Antigen Acquisition by Dendritic Cells: Intestinal Dendritic Cells Acquire Antigen Administered Orally and Can Prime Naive T Cells In Vivo

By L. M. Liu and G. G. MacPherson

From the Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE, United Kingdom

Summary

In the rat, mesenteric lymphadenectomy allows collection of dendritic cells (DC) derived from the small intestine after cannulation of the thoracic duct. We prepared rats this way and administered antigens by oral feeding or intraintestinal injection. DC enriched from the thoracic duct lymph collected over the first 24 h from these animals are able to stimulate sensitized T cells in vitro and to prime popliteal lymph node $CD4^+$ T cells after footpad injection, while B and T cells from the same thoracic duct lymph are inert in priming. 500 or less DC pulsed in vitro with antigen can prime T cells in vivo, whereas 100 times more B cells or macrophages pulsed in vitro are quite inert. 1 mg of ovalbumin administered orally is sufficient to load DC for in vivo priming of T cells. Antigen could not be detected directly in DC but was present in macrophages in the lamina propria. Direct presentation of antigen by DC to T cells was demonstrated by injecting F_1 recipients with parental DC and showing restriction of T cell sensitization to the major histocompatibility complex of the injected DC. Antigen-bearing DC do not induce a detectable primary antibody response but a small secondary antibody response can be detected after a boosting injection. These results show that acquisition of antigens by DC in the intestine is very similar to what occurs in vitro or in other tissues, suggesting that there may be no special difference in antigen handling at mucosal surfaces. One implication of these results is that hypotheses designed to explain oral tolerance must take into account the presence of immunostimulatory, antigen-bearing DC in animals that have received oral antigens.

The intestine is continually exposed to foreign antigens, but whereas it is critical that protective immune responses are made to potential pathogens, it is equally important that hypersensitivity responses are not made to dietary antigens. Antigens introduced into the small intestine can induce both local and systemic responses and in some circumstances may induce local immunity but systemic tolerance (1). The mechanisms underlying both stimulatory and tolerogenic responses are not understood and, in particular, the role of differential antigen handling and presentation has been rarely investigated. Several distinct potential APC are present in the small intestine. These include macrophages (2), B lymphocytes (3), epithelial cells (4), and dendritic cells (DC)[1] (5, 6), and it has been suggested that regulation of intestinal immune responses may be in part dependent on the nature of the cells involved in presentation to T cells, in particular that presentation by epithelial cells (7) or supposed I-J-positive APC (8) may be responsible for the generation of suppressor cells.

There is, however, increasing evidence that at least in rodents, DC are the only APC able to activate resting T cells efficiently and that other APC are able to present antigens only to activated T cells (9). One of the main functions of DC in vivo is thought to be the acquisition of antigens in peripheral tissues and their transport to draining lymph nodes for presentation as processed peptides to T lymphocytes. Thus, Mayrhofer et al. (10) showed that during a *Salmonella* infection, specific antigens were present in cells with the morphology of DC in lymph draining the intestine. Macatonia et al. (11) showed that after skin painting with FITC, DC in the draining lymph node expressed FITC, could stimulate sensitized T cells, and could induce sensitivity in a naive recipient. Bujdoso et al. (12) showed that after subcutaneous injection of antigen, DC in lymph draining the site of injection could present the antigen to sensitized T cells. Crowley et al. (13) showed that after intravenous injection of antigen, DC were the only cells in the spleen that contained immunogenic fragments.

We have developed a model in which after mesenteric lymphadenectomy (MLNX), DC derived from the small intestine can be collected in thoracic duct lymph (XTDL) (14). We have shown that after the direct injection of soluble an-

[1] *Abbreviations used in this paper:* DC, dendritic cells; HRP, horseradish peroxidase; L-DC, lymph-borne DC; MLNX, mesenteric lymphadenectomy; XTDL, thoracic duct lymph from lymphadenectomized rat.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/93/05/1299/09 $2.00
Volume 177 May 1993 1299-1307

tigen into the intestinal lumen, DC in the intestinal wall acquire the antigen and migrate into peripheral lymph, and that these lymph-borne DC (L-DC) can present the antigen to sensitized T cells in a MHC class II–restricted, CD4-dependent manner (15).

In this paper we have compared the effects of giving antigen orally or intraintestinally upon its acquisition by L-DC, and have examined the ability of such L-DC to induce immune responses in naive animals in comparison with DC pulsed with antigen in vitro. We show that oral antigen is acquired efficiently by intestinal DC, but not by other cells present in lymph, that antigen-bearing L-DC but not B cells or macrophages can prime naive T cells directly in vivo, and that such L-DC do not induce detectable primary antibody synthesis although weak secondary antibody responses are seen after a boosting injection. One implication of these results is that hypotheses designed to explain oral tolerance must take into account the presence of immunostimulatory, antigen-bearing DC in animals that have received oral antigens.

Materials and Methods

Animals. Rats were specific pathogen-free inbred strains bred in the MRC Cellular Immunology Unit, Sir William Dunn School of Pathology. The strains used were PVG-RT1^u, PVG-RT1^c, DA-RT1^a, and (PVG-RT1^u × PVG RT1^c)F_1.

Surgical Procedures. MLNX and thoracic duct cannulation were carried out as described previously (14).

Antigens. OVA (grade v) and horseradish peroxidase (HRP; for immunization, type VI-A; for pulsing, type II) were from Sigma Chemical Co. (St. Louis, MO).

Pulsing Cells with Antigens In Vitro. Cells were incubated with OVA or HRP (1 mg/ml) in RPMI 1640 containing 10% FCS, 1% sodium pyruvate, and 10^{-5} M mercaptoethanol (complete medium) at 37°C for 2.5–3 h. The pulsed cells were washed three times with RPMI 1640 plus 10% FCS and resuspended in PBS before use.

In Vivo Antigen Administration. In vivo antigen administration was carried out orally by gastric intubation of anesthetized rats or by injecting antigens directly into the gut lumen of MLNX rats at laparotomy. 1–30 mg OVA or HRP in 2 ml PBS was introduced to MLNX PVG rats via gastric intubation or intraintestinal injection (15). TDL cells were collected overnight after antigen administration.

