. Then the B cells become responsive to soluble helper factors needed for the development of PFC.

Cell Clusters Generate the Majority of the PFC in an Anti–Sheep RBC Response. Unfractionated spleen, or mixtures of DC and Sephadex G10–nonadherent spleen (B/T lymphocytes), were cultured for 2 d. Clusters formed and were readily isolated by velocity sedimentation (Fig. 1). Typically we retrieved 2×10^5 clustered cells and 2×10^6 nonclusters per culture of 5×10^6 spleen cells. If sheep RBC were added to the cultures from time 0, the clusters lacked PFC when isolated at day 1–2 (not shown) but gave rise to large numbers of antibody-secreting cells when cultured an additional 2–3 d. Nonclustered cells, even in

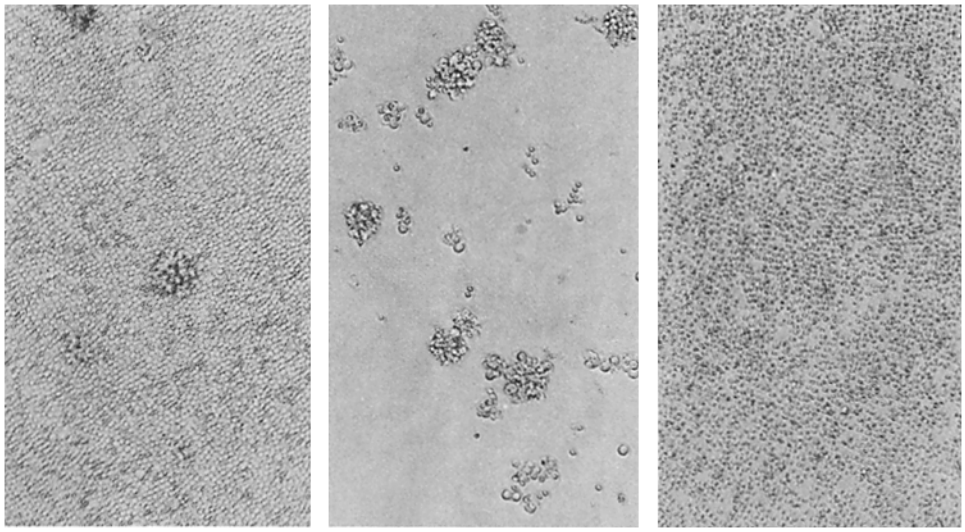

FIGURE 1. The appearance of clusters under the inverted microscope. Unseparated cultures of 2.5×10^4 DC and 5×10^6 speen B/T cells contain discrete clusters (*left*) and can be separated into nonclustered (*right*) and clustered components (*middle*) by velocity sedimentation in Percoll × 75.

TABLE I

Primary Anti–Sheep RBC Antibody Responses Are Generated from Cell Clusters

Exp. No.	Clusters separated from:	PFC per culture (+/− sheep RBC)		
		5×10^6 Unseparated	2×10^6 Nonclusters	2×10^5 Clustered cells
1	Whole spleen	827/15	20/11	1,005/4
	G10 spleen + DC	1,123/5	45/3	1,248/4
2	Whole spleen	651/40	43/5	659/16
3	Whole spleen	448/24	10/21	595/8
4	G10 spleen + DC	973/43	0/NT	3,323/NT
5	G10 spleen + DC	924/24 (6)	20/20 (0)	756/20 (12)
6	G10 spleen + DC	1,260/7 (0)	30/NT (0)	917/NT (0)
7	G10 spleen + DC	973/43 (10)	0/NT (0)	3,323/NT (3)
8	G10 spleen + DC	1,547/13 (0)	50/NT (20)	4,747/NT (3)

5×10^6 cells (whole spleen, or Sephadex G10–nonadherent spleen + 0.5% DC) were cultured for 2 d +/− sheep RBC and separated into cluster and noncluster fractions by velocity sedimentation (recovery of 2×10^5 and 2×10^6 viable cells per culture, respectively). The fractions were cultured an additional 2 d (Exps. 2 and 3) or 3 d +/− sheep RBC and PFC were enumerated. In Exps. 5–8, PFC to horse RBC were also measured (numbers in parentheses). NT, not tested.

much larger numbers (2×10^6), did not generate PFC. PFC yields from the cluster fraction were comparable to that seen in unseparated spleen (Table I).

Although clusters developed in the absence of exogenous antigens, the development of anti–sheep RBC responses was clearly antigen dependent (Table I). The PFC responses were also antigen-specific, since no anti-horse PFC were detected (Table I, Exps. 5–8). Specific anti-horse PFC were found in clusters if the cultures were stimulated with horse RBC (see below).

Cell Composition of Clusters: Cytology and Surface Markers. By phase contrast

microscopy, clusters isolated at 24 h each exhibited at least one to three irregularly shaped DC; the nonclusters were DC depleted (Fig. 2, *A* and *B*). The DC within the clusters appeared much larger and more irregularly shaped relative

