# Presence of Lymphoid Dendritic Cells in Thoracic Duct Lymph From Lewis Rats

A. O. Anderson, J. T. Warren, and D. L. Gasser

**V**ARIOUS forms of adherent cells bearing dendritic processes[1-4] have recently been described within lymphatic tissues and in cell cultures derived from them.[5-7] By implication, these cells have been proposed as possible antigen-presenting cells. While it is clear that monocytes or macrophages predominate among the adherent cells in cultures used to test antigen presentation,[8-10] it is also likely that dendritic cells (DC) might be responsible for triggering the immune response. If these peculiar nonphagocytic adherent cells are as potent presenters of exogenous antigens as they are stimulators of primary allogeneic mixed leukocyte reactions (MLR),[11] then these Ia antigen positive, Fc receptor (FcR) negative, non-T non-B cells might actually be true antigen-presenting cells of in vivo and in vitro immune responses.[9,12,13] Their ability to induce lymphocyte proliferation in syngeneic MLR suggested this possibility but if an exogenous antigen had been presented its identity was not known.[13] Induction of immunity to sheep red blood cells (SRBC) is highly dependent on the presence of "adherent" cell populations.[14] Howard was able to induce reactivity to SRBC in adoptive recipients using unadulterated thoracic duct lymph (TDL), and recently E. B. Bell described an antigen-laden cell present in low multiplicity in TDL that produced a secondary response to human serum albumin in adoptive recipients despite

simultaneous presence of large numbers of suppressor cells.[15] These observations suggested that some form of antigen-presenting cell might be found in TDL that is capable of entering the circulation and seeding the spleen and other tissues.

The present study documents the existence of small monocytes and Ia$^+$, FcR$^-$, dendritic cell precursors among the nylon wool nonadherent lymphoid cells in thoracic duct lymph from normal Lewis rats maintained under conventional laboratory conditions. DC show a greater than 100-fold increase in frequency following immunization with fluorescein isothyocyanate tagged bovine serum albumin (FITC-BSA) emulsified in complete Freund's adjuvant, while monocyte precursors increased 7-fold over normal. Normal DC were predominantly C3b receptor negative, but "immune" DC included 60%–80% C3b receptor positive cells. The possible implications of DC in TDL are discussed within the context of antigen-presenting cells associated with lymphatic tissues.

## MATERIALS AND METHODS

Adult male LEW rats (Microbiological Associates, Walkersville, Md.) were used for collection of thoracic duct lymphoid (TDL) cells via surgically placed Bollman fistulae.[16] TDL were collected for 24 hr in RPMl 1640 containing 10 U/ml of preservative-free heparin maintained at 4°C. TDL was depleted of lymphoblasts, B cells, and other adherent cells by brief incubation in nylon wool columns according to the method of Julius et al.[17] Effluent TDL were suspended in RPMl 1640 containing Pen/Strep and 5%–10% fetal calf serum (FCS, Grand Island Biological Co., Grand Island, N.Y.), and incubated in T-25 flasks (Falcon) at densities of 3–7 × 10$^6$ cells per flask. Each flask was examined by phase microscopy at daily intervals. After 3 days incubation, nonadherent lymphoid cells were removed, and the remaining cells were returned to the incubator in fresh media. Cells were characterized and counted through 25 randomly selected 1 sq mm fields, and the numbers calculated to represent percent of the 3 × 10$^6$ TDL added.

Morphology, motility, and capacity for phagocytosis of

carbon or zymosan particles were used to characterize living cells. Selected cultures were fixed in 2% phosphate-buffered glutaraldehyde, pH 7.2, for 1 hr at 37°C, washed, and treated overnight with $10^{-3}M$ glycine to prevent nonspecific protein binding to free aldehyde groups.

Surface markers were labeled with antibody or complement[18] linked to bacteria in a binding assay, which also yielded good morphology. Protein-A bearing staphylococci were used to localize antibodies bound to cell surface antigens. Surface immunoglobulin (sIg) was labeled by 7s rabbit anti-rat IgG (heavy chain specific, Miles Yeda); Fc receptors bound 7s rabbit anti-rat IgG/rat IgG immune complexes prepared at equivalence; and Ia antigens were labeled using an IgM rat–mouse hybridoma antibody H-76 characterized elsewhere.[19] This IgM anti-Ia reagent bound protein-A Staph at 3–7 cocci/Ia$^+$ cell. Addition of a rabbit anti-rat IgM sandwich increased the number of cocci labeling each cell to 18–25 but did not result in additional labeled DC. Killed *E. coli.* incubated for 15 min in fresh rabbit plasma were used to label C3b receptors.

