

# Immortalized Dendritic Cell Line Fully Competent in Antigen Presentation Initiates Primary T Cell Responses In Vivo

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## Summary

Dendritic cells (DC) can provide all the known costimulatory signals required for activation of unprimed T cells and are the most efficient and perhaps the critical antigen presenting cells in the induction of primary T cell-mediated immune responses. It is now shown that mouse cell lines with many of the features of DC can be generated using the MIB $\psi$ -2-N11 retroviral vector transducing a novel *env*<sup>AKR</sup>-*myc*<sup>MH2</sup> fusion gene. The immortalized dendritic cell line (CB1) displays most of the morphologic, immunophenotypic, and functional attributes of DC, including constitutive expression of major histocompatibility complex (MHC) class II molecules, costimulatory molecules B7/BB1, heat stable antigen, intracellular adhesion molecule 1, and efficient antigen-presenting ability. Granulocyte/macrophage colony-stimulating factor (GM-CSF) proved to be effective in increasing MHC class II molecule expression and in enhancing presentation of native protein antigens. In comparison with macrophages, CB1 dendritic cells did not exhibit phagocytic and chemotactic activity in response to various stimuli and lipopolysaccharide activation was ineffective in inducing tumor necrosis factor  $\alpha$  or interleukin  $1\beta$  production. CB1 cells, pulsed with haptens in vitro and injected into naive mice were able to induce delayed-type hypersensitivity responses, further increased with pretreatment with GM-CSF, indicating that these cells may represent an immature, rather than a mature DC. The ability of CB1 to prime T cells in vivo could provide a tool to design novel immunization strategies.

Dendritic cells (DC)<sup>1</sup>, first described by Steinman and Cohn in 1973 (1), are a population of widely distributed leukocytes that play a key role in the immune system (2, 3) given that they are: (a) highly specialized in antigen presentation; (b) the principal activators of resting T cells in vitro (4, 5); (c) the major source of immunogenic epitopes for specific T cell clones after administration of antigen in vivo (6, 7) and (d) the most potent initiators of primary T cell-mediated responses in vivo (8).

Several studies (2, 3) have suggested that DC provide naive T cells with all the necessary signals required for activation

and proliferation. These signals are generated by the interaction of complexes of MHC molecules and antigenic peptides with the TCR (9), and by the engagement of costimulatory molecules, including binding of B7/BB1 molecules on APC to CD28 receptor on the T cell surface (10, 11). The first signal alone elicits effector functions only in activated T cells and is unable to stimulate naive or resting T cells, which in the absence of costimulatory signals can enter a period of unresponsiveness (12–14). The expression of the costimulatory molecule B7/BB1 on DC populations has been recently reported and shown to be critical in DC-driven primary T cell responses (15–17).

Understanding the mechanisms underlying the potent stimulatory capacities of DC could explain how T cells are primed and how the immune response is initiated. With this knowledge one might try to manipulate immune responses at very early stages and provide a way for inducing immunity or tolerance. However, an important limitation in the study of DC biology has been the small numbers of cells

<sup>1</sup> Abbreviations used in this paper: CI, chemotaxis index; CS, contact sensitivity; DC, dendritic cell; DNBS, 2,4-dinitro benzene sulfonic acid; DTH, delayed-type hypersensitivity; fmlp, formyl peptide; HSA, heat stable antigen; ICAM-1, intracellular adhesion molecule 1; MCP-1, macrophage chemotactic protein 1; MIP-1 $\alpha$ , macrophage inflammatory protein 1 $\alpha$ ; PAF, platelet-activating factor; PI, phagocytosis index; rm, recombinant mouse; SpWMB, spermwhale myoglobin.

available from any tissue, given that no stable cell lines that are clearly similar to DC have been obtained so far. Three different tissues have been used as major sources of DC: mouse spleen, the epidermis, where DC are known as Langerhans cells, and human blood. In each case DC constitute a tiny fraction of the starting tissue, representing about 1% of crude spleen (18) or epidermal (19, 20) cell suspensions and 0.1–1% of PBMC (21). More recently, Inaba et al. (22) have described a method for generating DC from both peripheral blood and bone marrow precursors, but cell proliferation ceases within 1–3 wk.

A different approach is to generate cell lines of DC. Earlier studies from our laboratory (23, 24) have shown that, using a mixture of recombinant retroviruses transducing the avian  $\nu$ -myc<sup>MH2</sup> oncogene, the immortalization of macrophages could be readily achieved from several different tissues. The immortalized and cloned macrophages were shown to respond in a physiological way to activation signals and were able to exert all the expected functions of macrophages (25).

The retroviral recombinant genome was molecularly cloned and transfected into the  $\psi$ 2 packaging cell line. The novel MIB $\psi$ 2-N11 helper-free retrovirus was then used to infect mouse spleen primary cultures (Sassano, M., F. Granucci, P. Paglia, M. Foti, and P. Ricciardi-Castagnoli, manuscript submitted for publication). With this approach, immortalized DC lines were also generated. One clone, named CB1, was extensively characterized and shown to possess many of the features described for DC, including the expression of B7/BB1, heat stable antigen (HSA), intracellular adhesion molecules 1 (ICAM-1), and the reactivity with several anti-DC mAbs.

