Brief Definitive Report

DENDRITIC CELLS INDUCE T CELL PROLIFERATION TO SYNTHETIC ANTIGENS UNDER Ir GENE CONTROL

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There has been much debate as to the exact nature of the antigen-presenting accessory cell in various immunological reactions such as T cell proliferation and help (1). It has been suggested that the accessory cell bears Ia antigen on its surface (2). These antigens characterize some but not all macrophages; conversely, they are not unique macrophage markers (3). The dendritic cell of Steinman and Cohn has recently been described as expressing Ia antigens and being a potent stimulator of allogeneic T cell proliferation (4, 5). We were interested in determining if this dendritic cell might be important in presenting antigen to syngeneic T cells.

We have found that dendritic cells are strongly I-A positive and are highly efficient at presenting soluble antigens to syngeneic T cells. Furthermore this antigen presentation is under immune response (Ir) gene control as dendritic cells from nonresponder strains do not induce proliferation in responder × nonresponder F_1 T cells.

Materials and Methods

Mice. Mice were bred at the Imperial Cancer Research Fund colony in Mill Hill, London.

Antigens. Glu,Ala,Tyr^{10} (GAT) and poly(LTyr,LGlu)-poly(DLAla)--poly(LLys) [(T,G)-A--L] were the generous gifts of Dr. Paul Maurer, Jefferson University, Philadelphia, Pa. and Dr. Edna Mozes, The Weizmann Institute of Science, Rehovot, Israel, respectively. Purified protein derivative of tuberculin (PPD) was kindly supplied by the Ministry of Agriculture Fisheries and Food, Central Veterinary Laboratory, Surrey, England.

Cell Fractionation. Dendritic cell preparation was based on that of Steinman and Cohn (4–6) with minor modifications. Initial fractionation of spleen and lymph node suspensions was on discontinuous bovine serum albumin gradients (7) (Path-O-Cyte 4; Miles Laboratories, Inc., Kankakee, Ill.) rather than a single-step procedure. The lightest (A) layer, separating at the interface of 10 and 23% albumin after spinning at 18,000 g_{av} for 30 min, was used as the starting population. It constituted <1% of the original spleen and lymph node. A layer cells were cultured for 2 h at 37°C as described previously (4, 5); nonadherent cells were then removed by aspiration, and the adherent cells left for a further 18 h in culture.

Fc receptor (FcR) rosetting was performed on the nonadherent cells at 18 h using anti-sheep erythrocyte antibody (7S IgG; Cordis Laboratories Inc., Miami, Fla.) -coated sheep erythrocytes. This cell mixture was spun on a Ficoll-Hypaque gradient (20°C for 30 min at 400 g_{av}) to separate nonrosetted (FcR^-) from rosetted (FcR^+) populations. Both interface and pelleted populations were treated with 0.83% ammonium chloride in Tris buffer, pH 7.6, for 5 min to lyse erythrocytes and washed twice in supplemented medium (*vide infra*). Dendritic cells are the interface (FcR^-) cells and constitute between 0.2 and 0.5% of the original starting nucleated cell population in agreement with the previously described yield (6).

Cells adherent after 18 h in culture were removed by 10 mM EDTA and vigorous pipetting. These represent the 18-h-adherent fraction of the original population.

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Preparation of Spleen Adherent Cells (SAC). Spleen cell suspensions were cultured for 2 h on microexudate-coated Petri dishes that had been prepared by previously removing confluent BHK fibroblasts with 10 mM EDTA (8, 9). The adherent fraction, constituting ~2% of the original spleen population, was removed using 3 mM EDTA. This population was highly enriched for FcR^+ cells (>98% using EA rosetting) as found in human systems (8, 9).

