A Life Stage of Particle-laden Rat Dendritic Cells In Vivo: Their Terminal Division, Active Phagocytosis, and Translocation from the Liver to the Draining Lymph

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Summary

Initiation of an adaptive immune response against pathogenic organisms, such as bacteria and fungi, may involve phagocytic activity of dendritic cells (DC) or their immature precursors as a prelude to antigen processing and presentation. After intravenous injection of rats with particulate matter, particle-laden cells were detected in the peripheral hepatic lymph. Since it has been known there is a constant efflux of DC from nonlymphoid organs into the draining peripheral lymph, we examined whether these particle-laden cells belonged to the DC or macrophage lineage. The majority of particle-laden cells in lymph showed immature monocyte-like cytology, and the amount of ingested particles was small relative to typical macrophages. We identified these particle-laden cells as DC based on a number of established criteria: (*a*) they had a phenotype characteristic of rat DC, that is, major histocompatibility complex class I^{high+} and II^{high+}, intercellular adhesion molecule 1^+ and ~80% positive with the rat DC–specific mAb OX62; (*b*) they showed strong stimulating capacity in primary allogeneic mixed leukocyte reaction; (*c*) in vitro, they had little phagocytic activity; and (*d*) the kinetics of translocation was similar to that of lymph DC in that they migrated to the thymus-dependent area of the regional nodes. Furthermore, bromodeoxyuridine feeding studies revealed that most of the particle-laden DC were recently produced by the terminal division of precursor cells, at least 45% of them being <5.5 d old. The particle-laden DC, defined as $OX62^+$ latex-laden cells, were first found in the sinusoidal area of the liver, in the liver perfusate, and in spleen cell suspensions, suggesting that the site of particle capture was mainly in the blood marginating pool. It is concluded that the particle-laden cells in the hepatic lymph are recently produced immature DC that manifest a temporary phagocytic activity for intravascular particles during or after the terminal division and that the phagocytic activity is downregulated at a migratory stage when they translocate from the sinusoidal area to the hepatic lymph.

Dendritic cells (DC)[1] comprise a system of potent APC that occupy discrete portions of nonlymphoid and lymphoid organs that are interconnected by defined pathways of cellular traffic (1). There is a substantial continuous efflux of DC from various organs into the draining peripheral lymphatics (2). 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 LN for presentation as processed peptides to T lymphocytes (3). For adaptive immune responses to be mounted against pathogenic organisms, such as bacteria and fungi, however, it is not apparent how DC would acquire and present such particle-derived peptides. The requirement for endocytosis for antigen processing suggests that DC may be phagocytic at some stage in their life cycle (4). In vitro mouse studies support this by showing that proliferating DC precursors in the bone marrow (5) and freshly isolated Langerhans cells (4) readily phagocytose bacteria as well as latex microspheres. On the other hand, there is little in vivo experimental support regarding the phagocytic activity of DC lineage, although it has been reported that some interdigitating cells in the rat thymus or spleen ingest lymphocytes (6, 7).

Recently, we have demonstrated a continuous efflux of DC from the rat liver into the draining hepatic lymph under a steady state (8). In addition, when rats are intravenously injected with carbon particles, only the draining LN of the liver (the celiac nodes) turn black (9, 10). This is due

[1] *Abbreviations used in this paper:* BrdU, 5-bromo-2′-deoxyuridine; DC, dendritic cells; HRP, horseradish peroxidase; HX-TDL, thoracic duct leukocytes of celiac lymphadenectomized rats; ICAM, intercellular adhesion molecule; LCA, leukocyte common antigen; MX-TDL, thoracic duct leukocytes of mesenteric lymphadenectomized rats.

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to an accumulation of carbon-laden cells in the nodes that have migrated from the liver via the lymphatic pathways (10). Whereas peripheral hepatic lymph was originally thought to contain a relatively high proportion of macrophages in sheep (11), these cells were subsequently suggested to belong to the DC lineage (2). Thus, the migrating particle-laden cells may be Kupffer cells in the liver (9) or blood monocytes, or there is another possibility that they may represent cells of the DC lineage with phagocytic activity.

The studies presented here were designed to characterize these particle-laden cells by examining output, phenotype, allostimulating and phagocytic activity in vitro, and turnover and kinetics in vivo. On the basis of these analyses, we suggest that these particle-laden cells are recently produced members of the DC lineage with transient phagocytic activity in vivo. The site of particle capture and residence of this population will also be discussed.

Materials and Methods

Animals. Inbred DA ($RT1^a$) and BUF/Mna ($RT1^u$) rats, reared under specific pathogen–free conditions, were supplied by the Laboratory Animal Center for Experimental Research, Kumamoto University School of Medicine. Three to six rats were used for each experimental group at 5–12 wk of age. Male rats were used throughout except for the in vivo localization study, in which female rats were studied. BUF/Mna rats were used as a source of responder cells for allogeneic MLR.

Particulate Tracers and Antibodies. Carbon particles (diameter ~50 nm, dose of 25 mg/100 g body wt; C11-1431a, Pelikan AG., Hannover, Germany), polyvinyl toluene latex (2 μm diameter, 1 ml of 3.3% [vol/vol] suspension/rat; Seradyn Inc., Indianapolis, IN), and paramagnetic latex (0.9 μm diameter, 0.5 ml/200 g body wt, L1023; Sigma Chemical Co., St. Louis, MO) were injected intravenously as particulate tracers. Mouse mAbs specific for rat determinants included antibodies specific for MHC class I (OX18), MHC class II (OX6), CD5 (OX19), CD4 (OX35), CD8 (OX8), CD90/Thy1.1 (OX7), CD25/IL-2 receptor (OX39), CD45/leukocyte common antigen, (LCA) (OX1), CD54/ICAM1 (IA29), CD11a/LFA1α (WT1), and CD11b/CR3 (OX42). mAbs to the macrophage-related antigens ED1, ED2, and ED3 (12; Dr. C.D. Dijkstra, Free University, Amsterdam, The Netherlands), to a pan–B cell marker (13; HIS14; Dr. F.G.M. Kroese, University of Groningen, The Netherlands), and to a rat DC–specific marker (14; OX62; Dr. M. Brenan, Oxford University) were kindly donated. An mAb against the TdR analogue 5-bromo-2′-deoxyuridine (BrdU) (B44) was purchased from Becton Dickinson & Co. (Mountain View, CA), and IA29 and WT1 were purchased from Seikagaku Kogyo (Tokyo, Japan). All other mAbs were obtained from Sera-Lab Ltd. (Crawley Down, UK). A rabbit polyclonal antibody to bovine type IV collagen (Advance Co. Ltd., Tokyo, Japan) was also used. As secondary antibodies, an alkaline phosphatase–labeled goat Ig to mouse Ig (Sigma Chemical Co.), a horseradish peroxidase (HRP)-labeled rabbit Ig to mouse Ig (Dako Corp., Carpinteria, CA), and an HRP-labeled goat Ig to rabbit Ig (Bio-Rad Laboratories, Richmond, CA) were used.

Collection of Particle-laden Cells in the Hepatic Lymph. Collection of nonlymphoid cells from the hepatic lymph has been described recently (8). Briefly, the regional LN of the rat liver (celiac nodes) were surgically removed, which resulted in the direct influx of peripheral hepatic lymph into the thoracic duct after regeneration of lymphatic vessels. The rats were allowed to recover for >6 wk. Thus, particle-laden cells in the hepatic lymph could be directly obtained by cannulating the thoracic duct. Particles were injected intravenously immediately after the cannulation surgery. Thoracic duct leukocytes of celiac lymphadenectomized rats (HX-TDL) were collected in PBS containing 100 IU/ml heparin at room temperature for 2 d, and either overnight (16 h) or daytime (8 h) collections of lymph were used for the analysis. For control purposes, DC in intestinal lymph were collected in TDL of mesenteric lymphadenectomized rats (MX-TDL) without removal of the celiac nodes as described (8). As a further control, TDL of rats without lymphadenectomy were also collected.

Isolation of Particle-laden Cells. Two alternative methods were used. In the first, particle-laden cells were enriched on the discontinuous gradients of metrizamide (analytical grade; Nycomed Pharma AS, Oslo, Norway). The gradients were made by overlaying 2 ml of 15% metrizamide in PBS containing 0.1% BSA onto 4 ml of 17.5% metrizamide solution in a 15-ml conical centrifuge tube. HX-TDL were washed and resuspended to 1–2 × 10^8 cells/2.5 ml of PBS containing 0.1% BSA. The cell suspension was overlaid onto gradients and centrifuged at 400 *g* for 30 min at room temperature. Cells at the interfaces between medium and 15% metrizamide (fraction 1) or between 15 and 17.5% metrizamide (fraction 2) were collected. Particle-laden cells in hepatic lymph were concentrated in both metrizamide fractions 1 and 2. Alternatively, cells ingesting paramagnetic latex were purified using a magnetic cell separator (MPC-1; Dynal, Oslo, Norway) according to the manufacturer's instructions. Briefly, HX-TDL of rats that received an intravenous injection of paramagnetic latex were collected. A 15-ml test tube containing 1–3 × 10^8 HX-TDL/ml of PBS supplemented with 1% FCS was clamped in the separator and left for 10 min with occasional gentle agitation. After the supernatant was removed by pipetting, test tubes were released from the separator, and the remaining cells were resuspended in medium. This procedure was repeated once, and the partially purified cells were pooled. The pooled cells were then subjected to a further round of sorting.