The stimulatory capacity of the CB1 clone was shown in vitro, both in primary MLR and in antigen-specific T cell proliferative assay. GM-CSF increased the constitutive MHC class II molecule expression and was essential for antigen-specific APC activity in vitro. In addition, CB1 cells pulsed in vitro with haptens were able to induce a delayed-type hypersensitivity (DTH) immune response, thus indicating that T cell priming can be achieved using an antigen-loaded cell line as natural adjuvant.

## Materials and Methods

**Generation of Dendritic Cell Lines.** Spleen cell suspensions were prepared from newborn DBA/2 mice (Charles River, Como, Italy) in RPMI-1640 (Sigma Chemical Co., St. Louis, MO) supplemented with 10% FCS (GIBCO BRL, Gaithersburg, MD), glutamine, penicillin-streptomycin, and 0.5 mM  $\beta$ -ME. RBC were lysed and spleen cells were seeded at  $10^6$ /ml density in 35-mm petri dishes. Immortalization was carried out with the MIB- $\psi$ N11 retroviral vector generated by transfection of the packaging cell line  $\psi$ 2 with a molecularly cloned recombinant retrovirus (Sassano, M. et al., manuscript submitted for publication). This recombinant retrovirus was originally obtained by transfection of mouse fibroblasts with the avian MH2 and the mouse AKRv viral genomes and resulted in recombinant retroviruses carrying the *gag*, *pol*, *env* genes and LTR of AKRv and the avian  $\nu$ -myc<sup>MH2</sup> oncogene (23–25).

Infection with MIB $\psi$ 2-N11 was performed with filtered supernatant from a 24-h subconfluent culture of the viral producer cell line  $\psi$ N11 diluted 1:1 with complete medium containing 10  $\mu$ g/ml

polybrene (Sigma Chemical Co.). After 1 h incubation at 37°C in a 5% CO<sub>2</sub> incubator, half volume of fresh medium was added and then regularly changed twice a week. During the first week after infection, cells were fed with 10% L929.6C-conditioned media, reduced at 5% in the following 2 wk, and then gradually eliminated.

About 20–30 d after infection, multiple foci of dividing cells were observed in the petri dishes. Cultures could then be trypsinized and cells replated. The cell line was considered established after 10 passages, and then cloned by limiting dilution.

**Immunostaining Analysis of CB1 Cells.** Cell surface expression of leukocyte markers was assessed using the following mAbs: M1/9.3 (TIB 122), RA.3.3.A1 (TIB 146), F4/80 (HB198), 2.4G2 (HB 197), FD 441.8 (TIB 213), M1/70 (TIB 128), M1/42 (TIB 126), 33D1 (TIB 227); NLDC145, B5.5, RB6–8C5, B21–2, M1/69 (kindly provided by R. M. Steinman, The Rockefeller University, New York), and FA11, 3D6 (kindly provided by S. Gordon, Oxford University, Oxford, UK), 1G10 (PharMingen, San Diego, CA), followed by anti-rat Ig-FITC (Boehringer Mannheim, Mannheim, Germany); N418 (HB224), 145–2C11, 3E2, H57–597 (PharMingen), followed by anti-hamster Ig-PE (Southern Biotechnology Associates, Birmingham, AL), biotinylated MK-D6, 39–10–8, AMS-16, IM7, RM2–5, RM4–5, 53–6.7, RA3–6B2, 34–2–12 (PharMingen) mAbs, followed by Streptavidin-PE (Sigma Chemical Co.). Purified Igs (PharMingen) or unrelated isotype-matched Abs were used as controls.

CB1 cells ( $5 \times 10^5$ ) were incubated for 30 min at 4°C with hybridoma supernatants, affinity purified or biotinylated primary mAbs followed by either anti-rat Ig-FITC or anti-hamster Ig-PE, or Streptavidin-PE. For MHC class II expression, cells were stained before and after 48-h treatment with either 100 U/ml of recombinant mouse (rm) IFN- $\gamma$  or 200 ng/ml of rmGM-CSF (Genzyme Corp., Cambridge, MA).

Analysis was performed on FACSscan<sup>®</sup> cytofluorimeter (Becton Dickinson & Co., Mountain View, CA) after dead cells removal by propidium iodide gating.

Peroxidase immunostaining of CB1 cells was carried out to show reactivity for intracellular antigens recognized by the rat anti-DC mAbs 2A1 (26) and by the hamster anti-DC M342 (27) kindly provided by N. Romani (University of Innsbruck, Innsbruck, Austria). CB1 cells were grown on coverslips and fixed in cold acetone. Cells were incubated for 1 h with mAbs in the presence of 1% normal mouse serum, washed and stained with peroxidase mouse anti-hamster (Pierce, Rockford, IL) or anti-rat Ig (Boehringer Mannheim), and visualized using diaminobenzidine as substrate (Sigma Chemical Co.).

**Adherence, Phagocytosis, Chemotaxis, and Cytokine Production.** Phagocytic ability was assessed using zymosan particles either untreated or opsonized with normal mouse serum. Phagocytosis index (PI) was evaluated at the microscope. Chemotaxis index (CI) was kindly measured by P. Sacerdote (University of Milan) in response to  $10^{-7}$  M formyl peptide (fmlp), 10 ng/ml macrophage chemotactic protein (MCP-1) or  $10^{-10}$  M platelet activating factor (PAF). TNF- $\alpha$  secretion was measured by ELISA according to the suggestions of the supplier (Genzyme Corp.). IL-1 $\beta$  secretion was kindly measured by P. Gnocchi (Farmitalia-Carlo Erba, Nerviano, Italy) with a RIA method previously described (28).