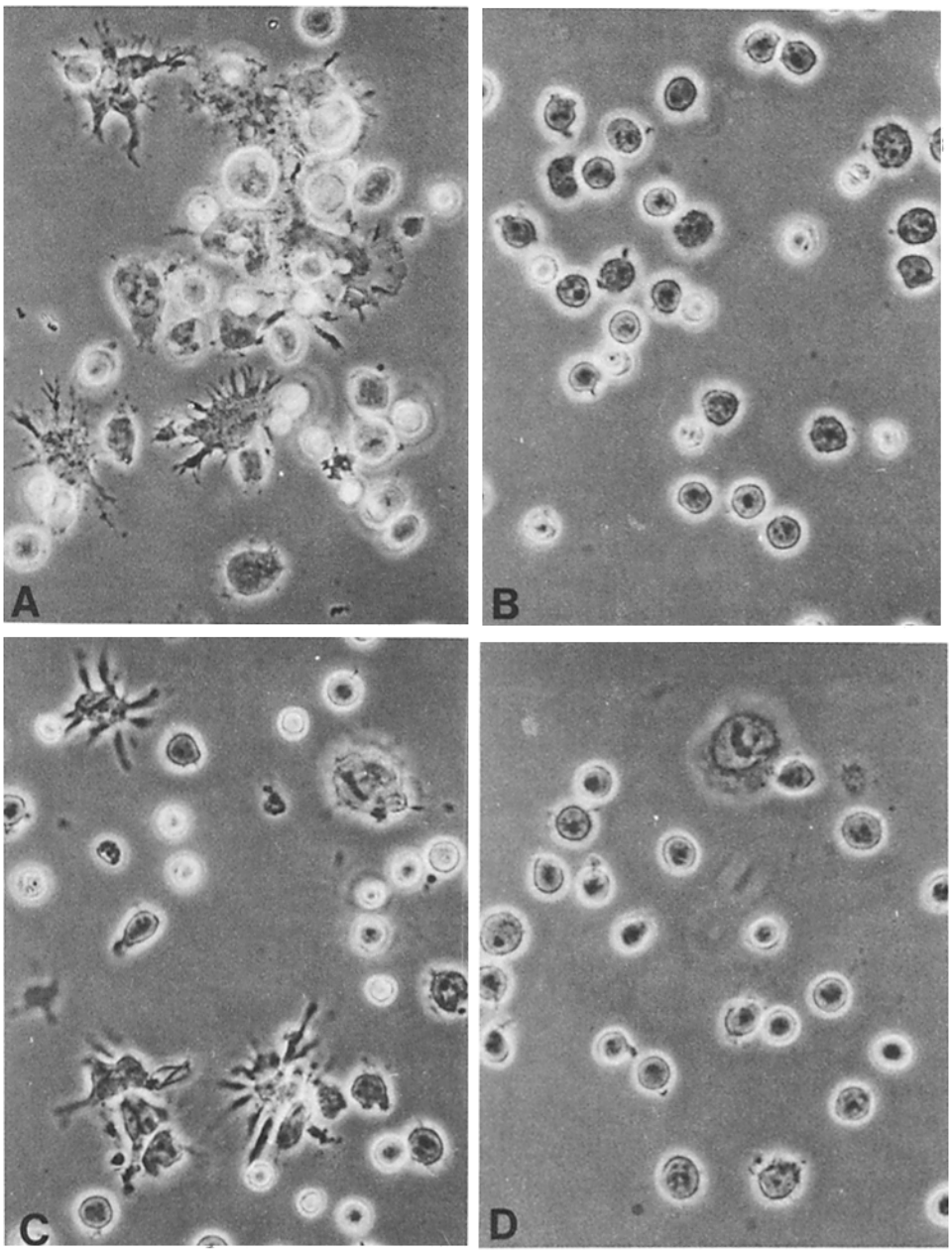

FIGURE 2. Phase contrast micrographs of spleen cells spun onto poly-L-lysine–coated coverslips and allowed to spread for 20 min at 37°C before fixation in 1.25% glutaraldehyde in phosphate-buffered saline. (*A*) Clusters at day 2; note four DC. (*B*) Nonclusters at day 2; no DC are evident. (*C*) Clusters at day 2 after treatment with no antibody or C; the DC are intact. (*D*) Clusters treated with 33D1 α-DC antibody and C; the DC have been eliminated. × 750.

TABLE II
Phenotype of Cells in Clusters

Monoclonal antibody	Number of experiments	Percent killed (Ab/C)
None	5	0
33D1, α-DC	5	10 ± 2
TIB 99, α-Thy-1.2	4	53 ± 5
C3PO, α-Lyt-1.2	2	46
TIB 150, α-Lyt-2.2	2	14
B21.2, α-$Ia^{b,d}$	3	37 ± 1

The phenotype of clustered cells was determined by cytotoxicity with monoclonal antibodies and rabbit C.

to companion cultures of purified DC. Lymphocytes in the day 1 clusters were small, but by day 2 lymphoblasts accounted for 5–15% of clustered cells.

Cytotoxicity (Table II) and indirect immunofluorescence tests with monoclonal antibodies showed that 20–50% of the clusters were T cells, primarily of the Lyt-1^+2^- phenotype (Table II). Most of the remaining cells reacted with the anti–B cell reagents, TIB 146 and TIB 145 (see below). The 33D1 anti-DC monoclonal killed 10% of the clustered cells; after 33D1 and C, the aggregates disassembled and cells with the cytology of DC were absent (Fig. 2, *C* and *D*).

Contribution of DC to Cluster Function. DC and B/T lymphocyte mixtures were cultured with and without sheep RBC for 1 d. Cluster and noncluster fractions were prepared and both fractions were cultured 3 more days. PFC developed exclusively from the clusters, but only if the clusters had been formed in the presence of sheep RBC (Table III). Treatment with the DC-specific antibody 33D1 and C killed ~10% of the clustered cells but ablated >90% of the PFC response (Table III). Treatment with 33D1/C also ablated >90% of the 1° MLR–stimulating capacity in clusters (not shown). PFC responses and MLR-stimulating capacity were restored when the 33D1/C-treated clusters were supplemented with DC (Table III).

When the experiment was repeated on clusters that had been isolated after 2 d of culture, treatment with 33D1 and C only reduced PFC and MLR-stimulating capacity by 50% (not shown). By phase contrast microscopy it was evident that 5–10% of the DC resisted killing with 33D1 and C. These few DC may have been sufficient for normal function or DC were not needed after 2 d.

The noncluster fraction also developed some PFC, but only when supplemented with DC or with DC/T cell clusters (Table III). The response was antigen dependent, but the specific activity (PFC/nonclustered cells) was <10% of that observed in the clusters (Table III). The nonclusters did not develop PFC if supplemented with T cells or with clusters that had been treated with 33D1 and C. Therefore, nonclusters contain some antigen-dependent PFC precursors, but lack sufficient numbers of DC.

