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Michel C. Nussenzweig

Ralph M. Steinman

Badma Gutchinov

Zanvil A. Cohn

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# DENDRITIC CELLS ARE ACCESSORY CELLS FOR THE DEVELOPMENT OF ANTI-TRINITROPHENYL CYTOTOXIC T LYMPHOCYTES\*

By MICHEL C. NUSSENZWEIG, RALPH M. STEINMAN,‡ BODMA GUTCHINOV,  
AND ZANVIL A. COHN

*From The Rockefeller University, New York 10021*

The adherent cell population from mouse spleen is a heterogeneous collection of cells that includes mononuclear phagocytes and a novel entity, the dendritic cell. As reviewed recently (1), dendritic cells and macrophages differ in many important respects, including their structure at the light and electron microscopic level, endocytic activity, behavior in *in vitro* systems, life history and phylogeny, and surface antigens and receptors. It is now possible to separate dendritic cells and macrophages from complex parenchymal mixtures, to monitor the purity of the preparations, and to cultivate each cell type for prolonged periods in a tissue culture environment.

With the availability of the above techniques, it has been possible to evaluate the separate roles of dendritic cells and macrophages in the induction of immune responses. We have reported that dendritic cells serve as powerful stimulators of both the allogeneic and syngeneic mixed leukocyte reactions (MLR),<sup>1</sup> whereas macrophages (M $\phi$ ) are either weak or inactive (2–4). We now compare the accessory cell function of dendritic cells (DC) and M $\phi$ , specifically their ability to support the generation of anti-trinitrophenol (TNP) cytotoxic T lymphocytes (CTL) in a primary *in vitro* response. The evidence will show that DC are the critical accessory cells, whereas M $\phi$ , regardless of source or expression of Ia antigen, are without significant activity. M $\phi$ , however, can serve as potent inhibitors of the DC-dependent production of CTL via an indomethacin-sensitive mechanism.

## Materials and Methods

*Mice.* C57BL/6 and B6D2F<sub>1</sub> mice were obtained from The Trudeau Institute, Saranac Lake, N. Y., and CBA/J mice from The Jackson Laboratory, Bar Harbor, Maine. Mice of both sexes, 6 wk–6 mo old were used.

### *Cell Preparations*

**T CELLS.** Dissociated spleen cells were either enriched by passage over nylon wool columns exactly as described by Julius et al. (5), or by depleting B cells on Petri dishes coated with sheep anti-mouse Ig according to Wysocki and Sato (6). Both T cell preparations contain >80–90%

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<sup>1</sup> *Abbreviations used in this paper:* BCG, Bacillus Calmette-Guérin; C', complement; CTL, cytotoxic T lymphocytes; DC, dendritic cell(s); EA, opsonized sheep erythrocytes; FCR, fetal calf serum; HKBCG, heat-killed BCG; LODAC, low-density adherent cells; MLR, mixed leukocyte reaction; M $\phi$ , macrophages; NyT, nylon wool-passed T cells; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; PHA, phytohemagglutinin A; sIg<sup>-</sup>, surface Ig-negative; TNP, trinitrophenol; UV, ultraviolet.

T cells as evaluated with cytotoxicity assays with a monoclonal rat anti-mouse Thy-1 (clone B5-3) and guinea pig or rabbit complement (Heme Lo; Accurate Chemical & Scientific Corp., Hicksville, N. Y.) at a final concentration of 1:15.

**M $\phi$  AND DC.** Low-density spleen cells, obtained by floatation on bovine plasma albumin columns, were used as an enriched source of adherent cells (7). The low-density adherent cells (LODAC) contain DC and M $\phi$  in varying proportions and <5% contaminating lymphocytes (8). After overnight culture, most of the LODAC can be dislodged from the culture surface and the mixture can be separated into DC and M $\phi$  fractions by either of two techniques. First, the mixture can be rosetted with opsonized sheep erythrocytes (EA) and fractions can be obtained after floatation in dense bovine plasma albumin columns (8). The EA<sup>-</sup> fraction consists of irregularly shaped DC that are Ig<sup>-</sup> and Thy-1<sup>-</sup>. The EA<sup>+</sup> fraction contains 60–70% rosetted cells, 20–30% nonrosetted DC, and small numbers of contaminating lymphocytes and dead cells.

A second separation procedure is based upon the readherence of M $\phi$  (3, 4).  $2 \times 10^5$ – $4 \times 10^5$  LODAC are applied to 13-mm diameter glass coverslips (Gold Seal 3550; Clay Adams, Div. Becton, Dickinson & Co., Parsippany, N. J.) in 0.1 ml medium and incubated for 1–3 h at 37°C. The nonadherent fraction is dislodged by gentle pipetting. It contains 60–90% irregularly shaped, EA<sup>-</sup> DC, 5–20% phagocytic M $\phi$ , and small numbers of contaminating lymphocytes and dead cells. The readherent fraction is >90–95% typical M $\phi$  capable of binding and interiorizing EA.

**Ia-BEARING M $\phi$ .** To obtain M $\phi$  that were rich in surface Ia antigens and that would continue to express Ia through a 4 to 5-d culture period (Results), we used mice that had been infected intravenously with  $10^6$  live *Bacillus Calmette-Guérin* (BCG; Pasteur strain, The Trudeau Institute) 1–3 mo before killing. Adherent spleen cells from these mice contain M $\phi$  that express substantial amounts of Ia which was quantitated and visualized as described below. The yield of DC and the amount of Ia on DC was not significantly altered in immunized animals. Suitable populations of Ia<sup>+</sup> spleen M $\phi$  could sometimes be obtained from mice reared in a facility that was not pathogen free. BCG-infected mice boosted with  $2 \times 10^6$  heat-killed BCG (HKBCG) intraperitoneally 2 d before killing were a reliable source of peritoneal M $\phi$  rich in Ia. BCG-immune boosted peritoneal cells were 95% typical phagocytic M $\phi$ . However, nonphagocytic adherent DC were present in this population (Results).

**MONOCYTES.** Heparinized peripheral blood was diluted 1:1 with saline and spun on a cushion of three parts 50% Hypaque and seven parts 9% Ficoll for 20 min at 400 *g*. The interphase was collected, washed three times, and the cells plated on 13-mm coverslips in 5% fetal calf serum (FCS) (Flow Laboratories, Inc., Rockville, Md.)-supplemented RPMI-1640 medium (Grand Island Biological Co., Grand Island, N. Y.). The nonadherent cells were dislodged with Pasteur pipettes after a 1-h incubation at 37°C.

