T CELL-DEPENDENT MAST CELL DEGRANULATION AND RELEASE OF SEROTONIN IN MURINE DELAYED-TYPE HYPERSENSITIVITY*

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In a previous study of murine delayed-type hypersensitivity (DTH)[1] we suggested that the release of serotonin (5-hydroxytryptamine) (5-HT) by local tissue mast cells was required for the elicitation of these responses (1). We noted that DTH was preferentially elicited in those cutaneous sites that were especially rich in 5-HT-containing mast cells, and that administration of the 5-HT-depleting agent, reserpine, abolished the ability to elicit DTH. Because the effect of reserpine could be prevented by administration of a monoamine oxidase (MAO) inhibitor, the attribution of the action of reserpine to depletion of a monoamine (such as 5-HT) was confirmed (1). Subsequent studies, which have employed two additional and independent pharmacological maneuvers (5-HT tachyphylaxis of the vasculature [2] and the use of 5-HT antagonists[2]), have been consistent with our hypothesis that in murine DTH, specifically sensitized T cells interact with tissue mast cells leading to the release of 5-HT, which acts on the local endothelium allowing the emigration of bone marrow-derived leukocytes from the intravascular to the extravascular space (3). The current study was undertaken to test our hypothesis by determining if we could obtain direct evidence for activation of mast cells to release 5-HT during murine DTH.

Materials and Methods

Immunization and Skin Testing (Challenge). BDF_1 mice (The Jackson Laboratory, Bar Harbor, Maine) received an optimal immunization for the elicitation of DTH (0.2 ml of 0.01% sheep erythrocytes [SRBC]; [Colorado Serum Labs, Boulder, Colo.] diluted in sterile saline) (4). 4 d later, the animals were tested for DTH by injecting 0.03 ml of 20% SRBC into the ventral surface of a rear footpad. The thickness of the footpad was measured with a micrometer before and after challenge with antigen, and the percent change calculated (5). Immunized controls were challenged by footpad injection of 0.03 ml saline. Nonimmunized controls received an intravenous injection of saline on day 0 and were tested by footpad injection of 20% SRBC on day 4. In some experiments, contact hypersensitivity reactions were elicited in the skin of the

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[1] *Abbreviations used in this paper:* DNFB, dinitrofluorobenzene; DTH, delayed-type hypersensitivity; 5-HT, 5-hydroxytryptamine (serotonin); MAO, monoamine oxidase; SRBC, sheep erythrocytes.

[2] Askenase, P. W., C. M. Metzler, and R. K. Gershon. Dependence on vasoactive amines for the localization of leukocytes in DTH reaction sites and lymph nodes. Manuscript in preparation.

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ear by applying a drop of 0.2% dinitrofluorobenzene (DNFB) (Eastman Organic Chemicals, Eastman Kodak Co., Rochester, N. Y.) in 4:1 acetone:olive oil 5 d after immunization by contact painting with DNFB (1).

Cell Transfer of DTH and Cyclophosphamide Treatment. The harvest of spleen cells, fractionation on nylon-wool columns, cell transfers of DTH, and treatment with cyclophosphamide were done as described previously (4–6).

Electron Microscopy. Mice were killed at 6 or 18 h after challenge. Additional immunized mice were used to verify that strong 24- and 48-h macroscopic reactions occurred after SRBC challenge. Mice were killed by cervical dislocation, their feet were then severed, and quickly immersed in Karnovsky's fixative (1% gluataraldehyde [EM Sciences, Fort Washington, Pa.] and 2% formaldehyde generated from paraformaldehyde buffered with 0.1 M sodium cacodylate [ICN, Life Sciences Division, K and K Laboratories, Plainview, N. Y.] at pH 7.3). Skin from the dorsal foot was excised, cut into 1-mm pieces, and placed for 4–5 h at room temperature in the fixative that contained sucrose which was added to bring the final osmolarity of the buffer to 310 mosm/liter. Tissues were then washed overnight in 0.1 M sodium cacodylate buffer that contained sucrose. The osmolarity of the buffered fixative was found to be very critical in preserving the ultrastructure of mast cells in the skin of the foot of mice. Specimens were post-fixed in buffered 1% OsO_4 (Polysciences, Warington, Pa.), stained en bloc with uranyl acetate (Fisher Scientific Co., Pittsburgh, Pa.), dehydrated in a graded series of ethanols, and embedded in Epon 812 (Ladd Research Industries, Inc., Burlington, Vt.). Sections were stained with uranyl acetate and lead citrate and examined in a Zeiss EM10 (Carl Zeiss, Inc., New York) or JEOLCO JEM 100C (JEOL USA, Electron Optics Div., Medford, Mass.) electron microscope.