The adherent cell monolayers were extensively washed with distilled water, stained with Wright's Giemsa, and air dried. The tops of each T-flask were cut off using a hot knife and coverslips were mounted on the cell preparations using immersion oil.

Twenty rats were inoculated in the footpads and legs with 0.2 ml of a 1:1 emulsion containing Freund's complete adjuvant and FITC-BSA. Prior to emulsifying, free fluorescein had been removed by passage over a Sephadex G-25 column. Bollman fistulae were established 1, 3, 7, 14, 21, and 29 days after inoculation, and the adherent cells in TDL were characterized as described above. In addition, glutaraldehyde-fixed unstained preparations were examined by fluorescence microscopy to detect bound or internalized FITC-BSA. Representative popliteal lymph nodes were excised, fixed, and prepared for transmission electron microscopy by

previously reported methods,[20] and the tissues were examined for possible lymphocyte DC interaction.

## RESULTS

Fresh TDL from normal LEW rats contained less than 0.1% monocytes, and none of these cells had dendritic processes. After 18–48 hr in culture 25,093 ± 958 in $3 \times 10^6$ TDL cells were clearly identifiable as motile monocytes or macrophages (Table 1). This eightfold increase in proportion of monocytes to 0.84% could have resulted from maturation of small monocytes that resembled lymphocytes initially. A few cells exhibiting multiple dendritic processes were found at this time, but there were not enough DC to count until 72–96 hr. After 4 days in culture, each flask contained 1130 ± 53 morphologically recognizable dendritic cells or 0.04% of the TDL inoculatum (Fig. 1). The cytoplasm of these stellate cells contained phase dense mitochondria but no discernible phagolysosomes. DC had small irregular nuclei that were smaller than those of monocytes and had heterochromatin markings similar to those of lymphocyte nuclei. These DC did not ingest carbon or zymosan particles as did the surrounding monocytes and macrophages. Monocytes made transient contact with stationary DC as they locomoted past. Syngeneic small lymphocytes bound avidly to the thin processes of DC when they were added back to some of these cultures (Fig. 2). After separating from

**Table 1. Macrophages and Dendritic Cells Present in Normal Rat TDL**

| Flask No. | Macrophages | Percent | Dendritic Cells | Percent |
|---|---|---|---|---|
| 1 | 28,610 | .95 | 1,200 | .047 |
| 2 | 24,650 | .82 | 1,200 | .047 |
| 3 | 20,570 | .69 | 1,175 | .039 |
| 4 | 22,240 | .74 | 800 | .026 |
| 5 | 28,500 | .95 | 1,100 | .033 |
| 6 | 21,930 | .73 | 975 | .042 |
| 7 | 24,280 | .81 | 1,400 | .040 |
| 8 | 25,680 | .86 | 1,200 | .047 |
| 9 | 29,420 | .98 | 1,250 | .032 |
| 10 | 25,050 | .84 | 1,000 | .037 |
| Mean ± 1 SEM | 25,093 ± 958 | .84 ± .1 | 1,130 ± 53 | .039 ± .002 |