**Expression of Ia Antigens.** Studies that required quantitation and visualization of Ia antigens utilized antibody secreted by clone B-21. B-21 is a monoclonal rat anti-mouse Ia reagent derived by fusing rat spleen cells, immunized to BALB/c DC, with the mouse myeloma P3U-1. As described previously (3), this clone appears to secrete anti-I-A<sup>b,d</sup> antibody. Ascites was obtained after growth in sublethally irradiated B6D2F<sub>1</sub> mice and the antibody purified by chromatography on DEAE-cellulose (DE52; Whatman, Inc., Clifton, N. J.) developed with 25 mM Tris, pH 8. The antibody was radioiodinated by the chloramine T method (9) with carrier-free Na<sup>[125I]</sup> (New England Nuclear, Boston, Mass.) to a level of 7  $\mu$ Ci/ $\mu$ gm protein. Briefly, 50  $\mu$ g of protein was iodinated with 0.5 mCi of Na<sup>[125I]</sup> and chloramine T at a 15-fold molar excess over protein for 30 min at 0°C. The reaction was terminated by addition of 0.1 ml of a 0.04% tyrosine solution followed by 0.01 ml of 1 M KI. Unreacted <sup>125</sup>I was removed by passing the mixture over 0.3 ml of Dowex-1 anion exchange resin (Sigma Chemical Co., St. Louis, Mo.) that was pre-equilibrated with phosphate-buffered saline. The iodinated antibody was used to quantitate and visualize Ia in various test populations. Quantitative binding studies were performed on cells adherent to 13-mm diameter glass coverslips, or in the case of nonadherent populations, the cells were attached to poly-L-lysine-coated (25  $\mu$ g/ml) (Sigma Chemical Co.) coverslips. Cells were exposed to antibody at 0.2  $\mu$ g/ml in RPMI-1640 supplemented with 5% newborn calf serum and 20 mM sodium azide for 1 h on ice. Coverslips were then washed, fixed in glutaraldehyde, and counted in a Packard Auto-Gamma Scintillation Spectrometer

(model 5220; Packard Instrument Co., Downers Grove, Ill.). Binding data are expressed as nanograms of anti-Ia bound at a concentration of 0.2  $\mu\text{g}/\text{ml}$ . Saturation occurred over an external concentration range of 1–3  $\mu\text{g}/\text{ml}$  for different populations, but the equilibrium binding constant is identical for all cell types. Nonspecific binding was  $\leq 0.05$  ng whether evaluated on coverslips that lacked cells or coverslips that bore cells of a nonspecific haplotype (Fig. 2C). To visualize Ia-bearing cells, the same coverslips were attached to glass slides, dipped in Ilford L4 emulsion (Ilford Limited, Basildon, Essex, England) exposed for 1 d, developed in Kodak D19 (Eastman Kodak Co., Rochester, N. Y.), and examined under phase-contrast and bright-field microscopy. Labeling was only seen on cells of the appropriate H2<sup>b</sup> or H2<sup>d</sup> haplotype (Results).

In experiments where Ia-bearing cells were removed from accessory or responding populations, the cells were treated with clone B-21 at 10  $\mu\text{g}/\text{ml}$  or alloantiserum ATH anti-ATL at a 1:100 dilution (kindly provided by Dr. John Ray, Division of Research Resources, National Institutes of Health, Bethesda, Md.) and rabbit complement (C') at a 1:20 dilution (LoTox, Accurate Chemical & Scientific Corp.) for 45 min at 37°C. DNase at 30  $\mu\text{g}/\text{ml}$  (type 1; Sigma Chemical Co.) was always added, and cells were washed extensively before culture. In experiments where LODAC were treated with antibody + C', coverslips were incubated in the appropriate reagents in 16-mm tissue culture wells (3524; Rochester Scientific Co., Rochester, N. Y.).

*Development of Anti-TNP CTL In Vitro.* Responders, usually nylon wool-nonadherent spleen cells, were cultured at  $5 \times 10^6$ /16-mm diameter tissue culture well in 1 ml RPMI-1640 medium supplemented with 5% FCS,  $5 \times 10^{-5}$  2-mercaptoethanol, 1 mM glutamine, 1,000 U/ml penicillin, and 20  $\mu\text{g}/\text{ml}$  gentamycin. TNP-modified stimulator cells were prepared by incubating cells in 10 mM/ml in trinitrobenzenesulfonate (Pierce Chemical Co., Rockford, Ill.) in phosphate-buffered saline for 10 min at 37°C and was used at a dose of  $2 \times 10^6$ /culture unless stated otherwise. Accessory cells were added either in suspension or adherent to glass coverslips. Both stimulator and accessory cells were irradiated with 1,000 rad from a Cs source (Gamma Cell 1000; Atomic Energy of Canada Limited, Ottawa, Canada) before use. The cultures were maintained for 4–5 d and then their cytotoxic activity assayed on <sup>51</sup>Cr-labeled targets as previously described (3). Phytohemagglutinin (PHA; Burroughs Wellcome & Co., London, England)-stimulated spleen cells, as well as cell lines, were employed as targets: the latter included TLX-9 (H2<sup>b</sup>), P815 mastocytoma (H2<sup>d</sup>), and the BW5147 lymphoma (H2<sup>b</sup>). Cytotoxicity was only seen on TNP-modified targets. In some experiments, the effect of indomethacin on CTL development was evaluated. Indomethacin (Sigma Chemical Co.) was dissolved in absolute ethanol at 10 mg/ml and 1  $\mu\text{g}$  was added daily to the appropriate 1-ml culture. In some experiments, accessory cells were exposed to ultraviolet (UV) radiation from a Mineralight 115-V source for 10 min at a distance of 18 cm (model R52; Ultra-Violet Products, Inc., San Gabriel, Calif.). Prostaglandin E<sub>2</sub> (P-5640; Sigma Chemical Co.) was diluted to  $10^{-4}$  M in ethanol and the culture medium was made  $10^{-9}$  M in appropriate experiments.

