Dendritic cells are accessory cells for the development of anti-trinitrophenyl cytotoxic T lymphocytes

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

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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), whereas macrophages (Mφ) are either weak or inactive (2–4). We now compare the accessory cell function of dendritic cells (DC) and Mφ, 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 Mφ, regardless of source or expression of Ia antigen, are without significant activity. Mφ, 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 B6D2F1 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%
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.

**Mφ 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 Mφ 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 Mφ 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− fraction consists of irregularly shaped DC that are Ig− and Thy−. The EA + fraction contains 60–70% rosetted cells, 20–30% nonrosetted DC, and small numbers of contaminating lymphocytes and dead cells. The readherent fraction is >90–95% typical Mφ capable of binding and interiorizing EA.

**Ia-bearing Mφ.** To obtain Mφ 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 10^9 live Bacillus Calmette-Guérin (BCG; Pasteur strain, The Trudeau Institute) 1–3 mo before killing. Adherent spleen cells from these mice contain Mφ 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 + spleen Mφ could sometimes be obtained from mice reared in a facility that was not pathogen free. BCG-infected mice boosted with 2 × 10^6 heat-killed BCG (HKBCG) intraperitoneally 2 d before killing were a reliable source of peritoneal Mφ rich in Ia. BCG-immune boosted peritoneal cells were 95% typical phagocytic Mφ. 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^k^ antibody. Ascites was obtained after growth in sublethally irradiated B6D2F1 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^125I^ (New England Nuclear, Boston, Mass.) to a level of 7 μCi/μg protein. Briefly, 50 μg of protein was iodinated with 0.5 mCi of Na^125I^ 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 125I 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 cover slips, or in the case of nonadherent populations, the cells were attached to poly-L-lysine-coated (25 μg/ml) (Sigma Chemical Co.) coverslips. Cells were exposed to antibody at 0.2 μg/ml in RPMI-1640 supplemented with 5% newborn calf serum and 20 mM sodium azide for 1 h on ice. Cover slips were then washed, fixed in glutaraldehyde, and counted in a Packard Auto-Gamma Scintillation Spectrometer.
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(model 5220; Packard Instrument Co., Downers Grove, Ill.). Binding data are expressed as nanograms of anti-Ia bound at a concentration of 0.2 µg/ml. Saturation occurred over an external concentration range of 1-3 µg/ml for different populations, but the equilibrium binding constant is identical for all cell types. Nonspecific binding was ≤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 H2b or H2d 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 µg/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 µg/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 × 10⁶/16-mm diameter tissue culture well in 1 ml RPMI-1640 medium supplemented with 5% FCS, 5 × 10⁻³ 2-mercaptoethanol, 1 mM glutamine, 1,000 U/ml penicillin, and 20 µg/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 × 10⁶/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 ⁵¹Cr-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 (H2b), P815 mastocytoma (H2d), and the BW5147 lymphoma (H2b). 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 µg 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 E₂ (P-5640; Sigma Chemical Co.) was diluted to 10⁻⁴ M in ethanol and the culture medium was made 10⁻³ 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-) 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- T responders, but not by NyT responders (Table I). The response obtained with sIg- 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- 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 \(^{51}Cr\) release obtained at effector:target ratios of 50:1, 10:1, and 2:1 |
|------------------------------------------------------|---------------------------------------------------------------------|
|                                                       | NyT responders | sIg\(^{-}\) responders |
|                                                       | 50:1 | 10:1 | 2:1 | 50:1 | 10:1 | 2:1 |
| Experiment 1                                          |      |      |     |      |      |     |
| 1.5 \times 10^8                                       | 0    | 0    | 0   | 66   | 39   | 14  |
| 1.5 \times 10^5                                       | 0    | 0    | 0   | 31   | 7    | 1   |
| 1.5 \times 10^4                                       | 0    | 0    | 0   | 15   | 1    | 0   |
| Experiment 2                                          |      |      |     |      |      |     |
| 3.0 \times 10^8                                       | 0    | 0    | 0   | 31   | 24   | 14  |
| 10^8                                                  | 0    | 0    | 0   | 34   | 19   | 9   |
| 3.0 \times 10^5                                       | 0    | 0    | 0   | 16   | 7    | 5   |

For experiment 1, 5 \times 10^8 CBA/J responders were cocultured with x-irradiated stimulators for 4 d. Cytotoxic activity was assayed on \(^{51}Cr\)-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%).

