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Immunology

Dendritic cells are critical accessory cells for thymus-dependent antibody responses in mouse and in man

(macrophages/monocytes)

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ABSTRACT **We report that dendritic cells (DC) are necessary and potent accessory cells for anti-sheep erythrocyte responses in both mouse and man. In mice, a small number of DC (0.3–1% of the culture) restores the response of B/T-lymphocyte mixtures to that observed in unfractionated spleen. An even lower dose (0.03–0.1% DC) is needed if the T cells have been primed to antigen. Responses are both antigen and T cell dependent. Selective depletion of DC from unfractionated spleen with the monoclonal antibody 33D1 and complement ablates the antibody response. In contrast to DC, purified spleen macrophages are weak or inactive stimulators. However, when mixed with DC, macrophages can increase the yield of antibody-secreting cells about 2-fold. In man, small numbers (0.3–1%) of blood DC stimulate antibody formation *in vitro*. Purified human monocytes do not stimulate but in low doses (1% of the culture) inhibit the antibody response. Likewise, selective removal of human monocytes with antibody and complement enhances or accelerates the development of antibody-secreting cells. We conclude that DC are required for the development of T-dependent antibody responses by mouse and human lymphocytes *in vitro*.**

Dendritic cells (DC) are specialized stimulators, or accessory cells, for the immune response (reviewed in refs. 1 and 2). Most of the work in this and other laboratories has focused on T-lymphocyte responses. We have now studied the primary antibody response to sheep erythrocytes (SRBC). DC prove to be effective stimulators of antibody formation in both mouse and human lymphocyte cultures. In contrast, mononuclear phagocytes are weak or inactive. Human monocytes in small numbers (1%) inhibit the development of antibody-secreting cells. This may explain the prior difficulties in initiating human B-cell responses *in vitro*.

MATERIALS AND METHODS

Animals. Mice of either sex, 6–10 wk of age, were used. Swiss mice were from The Rockefeller University, whereas (A × B6) F_1, (DBA/2 × BALB/c) F_1, and (BALB/c × C57BL/6) F_1 were from The Trudeau Institute (Saranac Lake, NY).

Antigen. SRBC and ox erythrocytes (Colorado Serum, Denver, CO) were stored in Alsever's at 4°C and used within 2 wk of shipment.

Antibodies. 33D1 (3, 4) and B21-2 (5) are rat monoclonal antibodies specific for mouse DC and I-$A^{b,d}$ antigens, respectively. TIB-99 is a monoclonal anti-mouse thy-1.2 (6) obtained from the American Type Culture Collection (Rockville, MD). 3C10, BA-1, and Leu-1 are mouse monoclonal antibodies that react with human macrophages (Mϕ), B cells, and T cells, respectively, but not with DC (7).

Table 1. DC stimulate the development of antibody-forming cells

Cells	PFC per culture					
	Exp. 1	Exp. 2	Exp. 3A	Exp. 3B	Exp. 4A	Exp. 4B
Spleen	518	288	410	1,847	491	3,026
G-10						
− DC	13	3	7	34	0	91
+ 0.01% DC	—	—	47	703	13	742
+ 0.03% DC	—	—	87	1,621	67	2,119
+ 0.10% DC	—	162	173	2,459	91	2,567
+ 0.15% DC	60	—	—	—	—	—
+ 0.30% DC	—	385	263	3,644	317	3,309
+ 0.50% DC	720	—	—	—	—	—
+ 1.0% DC	—	792	777	4,165	472	3,015
+ 1.5% DC	1,653	—	—	—	—	—
+ 3.0% DC	—	—	813	4,743	504	2,233

Unfractionated spleen cells or Sephadex G-10 nonadherent spleen cells (primarily B and T lymphocytes) (5×10^6) supplemented with varying doses of DC were cultured for 4 days with 5×10^6 SRBC as antigen. Values given are direct PFC; the standard deviation of the plaque assay was <15%. The PFC response in the absence of SRBC ranged from 3–136. In experiments 3 and 4, the DC were tested for accessory function on unprimed spleen (part A) or spleen from mice primed with 4×10^6 SRBC intraperitoneally 4 days previously (part B), a dose that primes T cells primarily (15).