Immunostaining. The single immunostaining for cytosmears or cryosections was performed by the indirect immunoalkaline phosphatase method and colored red with alkaline phosphatase substrate kit I (Vector red; Vector Laboratories, Inc., Burlingame, CA) as described previously (15, 16). The double immunostaining of TDL cytosmears was made first with a cocktail of HIS14 and OX19 and second with OX6 (8). The triple immunostaining was newly established as follows: the liver cryosections were fixed in pure acetone for 10 min, and then in formol calcium solution (16) for 2 min after rehydration in saline. After washing in saline and incubation with a blocking solution (Block Ace; Dainippon Seiyaku, Tokyo, Japan) for 10 min, sections were incubated with the first mAb, ED2, for 1 h at room temperature. Thereafter, each step was followed by washing three to five times with PBS for 2 min. Bound mAb was detected with an HRP-labeled second antibody for 40 min, and sections were fixed further with 1% glutaraldehyde (Nakalai Tesque, Tokyo, Japan) in PBS for 30 s. Labeled cells were colored black with the combination of an HRP substrate kit (True Blue; KPL Inc., Gaithersburg, MD) and 3,3′-diaminobenzidine hydrochloride (DAB; Dojin Chemical, Kumamoto, Japan) substrate with 0.03% $CoCl_2$ (Sigma Chemical Co.) and 0.01% H_2O_2 using a modification of the method described by Rye et al. (17). Sections were then incubated with the second mAb, OX62, and labeled cells were colored red by the in-

Table 1. *Yield of Particle-laden Cells in HX-TDL**

Particles injected intravenously	Total cell output‡	Percentage of particle-laden cells in original suspension	Yield of particle-laden cells after enrichment	
			Number	Purity
	cells/16 h/rat	%	*cells/16 h/rat*	%
Carbon	$2.2 \pm 0.2 \times 10^8$	1.07 ± 0.19	$6.3 \pm 2.2 \times 10^5$‖	13.7 ± 4.9
Polyvinyl toluene latex	$2.7 \pm 0.5 \times 10^8$	0.36 ± 0.13	$7.5 \pm 2.5 \times 10^5$‖	19.7 ± 8.8
Paramagnetic latex	$1.5 \pm 0.1 \times 10^8$	0.29 ± 0.13	$1.0 \pm 0.2 \times 10^5$¶	80.4 ± 2.7

*Collected overnight for first 16 h. Data are expressed as mean ± SE. Three to eight rats per group were examined.
‡Total cell output without particle injection was $2.1 \pm 0.3 \times 10^8$/16 h/rat.
§Proportion of particle-laden cells in enriched cell suspensions.
‖Particle-laden cells in fractions 1 and 2 of metrizamide gradients.
¶Paramagnetic latex–laden cells isolated by magnetic attraction.

direct immunoalkaline phosphatase method. They were reacted further with the third rabbit antibody to type IV collagen and colored brown by the indirect immunoperoxidase method with a DAB substrate. Sections were counterstained with hematoxylin and mounted in Aquatex (Merck; Darmstadt, Germany).

Experimental Design. In the first experiment, the isolated particle-laden cells in the hepatic lymph (particle-laden HX-TDL) were examined for yield, cytology, phenotype, in vitro allostimulating capacity, and phagocytic activity. In the second experiment, turnover of particle-laden cells was studied by feeding normal rats with BrdU before or after intravenous injection of carbon particles. In the third experiment, normal rats received an intravenous injection of latex particles, and cytosmears and cryosections of various tissues were immunostained to reveal in vivo localization and kinetics of particle-laden cells in lymph. Each value was cited as a mean ± SE, and for some data, statistical analysis was performed using Student's T test.

Yield and Cytology. Cytosmears were either stained with May Grünwald–Giemsa or immunostained. The proportion and yield of particle-laden HX-TDL on smears were estimated by examining 1,000 total cells/smears and total cell output. MX-TDL and control TDL were also examined for a presence of particle-laden cells. In addition, output of ordinary particle-free DC in HX-TDL, MX-TDL and control TDL were estimated by double immunostaining as described above. Using this, DC could be easily identified as non-B, non-T, and MHC class II highly positive (pan-B^-CD5^- MHC II^{high+}) cells (8). For electron microscopy, carbon-laden HX-TDL of first overnight collection were fixed in Karnovsky's fixative for 2 h at room temperature. Cells were postfixed in 2% osmium tetroxide for 1 h at 4°C and en bloc stained with 3% uranyl acetate. The specimens were then dehydrated, infiltrated in propylenoxide/Epon mixtures, and finally embedded in Epon 812. Ultrathin sections were cut and counterstained with 3% uranyl acetate and lead citrate, and specimens were viewed on a transmission electron microscope (JEM 100CX Nihon Densi, Tokyo, Japan).

Phenotype. Cytosmears of HX-TDL were immunostained with a panel of mouse mAbs to rat leukocyte antigens and colored red as above. Only clearly stained particle-laden cells were registered, and the proportion of positive cells was estimated by examining >200 particle-laden cells per animal. We compared the phenotype of the particle-laden HX-TDL to that of Kupffer cells in situ in the liver. 6-μm-thick cryosections of liver tissue recovered 1 h after latex injection were immunostained as above. Large latex-laden phagocytes with a long axis of >25 μm were designated as Kupffer cells, since in vivo localization study showed that cells corresponding to the particle-laden HX-TDL never exceeded that size on the liver sections.

Allogeneic MLR. MLRs were set up as previously reported (8). HX-TDL were incubated with mitomycin C, and then paramagnetic latex-laden cells were isolated and used as stimulator cells. Nylon wool–passed LN cells from Buffalo/Mna rats were used as responder cells. Intestinal DC in the metrizamide fraction 1 of MX-TDL without particle injection (8) as well as unfrac-

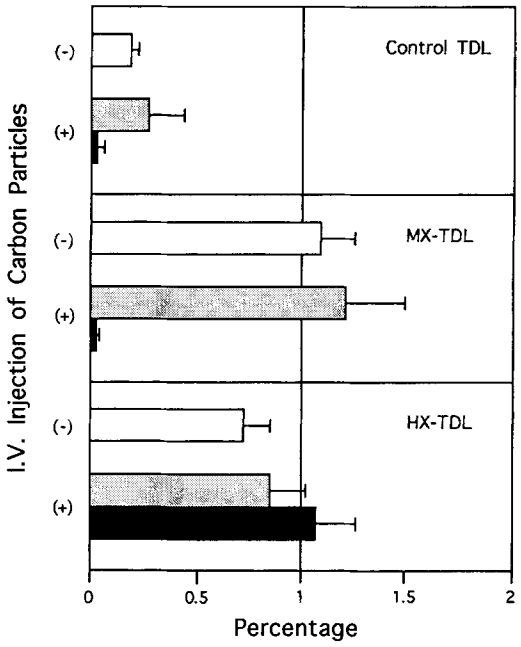
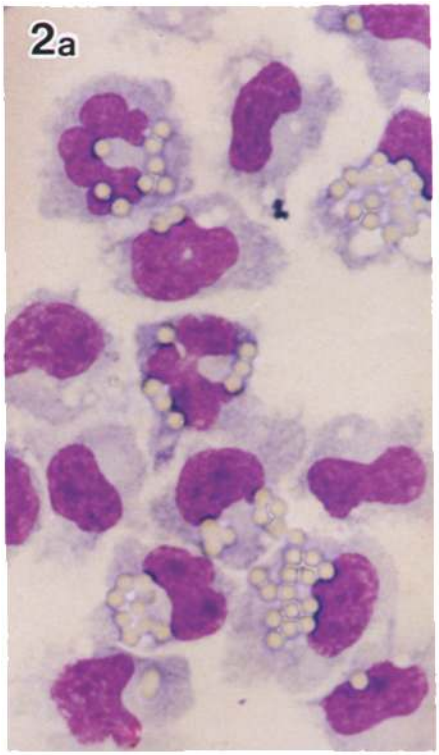