The finding that sIg\(^{-}\) 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 M\(_{\phi}\) (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 H\(^{2}\)\(^{k}\) targets, whereas C57BL/6 CTL showed some lysis of H-2k in addition to H\(^{2}\)\(^{k}\) 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\(^{4}\)–10\(^{5}\)/5 \times 10\(^{6}\) responders); in 5-d-cultures, peak levels of CTL were obtained with just 1 \times 10\(^{4}\)–3 \times 10\(^{4}\) 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 M\(_{\phi}\) as Accessory Cells. Because accessory cell function is generally attributed to M\(_{\phi}\), we compared spleen DC and M\(_{\phi}\) from high responder
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Table II

| Mouse strain | PHA blasts | CBA/J* PHA blasts | CBA/J PHA blasts | CBA/J BW5147 | CBA/J BW5147 | CBA/J BW5147 | C57BL/6 TLX9 | C57BL/6 TLX9 | C57BL/6 TLX9 | C57BL/6 TLX9 | C57BL/6 TLX9 |
|--------------|------------|-------------------|-----------------|---------------|---------------|---------------|--------------|--------------|--------------|--------------|--------------|
| C57BL/6      | 0          | 0                 | 0               | 43            | 16            | 0             | 32           | 25           | 7            | 62           | 42           |
| CBA/J*       | 0          | 0                 | 0               | 35            | 14            | 5             | 32           | 25           | 7            | 52           | 23           |
| CBA/J        | 0          | 0                 | 0               | 56            | 23            | 5             | 32           | 25           | 19           | 52           | 23           |
| CBA/J        | 0          | 0                 | 0               | 52            | 25            | 7             | 32           | 25           | 19           | 52           | 23           |
| CBA/J        | 0          | 0                 | 0               | 62            | 42            | 10            | 32           | 25           | 19           | 52           | 23           |
| CBA/J        | 0          | 0                 | 0               | 78            | 19            | 7             | 32           | 25           | 19           | 52           | 23           |
| CBA/J        | 0          | 0                 | 0               | 88            | 19            | 6             | 32           | 25           | 19           | 52           | 23           |

In all experiments, 5 x 10^6 NyT responders were cultured with 2 x 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

| Treatment | Percent specific 51Cr release at effector:target ratios of 50:1, 10:1, and 2:1 |
|-----------|---------------------------------------------------------------------------------
| 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   |
| --         | aIa + C'                    | 23   | 10   | 5   |
| --         | --                          | 39   | 19   | 7   |
| Experiment 3§ | C'                          | 27   | 12   | 4   |
| --         | aIa + C'                    | 7    | 0    | 0   |
| --         | --                          | 30   | 15   | 3   |

In all experiments, 5 x 10^6 NyT responders were cultured with 2 x 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^- DC were added to each culture.
‡ CBA/J mice were used and cytotoxicity was assayed on BW5147 lymphoma (spontaneous release = 8%). 10^5 EA^- DC were added to each culture. The anti-Ia (aIa) 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 aIa 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 Mφ obtained by readherence were weak or inactive (Table IV). The EA^+ fraction, which was 60-70% Mφ and 20-30% DC, exhibited accessory cell function proportional to DC content.
TABLE IV
Comparison of Spleen DC and Mφ as Accessory Cells

| Accessory cells | Purification | Percent specific 51Cr 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 |
| 10⁷ DC          | EA⁺         | 52 25 7 | 36 25 13 |
| 3 × 10⁶ DC      | EA⁺         | 27 12 0 | 20 12 4 |
| 10⁴ DC          | EA⁺         | 8 0 0   | 10 3 2 |
| 10⁶ Mφ          | EA⁺         | 25 5 0   | -- -- -- |
| 3 × 10⁴ Mφ      | EA⁺         | -- -- -- | 12 8 0 |
| 10⁵ DC          | Nonadherent | 57 28 13 | 52 34 19 |
| 10⁴ DC          | Nonadherent | -- -- -- | 10 7 0 |
| 2 × 10⁵ Mφ      | Readherent  | 18 4 0   | -- -- -- |
| 10⁸ Mφ          | Readherent  | -- -- -- | 9 0 0 |
| 10⁷ DC + 2 × 10⁴ Mφ | Readherent + | 59 36 11 | -- -- -- |