## Results

*DC-dependent Development of CTL.* Shearer (10) discovered that H-2-restricted anti-TNP CTL develop in response to chemically modified spleen cells. To determine if accessory cells are required for the formation of CTL, we prepared T cells either by passage over nylon wool (nylon wool-passed T cells [NyT]) or by removal of Ig-bearing cells which produced a population of surface Ig-negative (sIg<sup>-</sup>) spleen cells. Each population was tested as responders and stimulators, the latter were TNP-modified and x-irradiated. TNP-NyT stimulated anti-TNP CTL formation by sIg<sup>-</sup> T responders, but not by NyT responders (Table I). The response obtained with sIg<sup>-</sup> T responders was dependent on the dose of TNP-NyT and was comparable or greater in magnitude to that seen with unfractionated spleen responders. NyT did generate CTL in response to TNP-sIg<sup>-</sup> T stimulators (data not shown). These observations indicated that nylon wool filtration removes a cell type necessary for the induction of

TABLE I  
*Comparison of Two T Cell-enriched Populations as Responders for Anti-TNP Cytotoxic T Lymphocyte Development*

Number of TNP-modified NyT stimulators added/culture	Percent specific $^{51}\text{Cr}$ release obtained at effector:target ratios of 50:1, 10:1, and 2:1					
	NyT responders			sIg <sup>-</sup> responders		
	50:1	10:1	2:1	50:1	10:1	2:1
Experiment 1						
1.5 × 10 <sup>6</sup>	0	0	0	66	39	14
1.5 × 10 <sup>5</sup>	0	0	0	31	7	1
1.5 × 10 <sup>4</sup>	0	0	0	15	1	0
Experiment 2						
3.0 × 10 <sup>6</sup>	0	0	0	31	24	14
10 <sup>6</sup>	0	0	0	34	19	9
3.0 × 10 <sup>5</sup>	0	0	0	16	7	5

For experiment 1, 5 × 10<sup>6</sup> CBA/J responders were cocultured with x-irradiated stimulators for 4 d. Cytotoxic activity was assayed on  $^{51}\text{Cr}$ -labeled PHA blasts (spontaneous release = 25%). For experiment 2, the protocol was the same as experiment 1, but cytotoxicity was assayed on the BW5147 T cell line. BW5147 was less sensitive to cytolysis than PHA blasts when the same CTL were tested in parallel (spontaneous release = 10%).

anti-TNP CTL *in vitro*. The finding that sIg<sup>-</sup> T cells respond to TNP-NyT suggested that the accessory cell does not bear surface Ig and need not be TNP-modified directly.

The induction of CTL in cocultures of NyT responders and irradiated TNP-modified NyT stimulators was then used as an assay for accessory cell activity. Purified DC or mixtures of DC and Mφ (LODAC) were the first cell types tested for accessory cell function (Table II). In 10 consecutive experiments, NyT failed to respond unless a small number of DC were added (1–2 DC/100 responding T cells). This phenomenon was evident in both high- and low-responder strains. The amount of cross-reactive lysis on histoincompatible targets was as previously described (11); i.e., CBA CTL were highly restricted to H2<sup>k</sup> targets, whereas C57BL/6 CTL showed some lysis of H-2k in addition to H2<sup>b</sup> targets. Coculture of DC and NyT did not produce anti-TNP CTL in the absence of antibody.

As will become apparent in subsequent Tables, the level of CTL in 4-d cultures increased progressively with the number of added DC (10<sup>4</sup>–10<sup>5</sup>/5 × 10<sup>6</sup> responders); in 5-d-cultures, peak levels of CTL were obtained with just 1 × 10<sup>4</sup>–3 × 10<sup>4</sup> DC.

Several properties of interest in accessory cell biology were next examined. UV irradiation abolished DC accessory cell function, but ionizing irradiation (1,500 rad) did not. Medium from 1-d-culture LODAC or medium from 5-d cultures that contained an anti-TNP response did not substitute for DC. Treatment of DC with anti-Ia plus C' abolished accessory cell function, whereas similar treatment of responders had no effect (Table III). Finally, the CTL were T lymphocytes, because they were fully inactivated with anti-Thy-1 and C'. We concluded that DC are functional accessory cells in the development of anti-TNP CTL.

*Comparison of Spleen DC and Mφ as Accessory Cells.* Because accessory cell function is generally attributed to Mφ, we compared spleen DC and Mφ from high responder

TABLE II  
DC Enable NyT to Respond to NyT-TNP

Mouse strain	<sup>51</sup> Cr-labeled target	Percent specific <sup>51</sup> Cr release at effector:target ratios of 50:1, 10:1, and 2:1					
		No accessory cells			~10 <sup>5</sup> DC/culture		
		50:1	10:1	2:1	50:1	10:1	2:1
C57BL/6	PHA blasts	0	0	0	43	16	0
CBA/J*	PHA blasts	0	0	0	38	12	1
C57BL/6	PHA blasts	0	0	0	35	14	5
CBA/J	PHA blasts	0	0	0	56	23	5
CBA/J	BW5147	0	0	0	52	25	7
CBA/J	BW5147	0	0	0	52	34	19
C57BL/6	TLX9	0	0	0	52	25	7
C57BL/6	TLX9	0	0	0	62	42	10
C57BL/6*	TLX9	0	0	0	78	19	7
C57BL/6*	TLX9	0	0	0	48	19	6

In all experiments,  $5 \times 10^6$  NyT responders were cultured with  $2 \times 10^6$  TNP-modified x-irradiated NyT stimulators for 4 d. Irradiated accessory cells were added on cover slips (LODAC) or in suspension (DC). Spontaneous release was always <30% for PHA blasts and <20% for TLX9 and BW5147 cell lines.

\* Irradiated accessory cells were added on cover slips (LODAC).

TABLE III  
Some Properties of DC as accessory cells

	Treatment		Percent specific <sup>51</sup> Cr release at effector:target ratios of		
	Responders	DC	50:1	10:1	2:1
Experiment 1*	—	—	78	41	12
	—	UV	0	0	0
	—	1,000 rad xray	70	27	8
Experiment 2‡	C'	—	32	14	4
	$\alpha$ Ia + C'	—	23	10	5
	—	—	39	19	7
Experiment 3§	—	C'	27	12	4
	—	$\alpha$ Ia + C'	7	0	0
	—	—	30	15	3

In all experiments,  $5 \times 10^6$  NyT responders were cultured with  $2 \times 10^6$  x-irradiated TNP-modified NyT stimulators for 4 d. Cultures that did not receive DC did not develop CTL.

\* C57BL/6 mice were used and cytotoxicity was assayed on TLX9 (spontaneous release = 14%).  $10^5$  EA<sup>-</sup> DC were added to each culture.

‡ CBA/J mice were used and cytotoxicity was assayed on BW5147 lymphoma (spontaneous release = 8%).  $10^5$  EA<sup>-</sup> DC were added to each culture. The anti-Ia ( $\alpha$ Ia) reagent was ATH anti-ATL and killed ~8% of the NyT population.

§ Same as experiment 2, but LODAC on 13-mm cover slips were used as a source of DC. The  $\alpha$ Ia reagent kills 100% of DC.

CBA mice. Enriched populations of the two cell types were obtained from 1-d-cultures of LODAC by EA rosetting (8) or by readherence on glass or plastic (3, 4). DC enriched by either method were potent accessory cells. Spleen M $\phi$  obtained by readherence were weak or inactive (Table IV). The EA<sup>+</sup> fraction, which was 60–70% M $\phi$  and 20–30% DC, exhibited accessory cell function proportional to DC content.