Morphologic Methods. Aliquots from all samples were examined after either cytocentrifugation or adherence to polylysine-coated cover slips. Where required, fixation was in either 4% formol calcium or 2.5% glutaraldehyde. Enzymes examined by histochemistry were peroxidase (6), acid phosphatase (6), and nonspecific esterase (10). Phagocytic studies used 1.01-μm-diameter latex particles (Dow Chemical Co., Midland, Mich.) and dispersed *Candida albicans.*

For immunofluorescence, unfixed cells were examined using a monoclonal anti $I\text{-}A^k$ (10.2.16 from L. Herzenberg, courtesy of The Salk Institute, La Jolla, Calif.) and heteroantisera against $I\text{-}A^k$ (AS 760; from I. F. C. McKenzie, The Austin Hospital, Heidelburg, Victoria, Australia), Ia^d (AS 727 from I. F. C. McKenzie) and K^d (D5b; from the National Institutes of Health, Bethesda, Md.) determinants. Electron microscopy was performed on Epon-embedded cells using an HS9 electron microscope (Hitachi Ltd., London).

Purification of Primed T Cells. 50 μg antigen emulsified in complete Freund's adjuvant was injected into the footpads of a mouse, and the draining lymph nodes removed 10–20 d later (11). Single-cell suspensions were depleted of endogenous presenting cells and enriched for T cells by passage over a plastic tissue culture Petri dish (1 h at 37°C), a nylon-wool column (1 h at 37°C in RPMI-1640 medium + 20% fetal calf serum) and a column of Sephadex G-10 (12). The resultant population was highly enriched for Thy-1-bearing cells (98% lysed by monoclonal anti Thy-1.2 + complement).

Proliferation Assays. All proliferation assays were set up in triplicate in RPMI-1640 containing 5% heat-inactivated fetal calf serum, 10 mM Hepes, 2 mM L-glutamine, and 50 μM 2-mercaptoethanol for 96 h. Flat-bottomed microtiter plates (Falcon Labware, Div. of Becton, Dickininson & Co., Oxnard, Calif.) were used: each well contained 0.2 ml total. 1 μCi iododeoxyuridine (^{125}IUdR) in 10 μl was added from 90 to 96 h, and cells were harvested on an automated cell harvester (Dynatech Laboratories, Inc., Dynatech Corp., Alexandria, Va.). Radioactive macromolecular DNA was assessed by gamma-counting.

For mixed lymphocyte cultures, 6×10^5 responders were incubated with graded numbers of spleen stimulators that had been irradiated with 1,000 rad from a ^{60}Co source. In antigen-specific proliferation, 2×10^5 T cells were mixed with 50–100 μg/ml priming antigen and 1,000-rad-treated spleen cell fractions.

Results

Characterization of Dendritic Cells. Our first emphasis was to identify the morphologic features of the 18-h-cultured cells to see if our cells were comparable to those separated by Steinman and Cohn (4, 6). After FcR rosetting the nonadherent population, the FcR^- cells varied in both size and shape (range 7–15 μm) with long, extended blunt processes; fine surface processes were rare. The cytoplasm contained few granules (chiefly phase dense) and only occasional vacuoles. In some of the cells perinuclear granules stained by the acid phosphatase method as described previously (6). The peroxidase stain was also positive in 30–40% of the cells. None of the cells showed non-specific esterase activity. The cells did not interiorize either latex particles or opsonized *C. albicans.* Control whole spleen cells, spleen adherent cells, and peritoneal exudate cells all showed active phagocytosis under the same conditions. Other morphologic features (multivesicular bodies, nuclear appearance, Golgi apparatus) of this fraction were as described (4, 6). The FcR^+ nonadherent fraction was composed predominantly of typical macrophages. More than 80% of CBA FcR^- cells fluoresced with anti-$I\text{-}A^k$ antiserum compared with 60% of the FcR^+ cells. The 18-h adherent fraction showed only 20% fluorescence. Neither anti-Ia^d nor anti-K^d showed specific fluorescence.

Function of Isolated Cell Fractions. To further confirm that the 18-h nonadherent FcR^- cells isolated by our procedure had the same characteristics as the cell of Steinman and Witmer (5), we also showed that our FcR^- cell was a potent stimulator of a mixed lymphocyte reaction (Fig. 1). As few as 2×10^3 irradiated cells stimulated the proliferation of 6×10^5 allogeneic responders. In a series of experiments, stimulator dendritic cells showed a 10- to 50-fold increment in proliferation when compared with a stimulator whole spleen population.