For experiment 1, 5 × 10⁶ CBA/J NyT responders were cultured with 2 × 10⁶ x-irradiated TNP-modified NyT stimulators for 4 d. Spleen Mφ purified by readherence were >90% phagocytic for EA, and were added on 15-mm glass coverslips. Nonadherent DC were >90% DC by morphology and >90% EA-rosette negative. The EA⁺ LODAC fraction contained 20-30% EA⁺, morphologically typical DC, and 60-70% EA-rosetted Mφ. Cytotoxicity was assayed on ⁵¹Cr-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 Mφ induced the same level of CTL as purified DC alone. In most subsequent experiments, we used glass readherence to obtain highly enriched Mφ and EA rosetting to obtain highly enriched DC. However, we have consistently noted that separation of spleen LODAC by the readherence 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 Mφ. First, relative to DC, Mφ may express less Ia antigens, and second, Mφ may block their own accessory cell activity by producing prostaglandins. Populations of spleen Mφ 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 radiiodinated monoclonal anti-I-A 4 reagent, clone B-21.

Readherence purified Ia⁺ spleen Mφ 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, 10⁴ DC expressed one-tenth the amount of Ia as 1 × 10⁶ Mφ; the former provided accessory cell function and the latter did not. At higher doses, spleen Mφ slightly inhibited CTL development when added to DC-T cell cocultures. Addition of indomethacin did not enable Mφ to function as accessory cells. However, indomethacin removed the Mφ inhibition of
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### Table V

Comparison of Ia-bearing Spleen Mφ and DC as Accessory Cells

| Accessory cells | Nanograms of αIa Ab bound | Percent specific ⁵¹Cr release at effector:target ratios of |
|-----------------|---------------------------|----------------------------------------------------------|
|                 |                           | 50:1 | 10:1 | 2:1 |
| Experiment 1    |                           |      |      |     |
| 0               | 0                         | 0    | 0    | 0   |
| 10⁶ Mφ (adherent) | 1.4                       | 0    | 0    | 0   |
| 10⁶ Mφ + indomethacin | 1.4                     | 0    | 0    | 0   |
| 10⁵ DC (EA⁻)    | 1.5                       | 73   | 46   | 16  |
| 3 × 10⁴ DC (EA⁻) | 0.5                       | 52   | 22   | 6   |
| 10⁴ DC (EA⁻)    | —                         | 22   | 3    | 0   |
| Mφ + 10⁵ DC     | 2.9                       | 31   | 9    | 0   |
| Mφ + 10⁴ DC + indomethacin | 2.9                 | 70   | 34   | 12  |
| Experiment 2    |                           |      |      |     |
| 0               | 0                         | 0    | 0    | 0   |
| 10⁶ Mφ (adherent) | 0.39                      | 2    | 0    | 0   |
| 10⁵ Mφ (adherent) | —                         | 0    | 0    | 0   |
| 10⁵ Mφ + indomethacin | 0.39                    | 1    | 0    | 0   |
| 10⁴ DC (EA⁻)    | 1.8                       | 78   | 35   | 12  |
| 3 × 10⁴ DC (EA⁻) | 0.6                       | 67   | 34   | 9   |
| 10⁴ DC (EA⁻)    | —                         | 15   | 5    | 2   |
| 10⁵ DC (nonadherent BCG) | 2.0                     | 76   | 33   | 9   |
| 10⁶ Mφ + 10⁴ DC EA⁻ | 2.1                       | 64   | 18   | 5   |

For experiment 1, 5 × 10⁶ NyT B6D2F₁ responders were cultured for 5 d with 2.5 × 10⁶ x-irradiated TNP-modified NyT stimulators with or without accessory cells. Mφ were obtained from mice primed 12 wk previously with 10⁵ live BCG intravenously and boosted with 2 × 10⁶ HKBCG 1 wk before the experiment. The Mφ were all Ia⁺ (Fig. 1) and were added on 13-mm glass cover slips. DC were from normal B6D2F₁ mice. At the end of the culture period, Mφ remaining adherent to the cover slips bound 0.5 ng of ¹²⁵I-B-21 monoclonal Ia⁺ antibody (αIa Ab). Cytotoxicity was assayed on ⁵¹Cr-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 Mφ, i.e., mice primed to BCG 8 wk previously. Mφ 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 Mφ that bear substantial amounts of Ia are not active as accessory cells in the development of anti-TNP CTL.

### Peritoneal Mφ as Accessory Cells

Resident adherent Mφ 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 Mφ 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 Mφ express little surface Ia (e.g., Fig. 2 A), 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. 2 B) 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 Mφ (Fig. 2 D). However, a small subpopulation of the adherent cells had the cytologic features of DC and did not bind or interiorize EA (Fig. 2 D).