TABLE IV  
Comparison of Spleen DC and M $\phi$  as Accessory Cells

Accessory cells	Purification	Percent specific $^{51}\text{Cr}$ release at effector:target ratios of 50:1, 10:1, and 2:1					
		Experiment 1			Experiment 2		
		50:1	10:1	2:1	50:1	10:1	2:1
0		0	0	0	0	0	0
$10^5$ DC	EA $^-$	52	25	7	36	25	13
$3 \times 10^4$ DC	EA $^-$	27	12	0	20	12	4
$10^4$ DC	EA $^-$	8	0	0	10	3	2
$10^5$ M $\phi$	EA $^+$	25	5	0	—	—	—
$3 \times 10^4$ M $\phi$	EA $^+$	—	—	—	12	8	0
$10^5$ DC	Nonadherent	57	28	13	52	34	19
$10^4$ DC	Nonadherent	—	—	—	10	7	0
$2 \times 10^5$ M $\phi$	Readherent	18	4	0	—	—	—
$10^5$ M $\phi$	Readherent	—	—	—	9	0	0
$10^5$ DC + 2 $\times 10^5$ M $\phi$	Readherent + Nonadherent	59	36	11	—	—	—

For experiment 1,  $5 \times 10^6$  CBA/J NyT responders were cultured with  $2 \times 10^6$  x-irradiated TNP-modified NyT stimulators for 4 d. Spleen M $\phi$  purified by adherence were >90% phagocytic for EA, and were added on 15-mm glass coverslips. Nonadherent DC were >90% DC by morphology and >90% EA-rossette negative. The EA $^+$  LODAC fraction contained 20–30% EA $^-$ , morphologically typical DC, and 60–70% EA-rossetted M $\phi$ . Cytotoxicity was assayed on  $^{51}\text{Cr}$ -labeled BW5147 (spontaneous release = 18%). For experiment 2, the protocol was the same as experiment 1, but cytotoxicity assayed on PHA blasts (spontaneous release = 24%).

Finally, mixtures of DC and M $\phi$  induced the same level of CTL as purified DC alone. In most subsequent experiments, we used glass adherence to obtain highly enriched M $\phi$  and EA rosetting to obtain highly enriched DC. However, we have consistently noted that separation of spleen LODAC by the adherence technique yields accessory cell function almost exclusively in the nonadherent DC-rich fraction (Tables IV and V).

We next considered two possible explanations for our failure to observe significant functional effects of M $\phi$ . First, relative to DC, M $\phi$  may express less Ia antigens, and second, M $\phi$  may block their own accessory cell activity by producing prostaglandins. Populations of spleen M $\phi$  in which most cells expressed large quantities of surface Ia (Fig. 1 and Table V) were obtained from mice infected 6–15 wk previously with live BCG microorganisms intravenously. Ia antigens were quantitated and visualized with a radioiodinated monoclonal anti-I-A $^{b,d}$  reagent, clone B-21.

Adherence purified Ia $^+$  spleen M $\phi$  at all doses were unable to act as accessory cells, even when they expressed more Ia than effective doses of DC throughout the culture period (Table V). For example, in Table V, experiment 1,  $10^4$  DC expressed one-tenth the amount of Ia as  $1 \times 10^5$  M $\phi$ ; the former provided accessory cell function and the latter did not. At higher doses, spleen M $\phi$  slightly inhibited CTL development when added to DC-T cell cocultures. Addition of indomethacin did not enable M $\phi$  to function as accessory cells. However, indomethacin removed the M $\phi$  inhibition of

TABLE V  
Comparison of Ia-bearing Spleen M $\phi$  and DC as Accessory Cells

Accessory cells	Nanograms of $\alpha$ Ia Ab bound	Percent specific $^{51}\text{Cr}$ release at effector:target ratios of		
		50:1	10:1	2:1
Experiment 1				
0		0	0	0
$10^5$ M $\phi$ (adherent)	1.4	0	0	0
$10^5$ M $\phi$ + indomethacin	1.4	0	0	0
$10^5$ DC (EA $^-$ )	1.5	73	46	16
$3 \times 10^4$ DC (EA $^-$ )	0.5	52	22	6
$10^4$ DC (EA $^-$ )	—	22	3	0
M $\phi$ + $10^5$ DC	2.9	31	9	0
M $\phi$ + $10^5$ DC + indomethacin	2.9	70	34	12
Experiment 2				
0		0	0	0
$10^5$ M $\phi$ (adherent)	0.39	2	0	0
$10^4$ M $\phi$ (adherent)	—	0	0	0
$10^5$ M $\phi$ + indomethacin	0.39	1	0	0
$10^5$ DC (EA $^-$ )	1.8	78	35	12
$3 \times 10^4$ DC (EA $^-$ )	0.6	67	34	9
$10^4$ DC (EA $^-$ )	—	15	5	2
$10^5$ DC (nonadherent BCG)	2.0	76	33	9
$10^5$ M $\phi$ + $10^5$ DC EA $^-$	2.1	64	18	5

For experiment 1,  $5 \times 10^6$  NyT B6D2F $_1$  responders were cultured for 5 d with  $2.5 \times 10^6$  x-irradiated TNP-modified NyT stimulators with or without accessory cells. M $\phi$  were obtained from mice primed 12 wk previously with  $10^8$  live BCG intravenously and boosted with  $2 \times 10^6$  HKBCG 1 wk before the experiment. The M $\phi$  were all Ia $^+$  (Fig. 1) and were added on 13-mm glass cover slips. DC were from normal B6D2F $_1$  mice. At the end of the culture period, M $\phi$  remaining adherent to the cover slips bound 0.5 ng of  $^{125}\text{I}$  B-21 monoclonal Ia $^{b,d}$  antibody ( $\alpha$ Ia Ab). Cytotoxicity was assayed on  $^{51}\text{Cr}$ -labeled P815 mastocytoma (spontaneous release = 14%). For experiment 2, the protocol was the same as experiment 1 except two sources of DC were used. EA $^-$  DC were from normal mice. The nonadherent DC were obtained from the same LODAC preparation as the M $\phi$ ; i.e., mice primed to BCG 8 wk previously. M $\phi$  and nonadherent DC were separated by a single adherence step on 13-mm cover slips (spontaneous release = 15%).

DC-mediated CTL formation (Table V). We conclude that spleen M $\phi$  that bear substantial amounts of Ia are not active as accessory cells in the development of anti-TNP CTL.

*Peritoneal M $\phi$  as Accessory Cells.* Resident adherent M $\phi$  from unstimulated peritoneal cavity rosette with, and interiorize, EA. These cells showed no accessory cell activity at any dose in three consecutive experiments (Table VI). Resident M $\phi$  showed little or no inhibition of DC-mediated CTL when added up to a level of 2% of the cultured cells (not shown). Because many resident M $\phi$  express little surface Ia (e.g., Fig. 2A), we examined the properties of adherent elicited peritoneal populations from BCG-immune boosted mice. Most of these adherent cells exhibit large quantities of Ia (Fig. 2B) and this surface Ia is maintained throughout the culture period (Table VII). Most immune-boosted peritoneal adherent cells also have the characteristic cytologic and phagocytic properties of M $\phi$  (Fig. 2D). However, a small subpopulation of the adherent cells had the cytologic features of DC and did not bind or interiorize EA (Fig. 2D).

