

**MOUSE SPLEEN DENDRITIC CELLS PRESENT SOLUBLE
ANTIGENS TO ANTIGEN-SPECIFIC T CELL HYBRIDOMAS***

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The dendritic cell has been shown to be extremely effective at activating T lymphocytes involved in allogeneic, mitogenic, or TNP-specific responses (1–6). Previous studies (7, 8) have also suggested that the dendritic cell can participate in the presentation of soluble antigens to suitably primed T cells. These studies did not definitively indicate, however, whether the dendritic cells themselves were able to present soluble antigens to syngeneic T cells nor whether these effects could be seen in the absence of any other potential contaminating accessory cell in the indicator T cell population. We have now addressed these issues by using T cell hybridomas that proliferate autonomously in culture. When triggered by antigen in the presence of spleen cells of the appropriate haplotype, the hybridomas release interleukin 2 (IL-2) (9–11). Our studies were designed to test the idea that nominal antigen plus purified dendritic cells activate antigen-specific hybridomas to produce IL-2 in a genetically restricted fashion.

Materials and Methods

Mice. (CBA/N × BALB/c)F₁ male mice were the generous gift of Dr. Brigitte Huber, Tufts University Medical School. DBA/2 males were obtained from The Jackson Laboratory, Bar Harbor, ME and maintained in the Tufts University Medical School Animal Colony, as were CBA/Tufts mice.

Antigens. Ovalbumin (OVA) was obtained from the Sigma Chemical Co., St. Louis, MO; keyhole limpet hemocyanin (KLH) was the generous gift of Dr. Marvin Rittenberg, University of Oregon Health Science Center, Portland, OR.

Preparation of Spleen Dendritic Cell and Macrophage Fractions. Dendritic cells and spleen macrophages were obtained as previously described (2, 7). Briefly, spleen cells were spun on a discontinuous bovine serum albumin (Path-O-Cyte 4; Miles Laboratories, Inc., Kankakee, IL) gradient of densities 10, 23, 26, 29, and 35% for 30 min at 18,000 g. The interface of 10–23% was removed and cultured for 2 h in RPMI plus 5% fetal calf serum (fetal bovine serum; Dutchland Laboratories, Inc., Denver, PA) containing 5 × 10⁻⁵ M mercaptoethanol, 10 mM Hepes, and 2 mM L-glutamine (complete medium). Nonadherent (NAd) cells were discarded and the medium was replaced. After a further 18 h in culture, 90% of the cells were nonadherent. NAd cells were then separated by Fc receptor (FcR) rosetting, using rabbit anti-sheep cell antibody (7S IgG fraction; Cordis Laboratories, Inc., Miami, FL)-coated sheep erythrocytes (EA) at 37°C as previously described (2, 7).

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Since phagocytosis of EA may decrease macrophage viability (12), in some experiments EA rosetting was also performed at 4°C. FcR⁺ and FcR⁻ (dendritic) cells were then separated over Ficoll-Hypaque and bound EA were removed by hypotonic shock. The characteristics of the cells prepared by this method have been extensively analyzed previously (2, 7). The FcR⁻ cells were nonphagocytic and were 80–90% I-A⁺. The FcR⁺ fraction was phagocytic (>90% ingesting latex) and stained with nonspecific esterase; 50–60% of the cells were I-A⁺. Where indicated, FcR⁻ cells were rosetted with fresh EA for a further 45 min and respun on Ficoll-Hypaque. Recoveries of 70–80% were obtained in the twice-treated interface.

T Cell Hybridomas. The T cell hybridomas D011.10, AODH-3.4, and AODK-10.4, which secrete IL-2 when stimulated with OVA plus I-A^d, OVA plus I-A^k, and KLH plus I-A^d, respectively (9, 11), were used in these studies.

Anti-IA Reagents. Cells producing monoclonal anti-I-A^k 10.2.16 were kindly provided by the laboratory of Dr. L. A. Herzenberg via the Salk Institute Cell Distribution Center, La Jolla, CA. Cells producing monoclonal anti-I-A^d MK-D6 have been previously described (9). Culture supernatant from exhausted cell cultures was used as the source of antibody at a final concentration of 1:10 in our assays.