Cell Preparation. Spleen suspensions were prepared as described, including passage over Sephadex G-10 (Pharmacia) to yield accessory cell-dependent B/T lymphocyte mixtures (8). The accessory cells used to reconstitute antibody responses were purified DC (80–90% pure; ref. 9) or Mϕ-DC adherent cell mixtures (4). These adherent cells and unfractionated spleen were depleted of DC with 33D1 antibody and rabbit complement (C; ref. 4). Because the level of DC in spleen is so low (<1%), we monitored the efficacy of the depletion procedure by microscopic observation of the spleen adherent component (2, 4). 33D1 and C reduced the frequency of DC from 10–40% of adherent cells to 0–3%.

Human peripheral blood mononuclear cells and plastic-adherent cells were prepared as described (7, 10). Enriched populations (>97% pure) of monocytes were plastic-adherent cells that remained attached after overnight culture (7, 10). DC (30–70% pure by cytologic criteria) were obtained from adherent cells that had detached from the plastic surface after overnight culture and had been treated with monoclonal anti-monocyte and lymphocyte antibodies to enrich for DC (7). Selective depletion of monocytes from blood mononuclear cells was done with 3C10 antibody and C. This killed >95% of monocytes, as judged by nonspecific esterase staining and by cytology (7), and

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Abbreviations: DC, dendritic cell(s); SRBC, sheep erythrocyte(s); PFC, plaque-forming cell(s); C, complement; Mϕ, macrophages/monocytes.

Table 2. Antibody responses induced by DC are antigen dependent and specific

Erythrocytes used as antigen	Number of anti-sheep or anti-ox PFC in			
	G-10 nonadherent responders		G-10 nonadherent responders + 0.5% DC	
	Anti-sheep	Anti-ox	Anti-sheep	Anti-ox
None	25/8	38/10	61/10	75/19
Sheep	20/4	29/15	1,553/31	298/20
Ox	19/10	43/12	584/31	2,261/80

Sephadex G-10 nonadherent spleen cells were stimulated with SRBC or ox erythrocytes in the absence (left) or presence (right) of 0.5% purified DC. At day 4, PFC responses were measured in both SRBC and ox erythrocyte plaque assays. Data are means ± SD of triplicate cultures. The degree of sheep and ox crossreactivity *in vitro* is similar to that observed with immunization *in situ* (16).

the populations remained monocyte-depleted for 9 days in culture. Human B/T lymphocyte mixtures, which required accessory cells to generate antibody responses, were obtained by three techniques: sedimentation in self-generated Percoll (Pharmacia) gradients (11); passage over Sephadex G-10 columns as above (8); and two adherent cell depletions on plastic (10).

Culture Conditions. For mouse cultures we used RPMI 1640 (GIBCO) with 10% heat-inactivated fetal calf serum (HiClone, Sterile Systems, Logan, UT), 50 μM 2-mercaptoethanol, and 20 μg of gentamycin sulfate per ml. Graded doses of irradiated accessory cells were added to 5×10^6 responders and cultured for 4–5 days with or without 5×10^6 SRBC in 16-mm wells and a total volume of 1.2 ml.

For human cells we used RPMI 1640 with 20% fetal serum, gentamycin, 2-mercaptoethanol, 25 mM Hepes, and 2 mM L-glutamine. Although not shown in *Results*, isologous plasma [15% (vol/vol)] could be used instead of fetal calf serum for DC-mediated primary anti-SRBC responses. Cells (2.5×10^6) were cultured in 16-mm wells for 6–12 days with or without an equal number of SRBC in a total volume of 1.2 ml. After 5 days, the cultures were fed daily with 50 μl of culture medium.

Plaque-Forming Cells (PFC). Direct or IgM antibody-secreting cells were counted with a plaque assay in agarose gel as described (8).

RESULTS

DC Induce Plaque-Forming Responses in Nonadherent Mouse Spleen Cells. Spleen cells passed over Sephadex G-10 require accessory cells for the development of antibody responses (8, 12–14). Our first experiments showed that purified DC provided the accessory cell requirement for the generation of PFC (Table 1). As few as 0.3–1% DC induced responses comparable to or greater than that of whole spleen. If the T cells were primed with a low dose of SRBC *in situ*, the secondary response needed only 0.03–0.1% DC (Table 1, experiments 3 and 4).