Adherent Ia $^+$  peritoneal cells did not function as accessory cells at any dose



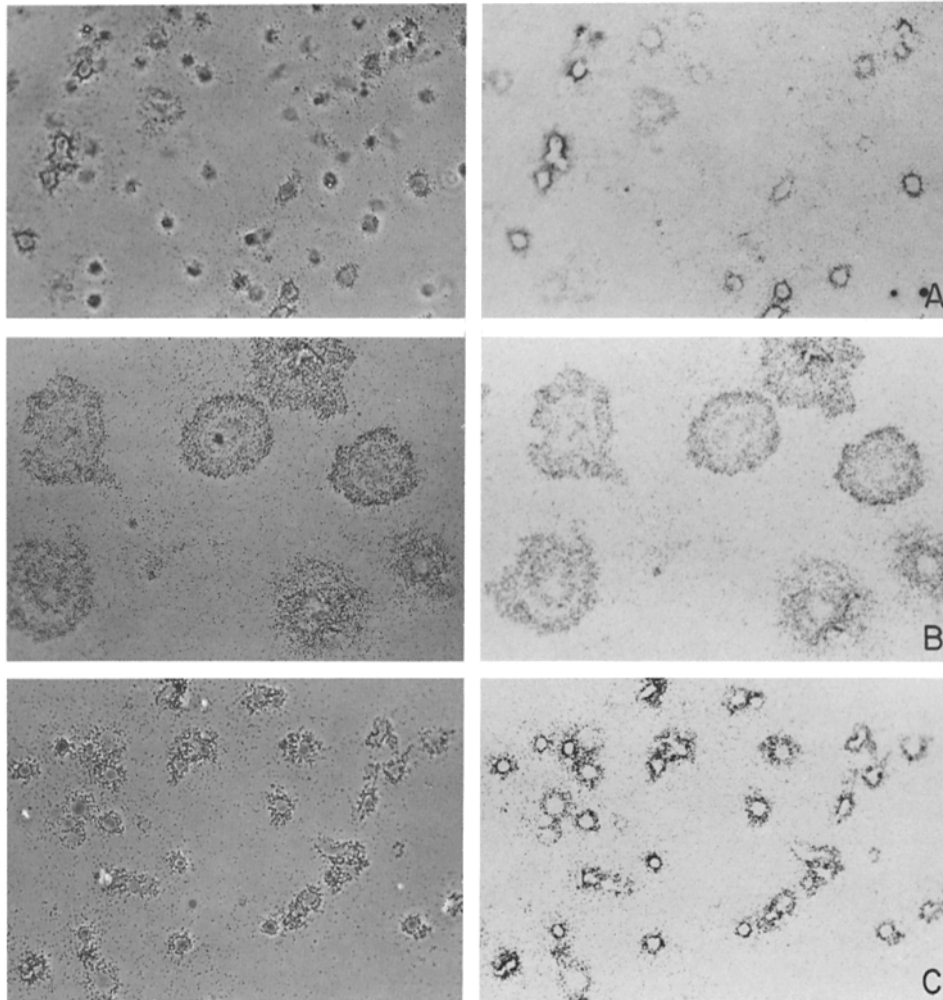


FIG. 1. Ia on spleen M $\phi$ . Radioautographs after binding of  $^{125}\text{I}$ -B21 at 4°C. The preparations were processed identically and simultaneously. Phase-contrast (left) and bright-field (right) micrographs are presented to visualize all cell profiles and the distribution of label respectively.  $\times 310$ . (A) Resident spleen M $\phi$  purified from LODAC by glass adherence after overnight culture. More than 90% of the cells in these preparations are typical M $\phi$  as determined by morphology and their ability to phagocytose EA. About one-third of the 60 profiles are labeled with anti-Ia. (B) Immune-boosted spleen M $\phi$  obtained from mice primed 8 wk earlier with BCG intravenously and boosted 2 d earlier with  $10^6$  HKBCG intraperitoneally. M $\phi$  are well spread and strongly Ia $^+$ . This preparation was used in Table V, experiment 1. (C) DC purified by the readherence technique. Most of the cells are strongly Ia $^+$ , which is a property of DC purified from unstimulated or BCG-immune mice. These are the same cells as in Table V, experiment 2.

employed (Table VII). However, the immune-boosted cells were potent inhibitors of CTL development when added to DC-T cocultures; e.g., 1 M $\phi$ /150 responders decreased the yield of lytic units by >80%. This inhibition was completely reversed by adding indomethacin (Table VII). Addition of low levels of prostaglandins ( $10^{-9}$  M prostaglandin E $_2$  [PGE $_2$ ]) reversed the indomethacin effect (Table VII). Indomethacin-supplemented cultures that contained only immune-boosted peritoneal cells as

TABLE VI  
Comparison of DC and Resident Peritoneal M $\phi$  as Accessory Cells

Accessory cells	Percent specific $^{51}\text{Cr}$ release at effector:target ratios of 50:1, 10:1, and 2:1								
	Experiment 1*			Experiment 2‡			Experiment 3		
	50:1	10:1	2:1	50:1	10:1	2:1	50:1	10:1	2:1
0	0	0	0	0	0	0	0	0	0
$10^5$ DC	52	25	7	—	—	—	62	42	10
$3 \times 10^4$ DC	27	12	0	31	12	6	—	—	—
$10^4$ DC	8	0	0	—	—	—	—	—	—
$10^5$ M $\phi$	0	0	0	0	0	0	0	0	0
$3 \times 10^4$ M $\phi$	—	—	—	0	0	0	—	—	—
$10^4$ M $\phi$	—	—	—	0	0	0	—	—	—

\*  $5 \times 10^6$  CBA/J NyT responders were cocultured with  $2 \times 10^6$  TNP-modified x-irradiated NyT stimulators for 4 d. Cytotoxicity was measured on  $^{51}\text{Cr}$ -labeled CBA PHA-stimulated spleen cells (spontaneous release = 25%).

‡ Same as experiment 1.