Induction of IL-2. 5–10 × 10⁴ T cell hybridomas were incubated in complete medium in duplicate in round-bottomed 96-well culture trays (Linbro 76-013-05; Flow Laboratories, Inc., McLean, VA) with graded numbers of accessory cells irradiated with 2,000 rad. Medium, 1 μg concanavalin A or 200 μg antigen, was added to each well to make a total volume of 200 μl. After 24 h at 37°C, 165 μl of supernatant (SN) was removed from each well and duplicate SN pooled. In some experiments, these SN were frozen at –20°C until the assay.

Assay for IL-2. An IL-2-addicted line, CTLL, originally derived by Gillis and Smith (13), was obtained from Dr. Paula Hochman, Tufts University Medical School. 8–10 × 10³ CTLL cells were incubated with 100 or 50 μl SN in triplicate in round-bottomed wells for 24 h as described above. 1 μCi [³H]thymidine ([³H]TdR) was included for the final 6 h. [³H]TdR counts were directly proportional to the number of hybridoma cells in the initial incubation as well as the volume of supernatant included in the CTLL assay (data not shown).

Results

Induction of IL-2 by Dendritic Cells. The T cell hybridoma D011.10 has previously been shown (11) to produce IL-2 only when cultured with OVA in association with products of I-A^d. Table I indicates that if D011.10 is incubated with 1 × 10⁶ DBA/2 spleen cells, high levels of IL-2 are produced in the presence but not the absence of OVA. Low numbers of unfractionated spleen cells induced little IL-2. Equally low numbers of DBA/2 dendritic cells (1–2 × 10⁴), however, induced very high levels of IL-2 production: 2 × 10⁴ dendritic cells were equivalent in effect to 1 × 10⁶ unfractionated spleen cells. Interestingly, the FcR⁺ fraction isolated under the same conditions as the dendritic cell population (by EA rosetting at 37°) had only a slight effect on IL-2 production at either 1 or 2 × 10⁴ cells per well when co-cultured with antigen. The levels of IL-2 produced approximated those induced by the same number of unfractionated spleen cells. Table I also indicates that induction of hybridoma IL-2 production by dendritic cells is genetically restricted since 2 × 10⁴ CBA (H-2^k) dendritic cells were totally ineffective in activating the hybridoma.

Presentation of KLH by Dendritic Cells. We also tested to see if a hybridoma specific for a much larger soluble antigen, KLH, could recognize antigen in association with dendritic cells. The first part of Table II illustrates that 1–2 ×

TABLE I

Dendritic Cells Induce IL-2 Production by an Ovalbumin-specific T Cell Hybridoma, D011.10, in a Genetically Restricted Fashion

Accessory cell present during incubation with D011.10	[³ H]TdR incorporation	
	No antigen	OVA
	<i>cpm ± SD</i>	
None	1,296 ± 126	1,132 ± 17
DBA/2 1 × 10 ⁶ spleen cells	1,041 ± 148	20,196 ± 3,580
DBA/2 1 × 10 ⁴ spleen cells	1,549 ± 78	2,546 ± 40
DBA/2 2 × 10 ⁴ NAd FcR ⁻ (dendritic cells)	1,102 ± 126	16,040 ± 2,981
DBA/2 1 × 10 ⁴ NAd FcR ⁻ (dendritic cells)	1,935 ± 782	9,017 ± 178
DBA/2 2 × 10 ⁴ NAd FcR ⁺	1,110 ± 308	2,700 ± 322
DBA/2 1 × 10 ⁴ NAd FcR ⁺	1,220 ± 164	1,987 ± 290
CBA 2 × 10 ⁴ NAd FcR ⁻	1,135 ± 52	1,370 ± 122

Irradiated accessory cells were incubated for 24 h with 10⁵ D011.10 cells in the presence or absence of 200 μg OVA. Supernatants were removed and 100 μl tested for IL-2 activity on 10⁴ CTLL cells. 1 μCi [³H]TdR was added for the last 6 h of a 24 h incubation. CTLL cells + medium: 1,356 ± 192; CTLL cells + 2½% T cell growth factor: 15,018 ± 772. Results shown are representative of three experiments.

