

EFFECT OF IMMUNOLOGICAL ADJUVANTS ON THE APPEARANCE OF MONOCYTE AND
DENDRITIC CELL PRECURSORS IN RAT THORACIC DUCT LYMPH

Arthur O. Anderson and Jonathan T. Warren

Departments of Pathology and Biology, G7
The University of Pennsylvania, Philadelphia, PA 19104

Successful induction of immune responses to thymic-dependent antigens such as sheep erythrocytes are absolutely dependent upon histocompatible, nonlymphoid antigen-presenting cells (1-3). Although thoracic duct lymph (TDL) exhibits less in vitro stimulatory activity than whole spleen cells (4), various in vitro and in vivo immune phenomena attributed to TDL suggest that antigen-presenting cells or their precursors must be present in normal TDL (5-7). This report describes adherent cell types in TDL of normal (8) and adjuvant-treated Lewis rats. The results indicate that macrophages and nonphagocytic dendritic cells differentiate in culture from small cells in TDL. These precursors pass through nylon wool and G-10 columns. Both cell types bear Ia-antigens but differ from each other with respect to complement and Fc receptor expression. Treatment with either complete Freund's adjuvant (CFA) or a new lipoidal amine adjuvant CP-20,961 (Pfizer) (9,10) increases the TDL output of both cells. One of the mechanisms of adjuvant action might be production and dissemination of precursors of antigen-presenting cells that complete differentiation in peripheral lymphoid tissues and provide continued antigenic stimulation for lodging daughter cells (11-13).

MATERIALS AND METHODS

Adult male Lewis rats were used for collection of thoracic duct lymphoid cells via Bollman fistulae. Smears were made of TDL taken directly from cannulae and of fresh TDL recovered from nylon wool or G-10 columns. The remaining cells were cultured as monolayers of 3×10^6 cells in T-25 flasks containing Earle's MEM with NEAA, 2 mM L-glutamine, 10% FCS, Pen/Strep, and Mycostatin.

Each flask was examined by phase microscopy at daily intervals and all nonadherent cells were removed after 96 hr of culture. Phagocytic activity of the adherent cells was assessed using colloidal gold, carbon and zymosan. Surface markers were labeled by antibodies or complexes sandwiched to killed Staphylococci (8). The antibodies were: 1. #H-76 hybridoma anti-rat Ia(14); 2. Rabbit anti-rat IgG (heavy chain); 3. Rabbit anti-rat IgG immune complexes for Fc receptors; 4. Rabbit anti-rat Thy-1; 5. E. coli incubated in rabbit plasma for C3b receptors. Adherent cells were fixed, washed, labeled, air dried and Giemsa stained for microscopy. CFA and CP-20,961 in soybean oil lipid emulsion were prepared as described previously (10) and volumes of 0.3 ml were injected into hind footpads and thighs. Bollman fistulae were established sequentially and collected TDL was passed through nylon wool prior to culturing. Representative lymphatic tissues were excised for electron microscopic evaluation of FDC and IDC.

RESULTS

Fresh unfiltered TDL from normal Lewis rats contained 0.1-0.3% mononuclear phagocytes (M); none of these had elongated processes. Nylon wool and G-10 filtration effectively removed fully differentiated M from TDL. There were only 0-0.001% M in the fresh sample. However, after 24-48 hr in culture, the same specimens contained 0.8-1.0% macrophages. After 4 days in culture additional cells, comprising 0.03-0.06% of the initial inoculum were found that exhibited multiple elongated dendrite-like processes and round eccentrically placed cell bodies. The cytoplasm of these stellate cells contained phase-dense mitochondria but no phagolysosomes (Fig. 1). These "dendritic cells" (DC) did not ingest carbon or zymosan neither did they produce phagokinetic halos with colloidal gold. Surrounding M exhibited active motility and phagocytosis. Monocytes often made transient contact with DC. The capacity of DC to bind small lymphocytes was about 100 times that of M because of the large surface area of the extended processes (Fig. 1, insert). Growth curves and culture characteristics of M and DC are listed in Table 1.