The first 24-hr collection of thoracic duct lymphocytes was "filtered" over nylon wool to remove adherent cells, and monolayers consisting of $3 \times 10^6$ cells were cultured in T-25 flasks. The cells that differentiated into adherent cells from small lymphoid cells were counted on the fourth day of culture after all the nonadherent cells were removed.
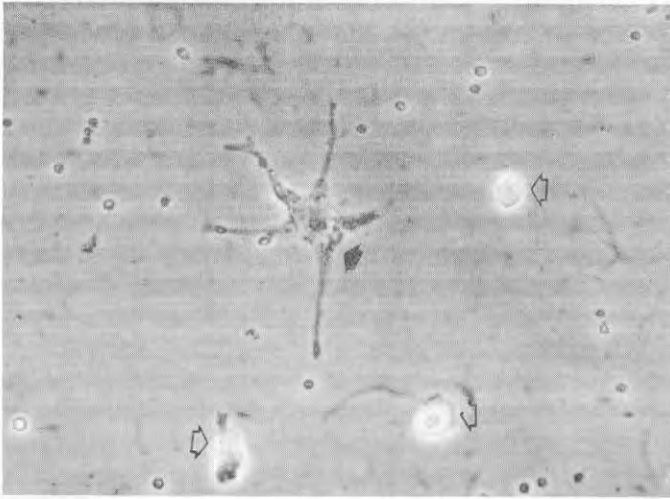
Fig. 1. Lymphoid dendritic cells (closed arrow) differentiate from small cells in rat TDL. Once the "dendritic" processes are formed, these cells become sessile. In contrast, monocytes and macrophages (open arrows) exhibit nearly constant locomotion in vitro.

DC, these lymphocytes did not readhere except for brief contacts. Since DC remained stationary after differentiation, individual cells were observed from day to day after marking their location on the bottom of the flask. After 9 days in culture, no new DC were found and counts remained constant until the cultures were discarded 5 weeks later. Macrophages, which had ingested zymosan granules prior to fixation, labeled with bacterial markers for C3b receptor, Fc receptor, and Ia surface antigens. Antiimmunoglobulin coupled staphylococci formed clusters on macrophages possibly because sIg bound to Fc receptors was distributed in discrete patches. Dendritic cells bound Ia-specific antibody along the distal portions of the cytoplasmic processes (Fig. 3A), but some DC labeled over cell bodies as well. Reagents for Fc receptor and surface immunoglobulins generally did not label DC, and less than 10% of DC from normal rat TDL were decorated with C3b-coated *E. coli*. C3b receptor positive DC were morphologically indistinguishable from the more prevalent C3b receptor negative DC (Fig. 3B). Surface marker char-
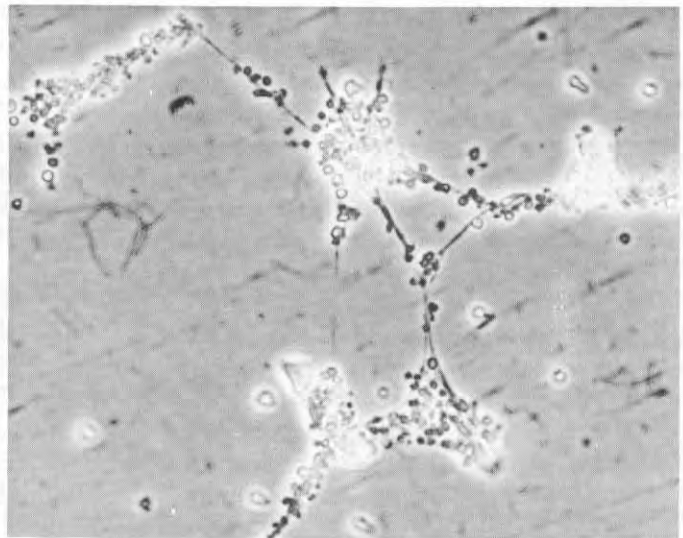