**Northern Blot Analysis.** Northern blot analysis of mRNAs from CB1 cells and a positive control was carried out using a specific myc<sup>MH2</sup> probe previously described (25). The myc<sup>MH2</sup> 3' probe was derived from a chicken genomic library and it represents the 3' region of the avian myc gene which does not crosshybridize with the murine myc genes.

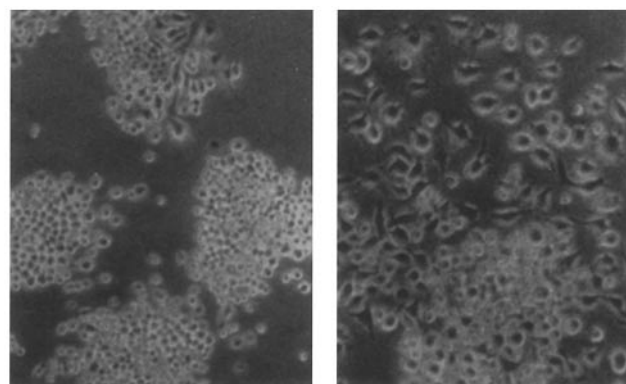
**Expression of B7/BB1 Molecule.** mRNA (polyA)<sup>+</sup> from LPS-activated splenocytes, LPS-activated A20 cells, and untreated CB1 cells was prepared from  $5 \times 10^6$  cells. Activation was carried out for 72 h with 10  $\mu\text{g/ml}$  of LPS (*Escherichia coli*, B5:055; Sigma Chemical Co.). First strand cDNA synthesis was obtained using AMV reverse transcriptase (Promega Corp., Madison, WI) and an oligo dT primer. PCR was performed in a 50- $\mu\text{l}$  vol using 2.5  $\mu\text{l}$  cDNA, 5  $\mu\text{l}$  10 $\times$  PCR buffer (Perkin Elmer Corp., Norwalk, CT), 1.5 mM Mg<sup>2+</sup>, 0.25 mM dNTPs, 1.5 U Taq polymerase (Boehringer Mannheim), and 25 pmol forward and reverse oligonucleotide primer. Nucleotides 249–266 and 1153–1169 of the B7/BB1 cDNA sequence were used as sense and antisense oligonucleotides, respectively, as previously described (15). PCR conditions were 40 cycles using a thermocycler (Perkin-Elmer Corp.): 1 min, 95°C; 45 s, 56°C; 1 min, 72°C. PCR products were analyzed by agarose gel electrophoresis and blotted to nylon membrane. Southern blot was probed with an internal B7/BB1 oligonucleotide (nucleotides 661–680) as described (15).

**In Vitro T Cell Stimulatory Capacity of CB1 Cells.** In an antigen-specific presentation assay, 10<sup>5</sup> CB1 or MT2 cells, treated with either 100 U/ml IFN- $\gamma$  or 200 ng/ml rmGM-CSF, or A20 cells, were used as APC for spermwhale myoglobin (SpWMB, 0–5  $\mu\text{M}$ ) to 10<sup>4</sup> antigen-specific T hybrid cells (13.26.8 H6.1 SpWMB-specific H-2<sup>d</sup> restricted T cells were kindly provided by A. M. Livingstone, Basel Institute of Immunology, Basel, Switzerland). Antigen, at the indicated doses, was present during all the time of the assay. After 24 h of incubation, 100  $\mu\text{l}$  of supernatant was transferred and tested for the IL-2 content on the HT.2 cell line ( $5 \times 10^3$  cells/well). Proliferation was measured using the MTT assay. In a MLR assay, GM-CSF treated or untreated dendritic CB1 cell line and MT2/1 macrophage cell line (23) were treated with mytomycin C (4  $\mu\text{g/ml}$ ) and used, at the indicated doses, as stimulators in coculture with  $3 \times 10^5$  allogeneic C57BL/6 T cells as responders. Syngeneic T cells were also used as controls. T cells were the nonadherent spleen cells purified on nylon wool columns. [<sup>3</sup>H]TdR incorporation (2.5  $\mu\text{Ci/ml}$ ) was measured 72 h later.

**Induction of Contact Sensitivity.** Contact sensitivity was induced by injection of 2,4-dinitro benzene sulfonic acid (DNBS; Eastman Kodak, Rochester, NY)-modified or FITC-modified CB1 cells pretreated or not with GM-CSF (200 ng/ml). CB1 cells were derivatized with either 200  $\mu\text{g/ml}$  of FITC (Sigma Chemical Co.) or with 1 mg/ml DNBS for 30 min at 37°C as described (29, 30) and inoculated subcutaneously into the dorsal skin of DBA/2 mice. Each mouse received 10<sup>4</sup>–10<sup>5</sup> cells suspended in HBSS in a final vol of 250  $\mu\text{l}$ . Recipient mice and nonsensitized controls were ear challenged 5 d later with either 25  $\mu\text{g}$  FITC or 15  $\mu\text{g}$  of 2,4-dinitro-1-fluorobenzene (DNFB, Sigma Chemical Co.) on each side of both ears. Mice sensitized epicutaneously were painted with 150  $\mu\text{g}$  DNFB or 2 mg FITC on the shaved abdomen, as described (29, 30). Ear thickness was measured with an engineer's micrometer.