§ Same as experiment 1, except C57BL/6 were used as the responders and stimulators and cytotoxicity assayed on  $^{51}\text{Cr}$ -labeled TLX9 (spontaneous release = 16%).

the source of accessory cells produced a small amount of cytotoxic activity (Table VII). The level of CTL produced by  $1.5 \times 10^5$  adherent peritoneal cells corresponded to less than one-fifth the level produced by  $3 \times 10^4$  DC, even though the amount of peritoneal M $\phi$  Ia was 3.5 times greater than that seen on DC. This low level of accessory cell function may be a result of contaminating DC, but we have been unable to separate peritoneal M $\phi$  and DC to test this directly. We conclude that peritoneal M $\phi$  are weak or inactive as accessory cells even when they express large amounts of Ia.

*Blood Monocytes as Accessory Cells.* DC are known to be bone marrow derived, but their lineage has not been defined in detail. Therefore blood monocytes, which are the direct progenitors of most tissue M $\phi$ , were compared to spleen DC as accessory cells. Populations of monocytes that were >90% Ia<sup>+</sup> were obtained from the peripheral blood of BCG-immune-boosted animals (Fig. 3). Monocytes remained adherent to glass over a 4–5-d-culture period, and continued to express Ia (Table VIII). However, monocytes did not act as accessory cells for the development of anti-TNP CTL at either high or low doses or after addition of iodomethacin (Table VIII). Inhibition of DC stimulated responses by monocytes was observed and could be reversed with indomethacin.

### Discussion

The ability of mouse DC and M $\phi$  to function as accessory cells during the development of anti-TNP CTL has been compared. Accessory cell dependence was achieved by using NyT as responders and TNP-modified x-irradiated NyT as stimulators. sIg<sup>-</sup> spleen cells, which are also enriched in T lymphocytes, when used either as responders or stimulators were not accessory cell dependent. We concluded that a nylon wool-adherent sIg<sup>-</sup> mouse spleen cell was necessary for the generation of anti-TNP CTL and that it need not be TNP modified. Pettinelli et al. (12) have presented a similar system using Sephadex G-10 instead of nylon wool adherence. The findings

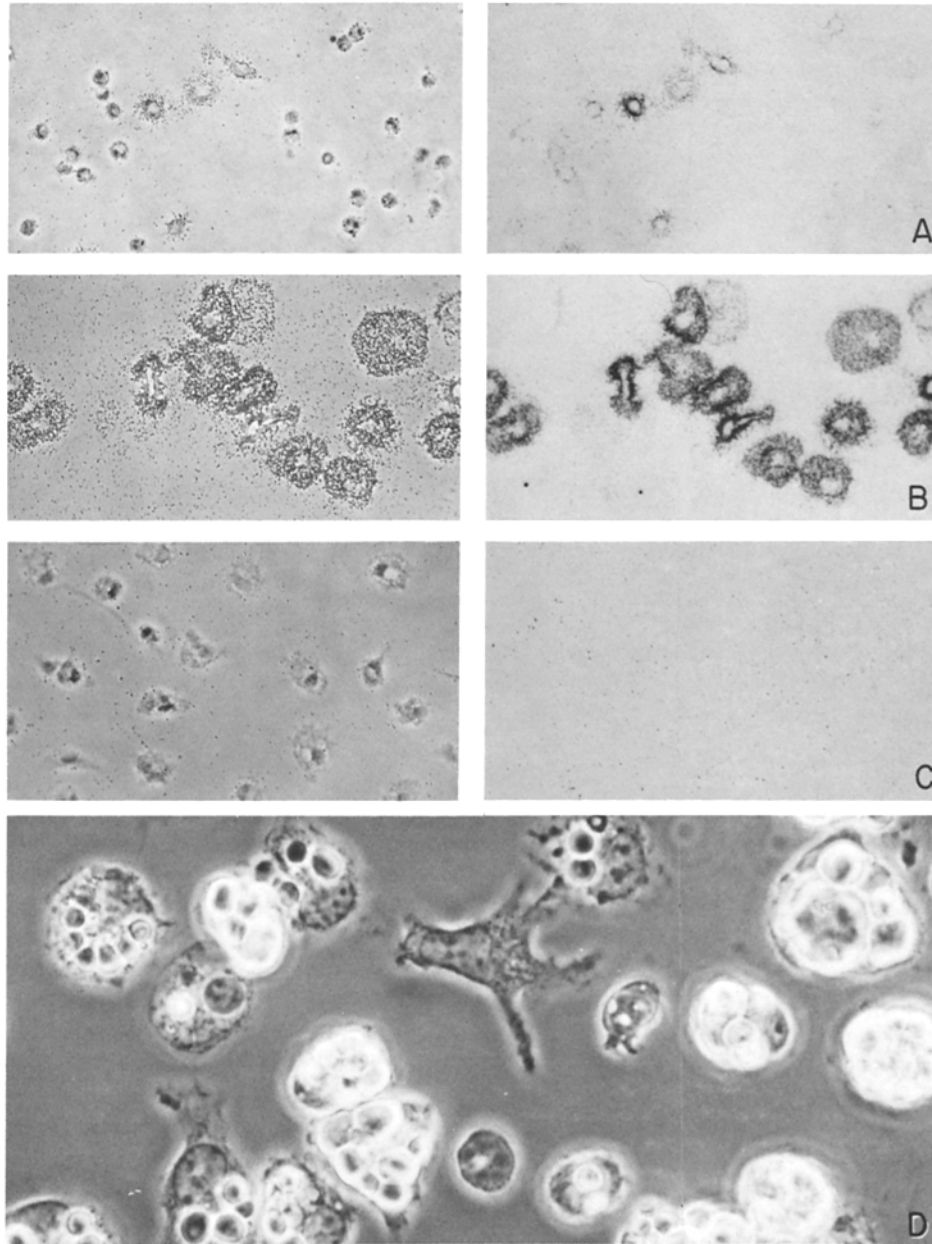


FIG. 2. Ia on peritoneal M $\phi$ . As in Fig. 1, phase-contrast (left) and bright-field (right) micrographs are shown. (A) Resident peritoneal M $\phi$  studied 3 h after explantation. A subpopulation exhibit Ia.  $\times 310$ . (B) Immune-boosted peritoneal M $\phi$  obtained 10 wk after priming mice intravenously with live BCG, and 2 d after boosting intraperitoneally with HKBCG. The cells are well spread and strongly Ia $^+$ .  $\times 310$ . (C) Control for the specificity of clone B-21 Ab binding. CBA (I-A $^b$ ) peritoneal (shown here) and spleen M $\phi$  (not shown) do not bind the anti I-A $^{b,d}$  antibody.  $\times 310$ . (D) Phase-contrast micrograph of immune-boosted peritoneal adherent cells exposed for 2 h to EA at 37°C after attachment to glass. Most of the cells phagocytose EA. Some well-spread cells, with abundant phase-dense mitochondria and few lysosomes or endocytic vacuoles, do not bind or interiorize EA. The presumptive DC population is a minor fraction (<10%) of the total adherent cells.  $\times 1,500$ .