Figure 1. Proportion of DC and carbon-laden cells in HX-TDL, MX-TDL, and control TDL. Cells were collected overnight for the first 16 h after carbon injection. Ordinary particle-free DC were defined as pan-$B^-CD5^-MHC\ II^{high+}$ (*gray column*) and carbon-laden cells were examined by May Grünwald-Giemsa staining (*black column*). For comparison, the proportion of ordinary DC without carbon injection (*white column*) was also included. Data are expressed as mean ± SE. Three to six rats per group were examined.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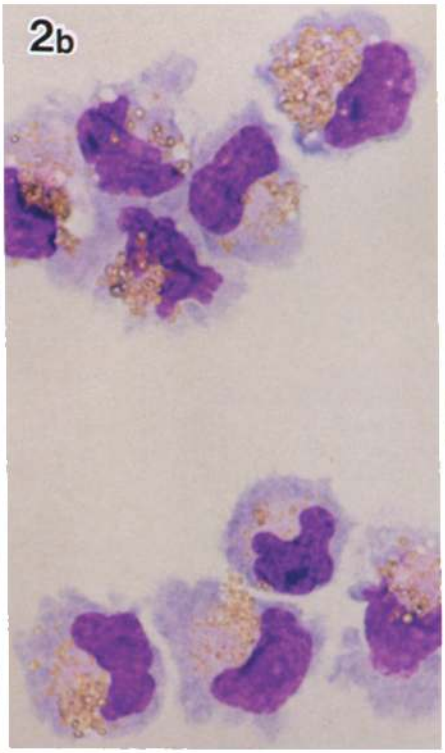
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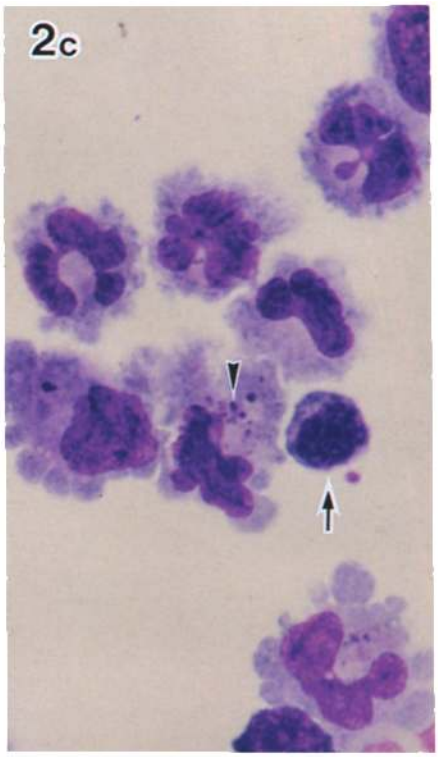
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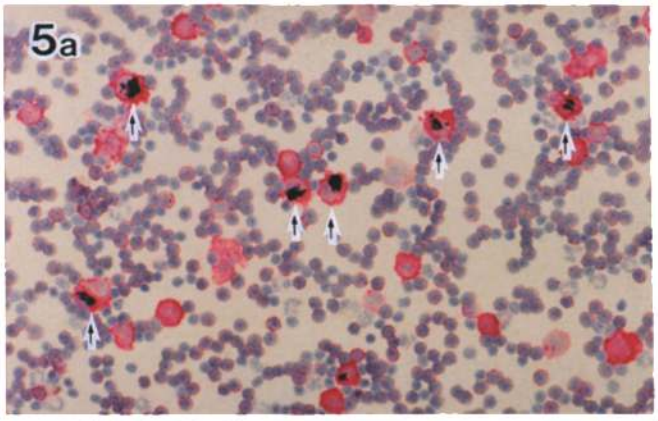
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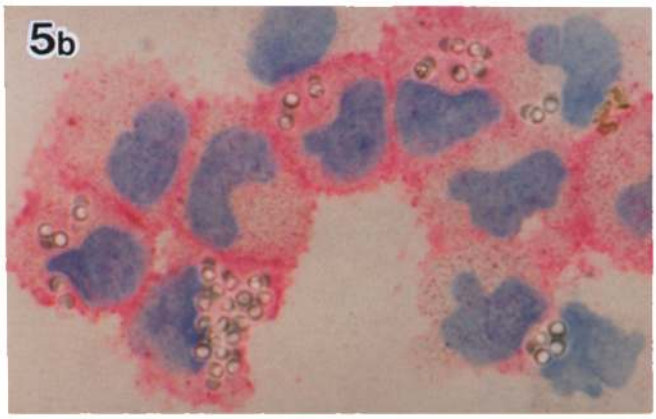
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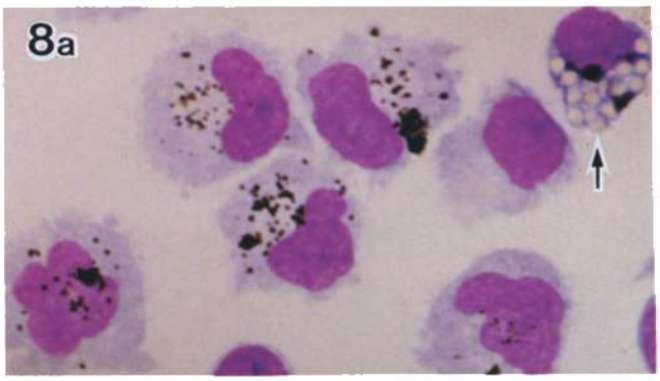
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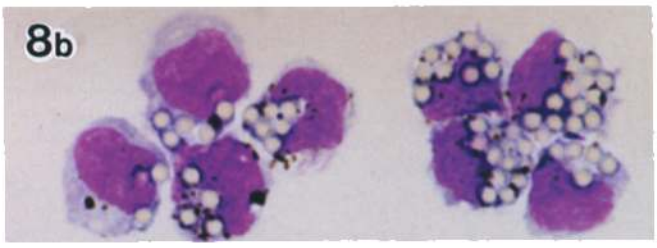
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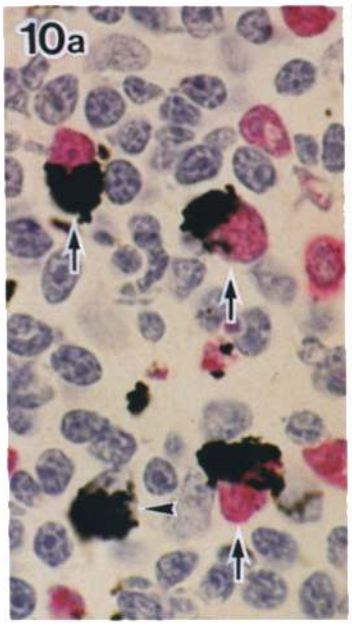
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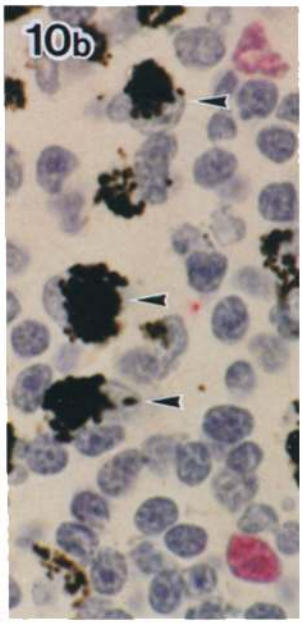
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tioned cells of both HX-TDL and MX-TDL were used as control stimulators.

In Vitro Phagocytosis of Latex Particles. Metrizamide fractions 1 and 2 containing enriched carbon-laden HX-TDL were pooled, and 2–15 × 10^5 cells/400 μl of RPMI medium were cultured with a 0.03% (vol/vol) suspension of 2-μm latex particles in a chamber (Lab-Tek; Nunc Inc., Naperville, IL) for 1, 4, and 10 h at 37°C in 5% CO_2 incubator. After this period, the cells were washed and prepared for light microscopy. As a control, peritoneal macrophages were studied. Normal rats received an intraperitoneal injection of carbon particles (8.5 mg in 10 ml saline), and 18 h later, peritoneal cells were collected by washing with 30 ml chilled PBS containing 100 IU heparin. The cells were treated and examined in the same manner as above. Intestinal DC in the metrizamide fraction 1 of MX-TDL without particle injection were also assessed for comparative purposes. Latex particles were used at concentrations that were saturating for normal peritoneal macrophages in similar assays. Cells ingesting more than five latex particles were designated as latex phagocytes, and the proportion of phagocytes that ingested both carbon and latex particles among total carbon-laden cells was estimated.

Turnover of Particle-laden Cells In Vivo. Proliferating cells were labeled in vivo with BrdU (Sigma Chemical Co.) delivered in drinking water ad lib. at a concentration of 0.8 mg/ml for 2.5 d. The feeding duration was predetermined as that required to label ~50% of blood monocytes. Immediately (0 d) and 1, 2, 3, 7 and 14 d after cessation of the BrdU feeding, rats received an intravenous injection of carbon particles, and the celiac nodes were removed 24 h later. In another group, rats were first injected intravenously with carbon particles, and then fed with BrdU for 24 h, and the celiac nodes were removed at the end of the BrdU feeding. Cryosections of the celiac nodes were immunostained with anti-BrdU mAb by the indirect immunoalkaline phosphatase method (15) and colored red with Vector red. We estimated the labeling index of carbon-laden cells in the celiac nodes by counting >200 cells per animal.