Immunization with Cells Pulsed with Antigens. Different numbers of antigen-pulsed cells resuspended in PBS were injected into the hind footpads of naive PVG rats (100 μl/footpad). 10 d later, cell preparations were made from the popliteal lymph nodes and used as a source of primed lymph node cells in an antigen presentation assay.

Immunization with Protein Antigens. Antigen in PBS (2 mg/ml) was emulsified with an equal volume of CFA (Sigma Chemical Co.). Rats were injected in the hind footpads with 100 μl emulsion containing 100 μg antigen. They were used as a source of antigen-primed spleen cells 12–20 d later.

Veiled L-DC. L-DC were enriched from XTDL by a single-step density separation. XTDL resuspended in RPMI 1640 plus 10% FCS (at a concentration of 5–10 × 10^6/ml) were overlaid over 14.5% metrizamide solution and centrifuged at 400 *g* for 20 min. The interface cells contained 60–80% L-DC. The major contaminating cells were B lymphocytes. There were ≤1% macrophages present as identified by morphology and the ability to phagocytose opsonized SRBC.

Peritoneal Macrophages. 15 ml cold PBS was injected into the peritoneal cavity. The abdomen was massaged and then opened. The cell suspension was collected by pipetting. The cell suspensions normally contained 70% macrophages.

T Cells. XTDL or spleen cells were incubated with OX6 (anti-Ia) (16) and OX12 (anti-Ig κ) (17) for 40 min at 4°C. After washing three times they were mixed with a 5% SRBC suspension coated with rabbit anti–mouse Ig antibody (Sigma Chemical Co.), rotated at 4°C for 20 min, and then separated by centrifugation over Isopaque-Ficoll. The interface contained ~90% T cells as identified by flow cytometry or immunocytochemistry.

B Cells. B cells were enriched by the rosetting technique described above. They were purified from XTDL cells or spleen cells by depleting OX19 (anti-CD5)- (18), OX52 (pan T)- (19), and W3/25 (anti-CD4)- (20) positive cells. The purity was usually 85–90%.

Popliteal Lymph Node Cells. Popliteal lymph cells were obtained by teasing and were washed two times with RPMI 1640 plus 0.1% BSA.

Antigen Presentation Assay. Assays were performed in triplicate in 96-well round-bottomed tissue culture plates (Flow Laboratories, McLean, VA). For antigen presentation to sensitized spleen T cells, 2 × 10^5 OVA-CFA-primed spleen cells in complete culture medium (containing 10% FCS) were cultured for 108 h in a total volume of 0.2 ml in the presence of L-DC from the rats challenged with OVA orally or intraintestinally (15). To measure the ability of antigen-pulsed cells to prime naive recipients, 2 × 10^5 draining popliteal lymph node cells were cultured for 120 h in complete culture medium (10% FCS was replaced with 5% dark agouti rat serum) in the presence of antigens (OVA or HRP). All the cultures were carried out at 37°C in an atmosphere of 5% CO_2 in air. [^{3}H]Thymidine (Amersham Corp.) (0.5 μci/well) was added to cultures 16 h before harvesting with a cell harvester (Skatron, Newmarket, England) and uptake was measured by scintillation counting. Data were expressed as mean gross counts per minute.

Immunocytochemistry. FITC-labeled canine albumin (Sigma Chemical Co.) was injected intraintestinally, and 1, 3, and 6 h later, tissues were taken from the small intestine and frozen in OCT compound (Tissue Tek, Elkhart, IN). Cryostat sections were stained with OX6 (anti-rat class II) or OX62 (21; a kind gift from Dr. M. Brenan, MRC Cellular Immunology Unit, Oxford) followed by tetramethylrhodamine B isothiocyanate–conjugated goat anti-mouse Ig antibody (Sera-lab, Crawley Down, England). In some experiments, cells were isolated from the small intestine by enzyme digestion after the injection of FITC-labeled canine albumin and stained as described above. FITC-labeled canine albumin was also injected intraintestinally into MLNX rats and L-DC enriched and examined for the presence of antigen under the fluorescence microscope.

ELISA. ELISA plates (Dynatech Dynal [UK], Merseyside, England) were coated by adding antigen (OVA or HRP; 100 μg/ml) in PBS (50 μl/well) and storing at 4°C overnight. The plates were washed three times with PBS/Tween (Koch Light Laboratories, Haverhill, England), and 50 μl of 1% BSA was added for 30 min at 4°C to block nonspecific binding. After washing three times, fivefold dilutions of the serum to be assayed were added and the plates were incubated at 4°C for 30 min. Negative, positive, and background wells were set up by adding negative serum, positive serum, or medium alone.

To detect anti-OVA antibody, 50 μl sheep anti-rat IgG or IgM (1:500 in PBS/Tween) was added after washing and the plates were

incubated at 4°C for 30 min. After three washes with 0.1 M citric acid/phosphate buffer (pH 5), 50 μl of peroxidase-conjugated swine anti-sheep Ig (1:500 in PBS/Tween) was added and the plates were incubated for 30 min at 4°C. After three washes with 0.1 M citric acid/phosphate buffer, 50 μl 1,2-phenylenediamine (OPD; Dako, High Wycombe, England) solution was added to each well and the plates were wrapped with foil and read at 405 nm in a ELISA reader (LKB Instruments, Inc., Gaithersburg, MD).

To detect anti-HRP antibody, alkaline phosphatase–conjugated rabbit anti-rat IgG (Sigma Chemical Co.) was added to the wells and the plates were incubated for 30 min at 4°C. After three washes with PBS-Tween, 50 μl *p*-nitrophenyl phosphate (Sigma Chemical Co.) in 9.7% diethylamine was added into the wells, and after incubation plates were read at 405 nm in a ELISA reader.