Table 3. Selective elimination of DC with 33D1 monoclonal antibody and C ablates antibody-forming responses by mouse spleen

Exp.	PFC response of spleen treated with					
	No Ab			33D1		
	No C	C heated	C fresh	No C	C heated	C fresh
1	—	—	1,390	—	—	17
2	—	—	2,483	—	—	37
3	714	—	561	—	—	123
4	518	—	429	—	—	19
5	203	128	212	164	168	23
6	1,094	894	1,110	1,372	1,230	10

Spleen cells were exposed to no antibody (Ab) or 33D1 anti-DC Ab with either no C, heated (56°C, ½ hr) C, or active C. The cells were washed and cultured for 4 days with SRBC as antigen. Values shown are PFC per culture. Standard deviations were all <15%. PFC responses in the absence of SRBC were <10% of those listed. In experiment 3, the 33D1/active C cells were also supplemented with 3×10^4 DC and shown to develop 408/39 PFC.

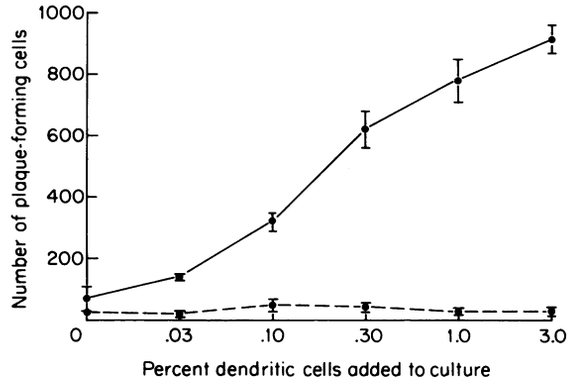

FIG. 1. DC reconstitute the response of 33D1/C-treated spleen cells. Graded doses of DC were added back to whole spleen that had been treated with 33D1 monoclonal antibody and C. PFC were measured with (●——●) or without (●- - -●) SRBC antigenic stimulation. Control PFC responses were 1,048 ± 190 (no antibody, no C) and 839 ± 203 (C only).

The DC-stimulated PFC response was antigen dependent (Table 1) and specific (Table 2), as demonstrated by comparing the responses to SRBC and ox erythrocytes. In 20 consecutive experiments (not shown), DC did not stimulate an anti-SRBC antibody response if T cells had been depleted from the Sephadex G-10 nonadherent population with anti-thy-1 monoclonal antibody and C. We conclude that DC are potent stimulators of antigen- and T-cell-dependent PFC responses.

Selective Removal of DC Ablates PFC Responses in Unfractionated Spleen. The need for DC was verified by killing these cells with a specific monoclonal antibody (33D1; refs. 3 and 4) and C. Selective elimination of DC with 33D1 and C ablated the antibody-forming capacity of mouse spleen (Table

Table 4. Selective elimination of DC with 33D1 monoclonal antibody and C ablates the accessory function of spleen adherent cells

Exp.	PFC in cultures of				
	Spleen	Sephadex G-10 nonadherent spleen			
	—*	—*	No Ab, No C†	C only†	33D1, C†
1	395	11	408	400	13
2	1,413	0	943	1,313	17
3	845	27	—	210	15
4	760	12	—	720	7
5	786	0	150	160	0
6	561	12	—	440	188
7	1,317	56	1,179	1,413	259
8	410	7	323	227	13

PFC responses to SRBC were measured after 4 days of culture. Responding cells (5×10^6 per culture) were whole spleen or Sephadex G-10 nonadherent spleen (B and T lymphocytes) reconstituted with spleen adherent cells ($3–10 \times 10^4$ per culture) that had been treated with no antibody (Ab) or 33D1 anti-DC and C. Responses in the absence of SRBC were 0–123 PFC. The spleen adherent cells were not irradiated, but in experiments 3 and 4, irradiated cells were compared to unirradiated cells and gave similar data. In experiments 2 and 8, adherent cells treated with 33D1 in the absence of C were also tested and gave 1,383 and 293 PFC, respectively.

* No adherents.

† Adherents.