Drugs Affecting Vasoactive Amines. In some experiments, mice were injected intraperitoneally with the monoamine-depleting drug reserpine (5 mg/kg) (Serpasil for parenteral injection; Ciba-Geigy Corp. Pharmaceuticals Div., Summit, N. J.) 18 h before skin testing. In other experiments, cyproheptadine (a competitive antagonist of 5-HT and histamine; Merck Sharp & Dohme Research Labs, West Point, Pa.) was injected subcutaneously at a dose of 0.3 mg in 0.5 ml saline at 6-h intervals, beginning 3 h before challenge with antigen.

Vascular Tracers

FLUORESCEINATED DEXTRANS (LIGHT MICROSCOPY). Fluoresceinated dextran (150,000 dalton; Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.), 80 mg/ml saline in a dose of 0.2 ml, was administered intravenously. Mice were injected with the tracer 18 h after challenge and were then killed 15, 30, and 60 min later. The animals did not exhibit any symptoms of distress after injection of this tracer. Tissues were excised and fixed in 0.1 M phosphate-buffered saline that contained 5% formaldehyde. Tissues were cleared in glycerine for 48 h, mounted in glycerine on slides, and viewed with a Leitz fluorescence microscope (E. Leitz, Inc., Rockleigh, N. J.) equipped with vertical illumination, TK 400 dichroic beam splitting mirror, and a K520 suppression filter (E. Leitz, Inc.).

COLLOIDAL CARBON (ELECTRON MICROSCOPY). Mice were injected intravenously with colloidal carbon (Pelikan C11/143Ia; Gunther Wagner Co., Hanover, Federal Republic of Germany) that contained 100 mg of carbon/ml in a dose of 0.2 ml/100 mg of body weight, and were killed 1 min after injection. The dorsal portion of the foot was quickly excised and fixed and processed for electron microscopy as described above.

Radioautography. To prevent catabolism of injected [^{3}H]5-HT, mice were injected intraperitoneally with the MAO-inhibitor pargyline (100 mg/kg, Abbott Diagnostics, Diagnostic Products, North Chicago, Ill.). Mice were challenged by injection of SRBC into their footpads 2 h later. Animals received [^{3}H]5-HT (1.2 mCi/mouse, 16 Ci/mmol; New England Nuclear, Boston, Mass.) intraperitoneally 1 h before challenge and then were killed 6 or 18 h after challenge. The dorsal footpad skin was removed into formaldehyde-glutaraldehyde and processed as above into Epon. Radioautographs were made from 1-μm sections placed on glass slides that had previously been coated with an adhesive chromium-alum-gelatine mixture. Slides were coated with Ilford L4 photographic emulsion (Polysciences) by dipping and were exposed in light-tight boxes over drierite at 0–4°C for 1–2 wk. Radioautographs were developed with Kodak D-19 (Eastman Kodak Co., Rochester, N. Y.), fixed, stained with toluidine blue, and examined by bright-field and dark-field microscopy.

Results

Depletion of [^{3}H]5-HT from Mast Cells in DTH. In a previous study of normal mice, we found that in the skin of the footpads radioactive 5-HT was exclusively localized to mast cells (Fig. 1 of reference [1]). In the present study, radioautography was performed 6 and 18 h after footpad challenge of mice injected systemically with [^{3}H]5-HT before challenge to determine if changes in the content of 5-HT in mast cells occurred in DTH. 6 h after challenge with SRBC, there was comparable accumulation of silver grains over the cytoplasm of mast cells in control and immune mice (similar to Fig. 1 a and b). Other cell types in the dermis were not labeled, except for occasional intravascular platelets.

At 18 h, mast cells from controls were still completely covered with silver grains (Fig. 1 a and b), whereas immunized and challenged mice showed fewer silver grains (Fig. 1 c, d, e, and f). This revealed metachromasia in the granules that was not evident in heavily labeled controls. Some mast cells had a small number of residual silver grains over them, suggesting that these cells had not completely released their 5-HT content. Thus, light microscopic radioautography confirmed that 5-HT is released from dermal and hypodermal mast cells in DTH.