Adherent Ia⁺ peritoneal cells did not function as accessory cells at any dose
Fig. 1. Ia on spleen Mφ. Radioautographs after binding of \(^{125}\)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. × 310.

(A) Resident spleen Mφ purified from LODAC by glass adherence after overnight culture. More than 90% of the cells in these preparations are typical Mφ 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 Mφ obtained from mice primed 8 wk earlier with BCG intravenously and boosted 2 d earlier with 10^6 HKBCG intraperitoneally. Mφ 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.

However, the immune-boosted cells were potent inhibitors of CTL development when added to DC-T cocultures; e.g., 1 Mφ/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\(_2\) [PG\(_E_2\)]) reversed the indomethacin effect (Table VII). Indomethacin-supplemented cultures that contained only immune-boosted peritoneal cells as
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**Table VI**

Comparison of DC and Resident Peritoneal MΦ as Accessory Cells

| Accessory cells | Percent specific 51Cr release at effector:target ratios of 50:1, 10:1, and 2:1 |
|-----------------|------------------------------------------------------------------|
|                 | 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   |
| 10^2 DC         | 52   | 25   | 7   | 62   | 42   | 10  |      |      |     |
| 3 x 10^4 DC     | 27   | 12   | 0   | 31   | 12   | 6   |      |      |     |
| 10^4 DC         | 8    | 0    | 0   |      |      |     |      |      |     |
| 10^5 MΦ         | 0    | 0    | 0   | 0    | 0    | 0   | 0    | 0    | 0   |
| 3 x 10^5 MΦ     |      | 0    | 0   | 0    | 0    | 0   |      |      |     |
| 10^6 MΦ         |      | 0    | 0   | 0    | 0    | 0   |      |      |     |

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

‡ Same as experiment 1, except C57BL/6 were used as the responders and stimulators and cytotoxicity assayed on 51Cr-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 x 10^8 adherent peritoneal cells corresponded to less than one-fifth the level produced by 3 x 10^4 DC, even though the amount of peritoneal MΦ 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 MΦ and DC to test this directly. We conclude that peritoneal MΦ 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 MΦ, were compared to spleen DC as accessory cells. Populations of monocytes that were >90% Ia^+ 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 MΦ 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. slg^- 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 slg^- 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 MΦ. As in Fig. 1, phase-contrast (left) and bright-field (right) micrographs are shown. (A) Resident peritoneal MΦ studied 3 h after explantation. A subpopulation exhibit Ia. × 310. (B) Immune-boosted peritoneal MΦ 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+. × 310. (C) Control for the specificity of clone B-21 Ab binding. CBA (I-Ak) peritoneal (shown here) and spleen MΦ (not shown) do not bind the anti I-Ak Ab. × 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 vacules, do not bind or interiorize EA. The presumptive DC population is a minor fraction (<10%) of the total adherent cells. × 1,500.
TABLE VII

Comparison of DC- and BCG-induced Peritoneal MΦ as Accessory Cells

| Accessory cells | Nanograms of αβ Ab bound | Percent specific ¹²⁵I release at effector:target ratios of |
|-----------------|--------------------------|----------------------------------------------------------|
|                 |                          | 50:1 | 10:1 | 2:1 |
| Experiment 1    |                          |      |      |     |
| 0               | 0                        | 0    | 0    | 0   |
| 0 + indomethacin| 1.3                      | 100  | 43   | 14  |
| 10⁸ DC          | 2.25                     | 0    | 0    | 0   |
| 10⁸ DC + indomethacin | 3.55                 | 38   | 9    | 2   |
| 1.5 × 10⁷ MΦ    | 2.25                     | 29   | 2    | 0   |
| 1.5 × 10⁷ MΦ + 10⁸ DC | 3.55                | 100  | 49   | 15  |
| 1.5 × 10⁷ MΦ + 10⁸ DC + indomethacin | 3.55     | 0    | 0    | 0   |
| Experiment 2    |                          |      |      |     |
| 0               | 0                        | 0    | 0    | 0   |
| 10⁸ DC          | 2.2                      | 92   | 46   | 15  |
| 3 × 10⁸ DC      | 0.7                      | 91   | 62   | 9   |
| 10⁷ DC          | 0.2                      | 32   | 20   | 3   |
| 1.5 × 10⁷ MΦ    | 2.5                      | 0    | 0    | 0   |
| 1.5 × 10⁷ MΦ + 10⁸ DC | 4.7                   | 18   | 7    | 0   |
| 1.5 × 10⁷ MΦ + indomethacin | 2.5                | 46   | 15   | 3   |
| 1.5 × 10⁷ MΦ + 10⁸ DC + indomethacin | 4.7              | 88   | 54   | 15  |