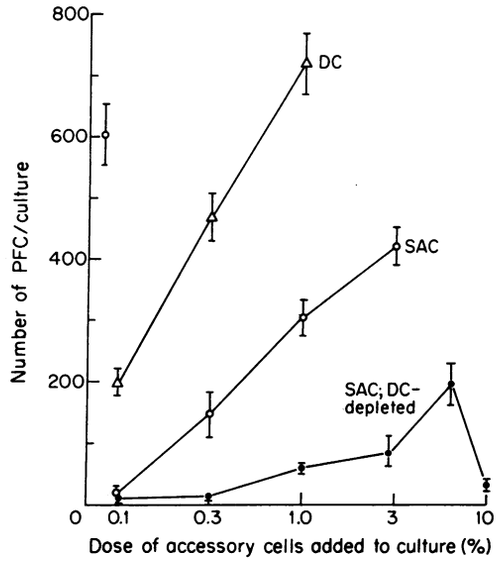

FIG. 2. Purified spleen Mϕ are not active accessory cells for the PFC response. Cultured spleen adherent cells (SAC; 80% Mϕ) were treated with no antibody and C (○) or 33D1 anti-DC and C (●), irradiated, and tested as accessory cells in PFC responses. Purified DC (△) were also studied. The percentage of Ia-bearing Mϕ in the spleen adherent population was 35%, as determined by indirect immunofluorescence with an Fab fragment of the monoclonal antibody B21-2. The control PFC response for whole spleen is shown at the top left.

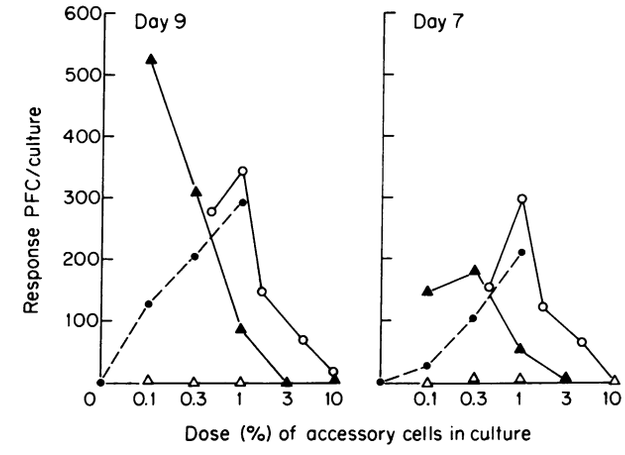

FIG. 4. DC stimulate and monocytes inhibit the human primary PFC response. Plastic nonadherent, peripheral blood mononuclear cells were passed over Sephadex G-10 columns to provide accessory cell-dependent mixtures of human B and T lymphocytes. Cells (2.5×10^6) were cultured in 16-mm wells in the presence of graded doses of accessory cells: △, purified monocytes obtained from 1-day cultures of adherent cells that remained attached to plastic; ○, adherent cells that were released from plastic after 1 day of culture (mixtures of monocytes, lymphocytes, and DC); ●, enriched populations of DC (>70% by cytologic criteria) that were depleted of monocytes and lymphocytes with monoclonal antibodies and C; ▲, a fixed number of DC (0.3%) supplemented with the doses of monocytes shown on the abscissa. PFC responses to SRBC were measured at 7 and 9 days of culture. No PFC were detected in the absence of SRBC.

3). The function of these 33D1/C-treated spleen cells was reconstituted with small numbers of DC enriched by a technique not requiring the 33D1 antibody (Fig. 1).

The Effects of Spleen Mϕ on Murine PFC Responses. The role of Mϕ in PFC development was studied with 33D1- and C-treated spleen adherent cells. Most of the remaining cells were Mϕ that expressed Fc receptors, Mϕ-restricted surface antigens, and phagocytic capacity (2–4). These Mϕ were inactive as accessory cells (Table 4). Suspensions of adherent cells obtained after overnight culture were also tested. These mixtures of Mϕ and DC were <20% as active as enriched DC (Fig. 2). Selective elimination of DC with 33D1 and C again removed most of the accessory function (Fig. 2).

To test for a possible synergism between Mϕ and DC, we added back-graded doses of purified DC to spleen Mϕ that had been treated with 33D1 and C. The DC-mediated response was increased about 2-fold when small numbers of Mϕ (0.5–1%) were present (Fig. 3).

Accessory Cell Requirements for Human PFC Responses. Accessory cell-dependent, human blood B and T cells were prepared in three ways (see *Materials and Methods*). As in mice, small numbers (0.3–1%) of DC induced an anti-SRBC PFC response, whereas enriched populations of Mϕ did not (Fig. 4, Table 5). However, very low levels (0.1–0.3%) of monocytes enhanced the DC-mediated response, whereas higher levels