TABLE II

Dendritic Cells Induce IL-2 Secretion by a KLH-specific Hybridoma AODK-10.4

Exp.	DBA/2 accessory cell present during incubation with AODK-10.4	[³ H]TdR incorporation	
		No antigen	KLH
		<i>cpm ± SD</i>	
1	1 × 10 ⁶ spleen	382 ± 127	6,927 ± 1,612
	1 × 10 ⁴ spleen	248 ± 163	190 ± 56
	2 × 10 ⁴ NAd FcR ⁻	177 ± 98	6,184 ± 1,965
	1 × 10 ⁴ NAd FcR ⁻	341 ± 97	5,667 ± 956
2	None	825 ± 46	1,029 ± 122
	1 × 10 ⁴ spleen	689 ± 33	1,057 ± 5
	2 × 10 ⁴ NAd FcR ^{-*}	1,250 ± 131	7,936 ± 1,975
	1 × 10 ⁴ NAd FcR ⁻	565 ± 283	7,300 ± 846
	0.5 × 10 ⁴ NAd FcR ⁻	720 ± 251	5,805 ± 973
	2 × 10 ⁴ NAd FcR ^{+‡}	1,186 ± 106	6,220 ± 656
	1 × 10 ⁴ NAd FcR ⁺	899 ± 355	2,087 ± 885
0.5 × 10 ⁴ NAd FcR ⁺	1,131 ± 567	1,255 ± 376	

Experimental conditions as in legend to Table I except 4 × 10⁴ hybridoma cells were used and 50λ supernatant tested on 8 × 10³ CTLL cells. Experiment 1, CTLL alone: 173 ± 26; CTLL + 2.5% cell growth factor (TCGF): 22,024 ± 2,362; experiment 2, CTLL alone: 235 ± 85; CTLL + 2.5% TCGF: 18,215 ± 1,054. Results for each experiment are representative of at least three repetitions.

* FcR⁻ population rosetted with a further round of EA for 45 min at 4°C.

‡ FcR⁺ population derived by EA rosetting for 45 min at 4°C.

10⁴ dendritic cells induced high levels of IL-2 in the presence of KLH; in several experiments these levels were as high or higher than those generated by 1 × 10⁶ unfractionated spleen cells. In the second part of Table II, FcR⁻ and FcR⁺ fractions were compared for their ability to induce hybridoma IL-2 secretion in the presence of antigen. To avoid potential problems of EA phagocytosis leading to decreased viability (12), EA rosetting was performed at 4°C. The FcR⁻ fraction was also subjected to a further round of EA rosetting at 4°C to remove any residual FcR⁺ cells. The resultant FcR⁻ population was still highly effective at inducing IL-2 secretion in the presence of KLH over the range 0.5–2 × 10⁴ (in some experiments as few as 1 × 10³ cells were effective). In a series of experiments, FcR⁺ cells prepared at 4°C induced significant IL-2 secretion at high

TABLE III
Monoclonal Anti-I-A Specifically Blocks Dendritic Cell-induced Hybridoma IL-2 Production

Accessory cell present during incubation with hybridoma	[³ H]TdR incorporation			
	Hybridoma tested			
	AODH-3.4		D011.10	
	No antigen	OVA	No antigen	OVA
	<i>cpm ± SD</i>			
None	521 ± 113	532 ± 94	361 ± 60	333 ± 40
2 × 10 ⁴ (CBA/N × BALB/c)F ₁ NAd FcR ⁻ cells*	1,436 ± 177	4,420 ± 274	737 ± 202	1,765 ± 316
Plus anti-I-A ^k	1,747 ± 192	1,564 ± 922	339 ± 57	2,649 ± 204
Plus anti-I-A ^d	649 ± 136	4,908 ± 5	410 ± 39	532 ± 36
1 μg concanavalin A alone	9,794 ± 628		3,578 ± 701	

Experimental conditions as in legend for Table I except that 6 × 10⁴ hybridoma cells were used and 50 μl supernatant was tested for IL-2 activity at 24 h. CTLL cells + medium: 391 ± 190; CTLL cells + 2.5% T cell growth factor: 14,421 ± 903. Results are representative of three experiments.

* Dendritic cells reosetted for 45 min with fresh EA.

levels but titrated out rapidly over the dose range at which dendritic cells were active.