Macrophages that had ingested zymosan particles prior to fixation and surface labeling displayed Fc receptors and C3b receptors; about 78% were also positive for Ia-antigen. Dendritic cells expressed Ia-antigen along distal portions of their cytoplasmic processes (Fig. 2). Reagents for Fc receptor, surface Ig, or Thy-1 antigen generally did not label DC and less than 10% of DC from normal rat TDL were decorated with C3b-coated E. coli (Fig. 3). Thy-1 positive lymphocytes associated with macrophages and C3b receptor negative DC, while Ig positive lymphocytes bound to DC bearing C3bR.

Inoculation of DFA resulted in a dramatic change in the number and character of DC and M precursors in TDL. CP-20,961 produced similar effects but they were of slightly less magnitude and were not associated with swelling and induration of the legs. Both adjuvants increased the output of thoracic duct lymphocytes in addition to increasing the proportion of lymphoblasts in TDL from 1.3% to 12% by the seventh day (Table 2). Precursors of M and DC rose to 5 and 150 fold baseline levels in TDL from CFA-treated rats by the fourteenth day. Output of M and DC precursors following treatment with CP-20,961 increased but a large incremental increase in DC was not seen.

Cell surface markers for M and DC did not change dramatically following adjuvant treatment. About 20% compared to 6% of the macrophages exhibited heavy labeling for Ia. Many of these phagocytes were larger and resembled activated M. Eighty percent of DC were Ia-antigen positive during peak output 14 days after adjuvant treatment. Surprisingly, the proportion of DC with C3b receptors rose to 59%. None of these cells were induced to phagocytose or display Fc receptors which differentiated them from M.

Transmission EM studies of selected adjuvant-draining lymph nodes revealed increased numbers of DC associated with M and dividing cells in the deep cortex. Both CFA and CP-20,961 produced similar cellular changes in lymph nodes, with the exception that mineral oil in CFA caused inflammation and scarring that was not found for CP-20,961. There were 2-3 DC per twenty grid spaces (100 spaces/mm²) in untreated control nodes, while 5-7 DC were found in comparable regions in nodes draining either adjuvant. Morphological changes associated with increased lymphocyte traffic were reported elsewhere (10). M and DC adhered to each other via long stretches of membrane (Fig. 4) and the extended processes of each DC made contact with more than 200 individual lymphoid cells. Although DC are deficient in phagolysosomes, rough endoplasmic reticulum and cytoskeletal structures needed for active phagocytosis, their prominent Golgi apparatus, mitochondria and tubulovesicular structures make DC ideally suited for glycosylation or phosphorylation of relevant moieties for surface membrane expression.

DISCUSSION

Our knowledge of the cellular basis of acquired immunity advanced rapidly following development of *in vitro* systems used to dissect the requirements for immune induction. The pivotal role of the "adherent mononuclear cell" in this response was appreciated relatively recently (1-3). Indeed, the mononuclear cell surface controls both antigen presentation and genetic restriction of helper cell interactions through phenotypic expression of Ia-