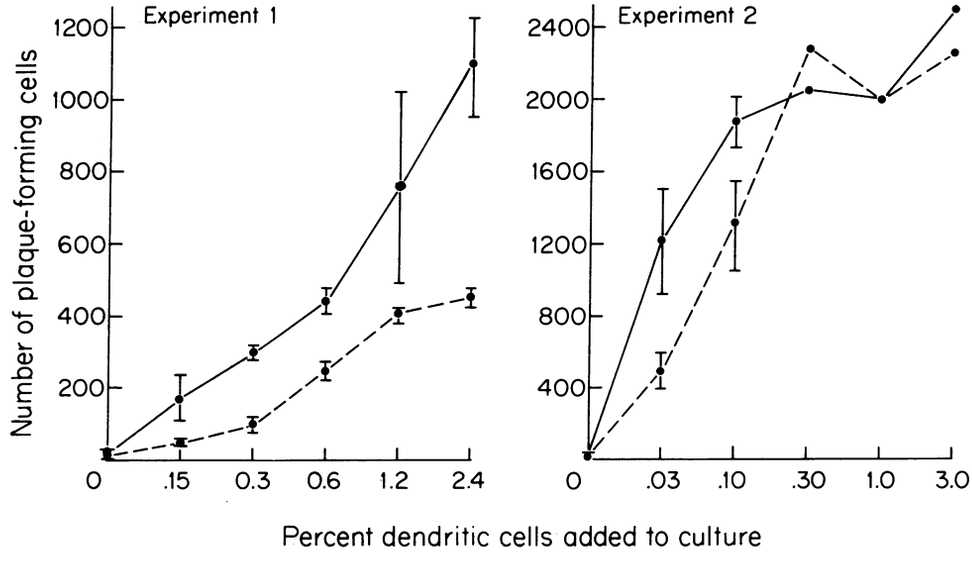

FIG. 3. Mϕ can enhance a DC-mediated PFC response. Sephadex G-10 nonadherent spleen was supplemented with graded doses of DC (●---●) or graded doses of DC and a constant number of Mϕ (●——●; spleen adherent cells treated with 33D1 antibody and C to eliminate DC). Other data from these experiments are given in Table 4, experiments 1 and 2. PFC responses in the absence of antigen were <90 PFC per culture.

Table 5. DC stimulate and monocytes inhibit the primary anti-SRBC response of human blood lymphocytes

		PFC response (with/without SRBC) stimulated by					
			DC-enriched populations		Monocyte-enriched populations		
Exp.	Day of assay	No accessory cells	0.3%	1.0%	1.0%	3.0%	DC + 1% monocytes
1	6	0/0	205/0	440/0	40/0	32/0	NT
2	8	0/3	78/0	130/0	0/3	3/0	30/3
3	8	0/0	33/0	93/0	NT	NT	NT
4	7	0/0	67/0	150/0	0/0	3/0	11/0
5	7	0/0	103/0	215/0	5/0	9/0	61/0
5	9	0/0	205/0	289/0	0/0	0/0	89/0

Accessory cell-dependent, human peripheral blood lymphocytes were prepared by adherence to plastic (experiment 1), sedimentation in dense Percoll (experiments 2 and 3), or passage over Sephadex G-10 columns (experiments 4 and 5). These B/T lymphocyte mixtures were cultured in the presence or absence of SRBC and no accessory cells, DC-enriched populations, monocyte-enriched populations (see text), or mixtures of DC (0.3% in experiments 2 and 5; 1% in experiment 4) and 1% monocytes. Direct or IgM PFC were measured on the indicated days. NT, not tested.

(1%) reproducibly inhibited PFC development (Fig. 4, Table 5).

The selective removal of monocytes enhanced PFC development in peripheral blood mononuclear cells; >95% of blood monocytes could be removed with the 3C10 monoclonal antibody and C as described (7). Monocyte depletion had two consequences that depended on the cell donor. One result was that the removal of monocytes allowed a PFC response to occur (e.g., Fig. 5, experiment 1). This finding was reproduced twice in this donor and with one other donor. In a third donor (Fig. 5, experiment 2), monocytes repeatedly delayed rather than blocked PFC development.

DISCUSSION

The antibody response to SRBC (17) was one of the first systems in which primary immune responses were generated in culture. In addition to lymphocytes, successful immunization required accessory cells, selected by adherence to glass or to plastic (18–22). DC were discovered in studies aimed at characterizing the active adherent cell (23). DC were distinguished and separated from other adherent cells, principally Mϕ, and were shown to be active stimulators of several T-cell responses (reviewed in refs. 1 and 2). We have returned to the primary anti-SRBC response to demonstrate that DC are essential for thymus-dependent B-cell development. Specifically, small numbers (<1% of the culture) of purified DC are sufficient for active PFC responses in both mouse and man (Tables 1 and 5). Selective elimination of mouse DC with 33D1 monoclonal antibody and C removes both the antibody response of whole spleen (Table 3 and Fig. 1) and the accessory function of heterogeneous adherent populations (Table 4 and Fig. 2).