Fig. 2. Lymphocytes adhere to dendritic cells obscuring their bodies and lining up along their processes.
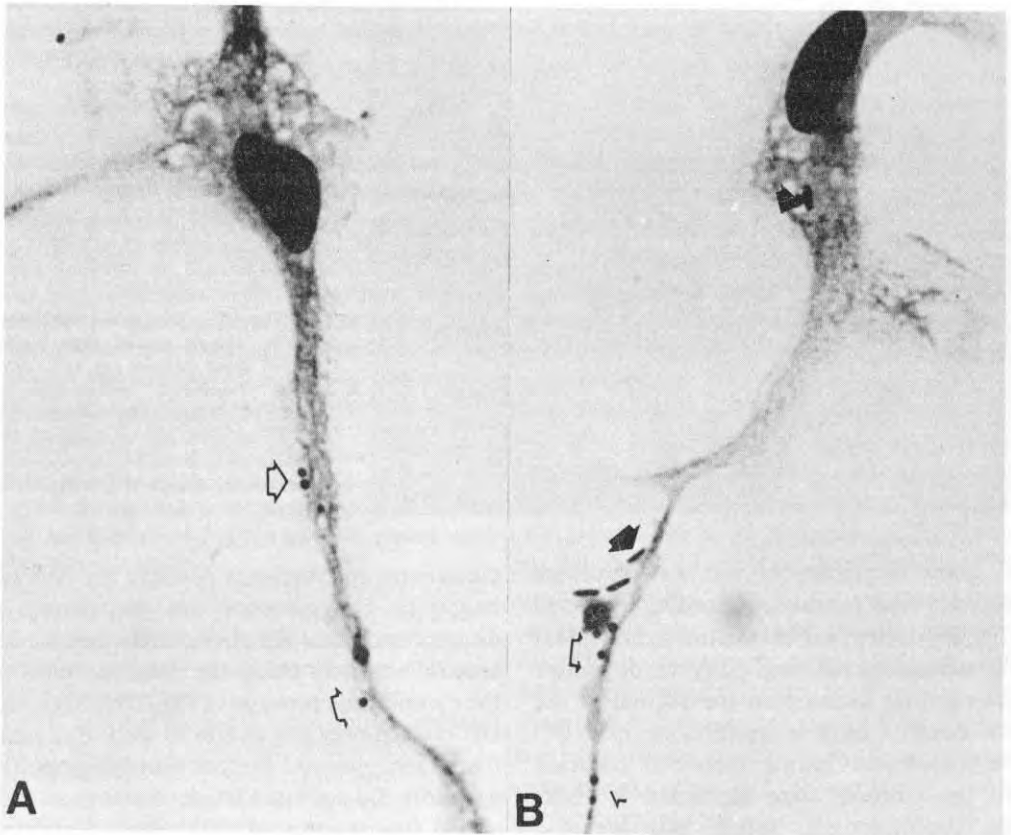
Fig. 3. These are two dendritic cells (DC) whose surfaces have been labeled with bacteria. (A) One type of DC is Ia-positive and C3b receptor negative. Note that cocci (open arrows) indicate the presence of bound antibody specific for rat Ia antigens. (B) Another type of DC is Ia-positive and C3b receptor positive. In this figure, cocci (open arrows) label Ia antibody, and rods (closed arrows) label C3b receptor.

Table 2. Characteristics of Adherent Cells

|  | Monocytes | Dendritic Cells | Langerhans Cells | MZ |
|---|---|---|---|---|
| Surface adher-<br>ence | + | + | + | ± |
| Motile | + | − | ? | ? |
| Phagocytic | + | − | ± | + |
| Produce 4 or<br>more dendritic<br>processes | − | + | + | + |
| Ia antigen | + | + | + | + |
| Fc receptor or<br>surface immu-<br>noglobulin | + | − | + | + |
| C3b receptor | + | ± | + | + |

Data for Langerhans cells and marginal zone cells were taken from Stingl et al.[7] and Humphrey,[4] respectively.

acteristics for DC compared to macrophages, Langerhans cells (LC), and marginal zone cells (MZC) reported here and in the literature are summarized in Table 2. In preparations where lymphocytes were present during surface staining, the predominant class of lymphoid cell spatially associated with C3b receptor-positive DC were sIg-positive B cells.

Inoculation of an emulsion of FITC-BSA in complete Freund's adjuvant resulted in a dramatic change in the number and character of DC and macrophage precursors in TDL. Following a 2-day period where monocytes and DC were depleted from TDL, the output of each of these cells increased to nearly 7% by 14 days postinoculation (Table 3).

**Table 3. Proportion of Dendritic Cells and Macrophages Present as Precursors in TDL From (FITC-BSA) CFA Immunized Rats**

| Days Past Inoculation | Percent DC x + lse | Percent M x + lse |
|---|---|---|
| 0 | 0.04 ± 0.002 | 0.84 ± 0.01 |
| 3 | 0.00 | 0.23 ± 0.06 |
| 7 | 3.97 ± 0.30 | 4.54 ± 0.08 |
| 14 | 7.10 ± 1.52 | 6.81 ± 0.41 |
| 21 | 3.69 ± 0.49 | 4.06 ± 0.32 |
| 30 | 3.21 ± 0.02 | 3.97 ± 0.26 |

These data show a marked increase in proportion of DC following immunization. The increases were actually greater because the volume and cell output also increased during these time periods.