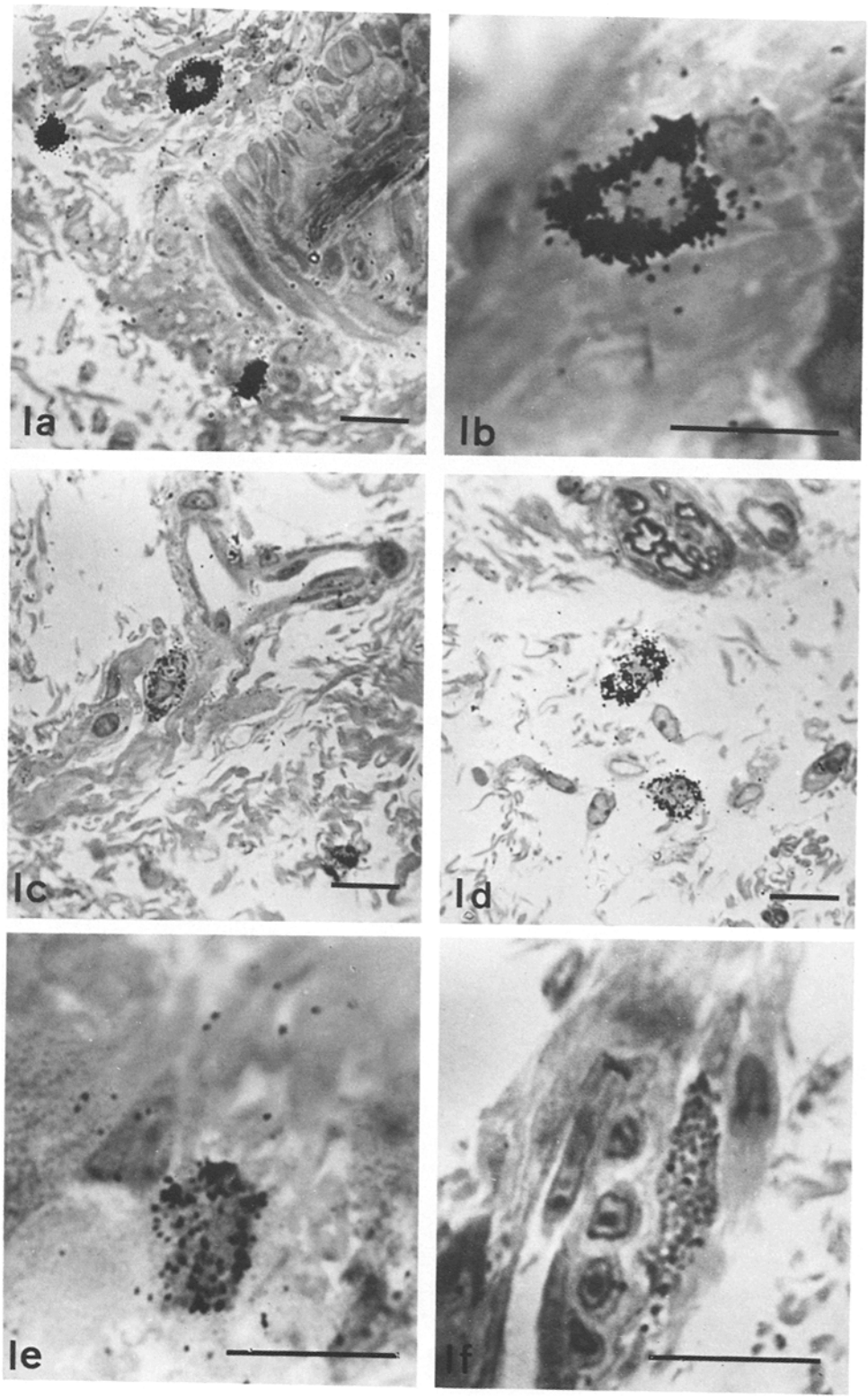
Ultrastructure of Mast Cells in the Footpads of Control Mice. Mast cells were frequently found in close proximity to blood vessels in the papillary and reticular layers of the dermis, as well as in the subcutaneous tissues. In nonimmune and unchallenged mice, mast cells were similar in appearance to those of nonimmune controls that were challenged with SRBC and examined 6 or 18 h later, and thus are described together.

Dermal mast cells were large (20–30 μm) and oval shaped with numerous short surface filopodia and characteristic dense cytoplasmic granules surrounded by a closely fitting plasma membrane (Fig. 2). The granules were highly osmiophilic and homogenous in shape and density, a feature characteristic of 5-HT-containing mast cells (7). A few granules were surrounded by a small perigranular space between the granule matrix and granule membrane. Some variation in the size and electron density of individual granules was observed, but disintegration of granules in mast cells from control mice was very infrequent (Table I). The filopodia at the surface of these mast cells were in close apposition to the cell surface membrane (Fig. 2).