In Vivo Localization of Particle-laden Cells. At 1, 6, and 18 h and 2 d after intravenous injection of 2-μm latex particles, the liver, spleen, lung, celiac and parathymic LN, and femoral bone marrow were examined for localization of particle-laden HX-TDL in other tissues. Aortic blood, sinusoidal free cells in the liver, and spleen cell suspensions were also collected, and content of the particle-laden cells was estimated. Under ether anesthesia, rats were injected intravenously with heparin (100 IU), and 2 ml of aortic blood was drawn, after which rats were killed by exsanguination. Sinusoidal free cells were collected by perfusion through the portal vein according to Bouwens et al. (18), except that the perfusion medium used was PBS without EDTA. Spleen cell suspensions were prepared by teasing spleen tissues gently without enzyme digestion. From the above cell suspensions, red blood cells, dead cells, and debris were removed using density separation medium (Lympholyte-Rat; Cedarlane Laboratories Ltd., Hornby, Ontario, Canada), and cytosmears were prepared. The femoral bone marrow plug was removed by splitting the diaphysis, and all excised tissues were embedded in cryomolds (Miles Laboratories Inc., Elkhart, IN) filled with OCT compound (Miles Laboratories Inc.). Tissues were snap frozen in liquid nitrogen without fixation, and 6-μm cryosections were cut. Cytosmears were immunostained singly with OX62 as described above. Cryosections of the liver were triple immunostained with ED2 (black), OX62 (red), and anti-type IV collagen (brown) as described above. $OX62^+$ cells containing more than two latex particles in the cytoplasm were regarded as cells corresponding to the latex-laden HX-TDL. About 15 mm^2 of stained sections were thoroughly examined for the presence of $OX62^+$ latex-laden cells within the portal, sinusoidal, and hepatic vein areas. The portal area represented the interlobular triangular area of connective tissues enclosing the triad of the bile duct, hepatic artery, and portal vein. The sinusoidal area covered the whole hepatic lobule except the central vein. The hepatic vein area included subendothelial spaces or connective tissues of the central, sublobular, and collecting veins. These three areas were clearly outlined by type IV collagen immunostaining and easily distinguished. The cryosections of tissues other than the liver were double immunostained with OX62 (red) and anti-type IV collagen (brown).

Results

Yield of the Particle-laden HX-TDL. Carbon-laden and polyvinyl toluene latex–laden HX-TDL of the first overnight collection (16 h) represented 1.07 and 0.36% of total HX-TDL, respectively. The yields in the pooled metrizamide fractions were 6.3 × 10^5 and 7.5 × 10^5/16 h per rat with a purity of 13.7 and 19.7%, respectively (Table 1). The yield of the paramagnetic latex–laden HX-TDL after magnetic attraction was 10^5/16 h per rat, ~25% of the starting cell suspension. Of the enriched cells, >80% contained paramagnetic beads, and contaminating cells were mostly PMN, small lymphocytes, and erythrocytes, but large cells such as

Figure 2. Typical morphology of enriched particle-laden cells and ordinary DC. (*a*) latex-laden HX-TDL, (*b*) paramagnetic latex-laden HX-TDL, and (*c*) typical lymph DC in MX-TDL. Note monocyte-like cytology of particle-laden HX-TDL (*a* and *b*), which differs from a typical DC having a cloverleaf or flowerlike nucleus and wide cytoplasmic veils (*c*). The arrowhead indicates azurophilic granules, and the arrow indicates a contaminating lymphocyte. May Grünwald-Giemsa staining. ×1,150.

Figure 5. Immunostaining of HX-TDL. Cytosmears of original HX-TDL after carbon injection (*a*) or enriched latex-laden HX-TDL (*b*), immunostained with (*a*) MHC class II (*red*) or (*b*) OX62 (*red*). Carbon-laden HX-TDL (*arrows*) are intensely positive for MHC class II, and most latex-laden HX-TDL are clearly positive for OX62. ×230 (*a*); ×1150 (*b*).

Figure 8. In vitro phagocytosis study. Cytosmears of carbon-laden HX-TDL (*a*) and carbon-laden peritoneal macrophages (*b*) after in vitro culture of 10 and 2 h with latex particles, respectively. One carbon-laden HX-TDL ingested latex (*arrow*) but not others, whereas most carbon-laden peritoneal macrophages ingested latex. May Grünwald-Giemsa staining. ×1,150.

Figure 10. BrdU immunostaining of the celiac nodes. (*a*) Rat was injected intravenously with carbon 3 d after cessation of BrdU and examined 24 h later. Three carbon-laden cells are $BrdU^+$ (*arrows*), whereas one cell is $BrdU^-$ (*arrowhead*). (*b*) Rat was injected with carbon, and then fed with BrdU for 24 h and examined immediately. Most carbon-laden cells are $BrdU^-$ (*arrowheads*). ×1,150.

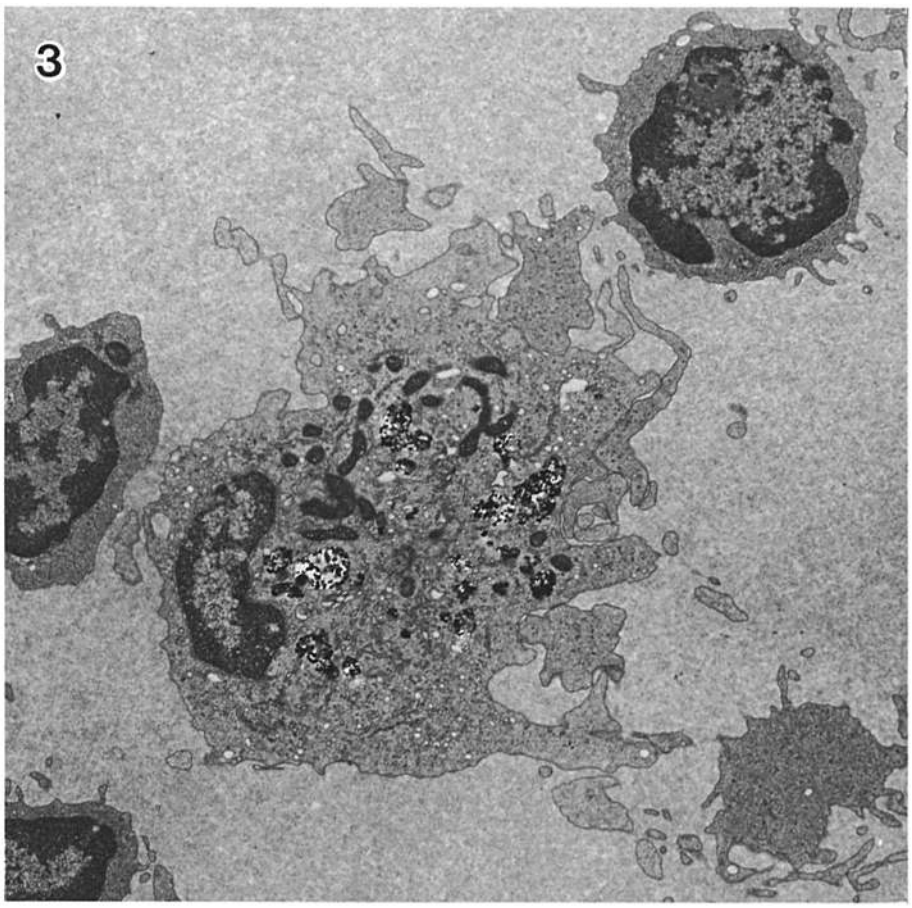


Figure 3. Transmission electron micrograph of a carbon-laden HX-TDL. Within the cytoplasm of the cell possessing irregular projections can be seen membrane-bounded phagosomes with engulfed carbon particles, multivesicular bodies, and other inclusion. Note that the cell contains an eccentric irregular nucleus, several round or fusiform mitochondria, ribosomal particles, and many small vesicles, often containing electron-lucent materials. The Golgi apparatus does not appear to be well developed. ×4,600.

ordinary particle-free DC were negligible. The viability of all particle-laden cells was >95% as assessed by trypan blue dye exclusion. In the ensuing collections of HX-TDL between 16 and 24, 24 and 40, and 40 and 48 h after the drainage operation, the proportion of polyvinyl toluene latex–laden cells decreased to approximately one-third, one-fourth, and one-sixth of the first overnight collection, respectively (data not shown). As for a presence of particle-laden cells in intestinal lymph (MX-TDL) and ordinary central lymph (control TDL), a negligible number was found (Fig. 1), confirming the previous finding (10). In addition, the output of ordinary particle-free DC in HX-TDL, MX-TDL, and control TDL did not change after particle injection and was similar to the normal steady state (Fig. 1).

Cytology. On cytosmears, the majority of particle-laden HX-TDL had an immature monocyte-like morphology (Fig. 2, *a* and *b*) and differed from typical DC in that they had a cloverleaf or flowerlike nucleus and wide cytoplasmic veils (Fig. 2 *c*). In general, the particle-laden cells had a large pale cytoplasm and an eccentric nucleus. They ingested a diverse number of particles, although obviously less than that of scavenger macrophages such as liver Kupffer cells. Phagosomes often localized around a clear perinuclear zone or centrosphere as shown by Inaba et al. (5). Ultrastructurally, the particle-laden HX-TDL had an eccentric irregular nucleus and a cytoplasm with ruffled projections (Fig. 3). The cytoplasm contained membrane-bounded phagosomes with engulfed particles, multivesicular bodies, small vesicles, and fusiform mitochondria. The Golgi apparatus does not appear to be well developed.

Phenotype. The phenotype of the particle-laden HX-TDL and Kupffer cells in situ is summarized in Fig. 4. Most particle-laden HX-TDL were LCA^+, MHC class I^{high+}, MHC class II^{high+} (Fig. 5 *a*), $ICAM1^+$, $CR3^+$, $ED1^+$, $ED2^-$, $ED3^-$, $CD5^-$, $CD8^-$, IL-2 $receptor^-$, $His14^-$, ~50% were $Thy1^+$ and $CD4^+$, and 20–30% were $LFA1\alpha^+$. Although there was no distinct difference in phenotype of carbon- and latex-laden HX-TDL, the proportion of $ED1^+$ carbon-laden cells was lower than that of latex-laden cells ($P < 0.01$). About 80% of particle-laden HX-TDL stained positive with the OX62 mAb (Fig. 5 *b*). In contrast, Kupffer cells in situ displayed a phenotype distinct from that of particle-laden HX-TDL. Most Kupffer cells were $ED1^+$, $ED2^+$, $ED3^+$, and 70–80% were LCA^+, $CR3^+$, $CD4^+$, and $LFA1\alpha^+$ but $OX62^-$ and $Thy1^-$.