## Results

**Generation of Dendritic Cell Lines.** Spleen cell suspensions from newborn mice were infected with the retroviral vector MIB $\psi$ 2-N11. About 3 wk after infection, foci were observed and proliferating cells, detaching from adherent aggregates, were easily cloned. Among several clones obtained, the clone named CB1 was selected because of its dendritic morphology. Fig. 1 shows typical aggregates of proliferating CB1 cells with, at the periphery of the clusters, cells possessing a dendritic appearance. Characteristically, these cells display sheetlike

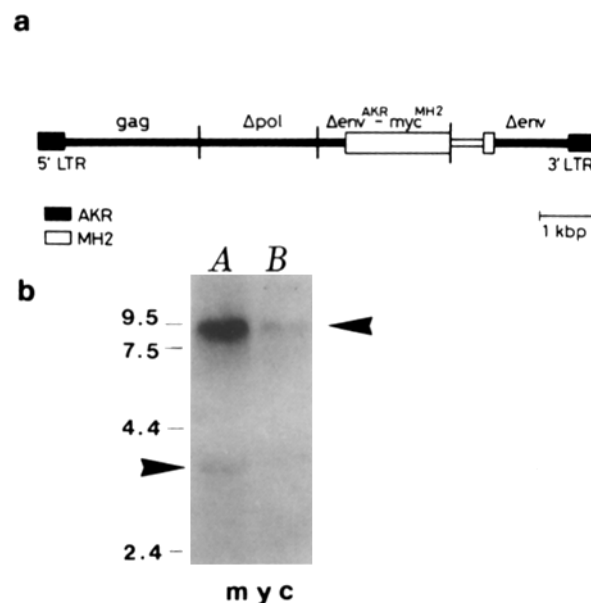


**Figure 1.** Morphological appearance of the cell line CB1 (phase contrast microscopy). Clusters of CB1 cells 48 h after seeding: at the periphery of the clusters, cells with a dendritic phenotype are observed.

processes, with a striking motility when observed at video microscopy (data not shown). This behavior is not exhibited by other leukocytes and may have a functional significance in the survey of T cells (2, 3).

The estimated doubling time of CB1 cell line was about 20 h (data not shown). Productive viral infection of CB1 cells with the MIB $\psi$ 2-N11 retroviral vector (described in Fig. 2 a) was tested by Northern blot analysis using a specific *myc*<sup>MH2</sup> probe (31): genomic and subgenomic transcripts of the expected size were detected (Fig. 2 b).

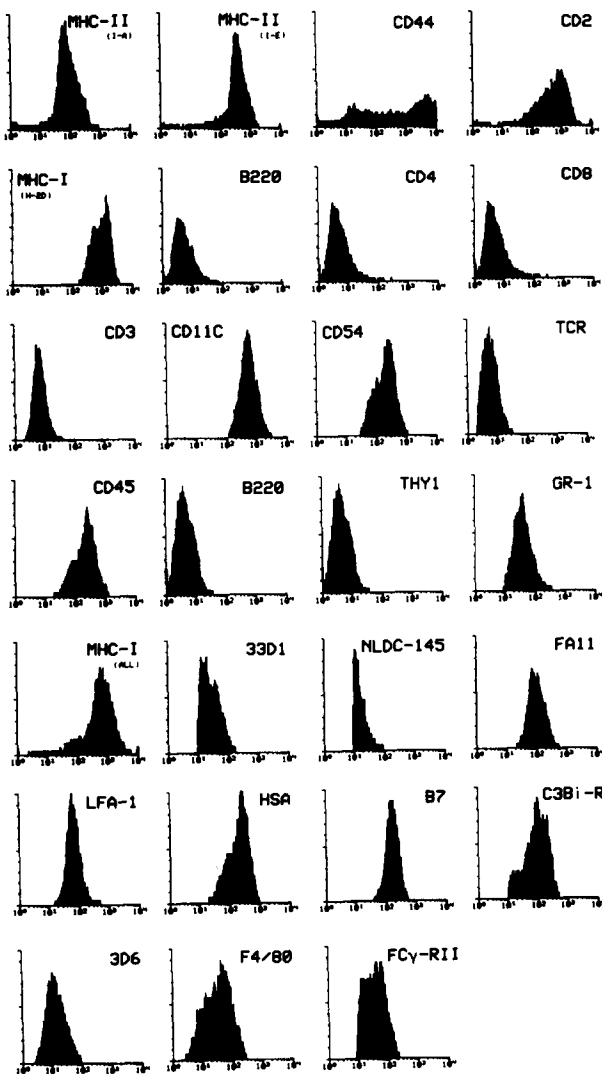
In addition to spleen, other tissues have been infected with the MIB $\psi$ 2-N11 retroviral vector. From infected cultures of bone marrow and skin tissues DC-like cell lines were generated (Paglia, P., manuscript in preparation). Moreover, from



**Figure 2.** (a) The MIB- $\psi$ N11 proviral genome. Viral immortalization with the MIB- $\psi$ N11 retroviral vector: Northern blot analysis of CB1 cells (A) and of a positive control (B) probed with a specific *myc*<sup>MH2</sup> probe. (b) The genomic and subgenomic transcripts with the expected size (arrowheads).