TABLE VII  
Comparison of DC- and BCG-induced Peritoneal M $\phi$  as Accessory Cells

Accessory cells	Nanograms of $\alpha$ Ia Ab bound	Percent specific $^{51}\text{Cr}$ release at effector:target ratios of		
		50:1	10:1	2:1
Experiment 1				
0	—	0	0	0
0 + indomethacin	—	0	0	0
$10^5$ DC	1.3	100	43	14
$10^5$ DC + indomethacin	1.3	98	40	12
$1.5 \times 10^5$ M $\phi$	2.25	0	0	0
$1.5 \times 10^5$ M $\phi$ + $10^5$ DC	3.55	38	9	2
$1.5 \times 10^5$ M $\phi$ + indomethacin	2.25	29	2	0
$1.5 \times 10^5$ M $\phi$ + $10^5$ DC + indomethacin	3.55	100	49	15
Experiment 2				
0	—	0	0	0
$10^5$ DC	2.2	92	46	15
$3 \times 10^4$ DC	0.7	91	62	9
$10^4$ DC	0.2	32	20	3
$1.5 \times 10^5$ M $\phi$	2.5	0	0	0
$1.5 \times 10^4$ M $\phi$	0.2	0	0	0
$1.5 \times 10^5$ M $\phi$ + $10^5$ DC	4.7	18	7	0
$1.5 \times 10^5$ M $\phi$ + indomethacin	2.5	46	15	3
$1.5 \times 10^5$ M $\phi$ + $10^5$ DC + indomethacin	4.7	88	54	15
$1.5 \times 10^5$ M $\phi$ + $10^5$ DC + indomethacin + $10^{-9}$ M PGE $_2$	4.7	21	6	0

For experiment 1,  $5 \times 10^6$  B6D2F $_1$  NyT responders were cocultured with  $2 \times 10^6$  x-irradiated, TNP modified NyT stimulators for 5 d  $\pm$  accessory cells. M $\phi$  were added adherent to 13-mm glass cover slips and EA $^-$  DC added in suspension. Surface Ia was measured with  $^{125}\text{I}$ -labeled B-21 monoclonal anti-Ia $^{b,d}$  antibody ( $\alpha$ Ia Ab). Cytotoxicity was assayed on P815 mastocytoma (spontaneous release = 17%). Indomethacin (1  $\mu\text{g}/\text{ml}$ ) was added daily to some cultures. For experiment 2, the protocol was the same as experiment 1, except PGE $_2$  at  $10^{-9}$  M was added to some cultures at the beginning of the culture period. After the culture period, M $\phi$  that remained adherent to cover slips bound 1.7 ng of  $\alpha$ Ia Ab. Cytotoxicity was assayed on P815 mastocytoma (spontaneous release = 10%).

of Vande Stouwe et al. (13) that non-T cells are required for CTL development in response to heat-killed stimulators in man, and the studies of Seldin et al. (14) in the TNP system appear analogous to the data in this paper.

Reconstitution of NyT anti-TNP responses was then used to assay accessory cell activity. Small numbers of DC served as accessory cells. 1 DC/500 responder T cells induced measurable levels of cytolytic activity, whereas maximal responses were obtained with 0.5–2 DC/100 responders. These levels correspond to our estimates of the incidence of DC in unfractionated spleen. In contrast, M $\phi$  obtained from spleen, peritoneal cavity, and blood consistently failed to function as accessory cells over a wide dose range (0.2–4% of responding cells). Expression of surface Ia antigens in quantities ranging from 30 to 100% of that found on DC did not influence the ability of M $\phi$  to achieve accessory cell function.

M $\phi$ , particularly those from the peritoneal cavity of BCG-immune-boosted mice were able to inhibit DC-mediated CTL development. These mononuclear phagocytes exhibited as much or more Ia than DC, but small numbers reduced cytolytic activity in DC-supplemented cultures by  $\geq 80\%$  (Table VII). The inhibitory activity of M $\phi$

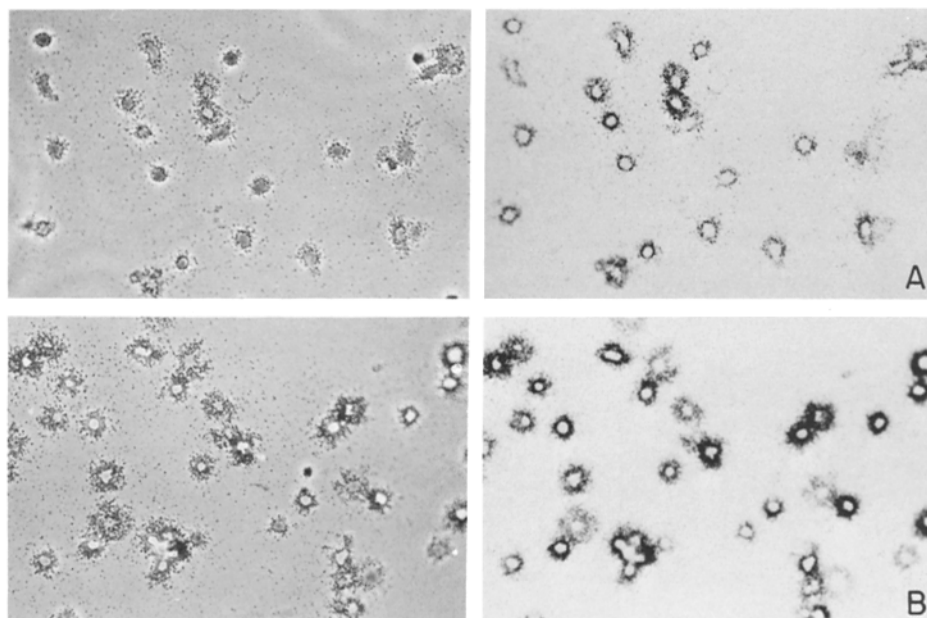


FIG. 3. Comparison of Ia on monocytes and DC. The cells were prepared as described in Fig. 1 and were used in Table VIII, experiment 1.  $\times 310$ . (A) Glass-adherent monocytes from BCG-immune boosted mice 2 h after explantation. Virtually all cells are Ia positive. (B) DC purified by the EA-rosetting procedure. The cells are strongly Ia positive.

was related to the release of prostaglandins because: (a) The inhibition was completely reversed by indomethacin; (b)  $\text{PGE}_2$  at  $10^{-9}$  M reversed the indomethacin blockade (Table VII); (c) The number of  $\text{M}\phi$  used in our experiments could generate this level of  $\text{PGE}_2$  in the culture medium (15). These inhibitory effects of  $\text{M}\phi$ -derived prostaglandins are consistent with previous work from several other laboratories (16).