Allogeneic MLR. As few as 30 paramagnetic latex–laden HX-TDL showed potent alloantigen-presenting capacity in a primary allogeneic MLR (Fig. 6). By comparison, 100 times more unfractioned cells were required to induce a similar level of responder cell proliferation. This allostimulating capacity was comparable to that of intestinal DC in

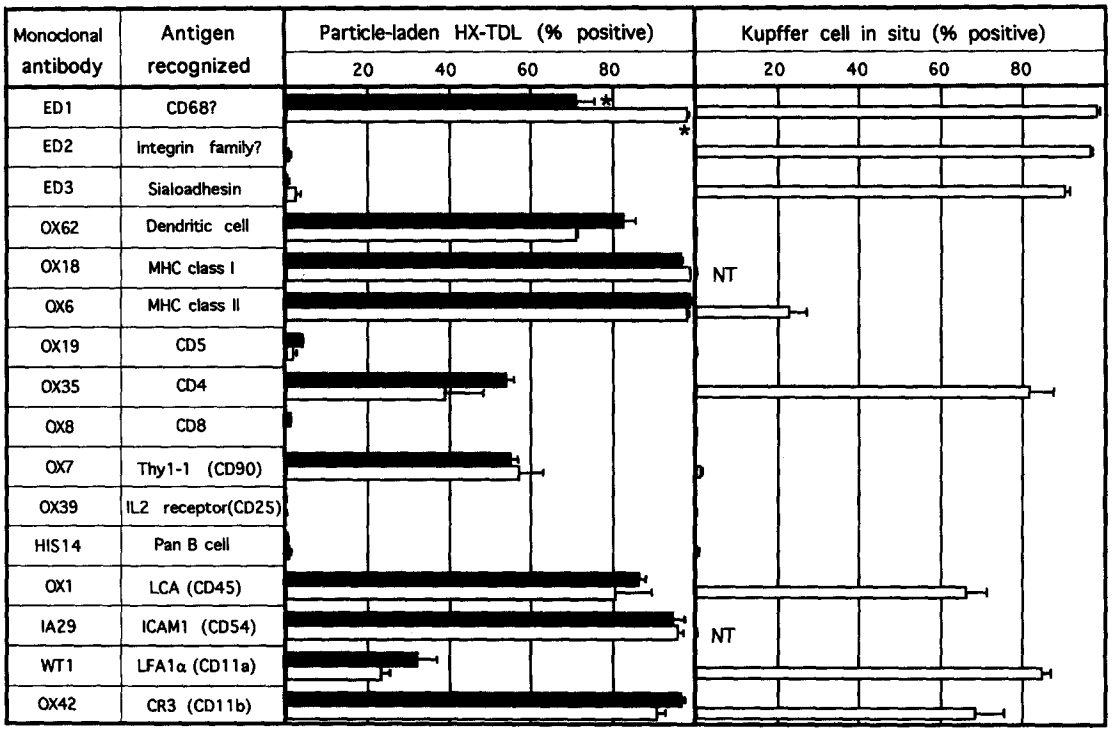


Figure 4. Phenotype of particle-laden HX-TDL and Kupffer cells in situ. Cytosmears of HX-TDL and cryosections of the liver obtained after intravenous injection of either carbon (*black column*) or latex (*white column*) particles were immunostained with mAbs by the indirect immunoalkaline phosphatase method. Kupffer cells after carbon injection were not examined. Large latex-laden phagocytes with a long axis of >25 μm were designated as Kupffer cells. Expression of MHC class I and ICAM1 by Kupffer cells was not estimated as mAbs to these antigens stained the sinusoids diffusely. Note a significant difference in proportion between $ED1^+$ carbon-laden cells and latex-laden cells ($^*P = 0.009$). Data are expressed as mean ± SE. Three rats per group and >200 particle-laden cells per rat were examined.

MX-TDL (Fig. 6). Since the magnetic attraction method precluded contamination with nonphagocytic DC, which is inevitable in ordinary density fractionation methods, the result clearly indicates that strong allostimulating capacity is possessed by the particle-laden HX-TDL.

In Vitro Phagocytosis. Less than 10% of carbon-laden HX-TDL ingested latex in vitro, whereas >50% of carbon-laden peritoneal macrophages showed strong phagocytic activity for latex in vitro (Figs. 7 and 8). This indicates that failure of the phagocytes in hepatic lymph (HX-TDL) to ingest latex in vitro was not due to the experimental conditions, but rather indicates that they have lost their phagocytic activity after entering the lymph. Intestinal DC in the metrizamide fraction 1 of normal MX-TDL were also nonphagocytic by the same method.

Turnover. Since particle-laden cells become $BrdU^+$ only if they have been synthesizing DNA when BrdU is available in the body, kinetic studies on the labeling index of particle-laden cells should clarify the rate of their turnover and the possibility for their proliferation after the ingestion of particles. For estimating the labeling index of particle-laden cells in the hepatic lymph, we examined the celiac nodes draining the liver because they accumulate in, and do not leave, the celiac nodes (10). Approximately 30% of carbon-laden cells in the celiac nodes were $BrdU^+$ if carbon was administered immediately after cessation of the BrdU feeding. The labeling index (Fig. 9) reached a peak of 45% at 3 d (Fig. 10 *a*) and decreased by 14 d. In contrast, when rats were fed with BrdU for 24 h after carbon injection, carbon-laden cells were scarcely labeled (Fig. 10 *b*). These results indicate that at least 45% of carbon-laden cells were produced by cell division within 5.5 d before ingestion of particles and that most of them did not proliferate after particle capture.

In Vivo Localization in the Liver and Other Tissues. Although OX62 detects only a subpopulation of resident DC in the liver, it does not recognize other macrophage subpopulations (14). As described above, OX62 also reacted with a majority of the particle-laden HX-TDL. Accordingly, we have regarded OX62 as the most exclusive and reliable marker to identify representatives of the particle-laden HX-TDL in other tissues. In the liver tissue using triple immunostaining with ED2 (black), OX62 (red), and anti–type IV collagen (brown), we could easily determine not only the localization of $OX62^+$ latex-laden cells with respect to the three structural domains but also their mutual relationship with Kupffer cells, which are $ED2^+$. In normal untreated animals, $OX62^+$ cells were found mainly in the portal area, and a few were found in the sinusoidal area and around the hepatic vein area, including the central vein (Figs. 11 and 12). 1 h after intravenous injection of latex particles, a few $OX62^+$ latex-laden cells appeared only in the sinusoidal area. Some of them obviously located intravascularly within the sinusoidal lumen (Fig. 13 *a*). The num-

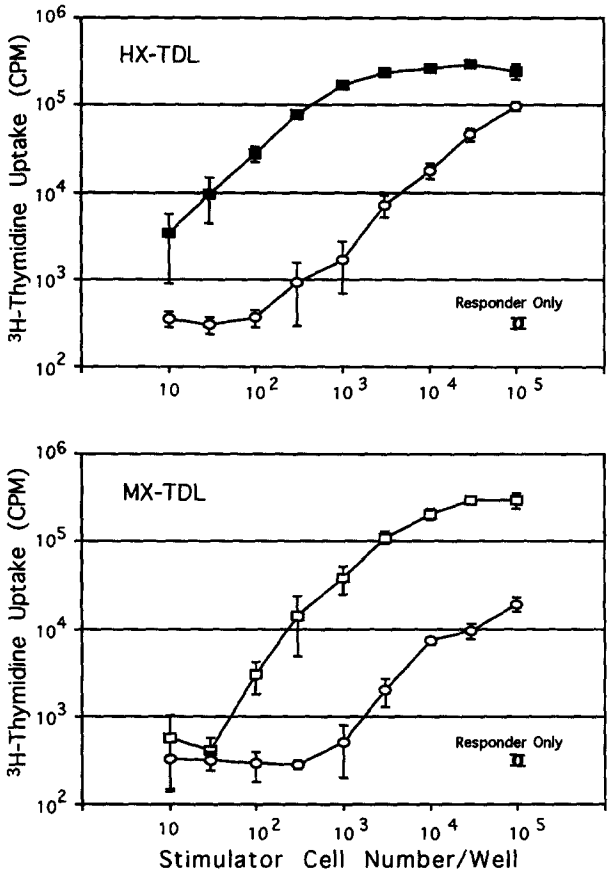


Figure 6. Immunostimulatory capacity of particle-laden HX-TDL in a primary allogeneic MLR. Note a very intense alloantigen-presenting capacity of paramagnetic latex–laden cell fraction of HX-TDL after intravenous injection of paramagnetic latex (■) and metrizamide fraction 1 of MX-TDL (□), compared with corresponding unseparated cell suspensions (○). Representative data from two experiments. Mean cpm ± SE (n = 3).