BALB/c spleen cultures, one additional dendritic cell line (D2SC/1) was established and extensively characterized (Paglia, P., manuscript in preparation), thus showing the reproducibility of the method.

**CB1 Cell Line Has Many of the Features of Splenic DC.** Cytofluorometric cell surface analysis with several mAbs (Fig. 3) and intracellular staining with the M342 (27) and the 2A1 (26) (Fig. 4) revealed a characteristic DC immunophenotype of the CB1 cell clone. CB1 cells express high levels of MHC-I molecules, of the common leukocyte antigen CD45, the adhesion molecules Pgp-1 (CD44) and CD11c, the latter shown to be a typical marker of spleen DC (32). A bright staining was also achieved with mAbs against B7/BB1, HSA, and ICAM-1 costimulatory molecules. Intermediate levels of expression were detected for 33D1, NLDC-145, FA11 antigens and LFA-1 and MAC-1 integrins. Moreover, CB1 cells lack



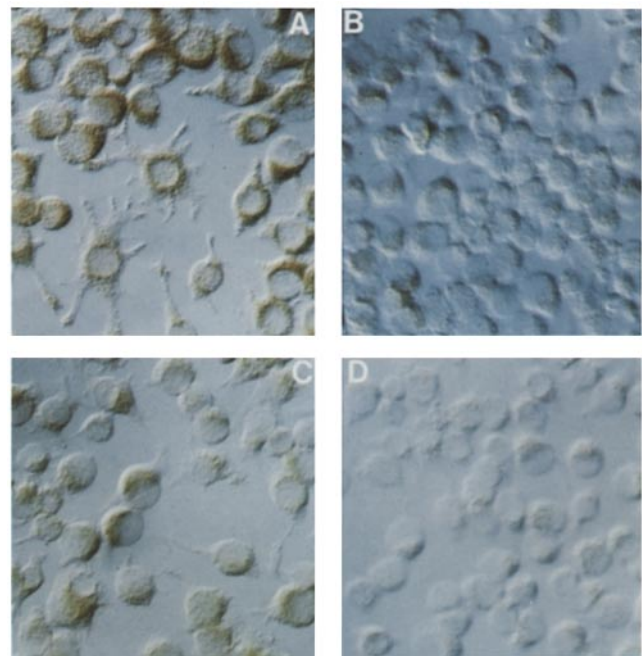
**Figure 3.** Cytofluorometric analysis of CB1 cells stained with a set of mAbs (described in Materials and Methods) recognizing various determinants specific for T and B lymphocytes, macrophages, granulocytes, and DC.

the B cell marker B220 and are negative for T cell-specific determinants: Thy1, CD3- $\epsilon$ , TCR, CD4, and CD8. These results are in agreement with those reported for spleen DC (26) and Langerhans cells (20, 33). Low levels of F4/80 and Fc $\gamma$ -R II were present on CB1 cell surfaces; however, comparing CB1 cells with macrophage MT2 cells we found that expression of F4/80 and Fc $\gamma$ -R II was fivefold higher on MT2 cells (data not shown). Finally, we detected high CD2 expression on CB1 cell surface. This result was unexpected and is intriguing because it suggests that homotypic interactions between DC and T cells may occur.

MHC-II expression on CB1 cells treated or untreated with GM-CSF was tested using a panel of anti-MHC mAbs recognizing various epitopes of I-A or I-E molecules. These mAbs (MK-D6, B21-2, 39-10-8, and AMS-16) detected constitutive expression of MHC-II molecules on CB1 cells with a high degree of variability (from low to high) depending on the mAb used (data not shown). Interestingly, in all cases the expression of MHC-II molecules was enhanced when CB1 cells were treated with GM-CSF. In contrast, IFN- $\gamma$  did not exert any influence on MHC-II expression on CB1 cells, whereas it was effective in inducing both I-A and I-E expression on MT2 macrophage cell line (data not shown).

Taken as a whole these results suggest that CB1 cells may represent an immature splenic DC precursor still responsive to GM-CSF.

**Functional Properties of CB1 Cells.** Spleen DC are loosely and transiently adherent to plastic surfaces (1-3); similarly,



**Figure 4.** Immunostaining of CB1 cells. Peroxidase immunostaining of CB1 cells showing reactivity for intracellular antigens recognized by the anti-DC mAbs 2A1 (A) and M342 (C) compared with the corresponding isotyped-matched control Ab (B and D).

CB1 cells were loosely adherent and pipetting was enough to detach them. Compared to macrophages (MT2/1 cells) which can be detached only in the presence of trypsin, CB1 cells were very much less adherent (Table 1).

Phagocytic and chemotactic activities were also tested. DC are known to be not actively phagocytic (2, 3) and, consistent with this, CB1 cells exhibited very little zymosan phagocytosis, whereas MT2/1 macrophages showed a strong phagocytic ability (Table 1).

Chemotaxis was measured in response to fmlp, MCP-1, and PAF. CB1 cells did not respond to any of these stimuli, even if previously activated with LPS or GM-CSF, whereas MT2/1 macrophages were able to migrate in response to all stimuli (Table 1). This finding is of particular interest since it suggests that DC have chemotactic and migratory properties different from those of macrophages.