T Cell Proliferation. The ability of the Ia^+ dendritic cell to activate allogeneic T cells suggested that it might also be able to present antigen to syngeneic T cells. This was tested by adding irradiated dendritic cells to purified immune T cells in the presence of priming antigen as described above. T cell proliferation was assessed 4 d later. Table I shows a typical experiment. The addition of dendritic cells, even at concentrations as lows as 1% ($2 \times 10^3 + 2 \times 10^5$ T cells), gave high levels of T cell proliferation. Comparable data have also been obtained using the antigens keyhole limpet hemocyanin and (T,G)-A--L. All the antigens have been tested in several strains of mice. Dendritic cells recovered after 18 h from similarly treated cultures of lymph node cells behaved in an identical manner (data not shown). Table I also indicates that no other spleen cell fraction recovered from the 18-h culture was able to present antigen to T cells. The only other cell that was comparably effective in antigen presentation was the adherent cell removed from the microexudate plate at 2 h.

Expression of Ir Gene Products by Dendritic Cells. The capacity of dendritic cells to present antigen to syngeneic T cells led us to investigate whether dendritic cells were under the control of Ir genes. CBA and BALB/c mice were primed with the synthetic polypeptides (T,G)-A--L and GAT in complete Freund's adjuvant. Fig. 2 illustrates that T cells from CBA mice [responders to GAT but not (T,G)-A--L] cultured with syngeneic dendritic cells respond to GAT and PPD but not to (T,G)-A--L. BALB/c

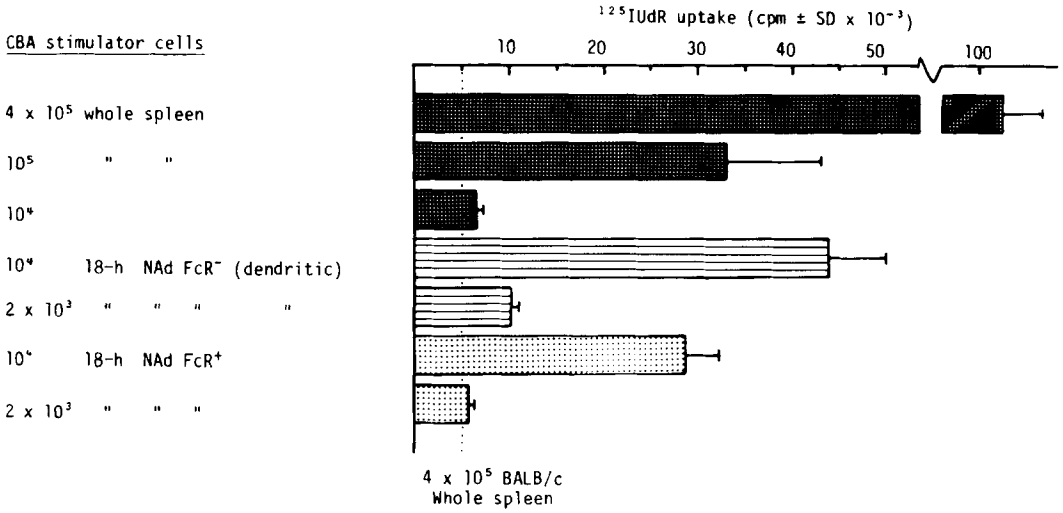

FIG. 1. Dendritic cells are potent stimulators of the mixed lymphocyte response. 6×10^5 BALB/c spleen cells were cultured with varying numbers of 1,000-rad-treated CBA spleen cell fractions: ⊞, whole spleen; ▤, dendritic cells; ⊠, 18-h nonadherent (Nad) FcR^+ cells. 96-h ^{125}IUdR incorporation expressed as mean cpm ± SD of triplicate cultures.