Because indomethacin prevented  $\text{M}\phi$  from inhibiting the accessory function of DC, we added the drug to enriched populations of  $\text{M}\phi$  to see if this would uncover accessory cell activity. Indomethacin did not enable Ia-bearing spleen  $\text{M}\phi$  or blood monocytes to function as accessory cells (Tables V and VIII). However, a small amount of accessory function was obtained with indomethacin-supplemented BCG-elicited adherent peritoneal cells. Specifically,  $1.5 \times 10^5$  adherent cells were 20% as active as  $3 \times 10^4$  DC (Table VII). We believe that the most likely explanation of these results is the presence of contaminating DC in the BCG-elicited population.

Several mechanisms are being considered to explain the potent accessory function of DC in generating anti-TNP CTL. The first of these focuses on the physical association of DC and responding T cells to form discrete cell clusters. Such aggregates have already been described in the syngeneic MLR (3) and are associated with the proliferative response. The conditions in the present experiments are similar to those employed in the syngeneic MLR, and in preliminary experiments, cell aggregates that are enriched in the capacity to form anti-TNP CTL have been recovered. An understanding of the cell-cell dynamics within the clusters may provide an understanding of DC function. Aggregates may allow responding T cells (helper T or CTL precursors) to have prolonged, intimate, and simultaneous contact with both antigen

TABLE VIII  
Comparison of DC and Monocytes as Accessory Cells

Accessory cells	Nanograms of $\alpha$ la Ab bound	Percent specific $^{51}\text{Cr}$ release at effector:target ratios of		
		50:1	10:1	2:1
Experiment 1				
$10^5$ Monocytes	0.5	0	0	0
$10^5$ Monocytes + indomethacin	0.5	0	0	0
$10^4$ Monocytes + indomethacin	—	0	0	0
$3 \times 10^4$ DC	0.77	64	29	6
$10^4$ DC	—	20	5	0
0	—	0	0	0
Experiment 2				
$10^5$ Monocytes	0.7	0	0	0
$10^5$ Monocytes + indomethacin	0.7	0	0	0
$10^4$ Monocytes	—	0	0	0
$10^4$ Monocytes + indomethacin	—	0	0	0
$10^5$ DC	2.09	79	44	11
$3 \times 10^4$ DC	0.7	52	16	5
$10^4$ DC	—	14	4	0
$10^5$ Monocytes + $10^5$ DC	—	44	16	17
$10^5$ Monocytes + $10^5$ DC + indomethacin	—	79	52	3

For experiment 1,  $5 \times 10^6$  B6D2F<sub>1</sub> NyT responders were cultured with  $2 \times 10^6$  x-irradiated TNP-modified syngeneic stimulators for 5 d with or without accessory cells. Monocytes were added adherent to 13-mm glass cover slips and EA<sup>-</sup> DC added in suspension. After the 5-d-culture period, monocytes remaining adherent to the to the cover slips bound 1.74 ng of  $^{125}\text{I}$  B-21 monoclonal  $\alpha$ la<sup>b,d</sup>. Cytotoxicity was assayed on P815 mastocytoma (spontaneous release = 20%). Whole spleen responders in parallel cultures generated 20%, 7%, and 3% specific  $^{51}\text{Cr}$  release at effector:target ratios of 50:1, 10:1, and 2:1, respectively. For experiment 2, the protocol was the same as experiment 1, but culture the period was 4 d and spontaneous release = 12%.

and DC products. Alternatively, the highly organized and tightly opposed elements of the cluster may sequester the responding population from excess antigen or suppressor cells.

A second possibility is that DC may produce or mediate the production of interleukins that enhance the formation of CTL. We have, however, been unable to replace the accessory cells with culture media obtained either from DC alone or from DC-NyT cocultures that generate CTL. Finally, we think it unlikely that DC function via a pathway that encompasses antigen processing or antigen presentation. DC exhibit little or no endocytic activity (7) toward a variety of antigens and have failed to induce significant CTL when pulsed with TNP-NyT for 1 d *in vitro*.

The formation of anti-TNP CTL is not an isolated example of the accessory function of DC. Preliminary data from our laboratory indicate that small numbers of DC enhance proliferation and lymphokine release from T cells sensitized to microbial antigens, and two groups have shown that DC act as accessory cells for periodate-induced blastogenesis in the mouse and the rat (17, 18). This suggests that DC are accessory cells in several immune responses and may represent the critical accessory cell for the *in situ* development of immune responses in lymphoid organs.

### Summary

This study establishes that dendritic cells (DC) are the critical accessory cells for the development of anti-trinitrophenol (TNP) cytotoxic T lymphocytes (CTL) *in vitro*. We developed a model in which nylon wool-nonadherent spleen cells were used both as the responding and stimulating cells, the latter having been TNP-modified and x-irradiated. Thy-1-bearing CTL developed in C57BL/6, B6D2F<sub>1</sub>, and CBA mice only when small numbers of DC were added. Maximal responses in 5-d cultures were achieved with 0.5–1 DC/100 responding T cells. The DC did not have to be TNP modified directly. Anti-Ia and complement inactivated accessory cells, whereas similar treatment of the responders had no effect. DC exposed to ultraviolet radiation were ineffective, but x-irradiated DC were fully active. Culture media from DC, or from DC-nylon wool-passed spleen T cell cocultures that contained abundant CTL, would not substitute for viable DC.

Enriched preparations of macrophages (M $\phi$ ) were obtained from blood, peritoneal cavity, and spleens of BCG-immune and unprimed mice. M $\phi$  added at doses of 0.2–4% were weak or inactive as accessory cells. The level of Ia antigens on test M $\phi$  populations was quantitated and visualized by binding of a radioiodinated monoclonal anti-I-A<sup>b,d</sup> antibody, clone B-21. M $\phi$  that bore substantial amounts of Ia from all organs were weak accessory cells.

Addition of M $\phi$  to DC-T cell cocultures produced inhibitory effects, usually at a dose of 2% M $\phi$ . In contrast, 0.5% Ia-bearing M $\phi$  from BCG-immune boosted mice inhibited >80% of the DC-mediated CTL response. Addition of indomethacin reversed M $\phi$  inhibition, and 10<sup>-9</sup> M prostaglandin E<sub>2</sub> in turn blocked the indomethacin effect. Indomethacin also restored a low level of accessory cell function in immune-boosted adherent peritoneal cells, but not in preparations of monocytes and spleen M $\phi$ . Small numbers of DC were identified in preparations of immune-boosted peritoneal cells and may have accounted for the observed accessory activity.

We conclude that the development of anti-TNP CTL is an immune response in which (a) DC are the critical accessory cells; (b) Ia-bearing M $\phi$  are weak or inactive; and (c) M $\phi$  can inhibit DC-mediated response by an indomethacin-sensitive mechanism.

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