Results

Orally Administered Antigens Are Acquired by Intestinal L-DC. Lymphadenectomized, cannulated rats were given graded doses of OVA by gastric intubation or, for comparison, by intraintestinal injection. TDL was collected overnight and DC were isolated by density gradient centrifugation. Different numbers of L-DC were added to sensitized spleen cells and proliferation was measured by thymidine incorporation. The results (a typical experiment is shown in Fig. 1) demonstrate that after doses of 1–10 mg, L-DC derived from the small intestine have acquired antigen and can present it to sensitized T cells. L-DC from orally challenged rats stimulated a much lower response than intraintestinally challenged rats. When, however, the ability of such L-DC to prime T cells in vivo was tested (see below), specific priming was seen with doses down to 1 mg.

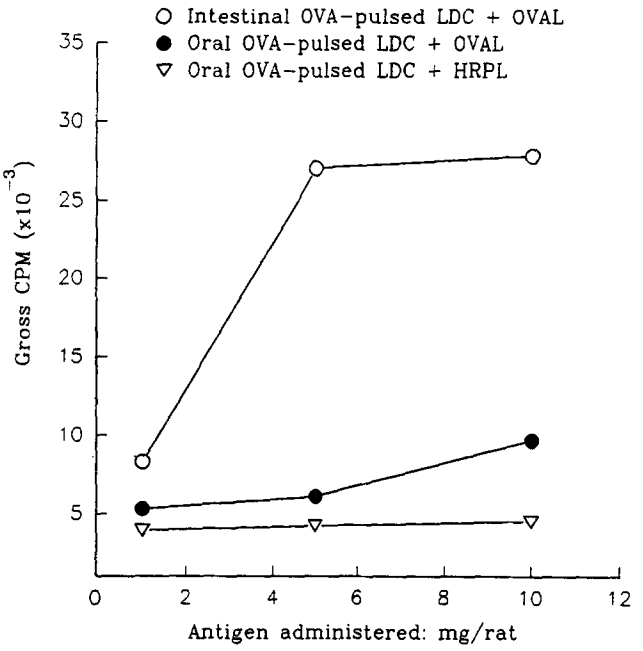

Figure 1. Acquisition of oral antigen by L-DC. PVG rats were cannulated and given different doses of OVA intraintestinally or orally (by gastric intubation). 18 h later, L-DC were enriched and cultured with OVA or HRP-primed spleen cells for 108 h as described in Materials and Methods. Proliferation was measured by thymidine incorporation and expressed as gross cpm. (*OVAL*) OVA-CFA-primed spleen cells; (*HRPL*) HRP-CFA-primed spleen cells. This experiment has been repeated twice with similar results.

Fate of Antigen after Intraintestinal Injection. FITC-labeled canine albumin was injected intraintestinally, and L-DC collected at intervals over the next 48 h were examined for the presence of fluorescent label. No labeled L-DC or other cells were seen. Frozen sections of small intestine taken 1, 3, and 6 h after injection, and cell suspensions prepared from the lamina propria, were stained for MHC class II or OX62, an antibody that labels DC and probably γ/δ T cells, but not macrophages, in the lamina propria (21). Antigen was clearly present in some large MHC class II–negative cells, presumed to be macrophages, but was not seen in $OX62^+$ cells.

In Vivo Priming of T Cells in Naive Rats by Antigen-bearing L-DC. L-DC, B cells, and T cells were enriched from normal XTDL and incubated with OVA (1 mg/ml) for 3 h and washed, or were enriched from XTDL after oral or intraintestinal antigen administration. Graded numbers of cells were

Table 1. *Priming of Naive Rats with In Vitro OVA-pulsed Cells from XTDL*

		Ag concentration in culture			
Cells injected	Ag	100 μg	50 μg	25 μg	No Ag
L-DC (2×10^5)	OVA	54,169	35,783	26,708	2,989
	HRP	6,424	1,689		
L-DC (2×10^4)	OVA	20,039	13,526		490
	HRP	916	1,001		
L-DC (2×10^3)	OVA	13,191	9,789		777
	HRP	536	489		
BC (2×10^6)	OVA	28,606	13,229	12,755	2,912
	HRP	1,618	2,750	3,063	
BC (2×10^5)	OVA	15,370	7,854	5,541	257
	HRP	1,246	1,649	1,744	
TC (2×10^6)	OVA	6,649	5,011	1,634	838
	HRP	1,381	2,060	2,903	
TC (2×10^5)	OVA	487	860	617	359
	HRP	312	769	971	

XTDL was collected from MLNX rats. L-DC were enriched to 70% purity by centrifugation over 14.5% Metrizamide, and B cells and T cells enriched by rosetting as described in Materials and Methods (85–90% purity for B cells; 90–92% for T cells). There were ~1–1.5% L-DC in the B cell fraction as identified by morphology and Ia staining. L-DC, B cells, and T cells were cultured with OVA (1 mg/ml) for 3 h, washed three times, and injected into the footpads of naive rats. 10 d after immunization, popliteal lymph node cells were prepared and cultured with OVA or HRP in different concentrations (100, 50 and 25 μg/ml) for 120 h. 16 h before harvesting, [^{3}H]TdR was added into the wells. Results are expressed as gross cpm. This experiment was performed three times with similar results.

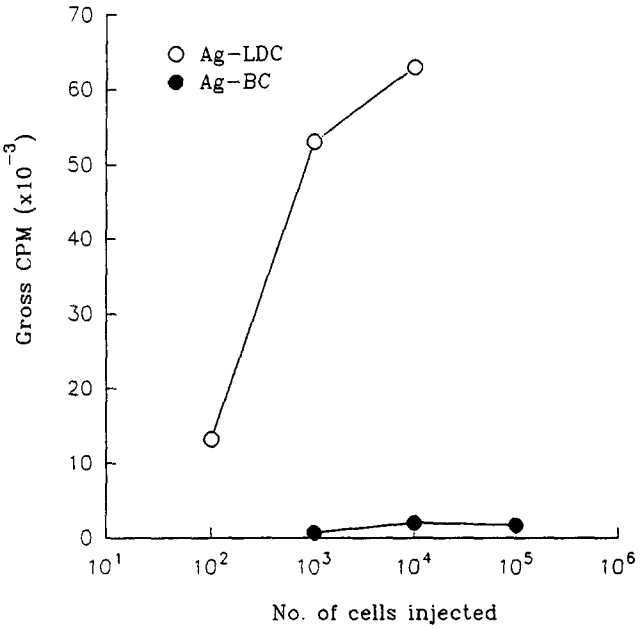

Figure 2. Ability of antigen-pulsed L-DC and B cells to prime naive rats. L-DC from MLNX rats and B cells from normal TDL were pulsed with OVA (1 mg/ml) and injected into the footpads of naive rats. 10 d later, popliteal lymph node cells were prepared and cultured with OVA (50 μg/ml) for 5 d and proliferation measured and expressed as gross cpm. This experiment has been performed twice with similar results.