PFC Development in Clusters Requires T Cells or T Cell–dependent Factors. T cells also were needed for cluster function, because elimination of T cells with α-Thy-1 or α-Lyt-1 and C, but not α-Lyt-2, ablated the PFC response (Fig. 3). The deficit produced by antibody and C could be reversed by the conditioned medium from DC/T cell co-cultures (syn MLR supernatant, Table IV), a known

TABLE III

Function of Clusters is Ablated After Treatment with 33D1 Anti-DC Antibody and C

Cells	Treatment/supplement	SRBC, days 0 → 1:	PFC/culture			
			−		+	
		SRBC, days 1 → 4:	−	+	−	+
Exp. 1						
2×10^5 clustered cells	No Ab, no C		4	104	272	1,048
2×10^5 clustered cells	No Ab, + C		0	44	220	980
2×10^5 clustered cells	33D1 Ab + C		0	0	4	88
2×10^5 clustered cells	33D1 Ab + C; plus 10^4 DC		0	88	160	876
2×10^6 nonclusters			0	0	0	0
2×10^6 nonclusters	Plus 10^6 T		0	0	0	4
2×10^6 nonclusters	Plus 10^4 DC		4	120	4	504
2×10^6 nonclusters	Plus T + DC		4	156	0	584
2×10^6 nonclusters	Plus 5×10^4 clustered cells treated with no Ab, no C		4	156	116	1,028
2×10^6 nonclusters	Plus 5×10^4 clustered cells treated with no Ab, + C		0	212	120	928
2×10^6 nonclusters	Plus 5×10^4 clustered cells treated with 33D1 Ab + C		0	4	0	44
Exp. 2						
2×10^5 clustered cells	No Ab, no C		0	40	816	2,088
2×10^5 clustered cells	No Ab, + C		0	28	740	2,064
2×10^5 clustered cells	33D1 Ab + C		0	0	4	136
2×10^5 clustered cells	33D1 Ab + C; plus 10^4 DC		0	48	564	1,648
2×10^6 nonclusters			0	0	0	0
2×10^6 nonclusters	Plus 10^4 DC		0	112	52	716
2×10^6 nonclusters	Plus 5×10^4 clustered cells treated with no Ab, no C		0	220	696	1,572
2×10^6 nonclusters	Plus 5×10^4 clustered cells treated with no Ab, + C		0	180	796	1,572
2×10^6 nonclusters	Plus 5×10^4 clustered cells treated with 33D1 Ab + C		0	8	20	84

Mixtures of 2.5×10^4 DC and 5×10^6 B/T cells (Sephadex G10–nonadherent spleen) were cultured for 1 d +/− sheep RBC (SRBC). Cluster and noncluster fractions were prepared and cultured separately +/− SRBC for 3 more days. Response of unseparated cultures +/− SRBC was 1,388/4 in Exp. 1 and 936/4 in Exp. 2. To assess the contribution of DC to cluster function, clusters were treated +/− 33D1 antibody (Ab) +/− C before culture. To show that nonclusters had anti-SRBC precursors but lacked DC, we tested the effects of various cell populations on noncluster function.

source of B cell helper factors (2). The noncluster fraction also developed some α-sheep PFC when supplemented with soluble factors and antigen (Table IV). Active syn MLR–conditioned media could be obtained from DC/T cell mixtures that were syngeneic or allogeneic with the clustered B cells (not shown), indicating that the function of the factors was not restricted by the MHC.

Previous work demonstrated that DC/T cell clusters released B cell helper factors and that helper factor release was blocked by α-Ia and α-IL-2 antibodies (2). It was shown that IL-2, released during DC/T cell interaction, acted as the trigger for helper factor release. Here we tested the effects of α-Ia antibodies and a rabbit α-IL-2 reagent on the function of preformed DC/T/B aggregates. Both reagents markedly inhibited the PFC response (Table V). In contrast, no blocking was observed with α-H-2 k or d, with normal rabbit Ig (Table V), or with α-Ia directed to the inappropriate haplotype (not shown). Therefore, the

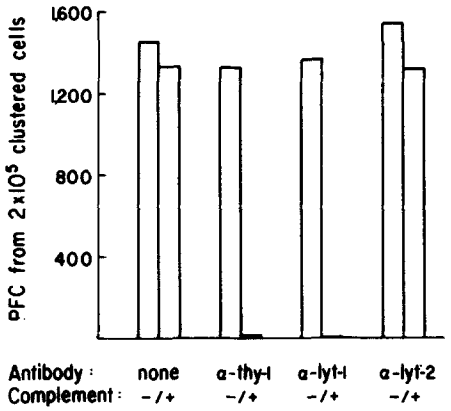

FIGURE 3. Thy-1$^+$, Lyt-1$^+$2$^-$ cells are needed for PFC development in clusters. Clusters were isolated on day 2 from cultures of DC, B/T lymphocytes, and sheep RBC. The clusters were treated with antibodies +/− C and cultured an additional 2 d. Killing in the presence of C was 45, 40, and 12% with α-Thy-1.2, α-Lyt-1.2, and α-Lyt-2.2. PFC in unseparated cultures were 1,957 and in nonclusters, 0.

TABLE IV

T Cell-depleted Clusters Will Generate PFC When Supplemented With Helper Factors from Syn MLR–conditioned Medium

Cells/treatment	PFC/culture		
	Exp. 1	Exp. 2	Exp. 3
G10–nonadherent spleen + DC	13	3	8
G10–nonadherent + DC + sheep RBC	1,547	990	1,016
Clusters from above (no Ab, + C)	4,747	1,630	1,580
Clusters (no Ab, + C) + syn MLR medium	4,820	5,057	1,572
Clusters (α-Thy-1 + C)	30	NT	4
Clusters (α-Thy-1 + C) + syn MLR medium	5,713	NT	2,424
Clusters (α-Lyt-1 + C)	NT	3	4
Clusters (α-Lyt-1 + C) + syn MLR medium	NT	5,623	2,244
Nonclusters (no Ab, + C)	43	0	0
Nonclusters (no Ab, + C) + syn MLR medium	550	610	1,272
Nonclusters (α-Thy-1 + C)	10	0	0
Nonclusters (α-Thy-1 + C) + syn MLR medium	637	890	1,612

Cluster and noncluster fractions were separated from 2-d cultures of DC, B/T lymphocytes, and sheep RBC. The fractions were treated with C +/− anti–T cell antibodies (Ab) and then recultured an additional 2 d +/− helper factors in 25% vol/vol syn MLR–conditioned medium. NT, not tested.

development of PFC in clusters is IL-2 dependent and appears to require recognition of Ia antigens.