Controversial results have been reported concerning the production of cytokines by different DC. Spleen DC have been shown to produce little or no IL-1 when activated with a variety of stimuli (34). In contrast, epidermal Langerhans cells have been identified as the major source of mRNA for IL-1 $\beta$  and macrophage inflammatory proteins, MIP-1 $\alpha$  and MIP-2, among unstimulated and hapten-activated epidermal cells, with a strong upregulation of the former upon short-

term culture (35–38). In contrast to MT2/1 macrophages, CB1 cells were shown to produce very small amounts of TNF- $\alpha$  and IL-1 $\beta$  upon stimulation with LPS (Table 1), confirming at a clonal level previous negative results on IL-1 expression by enriched spleen DC preparations (34).

**Expression of the Costimulatory Molecule B7/BB1.** APC need to provide two different signals in order to activate T lymphocytes: an antigen-specific interaction and accessory nonspecific signals. Recently, the costimulatory B7/BB1 molecule has been identified on APC (10, 11, 39, 40) and has proven to be essential for proliferation of unprimed allogeneic T cells in MLR assay and CD4<sup>+</sup> T cells in antigen-specific proliferative responses (15, 16).

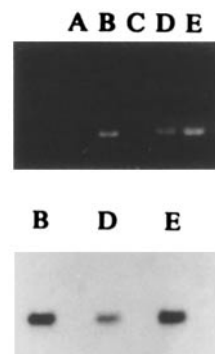
Spleen DC express constitutively the B7/BB1 molecule (15, 16) whereas the A20 cell line or B cells require activation by LPS or through cross-linking of either surface MHC class II molecules (41) or their cytoplasmic domain (11).

Transcription of the B7/BB1 gene was evaluated in LPS-activated splenocytes, in A20 cells, and in CB1 DC. PCR analysis was carried out using sense and antisense oligonucleotides derived from the murine B7/BB1 cDNA sequence (40, 42). PCR products are shown in the upper part of Fig. 5. Only CB1 cells have a constitutive transcription of the B7/BB1 gene whereas in A20 cells and in splenocytes transcription was induced only after treatment with LPS. To show specificity, PCR products were also analyzed in Southern blots (Fig. 5, bottom) probed with an internal B7/BB1 oligonucleotide. Cell surface expression of the protein was confirmed by cytofluorimetric analysis using the 1G10 mAb (11) which recognizes the mouse B7/BB1 molecule (Fig. 3).

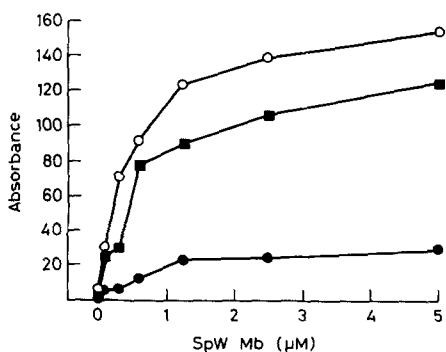
**Induction of T Cell Responses In Vitro and In Viva.** Fresh DC pulsed in vitro with native protein antigens or peptides can very efficiently stimulate antigen-specific primed and naive CD4<sup>+</sup> T cells (5, 12, 20, 43). In agreement with these reports, we found that a native protein such as SpWMB was presented by CB1 cells to specific T cell hybrids after incubation with rmGM-CSF (Fig. 6). The enhanced APC function induced by GM-CSF paralleled the GM-CSF-mediated induction of MHC-II molecules described above. GM-CSF inducibility of MHC-II molecules and accessory functions has been described in precursors of DC (44), strongly suggesting that CB1 cells may represent an intermediate stage of DC maturation.

**Table 1.** Adherence, Phagocytosis, Chemotaxis, and Cytokine Production by CB1 and MT2/1 Cell Lines

Functions	Stimuli	Cell line	Activity
Adherence to plastic surface		MT2/1	+++
		CB1	+
Phagocytosis	Zymosan	MT2/1	+++ (89 PI)
		CB1	+/- (5 PI)
Chemotaxis	fmlp	MT2/1	+
	PAF		++ (6.7 CI)
	MCP-1		+++ (11 CI)
	fmlp	CB1	-
	PAF		-
	MCP-1		-
Cytokine production	LPS	MT2/1	TNF- $\alpha$ (3.0 ng/ml)
			IL-1 $\beta$ (5.0 ng/ml)
	LPS	CB1	TNF- $\alpha$ (0.4 ng/ml)
			IL-1 $\beta$ (0.2 ng/ml)

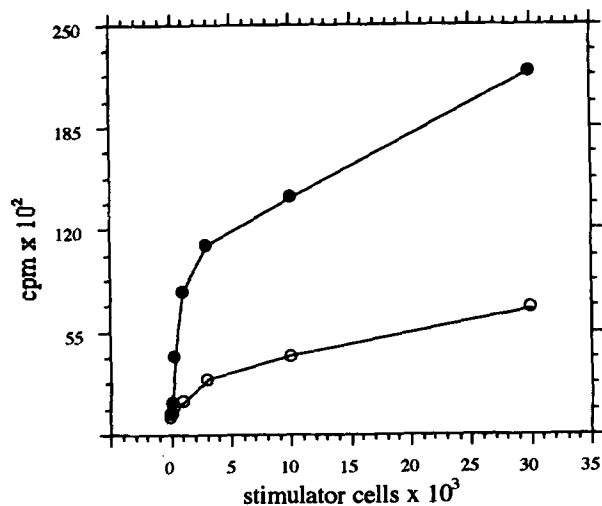


**Figure 5.** Expression of B7/BB1. (Top) Analysis of PCR products from untreated (A) or LPS-activated splenocytes (B), untreated (C), or LPS-activated A20 cells (D), and untreated CB1 cells (E). (Bottom) Southern blot analysis of PCR products hybridized with an internal B7/BB1 oligonucleotide. LPS-activated splenocytes (B), LPS-activated A20 cells (D) and untreated CB1 cells (E).