TABLE I

Presentation of Soluble Antigen by Fractionated Irradiated Spleen Cells

Cells	96-h ^{125}IUdR cpm	
	No antigen	100 μg/ml GAT
2×10^5 whole lymph node	2,306 ± 123	15,604 ± 2,304
2×10^5 purified T cells	1,502 ± 506	3,462 ± 789
2×10^5 purified T cells + 5×10^3 18-h nonadherent FcR$^-$	2,949 ± 876	20,857 ± 1,052
2×10^5 purified T cells + 2×10^3 18-h nonadherent FcR$^-$	2,003 ± 763	16,804 ± 269
2×10^5 purified T cells + 5×10^3 18-h nonadherent FcR$^+$	1,362 ± 204	2,631 ± 593
2×10^5 purified T cells + 5×10^3 18-h adherent	1,045 ± 265	952 ± 361
2×10^5 purified T cells + 5×10^3 SAC	3,598 ± 367	23,608 ± 3,542
2×10^5 purified T cells + 2×10^3 SAC	2,642 ± 1,052	12,538 ± 1,555

2×10^5 purified BALB/c T cells were incubated with graded numbers of 1,000-rad-treated spleen cell populations in the presence or absence of 100 μg/ml priming antigen (GAT) and T cell proliferation at 96 h assessed by ^{125}IUdR incorporation. The response to PPD in vitro showed an identical pattern to the GAT response and so is not included. 18-h nonadherent FcR$^-$ cells are the dendritic fraction. SAC were recovered from the microexudate plate at 2 h.

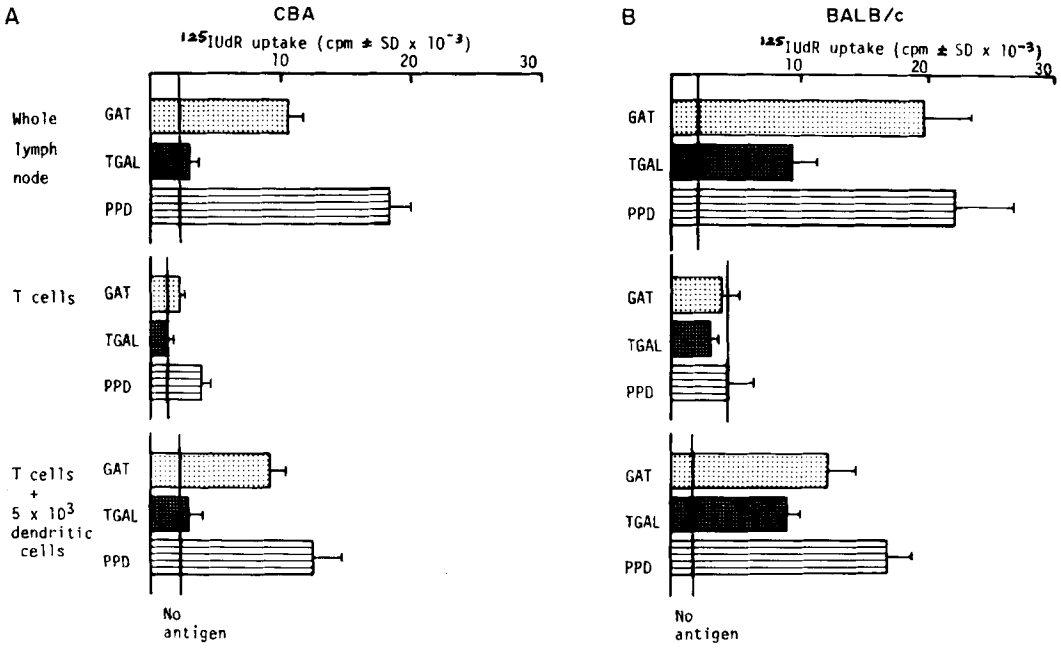

FIG. 2. Dendritic cells induce T cell proliferation to GAT but not to (T,G)-A--L in CBA mice. Draining lymph nodes from (A) CBA and (B) BALB/c mice primed with GAT and (T,G)-A--L were cultured for 96 h in vitro with 100 μg/ml GAT (▨); 100 μg/ml TGAL ⊞; or 50 μg/ml PPD (▤). ^{125}IUdR incorporation was measured over the final 6 h.