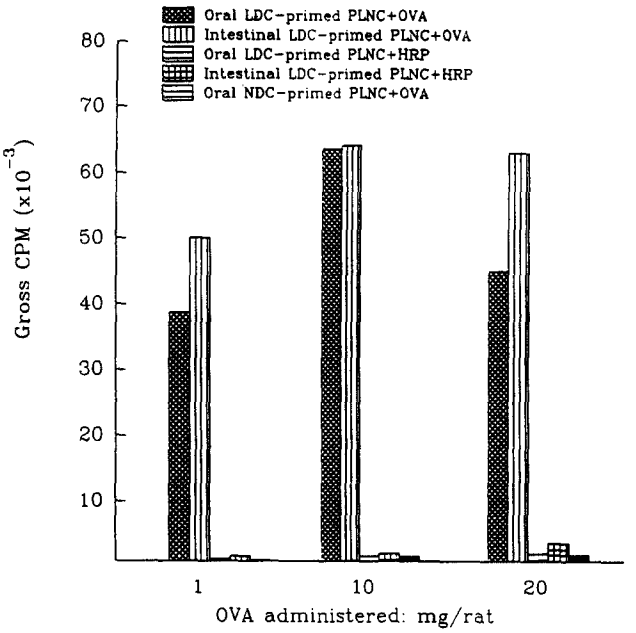

Figure 3. Ability of different doses of antigen to load L-DC for priming. MLNX rats were cannulated and challenged with different doses of OVA (1, 10, and 20 mg/rat) orally or intraintestinally. L-DC were enriched and injected into the footpad of naive PVG rats (5 × 10^4/footpad). 10 d later, popliteal lymph node cells were prepared and cultured with OVA (50 μg/ml) for 5 d and proliferation measured and expressed as gross cpm. This experiment was performed three times with similar results.

injected into the footpads of naive rats. 10 d later, popliteal lymph node cells were cultured with OVA or HRP for 5 d, and proliferation was measured.

Priming with In Vitro Pulsed Cells. The results from L-DC pulsed in vitro show that both the L-DC and B cell fractions sensitized specifically whereas T cells were inert (Table 1). 500–2,000 L-DC in different experiments sensitized naive rats specifically, whereas at least 100 times more enriched B cells fraction were needed to initiate a similar response. DC contamination in the B cell fraction was 1–1.5% as judged by morphology and Ia staining. To assess the role of DC in the Ag-pulsed B cell population from XTDL, we attempted to prime naive rats using Ag-pulsed B cells from normal TDL, which contains <0.1% DC. The results show that Ag-pulsed normal TDL B cells are completely ineffective in sensitization (Fig. 2), and thus the only cells in XTDL able to sensitize naive rats are DC.

Priming with DC from Animals after In Vivo Antigen Administration. DC enriched from XTDL collected for 24 h after oral or intraintestinal antigen administration were injected into naive rats, and 10 d later, popliteal lymph node cells were tested for specific sensitization. The results show that L-DC from rats challenged via both routes are effective at all doses of antigen tested (Fig. 3). Intraintestinal antigen gave rise to larger responses than oral antigen but the differences were not large.

Antigen-pulsed Macrophages Cannot Prime Naive Rats. Peritoneal macrophages were cultured with OVA for 3 h. After washing, they were injected into the footpads of naive rats. OVA-pulsed L-DC were used as a positive control. The results show that OVA-pulsed peritoneal macrophages were completely unable to sensitize naive rats (Table 2).

Only Live L-DC Can Prime Naive Rats. OVA-pulsed L-DC were either fixed with glutaraldehyde or frozen and thawed on dry ice. Fixed or killed L-DC were unable to stimulate a MLR (data not shown). When these cells were injected

Table 2. *Comparison of OVA-pulsed Peritoneal Macrophages and L-DC in Priming Naive Rats*

	Ag (50 μg/ml)		
Cells injected	OVA	HRP	No Ag
L-DC			
5 × 10^3	41,619	533	460
5 × 10^2	39,399	483	540
Macrophages			
5 × 10^4	492	218	427
5 × 10^3	110	282	260

Peritoneal macrophages were obtained as described in Materials and Methods. L-DC were enriched from XTDL. Macrophages and L-DC were cultured with OVA (1 mg/ml) for 3 h, washed three times, and injected into the footpads of naive rats. 10 d later, popliteal lymph node cells were prepared and a proliferation assay was carried out in the presence of OVA or HRP (50 μg/ml). This experiment was repeated once with similar results.