Increase in DC to 7% to TDL represented a 175-fold jump in concentration, while the increase to 6.8% macrophages was barely significant. Total TDL output was increased during this period (data not shown). During peak output of DC-containing TDL (day 14), between 60% and 80% of these cells were C3b receptor positive. Unstained monolayers were examined by fluorescence microscopy to detect whether cell-associated FITC-BSA was carried over into the TDL. Clumps of fluorescent material were found in monocytes and macrophages and dendritic processes of DC were faintly fluorescent. However, considerable free fluorescent material was present in the background, and it was possible that FITC had been enzymatically cleaved from the BSA carrier and was absorbed nonspecifically by DC.

Transmission electron microscopy of cortical tissue from popliteal lymph nodes removed 7–14 days after immunization revealed an increase in cells with lymphoid character and dendritic morphology. There were 2–3 DC per 20 grid spaces (300-mesh copper EM grids of about 3 mm diameter) in normal lymph nodes, while 5–7 DC were located within cortices of "immune" lymph nodes. These dendritic cells physically interacted with other nodal cells. Interlacing complexes of macrophage, lymphoblast, and multiple small lymphocytes were formed by nexus-like junctions between the processes of dendritic cells and the surrounding lymphoid and mononuclear cells (Fig. 4).

These dendritic cells did not contain phagolysosomes or other structures associated with endocytic activity. Instead, there were numerous mitochondria, tubulovesicular structures, and a golgi zone containing vessicles and bundles of microtubules. The near absence of rough endoplasmic reticulum and a relative paucity of polyribosomes suggested that these cells may carry out limited synthetic activity, but the extensive cytoplasmic equipment for energy metabolism and vessicle transport supported a secretory function for these cells. It remains to be shown whether these morphologically similar dendritic cells are the same or different from those described by other methods.[6]