Early Ultrastructural Alterations of Mast Cells in DTH. 4 d after immunization and 6 h after footpad challenge with SRBC, no increase in footpad thickness was detectable. However, ultrastructurally, an early and consistent change in local mast cells was surface activation that consisted of the extension of filopodia (Figs. 3 and 4 a). At 6 h after challenge, many mast cells that demonstrated surface activation showed no alteration in appearance of granules. These granules were rather closely housed by the perigranular membrane; however, a number of cells showed signs of granule

FIG. 1. Light microscopic radioautographs of the dermis of the skin of the footpads of mice injected with [^{3}H]5-HT. The animals were also injected with the monoamine oxidase inhibitor pargyline to prevent catabolism of [^{3}H]5-HT. The skin was removed 18 h after injection of SRBC to nonimmunized or immunized mice.

(a and b) Nonimmune controls; (c, d, e, and f) immune mice. Note the much lower intensity of labeling of the mast cells from the immunized animals. In the controls that were similarly challenged with SRBC, the overlying silver grains are so numerous that the metachromasia of the mast cell granules is obscured. In immunized and challenged mice, the reduction in radiolabeling allows the granules to be seen (compare b and f). All mast cells are heavily labeled in controls. The degree of depletion of labeling of mast cells after challenge of immunized mice is variable (compare c and d with e and f). The marker bar in each figure is 25 μm in length.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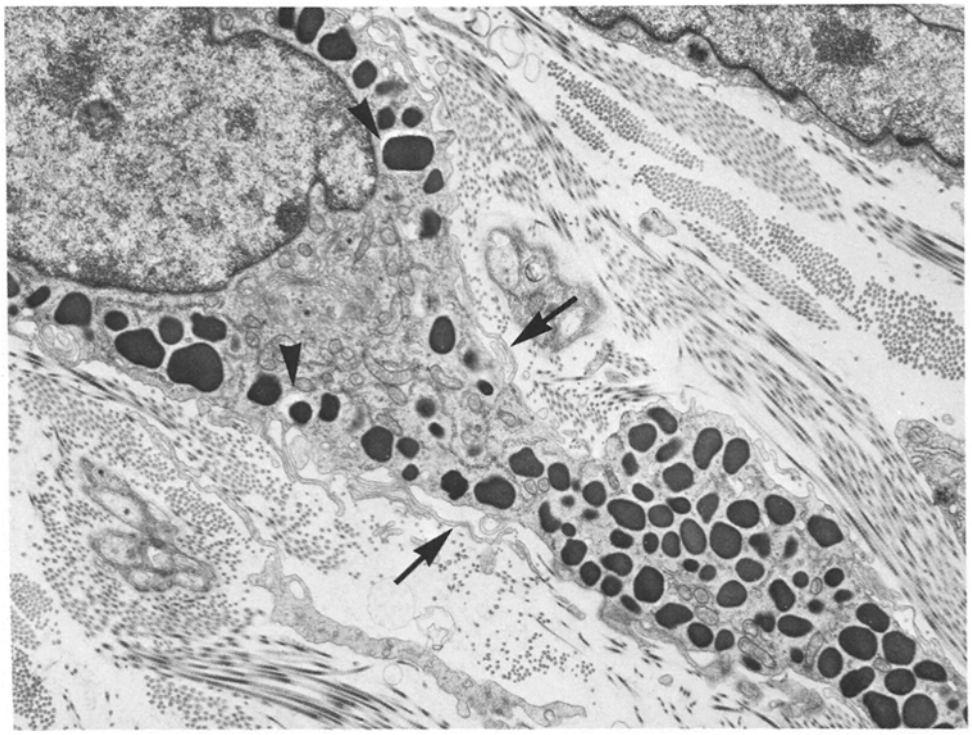

FIG. 2. An electron micrograph of a dermal mast cell from the footpad skin of an immunized but unchallenged mouse. The electron-dense granules of the cell are stained homogeneously. Occasionally, perigranular spaces can be seen (arrowheads). The surface filopodia (single arrows) appear flaccid and their distal tips lie in close proximity to the plasma membrane of the mast cell. × 7,200.

TABLE I

*Morphometry of Degranulating Mast Cells in DTH**

Electronmicrograph from	No. of mast cells counted	Total No. of granules/ mast cell ± SE	No. of normal granules‡/mast cell ± SE	No. of granules/mast cell demonstrating dissolution§ ± SE
Immunized and SRBC-challenged mice	25	52.4 ± 5	13.4 ± 2.6	39.0 ± 5.4
Control mice (immunized and not challenged or challenged only)	25	67.7 ± 3	67.0 ± 3.2	0.72 ± 0.2

* Granules were counted and evaluated in mast cells that had a cross section of their nucleus included in the plane of section and that were photographed at a magnification of 8,000.

‡ The matrix of normal granules is homogeneously stained.