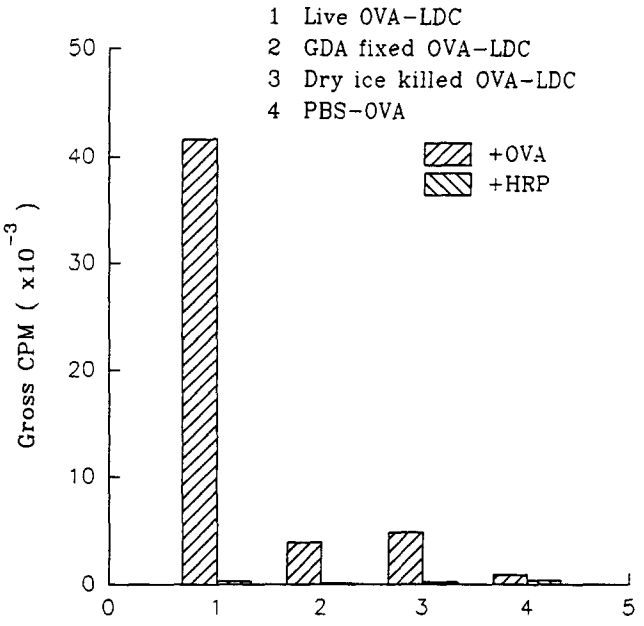

Figure 4. Only live L-DC can prime naive rats. L-DC were pulsed with OVA (1 mg/ml) for 3 h and washed. Some OVA-L-DC were fixed with 1% glutaraldehyde (GDA). Some OVA-L-DC were frozen and thawed several times on dry ice. Normal OVA-L-DC, GDA-fixed L-DC dry ice–killed L-DC, and OVA in PBS were injected into the footpads of naive rats (2 × 10^5/footpad). 10 d later, proliferation of popliteal lymph node cells to OVA or HRP (50 μg/ml) was measured. The results are expressed as gross cpm. This experiment was repeated twice with similar results.

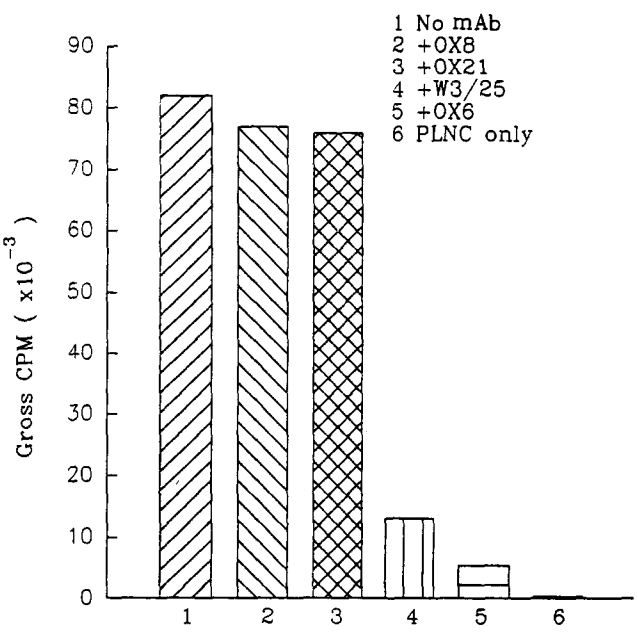

Figure 5. MHC class II restriction and CD4 dependence of primed cells. Popliteal lymph node cells of rats immunized with OVA-pulsed L-DC were cultured with OVA (50 μg/ml) in the presence of different mAbs (final concentration, 5 μg/ml) for 5 d. Proliferation of popliteal lymph node cells was measured and expressed as gross cpm. OX8, anti-CD8; W3/25, anti-CD4; OX6, anti-MHC class II; OX21, anti-human c3b INA. This experiment has been performed twice with similar results.

into the footpads of naive rats no significant sensitization was seen (Fig. 4).

Sensitized T Cells Are CD4$^+$ and MHC Class II Restricted. Popliteal lymph node cells from animals primed with antigen-pulsed L-DC were cultured with specific antigen in the presence of mAbs to MHC class II (OX6), CD4 (W3/25), CD8 (OX8) (22), and human C3b inactivator (INA) (OX21) (23). The results show that anti–MHC class II or anti-CD4 antibodies but not anti-CD8 or anti–human C3b INA inhibited the proliferation of sensitized lymph node cells to OVA (Fig. 5).

L-DC Present Antigen Directly to Naive T Cells In Vivo. Although indirect evidence (e.g., that only live L-DC are able to sensitize naive rats) suggests that antigen-pulsed L-DC may present Ag directly to host T cells, it was possible that host DC obtained antigen from donor L-DC for presentation to host T cells. To address this question, L-DC from PVG-RT1^u or congenic PVG-RT1^c rats, which differ at the MHC, were either pulsed in vitro with OVA or obtained from PVG-RT1^u or PVG-RT1^c rats that had been injected with OVA intraintestinally. These L-DC were injected into footpads of (PVG-RT1^u × PVG-RT1^c)F_1 rats. 10 d later, draining popliteal lymph node cells were depleted of B cells and MHC class II–bearing cells and cultured with irradiated PVG-RT1^c or PVG-RT1^u spleen cells in the presence of OVA or HRP. The results show that the responses were restricted to the MHC class II molecules on the immunizing L-DC (Table 3). T cells from F_1 animals injected with Ag-bearing L-DC from PVG-RT1^c rats respond to OVA in the presence of PVG-RT1^c spleen cells but not PVG-RT1^u spleen cells. Similarly, T cells from F_1 animals injected with Ag-bearing L-DC from PVG-RT1^u rats respond to OVA in the presence of PVG-RT1^u spleen cells but not PVG-RT1^c spleen cells. L-DC pulsed in vitro or from intraintestinally challenged rats (Table 3) gave similar results. These results show clearly that donor L-DC present OVA directly to host T cells.

Antigen-bearing L-DC Do Not Induce Primary Antibody Responses. The experiments mentioned above show that in vitro or in vivo antigen-pulsed L-DC could directly sensitize T cells in a naive rat. To determine whether antigen-bearing L-DC were able to induce an antibody response, naive rats were immunized with orally or intraintestinally challenged L-DC, in vitro antigen-pulsed L-DC, soluble antigens, or antigens emulsified with CFA. 10 d after priming, sera were obtained and the rats were boosted with soluble antigens subcutaneously (OVA or HRP). 10 d after boosting, rats were bled again to obtain sera. Serum antibody levels were measured using an ELISA technique. The results show that neither in vitro nor in vivo antigen-pulsed L-DC were able to induce a primary response. In vitro OVA-pulsed BC or peritoneal macrophages were similarly ineffective (Table 4). However, after boosting with soluble antigens, weak IgG antibody responses were present in all rats that had been primed with antigen-bearing L-DC (Table 4).