**Figure 6.** In vitro T cell stimulatory capacity of CB1 cells: antigen presentation. CB1 cells, treated with either 100 U/ml IFN- $\gamma$  (●-●), 200 ng/ml mrGM-CSF (■-■), or A20 cells (○-○) were used as APC for SpWMb to antigen-specific T hybrid cells (13.26.8 H6.1 SpWMb-specific H-2<sup>d</sup>-restricted T cells). IL-2 production was measured on the HT.2 cell line using the MTT assay.

A strong stimulating activity of DC in the primary MLR has been known since 1978 (45). We compared the stimulatory capacity of CB1 and MT2/1 macrophages in a primary dose-response MLR assay. As shown in Fig. 7, CB1 cells possess accessory function in the in vitro allogeneic T cell proliferative assay. This response does not have the expected amplitude typical of fully differentiated DC, but this may again reflect the immature state of CB1 cells. Antigen-pulsed DC, but not other APC such as B cells or macrophages, can be administered in vivo to prime T lymphocytes without additional adjuvants. DC are considered the most potent initiators of various primary T cell-mediated responses in vivo such as contact sensitivity (CS) (46), allograft rejection (8, 47), and activation of MHC-restricted T cells (6, 7). Moreover, it has been shown that antigen-pulsed DC are able to induce an Ab response in vivo (48). The ability of CB1 cells to prime



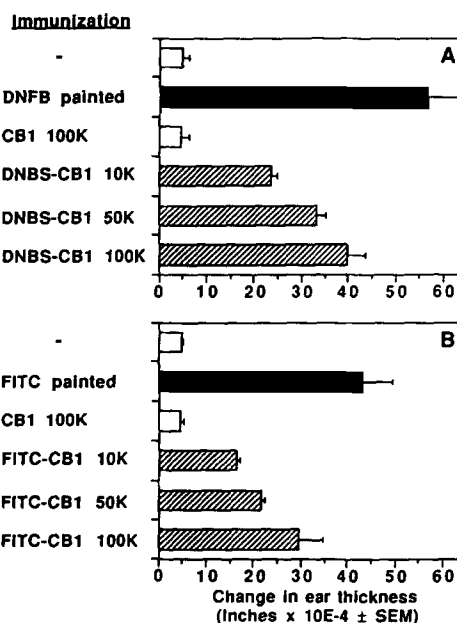
**Figure 7.** In vitro T cell stimulatory capacity of CB1 cells: MLR assay. Graded doses of CB1 (●-●) and MT2/1 (○-○) cells were used as stimulators in coculture with  $3 \times 10^5$  allogeneic C57BL/6 T cells as responders. Incubation of  $3 \times 10^4$  CB1 cells with  $3 \times 10^5$  syngeneic T cells resulted in 550 cpm [<sup>3</sup>H]TdR uptake (background level).

T cells in vivo was tested in the model of CS to haptens. CS is a particular type of delayed-in-time, T cell-mediated immune reaction occurring after epicutaneous immunization and challenge with reactive haptens (29, 46). Crucial to the development of CS reactions are epidermal Langerhans cells (30). CS also results when Langerhans or lymphoid DC are conjugated in vitro to haptens and injected into syngeneic mice (29, 46, 49).

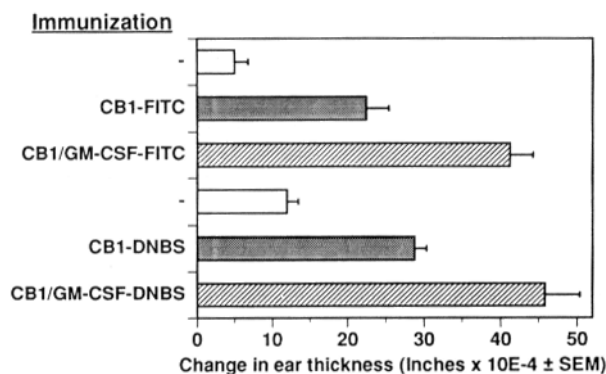
CB1 cells modified in vitro with either FITC or DNBS and injected subcutaneously into naive syngeneic mice were able to induce CS responses, indicating that CB1 cells can sensitize T lymphocytes in vivo. In contrast, injection of nonhapten-conjugated CB1 cells did not induce any significant ear swelling response (Fig. 8). The efficiency of CB1 cells to induce CS was increased when cells were pretreated for 48 h with GM-CSF (Fig. 9).