T cells [responders to both GAT and (T,G)-A--L] respond to GAT, (T,G)-A--L, and PPD in the presence of BALB/c dendritic cells.

These results indicated that the assay was susceptible to Ir gene influences. To study the cellular site of control, we purified GAT-primed T cells from F_1 mice derived from responder × nonresponder crosses (B10.A × B10.S and B10 × B10.G) and added irradiated parental dendritic cells in the presence of GAT. Table II demonstrates that only the dendritic cell of the responder parent (B10.A or B10 in the respective crosses)

TABLE II

Dendritic Cells from Parental Responder Mice Induce the Proliferation of Responder × Nonresponder F_1 T Cells

	Cells	96-h ^{125}IUdR cpm		
		No antigen	100 μg/ml GAT	50 μg/ml PPD
	B10 × B10G			
1.	Whole lymph node	2,632 ± 490	8,343 ± 999	10,948 ± 1,061
2.	T	1,782 ± 510	2,751 ± 273	3,142 ± 611
3.	T + 5 × 10^3 B10 dendritic cells	2,706 ± 360	6,752 ± 665	9,149 ± 1,129
4.	T + 5 × 10^3 B10.G dendritic cells	2,393 ± 439	2,532 ± 810	8,555 ± 1,542
	B10.A × B10.S			
5.	Whole lymph node	1,238 ± 402	3,054 ± 628	4,020 ± 198
6.	T	684 ± 162	902 ± 232	1,292 ± 284
7.	T + 5 × 10^3 B10.A dendritic cells	802 ± 190	2,228 ± 1,110	5,204 ± 654
8.	T + 5 × 10^3 B10.S dendritic cells	1,104 ± 798	584 ± 326	2,428 ± 1,064

Experimental conditions were as in the legend to Fig. 2.

is able to present GAT to F_1 T cells, whereas dendritic cells derived from nonresponder parents can present only PPD and not GAT to the F_1 T cells.

Discussion

These results clearly show that the dendritic cell is a site of expression of Ir gene products, firmly establishing it in the network of cell interactions involved in immune regulation. Many questions remain unanswered about these FcR^- dendritic cells. Other groups have found that Ia^+ FcR^+ cells are active in presenting antigens in helper and proliferation systems (2, 13). In this context it is interesting that the other effective antigen presenter in our system is the FcR^+-enriched spleen adherent population (Table II, lines 7 and 8). There are two possible explanations for these findings: the dendritic cell may be the only antigen-presenting cell for T cells and all accessory cell populations contain a small contaminating dendritic cell subpopulation, which is the active fraction. Alternatively, both the FcR^+ adherent cell with macrophage properties and the dendritic cell are effective antigen presenters. Investigations are underway to distinguish between these alternatives.

The mechanism of antigen presentation by the nonphagocytic dendritic cell is not known. It is possible that antigen becomes attached to the cell membrane and is presented without intracellular processing. As T cells can react to small peptides and fragments of antigens (14), a plasma membrane enzyme may cleave the antigen in such a way that the relevant fragment may associate with the Ia of the presenting cell.

The relationship of the dendritic cell to in vivo antigen-presenting cells is also unclear; in particular *vis-à-vis* the dendritic reticular cell of Nossal et al. (15) and the marginal zone macrophage (16), which may both be involved in antigen presentation to B cells. It is evident, however, that progress in understanding presenting cell-lymphocyte interactions is grossly hampered by not knowing the full diversity of macrophage-presenting cell lineages.

Summary

Dendritic cells prepared by a modification of the method of Steinman and Cohn are $I\text{-}A^+$ and FcR^-. They are extremely potent at activating not only allogeneic T cell

proliferation but also antigen-specific syngeneic T cell proliferation. Dendritic cells from nonresponder strains are unable to present antigens to responder × nonresponder T cells, suggesting that they may be a site of Ir gene product expression.

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