Table 1. Kinetics of TDL Adherent Cell Development In Vitro

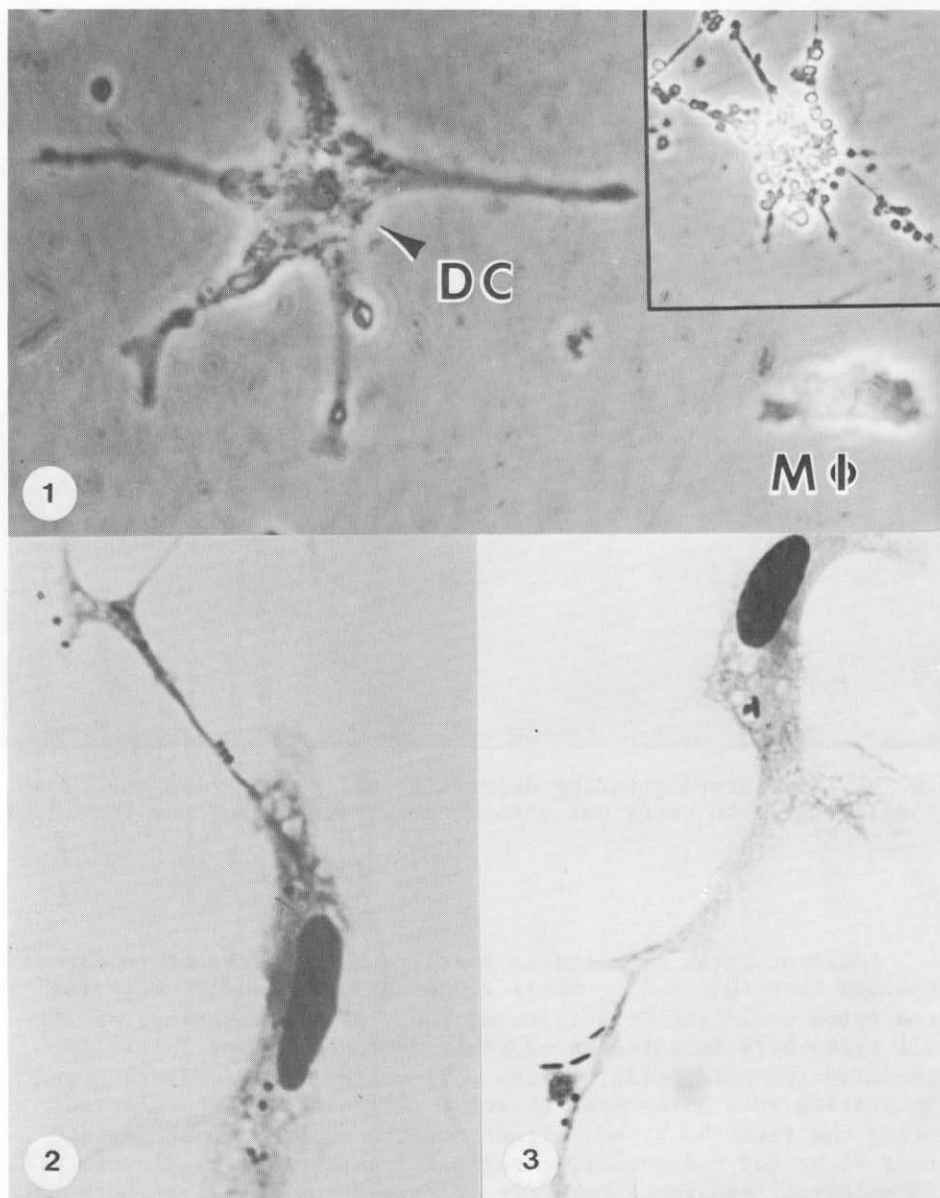
Cell Type	Number of Cells/10 ⁶ TDL								
	100	5640	8050	9800	10960	9910	9500	10820	
Macrophages	0	0	0	28	190	492	533	512	
Dendritic cells	0	0	0	0	0	20	420	2300	
Fibroblastic RC	0	1	2	3	4	7	14	21	
Culture days									

Table 2. Macrophage and Dendritic Cell Output in TDL After Adjuvants

Days	Percent of Thoracic Duct Lymphoid Cells					
	Complete Freund's			CP-20,961 (Pfizer)		
	%Mac	%DC	%LB	%Mac	%DC	%LB
0	0.84	0.04	1.3	0.80	0.03	1.3
3	0.07	0.00	6.5	1.34	0.39	3.1
7	3.19	2.82	11.9	2.54	1.85	4.9
14	4.74	6.04	7.2	2.83	2.54	2.2
21	3.17	4.01	1.2	1.15	1.35	2.0
29	2.89	2.28	1.2	0.79	1.52	1.4

The standard errors do not exceed 11% of any value (N=4/point).

antigens (20). We still do not know the precise identity of the cell or cells that carry out this function in vivo. Despite morphological heterogeneity of adherent cells types, the antigen-presenting cell in vitro has been assumed to be a monocyte or macrophage because of the prevalence of these phagocytic cells in spleen cell cultures. Interest in characterizing the structural and functional attributes of these cells in thoracic duct lymph was stimulated by various studies that suggested that TDL could provide antigen-presenting capacity to lethally-irradiated recipients (5-7) and studies of antigen-laden cells in TDL that overrode suppression in adoptive recipients and yielded a positive immune response despite presence in multiplicities of less than 1 in 5000 TDL (7).



FIGS. 1-3. Macrophage (M Φ) and dendritic cells (DC) from TDL precursors. 1. Phase dense mitochondria in cytoplasm of DC; insert, numerous TDL bind to DC and its processes. 2. Staph-A (cocci) binding to specific antibody marked sites of Ia-antigen. 3. Few DC from normal TDL label for both Ia (cocci) and C3b receptors (rods).