The effect of DC on antigen-specific responses most likely involves helper T lymphocytes that are induced to grow or function (or both) in response to DC and antigen. Some of the helper effects may not be antigen dependent, because DC are strong stimulators of the syngeneic mixed leukocyte reaction, which in turn results in the production of helper factors (24, 25). It is also possible that DC can interact directly with B cells. For example, it is known that DC and T cells can physically aggregate with developing B cells *in vitro* (ref. 26; unpublished data). However, we still do not know if DC and B cells will cluster independently of T lymphocytes.

The contribution of Mϕ to antibody responses is complex and entails three findings. First, enriched populations of mouse spleen Mϕ and human blood monocytes by themselves are weak or inactive accessory cells for anti-SRBC responses (Tables 4 and 5; Figs. 2 and 4). Many, if not most, of the Mϕ in our test populations expressed Ia antigens. Therefore, we could not confirm the conclusion of numerous previous studies that Ia$^+$

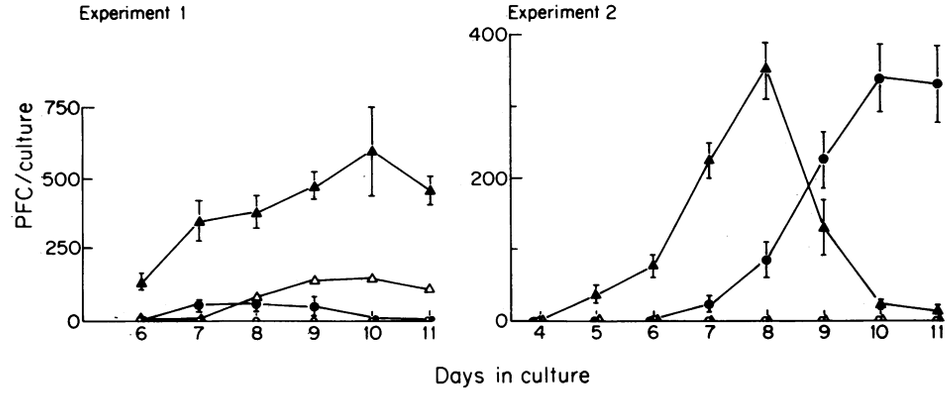

FIG. 5. Depletion of monocytes enhances human PFC responses. Blood mononuclear cells from two donors (experiments 1 and 2) were exposed to 3C10 anti-monocyte antibody and C (▲) to eliminate >95% of the monocytes (as judged by nonspecific esterase staining). Controls (●) were exposed to C only (or in other experiments to antibody alone). PFC responses to SRBC were measured on successive days in cultures of 2.5×10^6 cells. Viable cell recoveries (70% of the starting dose) were similar in all cultures. No PFC developed in the absence of SRBC in one donor (△, experiment 1), but a few were detected in the other (△, experiment 2).

Mϕ are essential for antibody responses. Instead, the active Ia^+ cell under our experimental conditions is the DC.

The second point is that a very small number of Mϕ (<1% of the culture) enhances the stimulation by DC (Figs. 3 and 4). This increase can even be mediated by Ia^- Mϕ (12) and perhaps by other Ia^- cells, such as cultured fibroblasts (27, 28). Synergism may involve the production of interleukin 1 (29) or additional "trophic" effects on cell viability (30).

The third finding is that Mϕ can inhibit the PFC response. Inhibition is known to require relatively high numbers in mice (2.5% of the culture; e.g., see refs. 22 and 30), but we find that inhibition occurs at very low doses in man (1%; Table 5 and Fig. 4). The realization that monocytes inhibit and DC stimulate antibody formation has led us to test the effect of selective monocyte depletion on human anti-SRBC responses. Responses are enhanced or accelerated after the elimination of most monocytes with a specific monoclonal antibody and C (Fig. 5). The human PFC response (100–700 PFC at days 6–8) seems comparable to the murine system, because the number of B cells that are cultured is about 10% of that used in murine systems. Although SRBC is the only antigen we have studied, our data indicate that the development of human antibody responses can best be achieved by removing rather than adding monocytes, as has been assumed (31–33). This change in approach toward *in vitro* immunization hopefully will permit the study of primary B-cell responses in clinical samples and aid in the development of B-cell hybridomas that secrete high levels of human monoclonal antibodies.

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