For experiment 1, 5 × 10⁸ B6D2F1 NyT responders were cocultured with 2 × 10⁶ x-irradiated, TNP-modified NyT stimulators for 5 d ± accessory cells. MΦ were added adherent to 13-mm glass cover slips and EA− DC added in suspension. Surface Ia was measured with ³²P-labeled B-21 monoclonal anti-Ia⁴ antibody (αβ Ab). Cytotoxicity was assayed on P815 mastocytoma (spontaneous release = 17%). Indomethacin (1 μg/ml) was added daily to some cultures. For experiment 2, the protocol was the same as experiment 1, except PGE₂ at 10⁻⁸ M was added to some cultures at the beginning of the culture period. After the culture period, MΦ that remained adherent to cover slips bound 1.7 ng of αβ Ab. Cytotoxicity was assayed on P815 mastocytoma (spontaneous release = 10%).

For experiment 1, 5 × 10⁸ B6D2F1 NyT responders were cocultured with 2 × 10⁶ x-irradiated, TNP-modified NyT stimulators for 5 d ± accessory cells. MΦ were added adherent to 13-mm glass cover slips and EA− DC added in suspension. Surface Ia was measured with ³²P-labeled B-21 monoclonal anti-Ia⁴ antibody (αβ Ab). Cytotoxicity was assayed on P815 mastocytoma (spontaneous release = 17%). Indomethacin (1 μg/ml) was added daily to some cultures.

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, MΦ 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 MΦ to achieve accessory cell function.

MΦ, 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 ≥80% (Table VII). The inhibitory activity of MΦ
was related to the release of prostaglandins because: (a) The inhibition was completely reversed by indomethacin; (b) PGE$_2$ at $10^{-8}$ M reversed the indomethacin blockade (Table VII); (c) The number of Mφ used in our experiments could generate this level of PGE$_2$ in the culture medium (15). These inhibitory effects of Mφ-derived prostaglandins are consistent with previous work from several other laboratories (16).

Because indomethacin prevented Mφ from inhibiting the accessory function of DC, we added the drug to enriched populations of Mφ to see if this would uncover accessory cell activity. Indomethacin did not enable Ia-bearing spleen Mφ 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

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**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. × 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.
DENDRITIC CELLS ARE ACCESSORY CELLS

### Table VIII
Comparison of DC and Monocytes as Accessory Cells

| Accessory cells | Nanograms of αα 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    |
| \(3 \times 10^5\) DC | 0.7                      | 52    | 16    | 5     |
| \(10^5\) DC       | —                        | 14    | 4     | 0     |
| \(10^5\) Monocytes + \(10^5\) DC | —   | 44    | 16    | 17    |
| \(10^4\) Monocytes + \(10^5\) DC + indomethacin | — | 79 | 52 | 3 |

For experiment 1, 5 \times 10^6 B6D2F1 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\(^+\) DC added in suspension. After the 5-d-culture period, monocytes remaining adherent to the cover slips bound 1.74 ng of \(^{125}\text{I} \) B-21 monoclonal αα\(^+\) IgG. 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 \textit{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, B6D2F1, 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 (Mφ) were obtained from blood, peritoneal cavity, and spleens of BCG-immune and unprimed mice. Mφ added at doses of 0.2–4% were weak or inactive as accessory cells. The level of Ia antigens on test Mφ populations was quantitated and visualized by binding of a radioiodinated monoclonal anti-I-A\(^{b,d}\) antibody, clone B-21. Mφ that bore substantial amounts of Ia from all organs were weak accessory cells.

Addition of Mφ to DC-T cell cocultures produced inhibitory effects, usually at a dose of 2% Mφ. In contrast, 0.5% Ia-bearing Mφ from BCG-immune boosted mice inhibited >80% of the DC-mediated CTL response. Addition of indomethacin reversed Mφ inhibition, and 10\(^{-6}\) M prostaglandin E\(_2\) 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 Mφ. 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 Mφ are weak or inactive; and (c) Mφ can inhibit DC-mediated response by an indomethacin-sensitive mechanism.

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