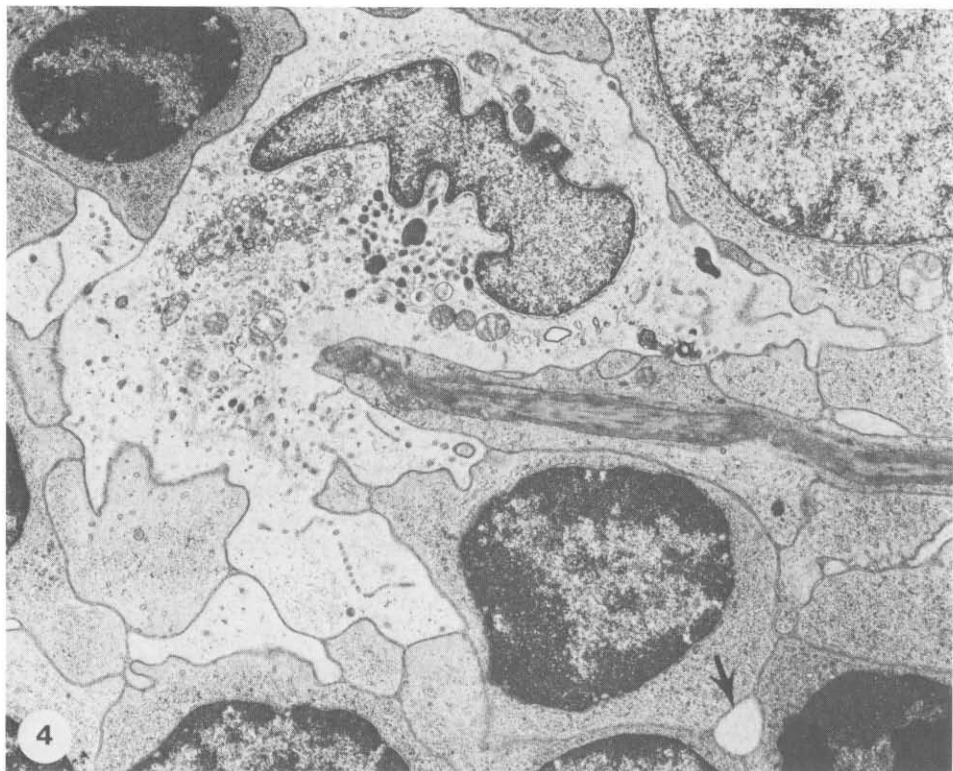


FIG. 4. The interdigitating dendritic cell personifies the type of cell likely to carry out antigen-presenting functions for T-cells.

Adherent cells of thoracic duct lymph were present in concentrations less than 1.0%. Small precursors of M and DC were isolated from nylon wool- and G-10 filtered TDL. High proportions of both cell types bore Ia-antigens on their surfaces. They initially resembled lymphoid cells, a form well-suited for circulating and immigrating into peripheral tissues. Differentiation occurred during the first 48 hr of culture for the small macrophages and after 96 hr for the dendritic cells. Treatment of the donors with immunological adjuvants markedly increased the output of both cell types in TDL. This adherent cell increase was highly reproducible and suggested that one of the many important effects of adjuvants on the immune system may be to stimulate production, traffic, or mobilization of Ia-antigen positive accessory cells. Similar effects of adjuvants on expression of Ia-antigens by peritoneal exudate macrophages (PEM) have recently been reported by Beller et al. (19).

Early cytological studies of TDL failed to convincingly demonstrate significant numbers of mononuclear phagocytes or related nonlymphoid cells (15-18). Indeed, it was proposed that the phagocytes that could be demonstrated in TDL probably were derived from the peritoneum (18). Close scrutiny of Roser's data suggested an alternative interpretation. If PEM could contaminate TDL directly, the PEM that were labeled in vitro with gold and the resident macrophages in the peritoneum that endocytosed the free colloidal gold injected i.p. should both have access to the thoracic duct. The results did not show this. Since the predominant route of cellular egress from the peritoneum is via transdiaphragmatic lymphatics which ultimately flow into the blood, the delayed appearance (3-6 days) of labeled cells in TDL after i.p. instillation suggested that the in vitro treatment may have selected for a phagocytic cell with a capacity to recirculate. The small phagocytic cell in Roser's study is very similar to the Ia-antigen positive M precursor described here and previously (8).