Discussion

The uptake, transport, and presentation of antigen in vivo are crucial stages in the initiation of immune responses but

Table 3. *Ag-bearing L-DC Present Ag Directly to Host T Cells*

	F_1 T cells	APC	OVA	HRP	No Ag
L-DC pulsed with OVA in vitro	cF_1T	cAPC	42,577	5,995	
	cF_1T	uAPC	9,156	637	
	cF_1T		3,855		
	cF_1T				880
	uF_1T	uAPC	25,076	4,431	
	uF_1T	cAPC	4,479	1,438	
	uF_1T		5,911		
	uF_1T				330
L-DC from rats challenged with OVA intraintestinally	cF_1T	cAPC	15,941	2,360	
	cF_1T	uAPC	1,412	214	
	cF_1T		600		
	cF_1T				630
	uF_1T	uAPC	16,388	1,848	
	uF_1T	cAPC	1,743	263	
	uF_1T		1,140		
	uF_1T				330

L-DC from either PVG-RT1^c or PVG-RT1^u rats were cultured with OVA (1 mg/ml) for 3 h, washed, and injected (2×10^4/leg) into the footpads of naive F_1 rats (PVG-RT1^c × PVG-RT1^u). L-DC from rats that had been injected with OVA (10 mg/ml) intraintestinally were injected into the footpads of naive F_1 rats (PVG-RT1^c × PVG-RT1^u). 10 d later, T cells were enriched from popliteal lymph node cells of those L-DC-injected rats, as described in Materials and Methods, and cultured with OVA or HRP in the presence of irradiated spleen cells from PVG-RT1^c or PVG-RT1^u rats for ~120 h. [^{3}H]TdR was added to the cultures 16 h before harvesting. The results are expressed as gross cpm. Concentration of Ag in culture is 50 μg/ml. Number of spleen cells as APC is 10^5/well; number of F_1 T cells is 10^5/well. cF_1T: T cells enriched from F_1 rats that had been injected with Ag-pulsed L-DC from PVG-RT1^c; uF_1T, T cells enriched from F_1 rats that had been injected with Ag-pulsed L-DC from PVG-RT1^u; cAPC, spleen cells from PVG-RT1^c rats; uAPC, spleen cells from PVG-RT1^u rats.

understanding of these processes is very limited. The rational design of vaccines, however, depends on a full comprehension of in vivo antigen handling. What is known of the functions of DC suggests that they may be the major APC involved in the initiation of primary in vivo responses, and there is considerable evidence that DC can acquire antigen in peripheral tissues and transport it for presentation to T cells in secondary lymphoid tissue (10–13). There is, however, little information on antigen handling at mucosal surfaces, despite these being the major sites for pathogen entry.

In this paper we examine the function of DC in the small intestine in the acquisition of antigens present in the intestinal lumen. We have previously shown that DC in the intestinal wall acquire soluble protein antigens injected directly into the intestinal lumen, and within a few hours, migrate into peripheral lymph, carrying antigen in a form that can be presented to sensitized T cells in an MHC class II–restricted, CD4-dependent manner (15). In this paper we extend these results to examine the handling of orally administered antigen and the ability of antigen-bearing APC to initiate primary responses in vivo.

Several different cell types expressing MHC class II can present antigens to activated T cells (3, 4, 9), but virgin or resting rodent T cells are far more responsive to antigens presented by DC than by other APC for both allogeneic and syngeneic conventional responses in vitro (11, 24, 25). It is, however, important to show that antigen-bearing APC can also stimulate primary responses in vivo. Several groups have shown that after the injection of antigen-bearing APC, the recipient animals become sensitized (11), but it is not possible to exclude presentation of antigen by host APC in these experiments. Direct presentation has, however, been shown by Inaba et al. (26), who found that after the injection of DC, either pulsed in vitro or in vivo, from one parental strain into F_1 recipients, T cells from these recipients gave a sensitized response only to antigen presented on APC from the strain from which the injected DC were obtained. We have similarly shown that after injection of F_1 rats with parental strain L-DC, either pulsed in vitro or from animals injected intraintestinally with antigen, the sensitization of T cells from the draining nodes is restricted to the MHC of the injected L-DC. We have also shown that only live DC are able to prime naive animals, and that antigen-pulsed B cells or peritoneal macrophages are completely inert in priming. Thus, it is clear that after oral or intraintestinal administration, DC in the wall of the intestine acquire antigen and are able to prime naive T cells in lymph nodes in vivo. Surprisingly small numbers of DC are required to initiate detectable responses

Table 4. *Serum Antibody Synthesis after Immunization with Antigen-bearing L-DC*

Priming	Primary		Secondary	
	IgG	IgM	IgG	IgM
Oral L-DC (OVA)	-	-	1/25	-
Intestinal L-DC (OVA)	-	-	1/25	-
Vitro L-DC (OVA)	-	-	1/25	-
Oral L-DC (HRP)	-	-	1/5	-
Intestinal L-DC (HRP)	-	-	1/25	-
Vitro L-DC (HRP)	-	-	1/25	-
Vitro-pulsed BC (OVA)	-	-	ND	ND
Vitro-pulsed Mϕ (OVA)	-	ND	ND	-
Soluble OVA	-	-	-	-
Soluble HRP	-	-	-	-
OVA-CFA	1/3,125	1/625	1/15,625	1/625
HRP-CFA	1/625	ND	1/15,625	ND

Naive PVG rats (three to four rats in a group) were immunized with cells from oral or intraintestinally antigen-challenged animals, cells pulsed in vitro with antigens, or antigens emulsified in CFA. 10 d after immunization, rats were boosted with soluble antigens subcutaneously. Sera were obtained from rats 10 d after immunization and 10 d after a boosting injection. Negative serum was from normal PVG rats. Serum anti-OVA or HRP antibody isotypes were measured by ELISA as described in Materials and Methods. Positive titers were defined as those whose OD value was higher than the OD of the negative control plus 2 SD. Oral L-DC, L-DC from rats that had been challenged with antigen (OVA or HRP; 10 mg) via intragastric tube. Intestinal L-DC, L-DC from rats that had been challenged with OVA or HRP intraintestinally (10 mg). Vitro pulsed, cells pulsed with OVA or HRP in vitro 1 mg/ml); -CFA, Antigen in PBS emulsified with an equal volume of CFA.

in vivo, the injection of as few as 500 in vitro antigen-pulsed DC is sufficient to give significant sensitization. Given that only a small percentage of injected DC are likely to migrate to the node (27), the process of sensitization is remarkably efficient. In vivo priming with antigen-pulsed DC is a more sensitive assay for antigen than the in vitro stimulation of proliferation in sensitized T cells in that after the oral administration of 1 mg of OVA, L-DC collected over the next 24 h are able to sensitize naive rats but do not stimulate significant proliferation of sensitized T cells.