Antigen Requirement for PFC Development within Clusters. Our initial experiments (Table III) indicated that sheep RBC had to be present during cluster formation for optimal PFC development. To test for antigen specificity we added

TABLE V

Effect of α-H-2 and α-IL-2 Antibodies on Cluster Function

Experiment	Cell line	Antibody (concentration)	PFC/culture
1	B6.H_2k (H-2^k)	None	2,204
		α-Ia^k (1/0.5/0.25%)	174/614/1,116
		α-K^k (1/0.5%)	2,480/2,620
		α-D^k (1/0.5%)	2,380/2,584
2	D2 × C (H-2^d)	None	1,104
		B21-2 (10 μg/ml)	68
		Rabbit α-IL-2 (40 μg/ml)	292
		Normal rabbit Ig (40 μg/ml)	3,320
3	D2 × C (H-2^d)	None	540
		α-Ia^d (1/0.5/0.25%)	8/24/36
		B21-2 (20/10/5 μg/ml)	0/0/16
		α-IL-2 (80/10/20 μg/ml)	0/0/30
		Control Ig (80/40/20 μg/ml)	556/512/504

Dendritic, B, and T cells were cultured 2 d with sheep RBC. Clusters were separated at day 2 and then recultured for 3 d +/− various antibodies.

either sheep or horse RBC to mixtures of DC and B/T lymphocytes, isolated the clusters at day 2, and recultured with either sheep or horse RBC. Clusters that formed in the presence of sheep RBC only developed α-sheep PFC, while clusters that formed in the presence of horse RBC only developed α-horse PFC (Table VI, Exp. 1). As in previous experiments (Table IV), the clustered B cells required intact T cells or T-dependent factors to develop into PFC (Table VI). Since horse and sheep RBC cross-react at the T but not B cell level (8), this experiment showed that B cells had to have encountered specific antigen to develop into PFC within clusters.

The experiment was extended in a second protocol in which we set up separate cultures of DC/T cells or B cells, each with and without sheep RBC. The former gave rise to DC/T clusters as expected (2), but the B cell cultures remained unaggregated. The 2-d cultures were then mixed for an additional 8 h, again with or without sheep RBC. Then the clusters were isolated and were maintained 3 d with or without sheep RBC. The first result was that only B cells exposed to sheep RBC from day 0 to 2 gave rise to PFC in clusters (Table VI, Exp. 2, groups 7, 8, 11, and 12). The addition of RBC at day 2–5 boosted the response but was not essential. In contrast, the DC/T cultures functioned effectively whether or not exposed to sheep RBC (Table VI *B*, groups 7, 8, 11, and 12). Finally, fresh DC/T cell mixtures did not form clusters with antigen-stimulated B cells (Table VI, Exp. 2, groups 1–4). Therefore, both B and T cells must be "primed" for effective clustering and PFC development.

PFC Responses to DNP-KLH Also Occur in Clusters and Require Helper Factors. The previous experiments showed that clustered B cells are responsive to soluble helper factors. However, some B cells will develop into α–sheep RBC PFC in the presence of syn MLR helper factors and antigen and in the absence of DC/T clustering (2). Thus, we could not determine if clustering was essential

TABLE VI

B Cells Developing Into PFC Within Clusters Need Exposure to Specific Antigen

Exp. 1	Cells/treatment	PFC/Culture					
		No RBC		Sheep RBC		Horse RBC	
		α-SRBC	α-HRBC	α-SRBC	α-HRBC	α-SRBC	α-HRBC
	G10-nonadherent spleen	10	7	6	50	0	3
	G10-nonadherent spleen + DC	3	3	629	6	0	377
	Clusters from above			560	6	38	315
	Clusters from above (α-Thy-1 and α-Lyt-1 + C)	NT		13	0	6	0
	Treated clusters from above + syn MLR medium			567	13	6	247

Exp. 2	Group	DC/T cells	SRBC in 48-h B cell culture	SRBC during clustering	Yield ($\times 10^{-5}$) of clustered cells	PFC per 2×10^5 clustered cells	
						−SRBC	+SRBC
	1	Not cultured	No	No	2.8	4	0
	2		No	Yes	2.6	4	0
	3		Yes	No	2.9	0	0
	4		Yes	Yes	2.7	8	100
	5	Cultured (−SRBC)	No	No	4.2	8	8
	6		No	Yes	4.5	4	20
	7		Yes	No	3.9	524	928
	8		Yes	Yes	3.7	212	548
	9	Cultured (+SRBC)	No	No	5.6	4	8
	10		No	Yes	6.1	8	148
	11		Yes	No	7.8	216	540
	12		Yes	Yes	7.3	976	1,908

Experiment 1: Cultures of DC and B/T lymphocytes were cultured for 2 d +/− sheep or horse RBC. Clusters were then prepared and recultured with the same RBC as initially. Plaques were tested on both sheep and horse RBC targets.

Experiment 2: Separate cultures of 2.5×10^4 DC/5×10^6 T cells or 3×10^6 B cells were maintained +/− SRBC for 0–1 d (DC/T) or 2 d (B cells). The cultures were then mixed (six different combinations) and allowed to cluster for 8 h +/− SRBC. Clusters were isolated (12 groups) and yields of clustered cells per culture tabulated. 2.5×10^5 clustered cells were then maintained 3 d +/− SRBC before PFC measurement. The cultures were treated with ammonium chloride to lyse red cells before and after clustering. Separate control cultures showed that no PFC developed in B cells or DC/T cells cultured separately. The PFC responses in cultures that were not separated into clusters and noncluster fractions, but were cultured with SRBC, were in all cases >400 PFC. All the noncluster fractions yielded 0–50 PFC and are not shown.

for other B cells to become responsive to factors. We next studied the primary response to another antigen, DNP-KLH, which should not be driven by antigen and factors (9, 10). Few α-DNP PFC developed in cultures of B cells, or B/T mixtures, that had been supplemented with syn MLR supernatant; in the presence of DC, antigen-dependent responses occurred (Table VII).