## Discussion

The potential use of elements of the immune system to prime naive animals offers great advantages over standard immunizations with artificial adjuvants. The “dirty trick of immunologists”, as stated by Charles A. Janeway, Jr. (50), which consists of the addition of mineral oil or killed bacteria (CFA) to highly purified or recombinant protein antigens to ini-



**Figure 8.** Induction of contact sensitivity by injection of DNBS- or FITC-modified CB1 cells.  $10^4$ - $10^5$  CB1 cells were derivatized with either FITC or with DNBS for 30 min at 37°C, and inoculated subcutaneously into the dorsal skin of DBA/2 mice. Recipient mice and nonsensitized controls were ear challenged 5 d later with either FITC or DNFB on each side of both ears. Mice sensitized epicutaneously were painted with DNFB or with FITC on the shaved abdomen. Ear thickness was measured with an engineer’s micrometer immediately before challenge and 24, 48, and 72 h after challenge. Data are expressed as the change (from prechallenge levels) in ear thickness  $\times 10^{-4}$  inches and represent the mean maximal increase within the 72-h observation period ( $n = 4$ ).



**Figure 9.** Induction of contact sensitivity by injection of DNBS- or FITC-modified CB1 cells (50,000) activated with GM-CSF. The procedure is the same as in Fig. 8, except that CB1 cells were pretreated or not for 48 h with 200 ng/ml GM-CSF before hapten derivatization.

tiate an immune response, can be overcome. Now we know that unprimed T lymphocytes recognize antigenic epitopes expressed on the cell surface of DC which possess all the costimulatory signals (e.g., B7/BB1, ICAM-1, LFA-3, and HSA) necessary for T cell proliferation and differentiation (2, 3, 5, 11). Moreover, priming with antigen-pulsed DC can also elicit a memory immune response. DC can thus be considered, as suggested by Ralph Steinman, “nature’s adjuvants” (2, 12) that can initiate *in vivo* both cell-mediated (DTH) and T cell-dependent Ab responses (3, 48).

In this paper we show the generation of a DC line using a novel retroviral vector, MIB $\psi$ 2-N11, transducing an activated *emv<sup>AKR</sup>-myc<sup>MH2</sup>* fusion gene. The established DC line, CB1, was shown to possess many of the phenotypic features of lymphoid DC, including expression of the B7/BB1, HSA, and ICAM-1 molecule.

The GM-CSF inducibility of class II expression in CB1 cells might mean that these cells represent a DC precursor, rather than a mature DC. The observation that bone marrow

and blood DC precursors derive from Ia<sup>-</sup> cells (26, 44) supports this hypothesis. In addition, immature DC, including freshly isolated epidermal Langerhans cells, express lower levels of class II molecules compared to a fully differentiated DC (2, 3, 51).

Functionally, CB1 exhibited both the ability of processing and presenting native protein antigens *in vitro* as well as the function of sensitizing T cells *in vivo*, as assessed by the ability to initiate a primary DTH response.

GM-CSF-increased expression of MHC class II molecules was necessary for the *in vitro* antigen-specific APC activity of CB1 cells, and enhanced the ability of CB1 cells to induce CS, confirming a key role of GM-CSF in the differentiation and activation of DC accessory functions. GM-CSF was also shown to support the growth and differentiation of DC from mouse blood or bone marrow cultures (22) and of early human cord blood and bone marrow progenitors into DC (51). In addition, GM-CSF in cooperation with TNF- $\alpha$ , promotes the *in vitro* differentiation of epidermal Langerhans cells into mature DC (2, 3, 52), and induces generation of Langerhans cells from human cord blood CD34<sup>+</sup> precursors (53).

Moreover, our results add further evidence to the notion that DC and macrophages can be distinguished for a variety of properties, including morphology, immunophenotype, phagocytosis, response to chemotactic stimuli, cytokine production profile, and cytokine activation requirements. It has been shown that APCs produce a limited number of predominant immunogenic peptides and that DC make the most immunogenic ligands for antigen-specific TCR (2, 3). In this regard, the CB1 cell line could be used to probe the immunogenicity of various antigens among which tumor-associated antigens are the most relevant candidates.

The stimulatory function of the DC line CB1 could also provide a tool to design novel immunization strategies. The role played by the route of administration, the organ where antigen presentation occurs, and the type of immune response elicited are questions that can now be addressed using in all experiments the same clonal population of DC.

We wish to thank R. Steinman for advice, critical review of the manuscript, and Abs; and C. A. Janeway, Jr. (Yale University, New Haven, CT), S. Gordon, M. Suter (University of Zurich, Zurich, Switzerland), and N. Romani for critical review of the manuscript, suggestions, discussion, and Abs. We also wish to thank A. M. Livingstone for the SpWMb-specific T cells; A. Mantovani (M. Negri Institute, Milan, Italy) for providing MCP-1; P. Sacerdote for chemotaxis measurement; P. Gnocchi for measuring IL-1 $\beta$  secretion; and V. Zacchi (University of Modena) for the experiments in mice. Special thanks go to M. Sassano and M. Righi (University of Milan) for characterization of the retroviral genome.

This work was partially supported by grants from the Italian Association for Cancer Research, Biotop and Consiglio Nazionale delle Ricerche (Biotechnology and Bioinstrumentation, Genetic Engineering and Clinical Applications in Oncology (ACRO)).

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Received for publication 11 June 1993 and in revised form 1 September 1993.



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