The site at which L-DC acquire antigen from the gut lumen is not known. M cells in Peyer's patches are specialized for antigen transport (28), but proteins can also cross normal epithelia. DC are present in Peyer's patches (29) and lamina propria (6), and L-DC could enter lymph from either or both sites. Endocytosis of antigen by DC can be shown after culture in vitro for long periods with relatively high concentrations of fluorescent antigen (26), but in vivo DC are probably exposed to relatively low concentrations for short periods, explaining our inability to detect antigen visually in DC in lamina propria, Peyer's patches, or lymph.

In many models, orally administered antigen leads to the development of specific systemic tolerance. The mechanisms hypothesized to underly oral tolerance include the generation of suppressor T cells (30) and circulating immune complexes (31), while a recent report has suggested the involvement of APC expressing I-J determinants (8). Others have suggested that presentation of enteric antigens by MHC class II–expressing epithelial cells may selectively generate suppressor cells (7). Why orally administered antigen should have these effects is not known, but Michael (32) has shown that whereas giving BSA orally induces tolerance in mice, intraintestinal injection leads to sensitization, but that peptides derived from the antigen after peptic digestion are tolerogenic on intraintestinal injection. Our results show that orally administered antigen is acquired by L-DC in a manner very similar to antigen injected intraintestinally. At all doses tested, down to 1 mg, L-DC collected after antigen administration by both routes are able to prime T cells in naive recipients although responses after oral administration are somewhat smaller. The antigen acquired by L-DC must be absorbed in the small intestine in both cases, as the lymph nodes draining the stomach are not removed during lymphadenectomy. Thus, orally administered antigens are made available to the immune system in a form able to stimulate virgin $CD4^+$ T cells, and any explanation of oral tolerance must take account of the presence of these highly immunostimulatory APC in mesenteric nodes.

We have shown that footpad injection of antigen-pulsed L-DC does not induce detectable levels of circulating antibody and that antigen-pulsed macrophages and B cells are similarly ineffective in stimulating primary antibody synthesis. However, when L-DC-primed rats are boosted with soluble antigens, weak secondary antibody responses can be detected. It is well recognized that small doses of antigen tend to stimulate cell-mediated responses selectively (33) and the L-DC that induce T cell sensitization do not carry directly detectable amounts of antigen. It is also likely that the bulk of the antigen acquired by L-DC is processed and expressed in the form of peptides and is therefore unable to stimulate an antibody response against whole native antigen. That weak responses are observed after challenge with soluble antigen may reflect the ability of $CD4^+$ T cells, sensitized in the primary response, to give increased levels of help to B cells.

Recently, Sornasse et al. (34) have shown that antigen-pulsed spleen DC can induce a primary antibody response in vivo. Although at first sight this seems in conflict with our results, they detected antibody only after boosting with the same soluble antigen 5 d later. The antibodies they measured were from sera taken 7 d after boosting. Thus, the antibody they measured may well reflect a secondary response.

We conclude that orally administered antigen is acquired efficiently in an immunostimulatory form by intestinal DC and that these early stages of the initiation of a primary immune response do not differ significantly from those that follow parenteral immunization.

We are extremely grateful for the excellent technical assistance of Chris Jenkins and ManHua Zhang. We thank the MRC Cellular Immunology Unit of the Sir William Dunn School of Pathology for their generous support both in mAbs and facilities.

This work was supported by a Sino-British TC Scholarship, the E. P. Abraham research fund, and the British Arthritis and Rheumatism Council.

Address correspondence to G. G. MacPherson, Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, UK.

Received for publication 15 December 1992 and in revised form 28 January 1993.