The DC we found in TDL bore many morphological, functional and surface receptor similarities to the splenic DC described by Steinman and associates (21-24). Steinman's DC behaved like the interdigitating DC first described by Veldman in this Ph.D. thesis in that it interacted predominantly with T-cells. These unique cells were nonphagocytic. Fc and C3b receptor (-) and Ia-antigen positive. Macrophages depleted of DC appear to be incapable of antigen presentation, and purified DC are 100 times as potent stimulators of murine mixed lymphocyte reactions and priming for cellular cytotoxicity as other leukocytes. It is not likely that the DC described in this paper represent Langerhans cells or marginal zone cells since both these cell types have well characterized Fc receptors. However, the DC in TDL that expresses C3b receptors may be a precursor of the follicular DC (13) since it preferentially interacts with B cells in culture; and, attempts to stimulate cellular cytotoxicity reactions with these cells have been unsuccessful (Anderson and Peters, unpublished data). This dichotomy of DC types suggested that these cells play a role in organizing lymphatic tissue microenvironments into T- or B-dependent areas. Selective depletion of one of the other cell type may be used to test this hypothesis. Failure to develop B-cell memory in complement depleted animals (12) may reflect a C3br(+)DC deficiency.

We should not discard the M as having a principal role in antigen acquisition and presentation because of the in vitro findings concerning DCs. In vivo studies that traced the distribution of labeled antigens clearly showed that the earliest sites of antigen localization appeared to be the lymphatic sinusoidal M. This type of antigen localization occurs long before antigen can be found on cells with dendritic morphology. Are sinus M removing

excess free antigen to prevent tolerance induction or are they involved in important "antigen-processing" functions? Since DC present antigens effectively but are not phagocytic, and M phagocytose antigens but are not efficient antigen-presenters, it is possible that processed antigen is somehow transferred from M to DC where it may be incorporated into the extensively ramifying membranes of the DC. Such an hypothesis has recently been presented by Cowing et al. (1), Anderson et al. (8), and Sprent (25).

Immunological adjuvants produce local, regional, and systemic effects that involve microvasculature, leukocyte migration and cell proliferation (10). A local inoculation of an adjuvant causes a significant increase in the influx and efflux of lymphoid cells passing through regional lymph nodes; if some of this traffic includes antigen-presenting cell precursors, it would be easy to see how quantitatively more cooperative cell interactions may take place. Dissemination of lymphoblastic daughter cells and accessory cells containing antigen to distant lymphatic tissues would provide most of the essential components of satellite germinal follicles or T-reactive nodules. In an extreme case (where local antigen may persist) establishment of antigen-presenting cells in tissue lying outside the lymphatic system may lead to autoimmune inflammation.

ACKNOWLEDGEMENTS

We are grateful to David L. Gasser and Ivan Otterness for providing generous supplies of H-76 hybridoma Ia-antisera and CP-20,961, respectively. This work was supported in part by BRSG #5S07RRO5415-20 and a grant from Pfizer Central Research Division.

REFERENCES

1. A.S. Rosenthal, and E.M. Shevach. *J. Exp. Med.* 138:1194 (1973).
2. C. Cowing, S.H. Pincus, D.K. Sachs, and H.B. Dickler. *J. Immunol* 121:1680 (1978).
3. A. Singer, C. Cowing, H.S. Hathcock, H.B. Dickler, and R.J. Hodes. *J. Exp. Med.* 147:1611 (1978).
4. J. Sprent, and J.F.A.P. Miller. *Cell Immunol.* 3:361, 1972.
5. D.D. McGregor, P.J. McCullagh, and J.L. Gowans. *Proc. R. Soc. Lond. B. Biol. Sci.* 168:299 (1967).
6. D.A. Rowley, J.L. Gowans, R.C. Atkins, W.L. Ford, and M.E. Smith. *J. Exp. Med.* 136:499 (1972).
7. E.B. Bell. *Immunol.* 38:797 (1979).
8. A.O. Anderson, J.T. Warren, and D.L. Gasser. *Transplant. Proc.* (1981) in press.