## DISCUSSION

Mononuclear cells with dendritic morphology have been described in various sites within lymphoid tissues. Because of their intricate associations with numerous surrounding cells, these unusual cells have been assumed to contribute something to the microenvironment. In B-cell areas, follicular dendritic cells (FDC) were the first cells to be described as "antigen-presenting" cells because radioactivity associated with the labeled antigen used for primary immunization persisted in these cells long after the label was lost from lymphatic sinusoidal macrophages.[1] Szakal and Hanna also demonstrated that FDC bound more antigen on secondary exposure than primary.[21] It has not been resolved to what degree "cytophilic antibody," Fc receptors, or complement receptors are involved in antigen binding to FDC. Because germinal follicles usually form late in a primary response it is believed that FDC might somehow function to maintain B-cell memory.[22] Interdigitating dendritic cells (IDC) with electron lucent cytoplasmic processes containing tubulovesicular structures have been found among the T cells in the paracortex of lymph nodes,[2] in the corticomedullary border of the thymus,[23] and
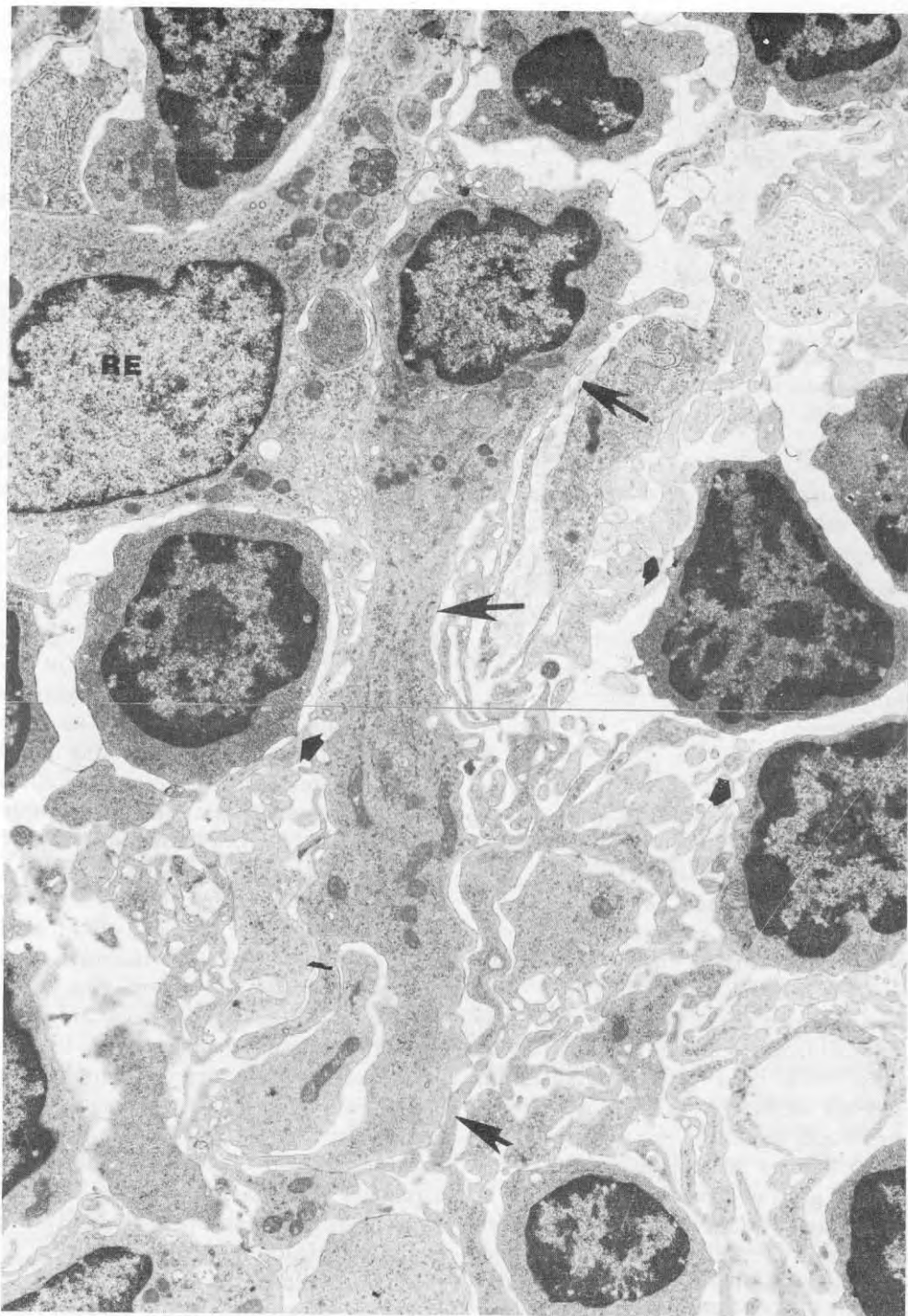
Fig. 4.    Lymphoid dendritic cells (long arrows) exhibit polarized elongated cytoplasmic processes from which thin blunt-ended "dendritic" processes emanate. These processes interact with surrounding lymphocytes via nexus-like junctions (short arrows). DC are frequently found pressed against lymph node macrophages (RE). This tissue arrangement can be seen repeatedly, which suggests some form of required interaction between DC and macrophages.

within the periarteriolar lymphatic sheath of the spleen. These cells resemble Langerhans cells, and some investigators described striate granules in the cytoplasm of IDC that are characteristic for LC. LC isolated from rabbit skin produce dendritic processes in vitro and stimulate T-cell proliferation in allogeneic MLR.[7] There are also suggestions that LC bind contact-sensitizing agents with high affinity and exhibit them on their surfaces after skin exposure.[3,25] Finally, marginal zone cells (MZC), which lie in the perimeter of the splenic periarteriolar lymphatic sheath, have recently been isolated after exposure to labeled starch antigens.[4] These cells also exhibit dendritic processes in vitro and concentrate antigens in the regions of the spleen that lie in the path of trafficking T and B cells. This list of possible antigen-presenting cells possessing dendritic morphology suggests a functional dichotomy, there may be DC that selectively interact with different classes of lymphocytes. For example, C3R + FDC may associate with B cells and IDC may interact with T cells.