Cluster and noncluster factors were then prepared on day 2 of an α-DNP-

TABLE VII

Anti-TNP B Cells Do Not Become PFC With Antigen and Syn MLR Supernatant

Exp. No.	Cells	Antigen	PFC		
			α-TNP	α-Horse RBC	α-Sheep RBC
1	5×10^6 whole spleen	None	94	12	8
		10 μg/ml DNP-KLH	588	8	12
		Horse RBC	88	944	8
	5×10^6 B/T cells	None	52	0	0
		10 μg/ml DNP-KLH	44	0	0
		10 μg/ml + syn sup	64	0	4
		Horse RBC	52	0	8
		Horse RBC + syn sup	56	1,232	8
	3×10^6 B cells	None	40	0	0
		10 μg/ml DNP-KLH	48	0	0
		10 μg/ml + syn sup	52	4	0
		Horse RBC	40	4	0
		Horse RBC + syn sup	60	1,728	4
2	5×10^6 whole spleen	None	40	0	0
		10 μg/ml DNP-KLH	533	0	6
		0.1 μg/ml DNP-KLH	69	0	0
	5×10^6 B/T, 2×10^4 DC	None	43	6	0
		10 μg/ml DNP-KLH	501	0	6
		0.1 μg/ml DNP-KLH	64	6	6
	5×10^6 B/T	None	37	0	0
		10 μg/ml DNP-KLH	43	0	0
		0.1 μg/ml DNP-KLH	32	0	0
		Syn sup	32	NT	NT
		plus 10 μg/ml DNP-KLH	37	NT	NT
		plus 0.1 μg/ml DNP-KLH	37	NT	NT
		plus horse RBC	64	1,520	6

Cells were cultured for 4 d +/− DNP-KLH or horse RBC as antigen. In some cultures, 25% vol/vol syn MLR–conditioned medium (syn sup) was added. NT, not tested.

KLH response. Most of the PFC developed from the clusters (Tables VIII and IX). As in the RBC system, cluster competence required that antigen be added during the first 2 d of culture, during the formation of the clusters. Subsequently, between days 2 and 5, additional DNP-KLH was not required (Tables VIII and IX).

If T cells were removed from the clusters, PFC did not develop (Tables VIII and IX). However, the response was now fully reconstituted with soluble helper factors (Tables VIII and IX). The α-TNP response was specific, since few α-RBC PFC were detected (Table IX). Therefore α-TNP B cells became responsive to soluble factor after clustering with DC and T cells.

Developing PFC Must Express the MHC to Which the Helper T Cell Is Restricted. To assess if B cell MHC products contributed to the PFC response

TABLE VIII

α-DNP-KLH PFC Develop in Clusters in an Antigen- and T Cell–Dependent Fashion

Cell fraction cultured from days 2 to 5	Supplement (days 2–5)	α-TNP PFC at day 5	
		No DNP-KLH (days 0–2)	Plus DNP-KLH (days 0–2)
Clusters	—	85	1,667
	DNP-KLH, 10 μg/ml	125	1,880
Clusters, T cell depleted	—	65	123
	DNP-KLH, 10 μg/ml	55	133
	Syn sup, 40%	85	1,433
	DNP-KLH + syn sup	55	1,727
Nonclusters	—	40	110
	DNP-KLH, 10 μg/ml	48	95
Nonclusters, T cell depleted	—	48	75
	DNP-KLH, 10 μg/ml	56	60

Mixtures of 2.5×10^4 DC and 5×10^6 B/T cells were cultured 2 d +/− 10 μg/ml DNP-KLH. Cluster and noncluster fractions were prepared and cultured 3 more days +/− antigen. Companion cultures were also tested after T cell elimination with α-Thy-1 and α-Lyt-1 plus C. Anti–sheep RBC PFC were negligible (not shown). Syn sup, syn MLR–conditioned medium.

within clusters, we added B cells that were either MHC compatible or incompatible with the test DC/T cell mixtures. These experiments were feasible because allogeneic small and large B cells did not stimulate a 1° MLR (Inaba and Steinman, manuscript in preparation).

Only syngeneic B cells developed into PFC within clusters of H-2^s or H-2^a DC and T cells (Table X, Exps. 1 and 2). Allogeneic B cells (full strain differences or MHC differences only) did not participate in the cluster response but would generate PFC when exposed to helper factors (Table X, Exp. 1). Since allogeneic B cells might have been suppressive, or might have entered the clustered but not induced the release of helper factor, we cultured mixtures of MHC-identical and -incompatible B cells and then H-2 phenotyped the resulting PFC. Allogeneic B cells were not suppressive, and only syngeneic B cells became PFC (Table X, Exp. 2).

The experiment was then redesigned to test T cells that were capable of recognizing allogeneic B cells. In the previous protocol, the T cells would be restricted to self, but we have found that allorestricted, Lyt-1^+2^- helper T cells can be generated from clusters of DC and allogeneic T cells (Inaba and Steinman, manuscript in preparation). Therefore we cultured B6.H-2k DC, B6 (H-2b) Lyt-2^- T cells, and B6 and/or B6.H-2k B cells. Clusters were isolated at day 2 and PFC were H-2 phenotyped at the time of assay, day 4. Now, B6.H-2k cells

TABLE IX

Development of α-TNP PFC in Clusters Requires Antigen and Soluble Helper Factors

Antigen (day 0–2)	Treatment (day 2)	Antigen (day 2–5)	PFC in clusters			PFC in nonclusters		
			TNP	HRBC	SRBC	TNP	HRBC	SRBC
None	No Ab, + C	None	0	0	0	0	0	0
		DNP-KLH, 10 μg/ml	11	0	0	13	0	6
		DNP-KLH, 0.1 μg/ml	0	0	0	0	0	0
		HRBC	5	0	0	5	4	6
		HRBC + syn sup		NT		23	1,040	18
DNP-KLH 10 μg/ml	No Ab, + C	None	224	0	6	43	6	18
		DNP-KLH, 10 μg/ml	304	18	30	53	0	24
		DNP-KLH, 0.1 μg/ml	549	6	12	21	0	0
		HRBC	149	68	12	16	4	6
		HRBC + syn sup		NT		16	772	6
None	α-Thy-1, Lyt-1 + C	None	5	6	0	11	0	0
		DNP-KLH, 10 μg/ml	11	0	0	27	0	0
		DNP-KLH, 0.1 μg/ml	5	6	0	32	6	6
		Syn sup	16	6	6	64	18	30
		DNP-KLH, 10 μg/ml + syn sup	27	0	6	53	18	42
		DNP-KLH, 0.10 μg/ml + syn sup	32	0	6	53	0	18
		HRBC		NT		59	0	18
		HRBC + syn sup		NT		80	1,318	36
DNP-KLH 10 μg/ml	α-Thy-1, Lyt-1 + C	None	107	0	6	5	0	0
		DNP-KLH, 10 μg/ml	96	6	12	11	6	6
		DNP-KLH, 0.1 μg/ml	107	6	12	5	0	6
		Syn sup	907	18	6	64	6	18
		DNP-KLH, 10 μg/ml + syn sup	1,216	12	18	331	18	24
		DNP-KLH, 0.1 μg/ml + syn sup	1,419	12	12	235	6	18
		HRBC		NT		21	3	0
		HRBC + syn sup		NT		33	1,344	6