References

1. Challacombe, S.J., and T.B. Tomasi, Jr. 1980. Systemic tolerance and secretory immunity after oral immunization. *J. Exp. Med.* 152:1459.
2. MacDonald, T.T., and P.B. Carter. 1982. Isolation and functional characteristics of adherent phagocytic cells from mouse Peyer's patches. *Immunology.* 45:769.
3. Kammer, G.M., and E.R. Unanue. 1980. Accessory cell requirement in the proliferative response of T lymphocytes to hemocyanin. *Clin. Immunol. Immunopathol.* 15:434.
4. Bland, P.W., and L.G. Warren. 1986. Antigen presentation by epithelial cells of the rat small intestine. II. Selective induction of suppressor T cells. *Immunology.* 58:9.
5. Spalding, D.M., W.J. Koopman, J.H. Eldridge, J.R. McGhee, and R.M. Steinman. 1983. Accessory cells in murine Peyer's patch. I. Identification and enrichment of a functional dendritic cell. *J. Exp. Med.* 157:1646.
6. Pavli, P., C.E. Woodhams, W.F. Doe, and D.A. Hume. 1990. Isolation and characterization of antigen-presenting dendritic cells from the mouse intestinal lamina propria. *Immunology.* 70:40.
7. Bland, P.W., and L.G. Warren. 1986. Antigen presentation by epithelial cells of the rat small intestine. I. Kinetics, antigen specificity and blocking by anti-Ia antisera. *Immunology.* 58:1.
8. Mowat, A.M., A.G. Lamont, and D.M. Parrott. 1988. Suppressor T cells, antigen presenting cells and the role of I-J restriction in oral tolerance to ovalbumin. *Immunology.* 61:141.
9. Metlay, J.P., E. Pure, and R.M. Steinman. 1989. Control of the immune response at the level of antigen presenting cells: a comparison of the function of dendritic cells and B lymphocytes. *Adv. Immunol.* 47:45.
10. Mayrhofer, G., P.G. Holt, and J.M. Papadimitriou. 1986. Functional characteristics of the veiled cells in afferent lymph from the rat intestine. *Immunology.* 58:379.
11. Macatonia, S.E., S.C. Knight, A.J. Edwards, S. Griffiths, and P. Fryer. 1987. Localisation of antigen on lymph node dendritic cells after exposure to the contact sensitizer fluorescein isothiocyanate. Functional and morphological studies. *J. Exp. Med.* 166:1654.
12. Bujdoso, R., J. Hopkins, B.M. Dutia, P. Young, and I. McConnell. 1989. Characterization of sheep afferent lymph dendritic cells and their role in antigen carriage. *J. Exp. Med.* 170:1285.
13. Crowley, M., K. Inaba, and R.M. Steinman. 1990. Dendritic cells are the principal cell in mouse spleen bearing immunogenic fragments of foreign proteins. *J. Exp. Med.* 172:383.
14. Pugh, C.W., G.G. MacPherson, and H.W. Steer. 1983. Characterization of nonlymphoid cells derived from rat peripheral lymph. *J. Exp. Med.* 157:1758.
15. Liu, L.M., and G.G. MacPherson. 1991. Lymph-borne (veiled) dendritic cells can acquire and present intestinally administered antigens. *Immunology.* 73:281.
16. McMaster, W.R., and A.F. Williams. 1979. Monoclonal antibodies to Ia antigens from rat thymus: cross reaction with mouse and human and use in purification of rat Ia glycoproteins. *Immunol. Rev.* 47:117.
17. Hunt, S.V., and M.H. Fowler. 1981. A repopulation assay for B and T lymphocyte stem cells employing radiation chimeras. *Cell Tissue Kinet.* 14:445.
18. Dallman, M.J., M.L. Thomas, and J.R. Green. 1984. MRC OX19: a monoclonal antibody that labels rat T lymphocytes and augments in vitro proliferative responses. *Eur. J. Immunol.* 14:260.
19. Robinson, A.P., M. Puklavec, and D.W. Mason. 1986a. MRC OX52: a rat T cell antigen. *Immunology.* 57:527.
20. Jefferies, W.A., J.R. Green, and A.F. Williams. 1985. Authentic T helper CD4 (W3/25) antigen on rat peritoneal macrophages. *J. Exp. Med.* 161:117.
21. Brenan, M., and M. Puklavec. 1992. The MRC OX-62 antigen: a useful marker in the purification of rat veiled cells with the biochemical properties of an integrin. *J. Exp. Med.* 175:1457.
22. Brideau, R.J., P.B. Carter, W.R. McMaster, and A.F. Williams. 1980. Two subsets of rat T lymphocytes defined with monoclonal antibodies. *Eur. J. Immunol.* 10:609.
23. Hsiung, L.M., A.N. Barclay, M.R. Brandon, E. Sim, and R. Porter. 1982. Purification of human C3b inactivator by monoclonal antibody affinity. *Biochem. J.* 203:293.
24. Inaba, K., and R.M. Steinman. 1984. Resting and sensitized T lymphocytes exhibit distinct stimulatory (antigen-presenting cell) requirements for growth and lymphokine release. *J. Exp. Med.* 160:1717.
25. Croft, M., D.D. Duncan, and S. Swain. 1992. Response of naive antigen-specific $CD4^+$ T cells in vitro: characteristics and antigen-presenting cell requirements. *J. Exp. Med.* 176: 1431.
26. Inaba, K., J.P. Metlay, M.T. Crowley, and R.M. Steinman. 1990. Dendritic cells pulsed with protein antigens in vitro can prime antigen-specific, MHC restricted T cells in situ. *J. Exp. Med.* 172:631.
27. Fossum, S. 1988. Lymph-borne dendritic leucocytes do not recirculate, but enter the lymph node paracortex to become interdigitating cells. *Scand. J. Immunol.* 27:97.
28. Owen, R.L., N.F. Pierce, R.T. Apple, and W.C. Cray, Jr. 1986. M cell transport of *Vibro cholerae* from the intestinal lumen

into Peyer's patches: a mechanism for antigen sampling and for microbial transepithelial migration. *J. Infect. Dis.* 153:1108.
29. Wilders, M.M., H.A. Drexhage, E.F. Weltervreden, H. Mullink, A. Duijvestijn, and S.G.M. Meuwissen. 1983. Large mononuclear Ia-positive veiled cells in Peyer's patches. Isolation and characterization in rat, guinea pig and pig. *Immunology.* 48:453.
30. Asherson, G.L., M. Zembala, M.A. Perera, B. Mayhew, and W.R. Thomas. 1977. Protection of immunity and unresponsiveness in the mouse by feeding contact sensitizing agents and the role of suppressor cells in the Peyer's patches, mesenteric lymph nodes and other lymphoid tissues. *Cell. Immunol.* 33:145.
31. Kagnoff, M.F. 1987. Antigen handling by intestinal mucosa: humoral and cell-mediated immunity, tolerance, and genetic control of local immune responses. *In* Immunopathology of the Small Intestine. M.N. Marsh, editor. John Wiley & Sons, Inc., New York. 74–102.
32. Michael, J.G. 1989. The role of digestive enzymes in orally induced immune tolerance. *Immunol. Invest.* 18:1049.
33. Lamont, A.G., A.M. Mowat, and D.M. Parrott. 1989. Priming of systemic and local delayed-type hypersensitivity response by feeding low doses of ovalbumine to mice. *Immunology.* 66:595.
34. Sornasse, T., V. Flamand, G.D. Becker, H. Bazin, F. Tielemans, K. Thielemans, J. Urbain, O. Leo, and M. Moser. 1992. Antigen-pulsed dendritic cells can efficiently induce an antibody response in vivo. *J. Exp. Med.* 175:15.