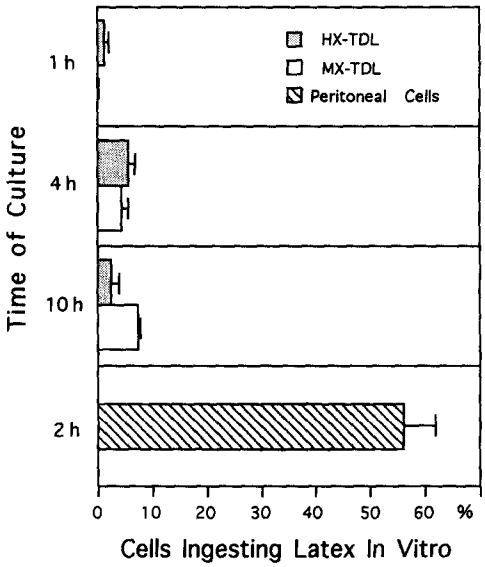


Figure 7. In vitro phagocytosis of latex by carbon-laden HX-TDL and peritoneal cells. Less than 10% of carbon-laden HX-TDL and normal DC fraction in MX-TDL phagocytosed latex particles during 1–10 h cultures. In comparison, >50% of carbon-laden peritoneal macrophages ingested latex.

ber of OX62$^+$ latex-laden cells reached a plateau at 6 h in the sinusoidal area. While, OX62$^+$ latex-laden cells first appeared in the portal area at 6 h and in the hepatic vein area at 18 h (Fig. 11). A close association between OX62$^+$ cells and Kupffer cells (ED2$^+$) was often observed in normal liver and liver after latex injection (Fig. 12, *b* and *c*, and Fig. 13 *b*).

In the liver perfusate, ~10^4 OX62$^+$ latex-laden cells/rat were found 1 h after intravenous injection of latex, and the number increased to 5 × 10^4 cells/rat at 18 h (Fig. 14). In spleen cell suspensions, ~7–8 × 10^4 OX62$^+$ latex-laden cells/rat were found from 1 to 18 h after intravenous injection of latex (Fig. 14). The total number of OX62$^+$ cells did not change significantly in the liver sections and perfusates, but decreased in the spleen cell suspensions 6 and 18 h after latex injection ($P < 0.01$). Furthermore, OX62$^+$ latex-laden cells were found not only in the red pulp cord and the marginal zone but also in the splenic sinus and trabecular vein from 1 h to 2 d after latex injection (Fig. 13 *d*). In contrast, OX62$^+$ latex-laden cells did not appear in the white pulp until 18 h after latex injection. In the celiac nodes, a few OX62$^+$ latex-laden cells appeared in the marginal sinus and the interfollicular area at 6 h, and they heavily accumulated in the paracortex 18 h after latex injection. In the parathymic nodes, a few OX62$^+$ latex-laden cells were observed in the marginal sinus and paracortex at 18 h and 2 d after latex injection. Other tissues such as the lung and aortic blood contained a negligible number, but a few

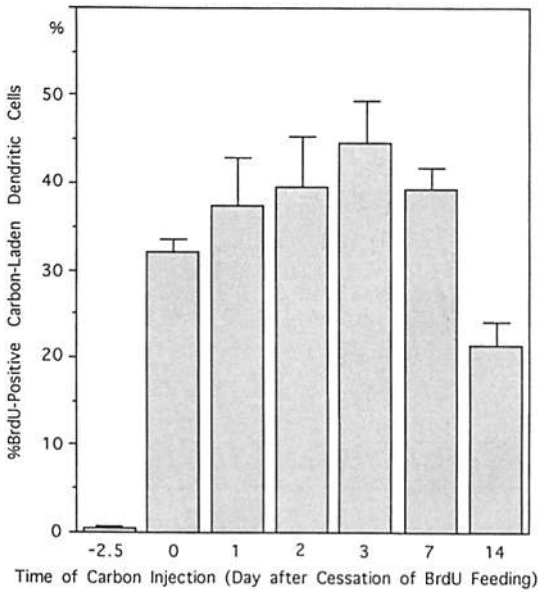


Figure 9. Proportion (labeling index) of BrdU$^+$ carbon-laden cells in the celiac nodes 24 h after intravenous injection of carbon particles. Particles were injected at various time points after the cessation of BrdU feeding for 2.5 d. In group −2.5 d, rats were injected with carbon, and then fed with BrdU for 24 h and examined immediately.

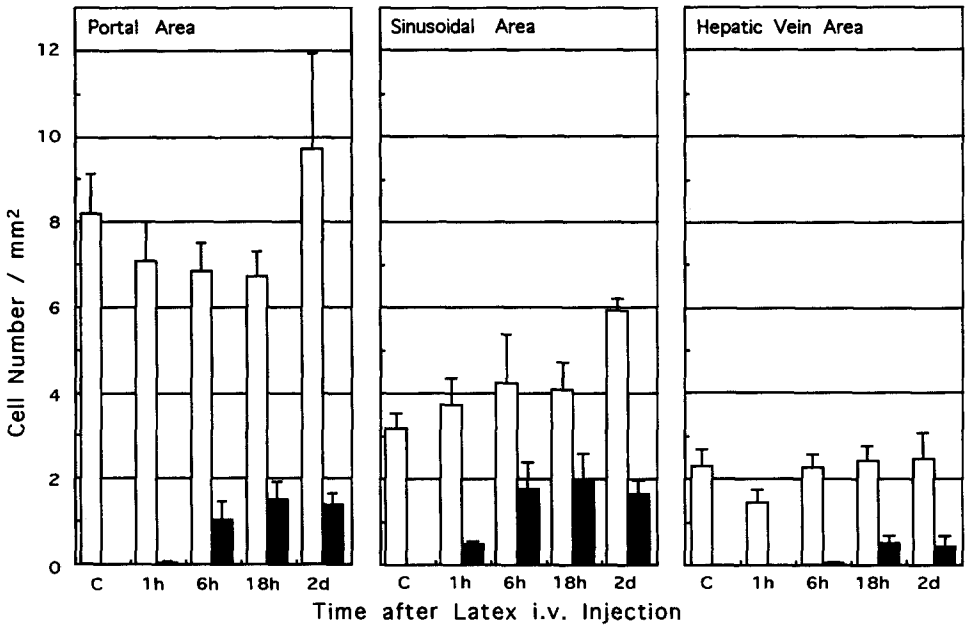


Figure 11. In vivo kinetics of total $OX62^+$ cells (*white column*) and $OX62^+$ latex-laden cells (*black column*) in the liver after intravenous injection of latex particles. Cryosections were triple immunostained with ED2, OX62, and anti-type IV collagen, and the proportions of total $OX62^+$ cells and $OX62^+$ latex-laden cells were estimated by thoroughly examining the portal, sinusoidal, and hepatic vein areas. Data are expressed as mean ± SE. Bars represent SE. Four to six rats per group were examined.

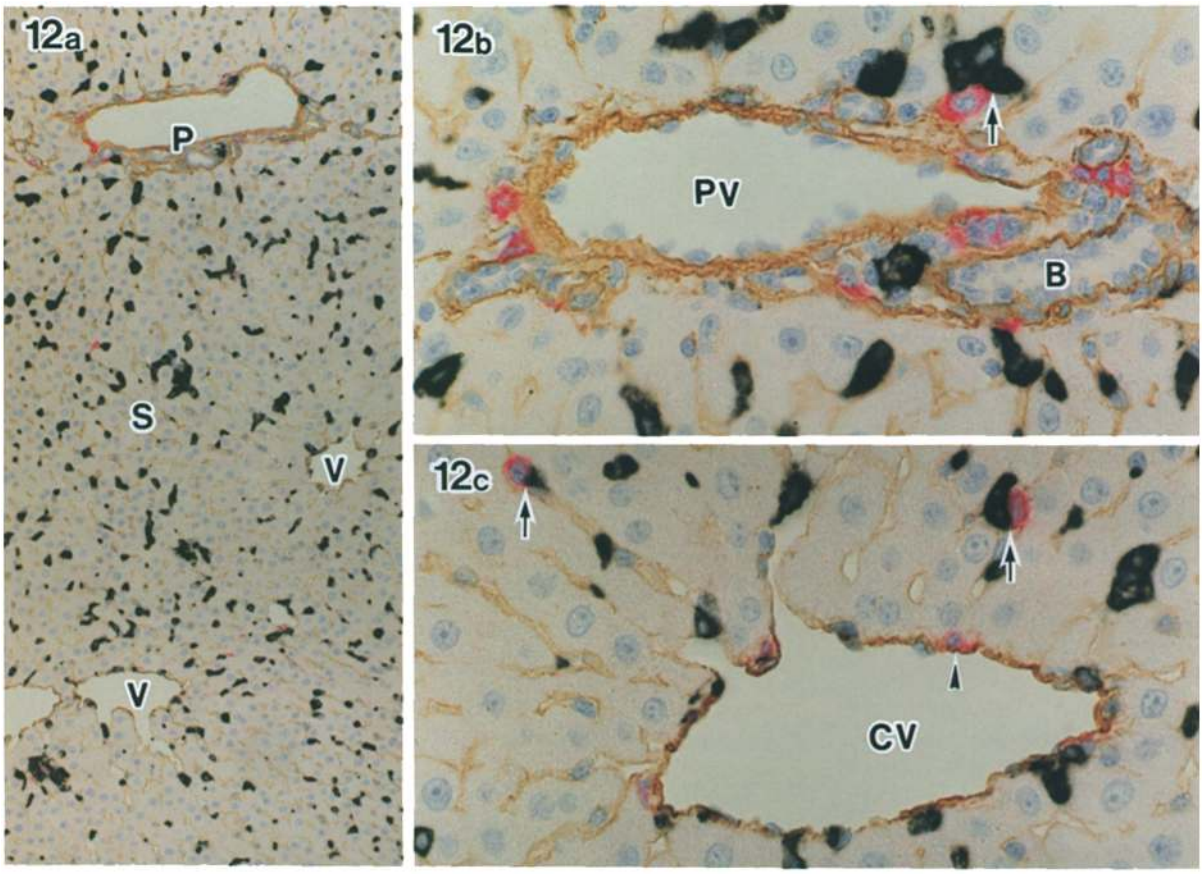
$OX62^+$ latex-laden cells were observed in and around the venous sinus of the bone marrow from 6 h to 2 d (data not shown).