HRBC, horse RBC; SRBC, sheep RBC; syn sup, syn MLR medium.

(allogeneic to the T cells) were the principal source of PFC within clusters, even when mixtures of B6 and B6.H-2k B cells were tested (Table IX, Exp. 3).

The MHC Restricts the Entry of B cells into DC/T cell clusters. To determine if MHC restriction governed B cell aggregation itself, we cultured DC/T cell mixtures with syngeneic or allogeneic B cells. Entry of B cells into the clusters was monitored by indirect immunofluorescence with the B cell–specific antibodies, TIB 146 and TIB 145. More cells clustered when the cultures contained syngeneic B cells and this increase was almost entirely due to a sixfold increase in the entry of syngeneic B cells (Table XI, Fig. 4). Interestingly, the allogeneic B cells that were found in clusters were to a large extent blast transformed (Fig. 4), suggesting that there is an MHC-unrestricted component to B cell activation within clusters.

Discussion

Composition of Clusters That Form During Antibody Responses In Vitro. At a minimum, the cellular requirements for T-dependent antibody responses are DC, helper T lymphocytes, and B cells precommitted to respond to a specific antigen. We have found that these three cell types are physically associated in

TABLE X

B Cells Developing into PFC in Clusters Are Recognized in an MHC-restricted Fashion

Exp. No.	Strain, cell type, treatment	PFC (+/− SRBC) with B cells from:		
	Swiss ($H\text{-}2^s$) DC/T	Swiss	B6.H-2k	Both
1	Unseparated cells	2,629/27	1,104/21	NT
	2×10^5 clustered cells	3,824	256	NT
	2×10^6 nonclustered cells	136	20	NT
	2×10^6 nonclustered cells + syn sup	1,064	1,604	NT
2	Strain A DC/T	A (K^k)	A.SW (K^s)	Both
	2×10^6 T, 3×10^6 B	16/4	8/4	16/12
	2×10^4 DC, 2×10^6 T, 3×10^6 B	928/0	748/0	796/0
	2×10^5 clustered cells	656	96	792
	2×10^5 clustered cells; R_x PFC with α-K^k + C	32	36	8
	2×10^5 clustered cells; R_x PFC with α-K^s + C	552	96	608
	2×10^6 nonclustered cells	28	40	40
	2×10^6 + syn sup	362	432	698
	2×10^6 + syn sup; R_x PFC with α-K^k + C	40	452	312
	2×10^6 + syn sup; R_x PFC with α-K^s + C	440	20	352
3	B6.H-2k DC, B6 Lyt-1^+2^- T	B6.H-2k (K^kD^K)	B6 (D^b)	Both
	2×10^5 clustered cells; R_x PFC with no Ab or C	1,212	344	936
	", R_x PFC with α-K^k + C	64	324	312
	", R_x PFC with α-D^k + C	48	348	304
	", R_x PFC with α-D^b + C	1,224	24	676
	2×10^6 nonclustered cells	0	0	0
	Plus syn sup, R_x PFC with no Ab or C	420	493	553
	", R_x PFC with α-K^k + C	27	506	367
	", R_x PFC with α-D^k + C	0	533	347
	", R_x PFC with α-D^b + C	467	7	243

DC and T lymphocytes were cultured for 2 d with B cells from different strains. Cluster and noncluster fractions were prepared and cultured an additional 3 d. The nonclusters were cultured +/− syn MLR supernatant (syn sup) as a source of helper factor. In Exps. 2 and 3, the cells were treated (R_x) with alloantibodies plus C at the time of the plaque assay to document the H-2 type of the PFC. Data with antibody only were comparable to that for untreated cells and are not shown. In Exps. 1 and 2, the T cells were not rigorously B cell–depleted (α-Ia + C), so that the small PFC response in allogeneic B cell clusters was likely due to contaminating B cells in the DC/T cell component.

discrete clusters, and that most antibody-forming cells develop therein under standard culture conditions. The B cells that cluster do so after exposure to antigen in vitro. It was evident some time ago that PFC precursors that had been antigen primed in situ, entered into clusters with DC (11). At that time, we lacked adequate cytotoxic antibodies to DC and to T lymphocytes and therefore were unable to characterize the cellular interactions that were required for the development of antigen-selected B cells into PFC. Both DC and Lyt-1^+2^- helper T cells prove to be necessary for PFC development in clusters (Fig. 3, Tables III, IV, VIII, and IX). Interestingly, significant helper activity can be generated

TABLE XI
Syngeneic B Cells Preferentially Enter DC/T Cell Clusters

Time	Monoclonal antibody (specificity)	Percent cells stained in clusters formed with:	
		Syngeneic B ($Ia^{b,d}$)	Allogeneic B (Ia^{k})
24 h	TIB 145 (α-B cell)	57	18
	TIB 146 (α-B cell)	59	19
	B21-2 (α-$Ia^{b,d}$)	20	23
	B5-3 (α-Thy-1.2)	15	53
48 h	TIB 145 (α-B cell)	28	10
	TIB 146 (α-B cell)	29	9
	B21-2 (α-$Ia^{b,d}$)	14	14
	B5-3 (α-thy-1.2)	51	63

This experiment is the same as described in Fig. 4 and was repeated once. The yield of clusters formed in the presence of syngeneic and allogeneic B cells was, at 24 h, 2 and 1×10^5 per culture, while, at 48 h, the yield was 2.6 and 1.3×10^5 per culture. Therefore, the total number of clustering syngeneic B cells was six times the allogeneic level. The data for B21-2 primarily reflect brightly stained DC and enlarged B cells (syngeneic only).

in the syn MLR between DC and T lymphocytes, in the apparent absence of antigen (e.g., Table VI, Exp. 2).