In the present study, cells with dendritic morphology but segregating into two types, based on ability to bind complement-coated *E. coli.,* were found to differentiate from small lymphoid cells in rat TDL. These DC were initially nylon wool nonadherent, but adhered by 72–96 hr as they produced their dendritic processes. Both forms of DC were easily distinguished from TDL-derived monocytes or macrophages by their multiple elongated "dendrites," failure to phagocytose carbon and zymosan particles, and sessile nature. Lymphocytes adhered to DC processes with high affinity and migrating monocytes often explored their surfaces. Staph protein-A specific antibody sandwich techniques failed to identify surface immunoglobin or Fc receptors on DC. However, both DC and macrophages labeled with hybridoma antibody directed to rat Ia antigens. When tested for complement receptors, two forms of DC could be distinguished: (1) Ia$^+$ FcR$^-$ C3bR$^-$ or (2) Ia$^+$ FcR$^-$ C3bR$^+$. Since LC,

MZc, and macrophages are FcR and C3R positive, it is not likely that the DC in TDL are any of these cell types. However, DC did express C3b receptors after FITC-BSA in complete Freund's adjuvant was used to immunize the rats used as sources to TDL.

Lymphoid dendritic cells (DC) of splenic origin have been described as potent stimulators of the primary allogeneic mixed leukocyte reaction (MLR), and they prime for production of allospecific cytotoxic T cells.[11,13] Many of the characteristics we see in differentiated DC from TDL resemble those of DC isolated from mouse spleens. Although purified DC have not been shown to present soluble antigens, their ability to stimulate lymphocyte proliferation in syngeneic MLR suggests that DC display environmental antigens or that contact with these cells nonspecifically enhances cell division. It cannot be directly stated that DC are the antigen-presenting cells, but circumstantial evidence for this function has been accumulated. Maneuvers designed to eliminate monocytes and macrophages from cultures also remove dendritic cells and result in diminished immune responses. In addition, DC share with antigen-presenting accessory cells the characteristics of surface adherence, radiation resistance, Ia antigen positivity, and absence of surface markers specific for T or B lymphocytes.[9] These "null" cells, which expresses I-A and I-E subregion determined surface antigens, could as easily be DC as monocytes or macrophages, and a pressing current question is whether one or both cell types are needed for antigen acquisition and presentation in vitro.[9] Our ultrastructural studies[30] confirm observations of others[29] that cells with dendritic morphology are usually found in contact with macrophages and lymphocytes. This physical association could be a coincidence, but it might also suggest that macrophages and DC cooperate to achieve both antigen processing and presentation.

Functional studies of these TDL-derived DC are still preliminary, but recent data

(Anderson and Peters, unpublished) suggest that the (Ia$^+$, FcR$^-$, C3bR$^+$) DC enhance B-cell proliferation and differentiation into IgG or IgA secreting cells. Thoracic duct lymph is known to receive a large contribution of lymphoid cells from the intestinal lymphatics and mesenteric lymph nodes.[26] It is possible that the DC present in normal rat TDL reflect background immune responses to environmental antigens in the intestine and Peyer's patches.[27] This is also supported by observations that TDL often contain B cells that differentiate into IgA-forming cells in culture.[28]

Macrophages and small cells with phagocytic capacity have been demonstrated in rat TDL using radiolabeled colloidal gold,[31] and earlier studies documented the ability of cells resembling lymphocytes in TDL to differentiate into monocytes and macrophages after transfer.[32,33] While small numbers of these cells are known to be present in TDL, it has generally been assumed that they would be filtered out by passage over nylon wool. The present study demonstrates that the differentiated monocytes but not the small "precursor" cells are removed by nylon wool passage. This may relate to temporary effects of the heparin used in the collection media on phagocytosis and cell surface adhesion. Heparin has been shown to contribute to "activation" of macrophage proliferation and heparin treatment blocks phagocytosis of some, but not all substances. It is unlikely that DC are an artifact of heparin treatment rather than a distinct cell type because normal monocytes and macrophages present in TDL recovered from heparin treatment and behaved normally after overnight incubation.

Normal TDL has been described as the least effective source of antigen-presenting cells,[35] but low levels of antigen-induced proliferation can be initiated with TDL cells, reflecting with antigen-presenting cells may be present but in low concentrations. Certainly 0.04% of TDL is a low concentration of cells, and if TDL were collected at room temperature rather than at 4°C, adhesion to the vessel surface might further deplete DC and macrophages.