Monoclonal Anti-I-A Sera Block Dendritic Cell Induction of IL-2. To test for the site of the genetic restriction in the interaction of dendritic cells and T cells, we assayed two hybridomas, D011.10 and AODH-3.4, which respond to OVA in association with Ia^d and Ia^k products, respectively. (CBA/N × BALB/c)F₁ spleen cells, which express both I-A^k and I-A^d determinants, were used as the source of dendritic cells for these experiments. Table III shows that in the presence of F₁ dendritic cells (prepared by reosetting the FcR⁻ with fresh EA) and ovalbumin, significant levels of IL-2 were induced in both hybridomas. (It should be stressed that IL-2 values in this table are ~25% those found in Table I. This is because both supernatant volume and hybridoma number were reduced by 50%.) If anti-I-A^d was also added, however, dendritic cell presentation of OVA to D011.10 was completely inhibited, whereas anti-I-A^k had no effect on IL-2 production. This pattern of anti-I-A blocking was reversed for OVA presentation to AODH-3.4, demonstrating not only the importance of I-A on the dendritic cell but also the specificity of the hybridoma for the relevant I-A plus antigen moiety.

Discussion

One of the major problems in asking which cells are able to present antigen to T cells has been the difficulty in totally depleting the indicator T cell population of endogenous accessory cells. This difficulty has been circumvented by the recent use of antigen-specific T cell hybridomas that are self-replicating in the absence of any "filler" cell (9-11) and which, when triggered by either concanavalin A or antigen plus spleen cells of the appropriate haplotype, produce the soluble mediator IL-2. The production of this mediator in this system has been used in several laboratories as an index of antigen presentation (9-11, 14-16).

We have found that in the presence of soluble antigen, low numbers of splenic dendritic cells can induce IL-2 production by hybridomas. This response is haplotype restricted and involves I-A determinants on the surface of the dendritic

cell, since appropriate anti-I-A sera block IL-2 induction. Alternative explanations for our findings have also been considered. One possibility is that a small contaminating population of macrophages within the dendritic cell fraction is responsible for all the effects described here. We feel this is unlikely since, as described above, the FcR⁺ population isolated at the same density as dendritic cells is not as effective at presenting antigen as dendritic cells over the effective titration range. Furthermore, in two sets of experiments, the FcR⁻ fraction was reresetted with EA and the remaining FcR⁻ cells still presented antigen to the hybridoma (Tables II and III). Indeed, this FcR⁻ population also induced hybridoma IL-2 secretion when preincubated rather than co-cultured with high concentrations of ovalbumin or KLH (data not shown). We therefore feel that the simplest explanation for our data is that dendritic cells are presenting exogenous soluble antigens in association with surface I-A products to T cells.

The low density macrophage population that we have isolated is less effective at presenting soluble antigens than the dendritic cell fraction and it is possible that this lower level of activity may be contributed by contaminating dendritic cells. Why the NAd FcR⁺ fraction used in these and previous studies is not as effective at presenting protein antigens is unclear. It contains a high proportion of I-A⁺ cells (7), and other I-A⁺ cells such as macrophage tumors and B cell lymphomas have been shown to present antigen to the hybridomas (14–16). There may, however, be subtle biochemical differences between the Ia determinants of these populations, as has been documented for B cell versus macrophage I-A (17), and this may be functionally relevant. It is unlikely that the FcR⁺ cells are actively suppressing the antigen-specific response since preliminary experiments indicate that mixing the FcR⁺ and FcR⁻ populations even at 1:1 ratios does not inhibit the dendritic cell-induced response. Experiments are underway to clarify these issues.

The fact that dendritic cells do not phagocytose, have few lysosomes, and are poorly pinocytic (18) leaves open the question of how these cells present polypeptide antigens to T cells. Since cells with similar characteristics have been described in vivo (19), we suggest that these cells may play a key physiological role in the activation of syngeneic antigen-specific T cells.

Summary

In the presence of the soluble polypeptide antigens ovalbumin and keyhole limpet hemocyanin, purified mouse spleen dendritic cells induce the secretion of IL-2 by antigen-specific T cell hybridomas. This response is H-2 restricted and can be specifically inhibited by monoclonal anti-I-A. These data indicate that dendritic cells can present soluble antigen to H-2-compatible T cells.

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