Strikingly, it is primarily MHC-appropriate B cells that develop into PFC within clusters. When DC and T cells are syngeneic, the developing PFC also must be syngeneic; when the T cells are responding to allogeneic DC, then allogeneic B cells preferentially become PFC (Table X). The entry of the B cells into the clusters is one step that is MHC restricted (Table XI, Fig. 4), and we suspect that the subsequent stimulation of B cells by clustered T cells requires MHC recognition. The cluster therefore represents a new and direct means for identifying B cells that respond in an antigen-dependent, MHC-restricted fashion.

Formation of Clusters. Cluster formation is probably initiated by the interaction of DC and T cells. We noted previously that DC efficiently aggregate with a small fraction of T cells (<5% nylon wool–nonadherent spleen cells) that proliferates in the syn MLR (2, 12). These DC/T cell clusters generate large amounts of B cell helper factor via an IL-2-dependent mechanism (2). In contrast, we have not observed direct DC/B cell or B/T cell clustering.

In recent studies of the syn and allo MLR (Inaba and Steinman, manuscript in preparation), we have found that the clustering T cells are restricted to recognizing the MHC of the stimulating DC. T cells isolated from DC/T clusters proliferated when cultured with DC bearing the same MHC used to initiate the syn or allo MLR. We presume that these clustered stimulated T lymphocytes mediate a second cell-cell interaction involving B cells of the appropriate MHC. T cells that have not been cultured with DC do not form productive aggregates (Table VI, Exp. 2) and the B cells also have to be stimulated in some way. For example, the vast majority of MHC-compatible B cells (>90%) do not cluster even with excess DC (not shown), and the development of PFC within clusters is clearly antigen dependent (Tables III, VI, VIII, and IX). Therefore, stimulated

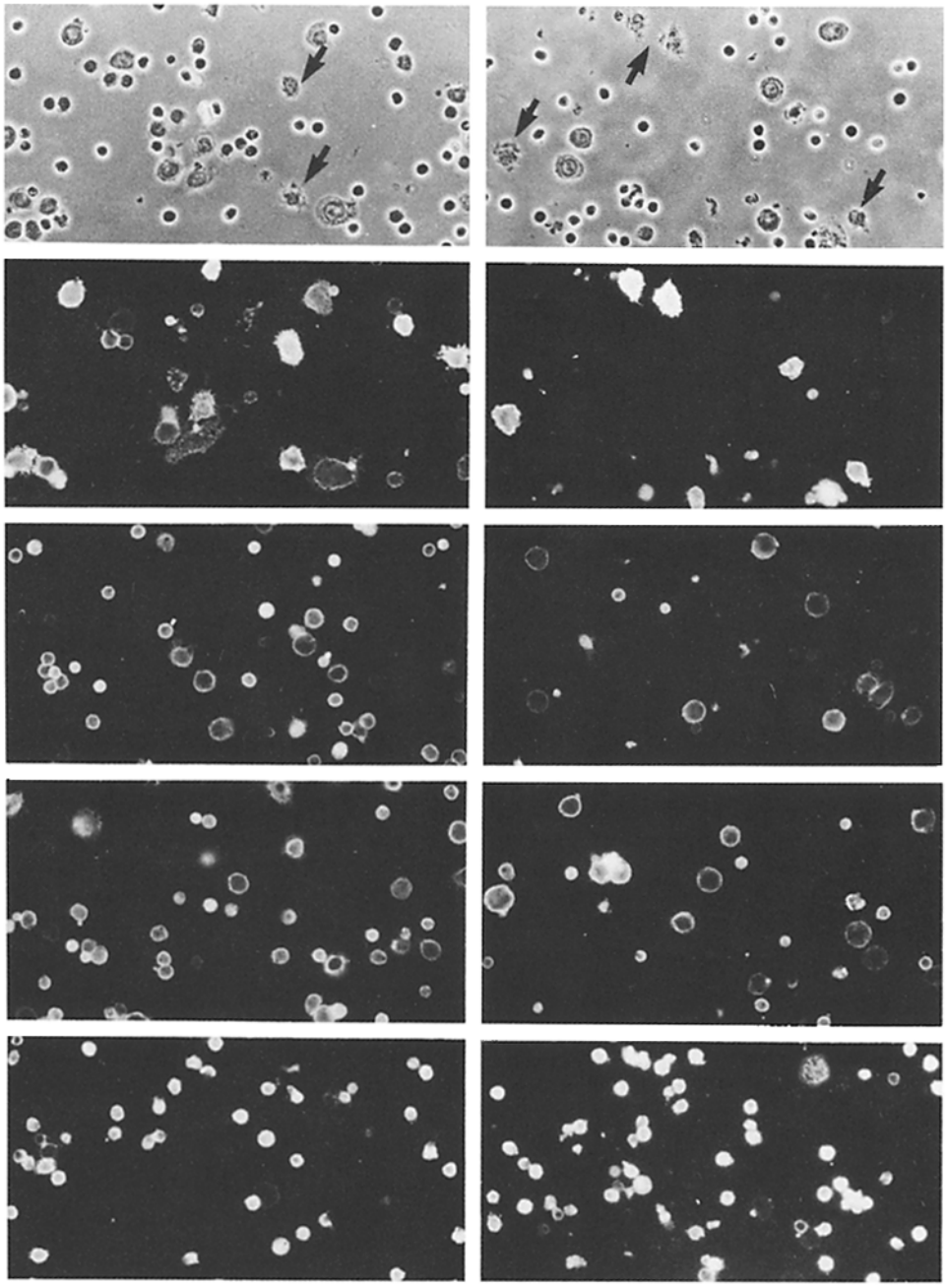

FIGURE 4. Syngeneic B cells preferentially cluster with DC and T cells. B6D2F$_1$ DC (2.5×10^4) and T cells (2×10^6; treated with α-Ia and C) were cultured for 2 d with 3×10^6 syngeneic (B6D2F$_1$) or allogeneic (B6.H-2k) B cells. Clusters were isolated, dissociated by pipetting, and spun onto coverslips to be stained with monoclonal antibodies, biotin mouse α-rat Ig, and fluorescent avidin. The total number and frequency of cells stained with each antibody is given in Table X. All fields contained ~50–60 profiles. On the *left*, clusters were formed with

T cells preferentially recognize B cells that have been exposed to antigen. The alternative, that DC in clusters differentiate to recognize B cells, seems unlikely. Specifically, we do not observe the reformation of clusters or the development of PFC when T cells have been depleted, as in the experiments of Fig. 3 and Table IV.