The significance of finding low levels of dendritic cells in normal thoracic duct lymph is not yet clear, but these cells might have some role in either the induction or maintenance of immunity since they increase in concentration 100-fold following immunization with adjuvantized FITC-BSA. It is not known whether cell division, increased traffic, or increased mobilization results in the enhanced TDL output of DC. It is tempting to propose that these cells may present antigens, but more direct observations are necessary. The faint fluorescence we observed in DC processes following FITC-BSA immunization might be artifactual if reticuloendothelial cells cleaved FITC from BSA.

Dendritic cells neither divided nor died during the 5 weeks of study. The maximum longevity of DC has not yet been determined, but ongoing studies suggest the possibility that these cells may be long-lived. Since DC label readily with $^3$H-uridine,[5] it is also possible that their traffic and sites of localization might be followed. Cells that are in states of transformation such as intermediary lymphocytes and lymphoblasts usually become sequestered in the spleen nonspecifically by filtration from the blood. Perhaps the fate of DC is to accumulate in the spleen and, either by themselves or together with macrophages, provide this tissue with a microenvironment conducive to antigen presentation.[35]

## REFERENCES

1. Nossal GJV, Abbot A, Mitchell J, et al: J Exp Med 127:277, 1968

2. Steinman RM, Cohn ZA: J Exp Med 137:1142, 1973

3. Silberberg-Sinakin I, Thorbecke GJ, Baer RL, et al: Cell Immunol 24:137, 1976

4. Humphrey JH: Adv Exp Med Biol 114:381, 1979

5. Steinman RM, Cohn ZA: J Exp Med 139:380, 1974

6. Steinman RM, Kaplan G, Witmer MD, et al: J Exp Med 149:1, 1979

7. Stingl G, Katz SI, Shevack EM, et al: J Immunol 120:570, 1979

8. Rosenthal AS, Shevach EM: J Exp Med 138:1194, 1973

9. Cowing C, Pincus SH, Sachs DK, et al: J Immunol 121:1680, 1978

10. Singer A, Cowing C, Hathcock HS, et al: J Exp Med 147:1611, 1978

11. Steinman RM, Witmer MD: Proc Natl Acad Sci USA 75:5132, 1978

12. Sprent J: J Immunol 125:2089, 1980

13. Nussenzweig MC, Steinman RM: J Exp Med 151:1196, 1980

14. Nelson DS: Immunobiology of the Macrophage. New York, Academic, 1976, pp 1–633

15. Bell EB: Immunology 38:797, 1979

16. Gowans JL: J Physiol 146:54, 1959

17. Julius MH, Simpson E, Herzenberg LA: Eur J Immunol 3:645, 1973

18. Gelfand JA, Fauci AS, Green I, et al: J Immunol 116:595, 1976

19. Gasser DL, Winters BA, Haas JB, et al: Proc Natl Acad Sci USA 76:4636, 1979

20. Anderson AO, Anderson ND: Immunology 31:731, 1976

21. Szakal AK, Hanna MG Jr: Exp Mol Pathol 8:75, 1968

22. Humphrey JH: Adv Exp Med Biol 66:711, 1976

23. Kaiserling E, Stein H, Mueller-Hermelink HK: Cell Tissue Res 155:47, 1974

24. Hoefsmith ECM, Balfour BM, Kamperdijk, et al: Adv Exp Med Biol 114:389, 1979

25. Shelley WB, Juhlin L: Nature 261:46, 1976

26. MacPherson GG, Steer HW: Adv Exp Med Biol 114:433, 1979

27. Cebra JJ: in Ogre PL, Dayton D (eds): Immunology of Breast Milk. New York, Raven, 1979, pp 1–18

28. Husband AJ, Gowans JL: J Exp Med 148:1146, 1978

29. Friess A: Cell Tissue Res 170:43, 1976

30. Anderson AO, Anderson ND: in Oppenheim JJ, Roesnstreich DA, Potter M (eds): Cell Biology of Immunity and Inflammation. (in press)

31. Roser B: Aust J Exp Biol Med Sci 54:541, 1976

32. Howard JG, Boak JL, Christie GH: Ann NY Acad Sci 129:327, 1966

33. Howard JG, Christie GH, Boak JL, et al: Br J Exp Pathol 50:448, 1969

34. Jacques LB: Science 206:528, 1979

35. Sprent J, Miller JFAP: Cell Immunol 3:361, 1972