Discussion

In this report, we have collected and analyzed particle-laden cells in rat hepatic lymph after intravenous injection of various particulate matters and demonstrated that these cells bore characteristics of DC, although they had immature monocyte-like morphology. The identification of the particle-laden HX-TDL as DC was based on a number of established criteria (1): (*a*) they had a phenotype typical of rat DC, that is, MHC class I^{high+} and II^{high+}, $OX62^+$, and $ICAM1^+$; (*b*) they showed a strong stimulating capacity in a primary allogeneic MLR; (*c*) they had little phagocytic activity in vitro; and (*d*) the kinetics of translocation was similar to that of lymph DC (2), in that, after entering the lymph, they migrated via the interfollicular area to the thymus-dependent paracortex area of the regional celiac LN. As for phenotype, although OX62 also recognizes intraepithelial $CD3^+MHC\ II^-$ cells, probably γ/δ T cells (14), it is clear that $OX62^+$ particle-laden cells differ from γ/δ T cells since lymphocytes hardly ingest particles. The expression of other markers on the particle-laden DC was equivalent to that of intestinal lymph DC, that is, LCA^+, $Thy1^+$ (50%), IL-2 $receptor^-$, and $CD5^-$ (19).

Ingestion of either polyvinyl toluene or paramagnetic latex particles of 2 or 0.8 μm in diameter, respectively, indicates that the particle-laden DC had a distinct phagocytic activity since the cutoff for fluid phase pinocytosis is conventionally taken to be ~0.5 μm (20). A high proportion of $ED1^+$ cells among latex-laden DC also confirms this, since ED1 recognizes a CD68-like antigen, and the amount of ED1 expression reflects recent phagocytic activity (21). This study, therefore, provides the first in vivo evidence for phagocytic activity in the rat DC lineage other than that previously reported for the interdigitating cells (6, 7). In contrast, $ED1^-$ carbon-laden DC taking 20% of total carbon-laden DC may represent more mature DC that have already lost phagocytic activity together with ED1 antigen when they encounter the carbon particles. Considering the carbon particle is only 50 nm in diameter, even nonphagocytic DC could accumulate the carbon particles in the cytoplasm by pinocytosis. Together with the fact that the particle-laden DC ingested diverse amount of particles, we consider that heterogeneity in phagocytic activity may exist among the particle-laden DC. The phagocytic DC appeared to represent a transitional stage as well (22, 23), with a number of characteristics of the mononuclear phagocyte system (12, 24). Phagocytosis may be important for ingestion and processing of particulate microorganisms such as bacteria and fungi. Inaba et al. (5) have examined cultures of proliferating mouse DC and found that DC that are pulsed and chased with Bacillus Calmette-Guerin mycobacteria are the most effective APC for mycobacteria and contain the most particles. The number of particles ingested by the particle-laden DC was small, relative to typical scavenger phagocytes such as Kupffer cells. The machinery required for antigen presentation may differ from that required for scavenging, both quantitatively and qualitatively (5). APC activity of the particle-laden DC for phagocytosed particulate antigens is currently under study.

The BrdU study has demonstrated that a turnover of the particle-laden DC is fast, the majority of them being recently produced within several days by division of precursor cells. Furthermore, most particle-laden DC were monocytoid cells and did not display a typical DC cytology. These results suggest that the particle-laden DC are still at an immature postmitotic stage when they appear in hepatic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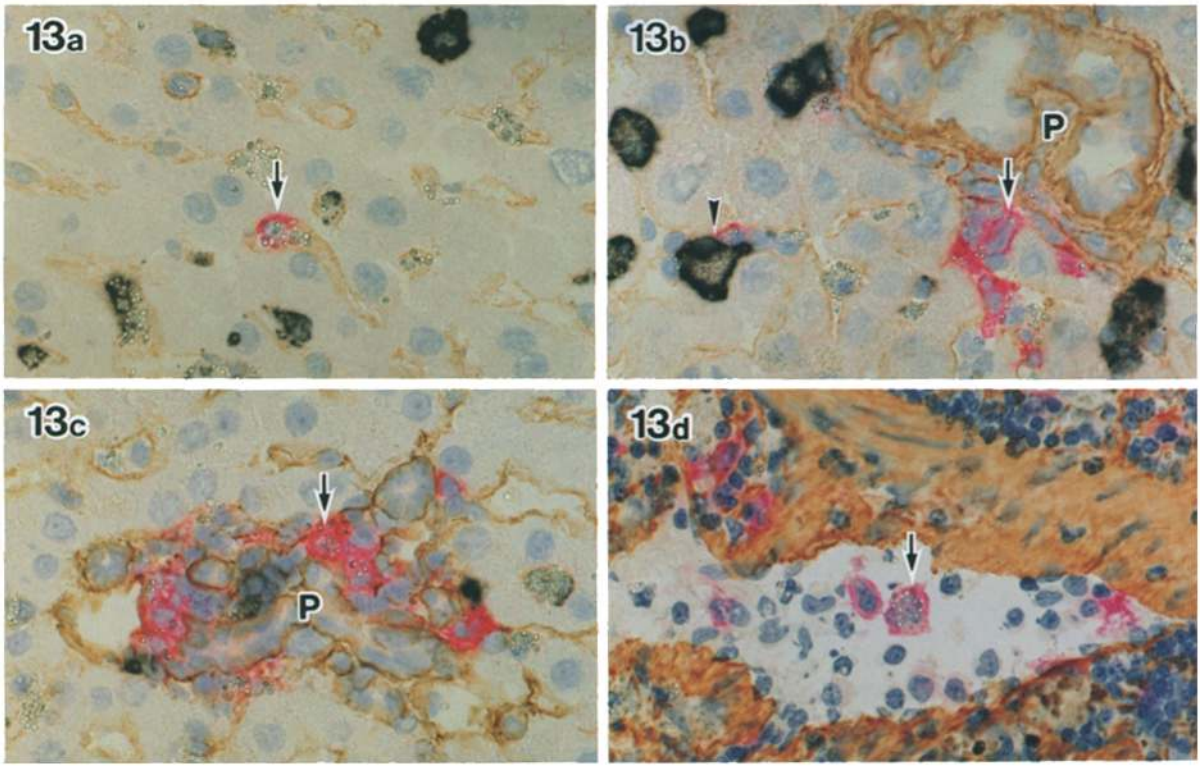
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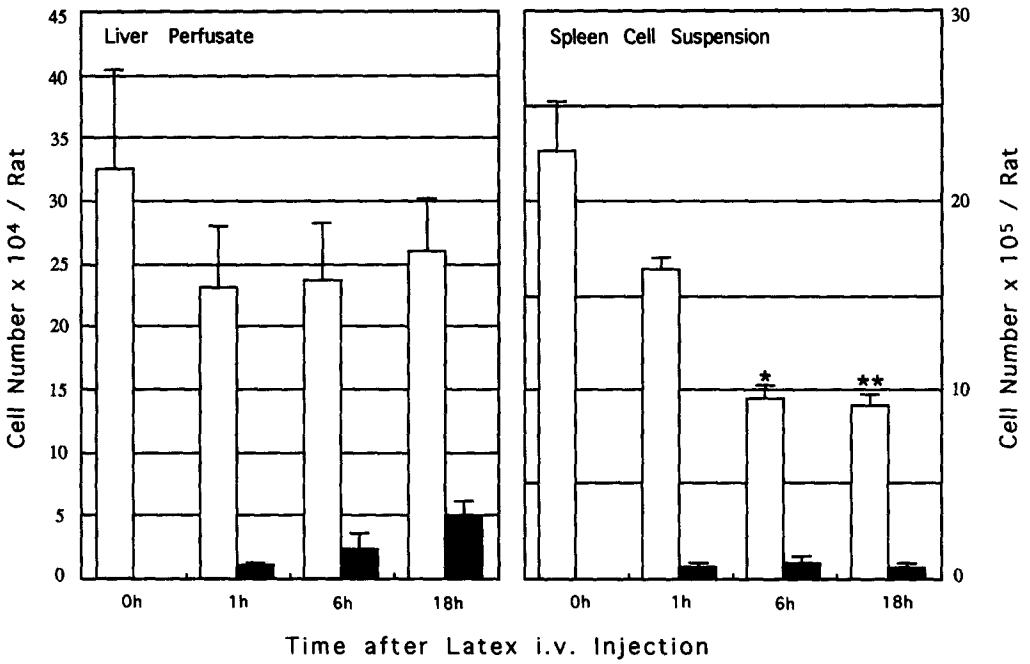