Significance of DC/T/B Clusters. Once B cells have been part of the cluster (for as little as 1–2 d), substantial PFC responses can be mediated by soluble factors in the absence of intact T cells. The helper factors are antigen nonspecific and are present in adequate quantity in the conditioned medium of DC/T cell co-cultures. However, acquisition of responsiveness to helper factors is antigen specific. This was particularly evident in the DNP-KLH response, which could be driven by soluble factors only after the B cell had been exposed to antigen and had entered the cluster (Tables VIII and IX). We regard the cluster as a long-sought source of specific, MHC-compatible B cells that become capable of differentiating into PFC in the presence of polyclonal stimulating factors. This phenomenon is more difficult to establish in the anti-RBC response, since some B cells develop into PFC in the presence of syn MLR factors and antigen but in the absence of a clustering step (2). However, recent experiments (Inaba, manuscript in preparation) have demonstrated that α-RBC PFC also require clustering. Neonatal and immunodeficient CBA/N × DBA/2 male mice were used. B cells from these strains were not stimulated by RBC and soluble factors, but became responsive to factors after clustering with DC and T cells.

Previous studies have indicated that resting or small B cells do not respond to polyclonal B cell–stimulating factors (13) and that resting B cells can be induced to become PFC after direct interaction with helper T cells (14). Our study of clusters has allowed us to establish, in tandem, the two main stages in a T-dependent B cell response, i.e., direct B-T interaction followed by factor-mediated PFC development. Thymus-dependent antigens are those that cannot directly induce the B cell to grow and differentiate, or to become responsive to growth and differentiation factors. Instead, additional stimuli must be provided by MHC-restricted T cells that recognize antigen and Ia on specific B cells. Such B-T interaction is feasible since B and T lymphocytes recognize different epitopes on complex antigens (15) and because B cells express enhanced levels of Ia after an encounter with antigen (16).

Anatomical studies indicate that there is an opportunity to form clusters in situ. It is known that B and T cells begin their circulation through lymphoid tissues from common sites, specifically the marginal zone of spleen and the

syngeneic B cells and, on the *right*, allogeneic B cells. Similar results were obtained with day 1 clusters in the same experiment, except that B lymphoblasts had not yet developed. All fields were photographed and developed with identical exposure times. × 275.

The *top* two frames (B21-2, α-Ia^{b,d}) are phase contrast and fluorescent views of an identical field. In the syngeneic system, both DC (arrows) and large B cells were stained strongly. Small B cells also stained, but too weakly to be visualized under the photographic conditions used. In the allogeneic system (*right*), only the DC (arrows) could stain since the B6.H-2k B cells were B21-2-negative. The *third* and *fourth* panels are fields stained with two distinct B cell–specific antibodies, TIB145 and TIB146. Syngeneic B cells, both large and small, were frequent in the cluster fraction, while allogeneic B cells were infrequent and consisted primarily of lymphoblasts. The *bottom* panel was stained with B5-3, rat α-mouse Thy-1.2. As expected, the frequency of T cells was increased on the right due to the paucity of allogeneic B cells.

postcapillary venules of nodes and Peyer's patch. Furthermore, B cells (presumably circulating B cells) can be found in T areas containing Ia^+ DC (4). Presumably, antigen enters the T area and, in concert with DC, initiates the development of helper T cells. The latter recognize MHC-compatible, antigen-stimulated B cells recirculating in the vicinity, thus forming the cluster. Experiments in situ first demonstrated the need for MHC compatibility between B and T cells during antibody responses (17), particularly the responses to hapten-carrier conjugates and heterologous RBC (17, 18) that we have studied here. DC-initiated T/B clusters likely represent the physiologic units in which the MHC contributes to antibody responses.

Summary

Mouse spleen suspensions generate discrete cell clusters within 1–2 d of culture. We have isolated these clusters by velocity sedimentation to study their contribution to primary antibody responses. Clusters represent ~5% of the starting spleen cells and consist of 20–50% B cells, 20–50% T cells, and 10–20% dendritic cells (DC). When the cultures are stimulated with thymus-dependent antigens, like heterologous red cells or dinitrophenyl–keyhole limpet hemocyanin (DNP-KLH), the clusters are the principal site for the development of plaque-forming cells (PFC). Noncluster fractions form few PFC and only when supplemented with fresh DC. PFC responses in all cases are antigen specific.

B cells cluster only in the presence of T cells and DC (1 DC/200 B-T cell mixtures) and only after encountering specific antigen. The elimination of either DC or Lyt-1^+2^- T cells, with monoclonal antibody and complement, ablates B cell development into PFC. PFC responses are restored with antigen-nonspecific helper factors formed in the syngeneic mixed leukocyte reaction between DC and T cells. Since PFC to DNP-KLH do not develop de novo when B cells are exposed to antigen and helper factors, anti-DNP PFC precursors must be stimulated within clusters to become responsive to helper factors.

PFC development within clusters is restricted by the major histocompatibility complex (MHC). When DC and T cells are from strain P_1, then P_1 but not P_2 B cells develop into PFC; when DC are from strain P_2 and T cells from strain P_1, strain P_2 B cells are selected to become PFC in clusters. The entry of B cells into clusters is itself MHC restricted, since P_1 DC/T cells aggregate six times as many B cells from strain P_1 as strain P_2.

Thus, clusters are the site in which DC, B, and T cells interact to generate PFC. One can use clusters to retrieve B cells that have been selected in an antigen-dependent, MHC-restricted fashion and to show that clustering B cells become responsive to soluble, polyclonal helper factors.

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