Figure 14. In vivo kinetics of total OX62$^+$ (*white column*) and OX62$^+$ latex-laden (*black column*) cells in the liver perfusate and spleen cell suspensions. Note a significant decrease of total OX62$^+$ cells in the spleen 6 ($^*P = 0.008$) and 18 h ($^{**}P = 0.007$) after latex injection. Data are expressed as mean ± SE. Bars represent SE. Three to six rats per group were examined.

lymph, although their allostimulating capacity is competent. It is widely accepted that DC are end-stage cells, incapable of cell division, since mitotic figures or DNA synthesis for cell division have never been seen in DC of peripheral lymph and the gut lamina propria or in interdigitating cells (2). Carbon-laden HX-TDL ended up in the celiac nodes (10), and the BrdU feeding study revealed that most of them did not proliferate after ingestion of particles. Together, these results suggest that the particle-laden DC are immature end-stage cells recently produced by the terminal division of precursor cells.

DC at migration stage (1), e.g., lymph veiled cells, rarely phagocytose particles (25; this study). The fact that the particle-laden DC became nonphagocytic after entering the hepatic lymph provides evidence that the endocytotic activity of DC is downregulated in vivo. This may imply that the particle-laden DC have differentiated to achieve a migratory function (1). The mechanism that turns off the phagocytic activity is unclear, but it may be autochthonously downregulated as they age. Some cytokine signals such as TNF-α, which induces migration of Langerhans cells to draining LN in mice (26), or GM-CSF (27) might accelerate this downregulation. This observation, when considered in the context of the BrdU feeding and cytological study, strongly suggests that the majority of particle-laden DC are recently produced cells that have a phagocytic activity at an immature stage during or within several days after the terminal division, and the phagocytic activity is downregulated at a migratory stage. In this respect, phagocytic activity of immature DC has previously been suggested (4, 5, 27). On the other hand, the particle-laden DC are cytologically immature cells, which usually do not appear in hepatic lymph. This may indicate a premature migration of the particle-laden DC into lymph, which is possibly induced by the phagocytic event.

The time kinetic study of OX62$^+$ latex-laden cells indicated that the particle-laden DC migrated from the sinusoidal area to the portal area, being in opposite direction to

Figure 12. In vivo localization of OX62$^+$ cells in the normal liver. Triple immunostaining with ED2 (*black*), OX62 (*red*) and anti-type IV collagen (*brown*). (*a*) Under low magnification, ED2$^+$ cells (Kupffer cells) are distributed diffusely, whereas OX62$^+$ cells are mainly found in the portal area (*P*) and fewer in the sinusoidal area (*S*) or the hepatic vein area (*V*). (*b*) At a higher magnification of the portal area, several OX62$^+$ cells are seen in the connective tissue surrounding the portal triads. Bile ducts (*B*) and the portal vein (*PV*) are indicated. (*c*) In the sinusoidal area, two OX62$^+$ cells (*arrows*) are located in the sinusoidal lumen, which is outlined by type IV collagen staining. Beneath the central vein (*CV*), a part of the hepatic vein area, an OX62$^+$ cell (*arrowhead*) is found. A close association between OX62$^+$ and Kupffer cells (ED2$^+$) is often observed (*arrows* in *b* and *c*). ×118 (*a*); ×420 (*b* and *c*).

Figure 13. In vivo localization of OX62$^+$ latex-laden cells. Triple immunostaining of the liver (*a–c*) and double immunostaining of the spleen (*d*) after intravenous injection of latex. At 1 h (*a*), OX62$^+$ latex-laden cells (*red, arrow*) were restricted to the sinusoidal area, but subsequently appeared in the portal area (*P*) at 6 h (*b*) with a further slight increase in their frequency at 18 h (*c*). Other latex-laden cells in the liver are Kupffer cells (ED2$^+$, *black cells*), monocytes, or PMN. Also note a close association between the OX62$^+$ cell and Kupffer cell (*b, arrowhead*). In the spleen (*d*), OX62$^+$ latex-laden cells (*red, arrow*) were found in the red pulp sinus 1 h after injection. ×420.

the overall blood flow, then translocated to the celiac nodes via the hepatic lymphatics (descending lymphatics; 28). The particle-laden DC in the sinusoidal area may have passed through the space of Disse to the connective tissue stroma of the portal area, then entered the initial lymphatic ducts located there (10, 28). In contrast, lymph accumulated in the hepatic vein area may enter ascending lymphatics, which are less developed than descending lymphatics, and drain into the intrathoracic nodes either deeply along with the inferior vena cava or superficially via diaphragmatic lymphatics (28). The parathymic nodes are regional nodes of the peritoneal cavity and diaphragm (29). Appearance of a few $OX62^+$ latex-laden cells in the hepatic vein area and in the parathymic nodes, therefore, may indicate an existence of this minor route.

The site of particle capture is still unknown, however, it may be either in the liver interstitium close to blood vessels or within blood. The initial appearance of $OX62^+$ latex-laden cells in the sinusoidal area, in the liver perfusate, and in spleen cell suspension suggests that the particle-laden DC may be recruited to the liver from systemic circulation. This is supported by a report that intravenously injected rat DC readily translocate to the hepatic lymph and then to the celiac nodes (30). Microvasculatures in the body such as the liver sinusoid and the portal system, including the splenic open circulation, constitute the blood marginating pool (31) where blood flows slowly, and some blood cells attach loosely to the vascular endothelium. The marginating pool contains a significant number of activated monocytes (32), both $OX62^+$ particle-free and particle-laden DC (this study) and NK cells (18), whereas much fewer numbers of the same cell populations are lodged in the peripheral blood (circulating pool). Therefore, it is probable that the particle-laden DC might have first ingested particles in the marginating pool and then performed blood–lymph translocation from the sinusoid. A significant decrease in number of total $OX62^+$ cells in the spleen cell suspension 6 and 18 h after latex injection and a presence of $OX62^+$ latex-laden cells in the splenic vein, which directly pours into the portal vein, indicates that a site of particle capture may be at least partly within the spleen. Brenan and Puklavec (14) have also observed a considerable number of $OX62^+$ cells in the splenic red pulp in addition to the thymus–dependent area of the white pulp. These cells may correspond to lymphoid DC in mice that can be isolated from the spleen (1) and also to $OX62^+$ cells isolated from the spleen in the present study. Alternatively, some particle-laden DC may correspond to resident DC in the liver interstitium. Resident DC are considered to translocate to hepatic lymph as ordinary particle-free DC in a steady state (8). Since there was no significant decrease in number of total $OX62^+$ cells in the liver sections, and output of ordinary particle-free DC in hepatic lymph did not change after particle injection, only small fractions of particle-laden DC, if any, may be derived from resident DC. We also cannot exclude the possibility of the particle capture by resident DC in the perivascular area of the bone marrow because of the finding of a few $OX62^+$ particle-laden cells in that area.

Frequent observation of close association of DC and Kupffer cells may imply some unknown interactions between two cell types. Association between NK and Kupffer cells is also reported (18). Since the particle-laden DC are $ICAM1^+$ and Kupffer cells are mostly $LFA1\alpha^+$, DC could attach to Kupffer cells via complementary binding of these adhesion molecules. One possibility is that the particle-laden DC in the blood marginating pool may perform the blood–lymph translocation by attaching to Kupffer cells via these adhesion molecules, and then entering the space of Disse, which is continuous with the tissue space in both the portal and hepatic vein areas.

The site for the terminal division of DC precursors may be mainly the bone marrow (1, 2), whereas, proliferating precursors must be situated in or close to the blood vessels if some DC are to phagocytose intravascular free particles simultaneously with or just after the terminal division, as suggested in the BrdU study. It is therefore speculated that some proliferating precursors might also exist at the site of particle capture, namely, at the blood marginating pool. Demonstration of proliferating DC progenitors among the liver nonparenchymal cells (33) may support this possibility.

In conclusion, the data presented here demonstrate that particle-laden cells in the rat hepatic lymph are relatively immature DC and that most of them are less than several days old. They bear a phenotype of rat DC plus some markers suggesting recent phagocytic activity, and they have a strong stimulating capacity in the primary allogeneic MLR. They carry out lymph-borne translocation from the portal tract of the liver to the paracortex of regional celiac nodes after phagocytosis of intravenously injected particles. They possess a temporary but obvious phagocytic activity in vivo, during or within several days after the terminal division, and the activity is downregulated at a migratory stage when they translocate to the lymph. The site of particulate capture may be mainly in the blood marginating pool. These cells may phagocytose and process microorganisms such as bacteria and fungi in situ and transfer and present antigenic information within the regional lymphoid tissues. The site of the terminal division, antigen-presenting capacity for phagocytosed particulate antigens, and variation in phagocytic activity of DC in different tissues and in different species are currently under study.

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