§ Dissolution of homogenous matrix of granule core, leading to heterogeneity of staining.

release. These included fusion of granules with each other and with the plasma membrane. The affected granules showed dissolution of their matrices so that the homogeneous osmiophilia of the granular matrix was replaced by a coarse stippling of material. (Fig. 3, inset). These changes, which were seen more extensively at 18 h (Table I) (see below), were noted in less than one-half the mast cells examined at 6 h, and these cells were located principally in the deep dermis.

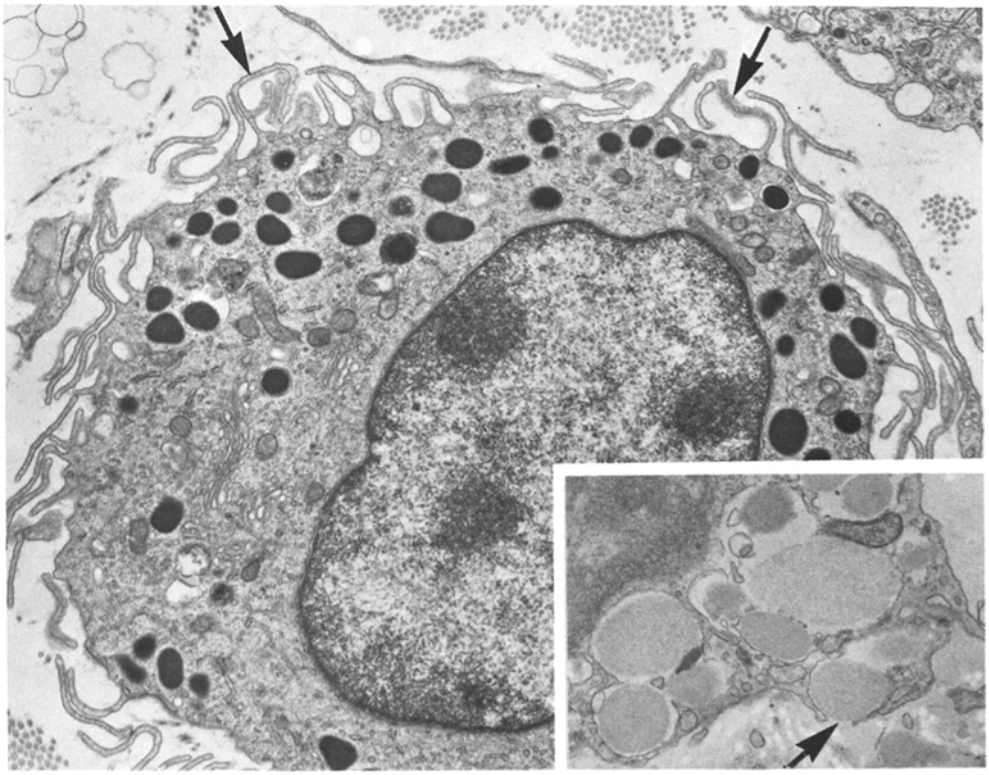

FIG. 3. A dermal mast cell from the footpad skin of an immunized mouse obtained 6 h after challenge with SRBC. Note the erection of the surface filopodia (arrows). The mast cell granules generally retain their homogeneous staining. × 11,000. Inset: occasional mast cells, even at 6 h, show fusion of granules with each other and apparent exocytosis (arrow). Inset × 18,000.

Later Stages of Mast Cell Activation in DTH. Almost all mast cells were in various stages of obvious degranulation in skin obtained from immunized mice 18 h after footpad challenge (Table I). Degranulation seemed to proceed in a sequence of steps (Figs. 4 and 5). At first, the granules enlarged and became reduced in electron density (dissolution). The plasma membrane of the granules sometimes showed even greater expansion, resulting in an open space between the granule and perigranular membrane (Fig. 4). Granule enlargement was followed by coalescence of granules with eventual fusion of their perigranular membranes (Figs. 4b and 5a). These fused granules had a matrix that exhibited swelling, and even greater loss of electron density (disintegration) (Fig. 5). As the granule matrix disintegrated, discrete particles replaced the formerly homogenous electron density. Occasionally, fusion of the granule membrane with plasma membrane was observed (Fig. 5b). The coalescence of granules resulted in great expansion of apparent diameter of granules, led to a decrease in their number (Table I), and to the formation of long-branched intracellular channels.

Not all granules of individual mast cells were at the same stage of dissolution. Granules with an electron density between that of normal granules and that of altered granules could at times be seen (Figs. 4 and 5). These were found close to altered granules as well as close to completely normal-appearing granules. The altered

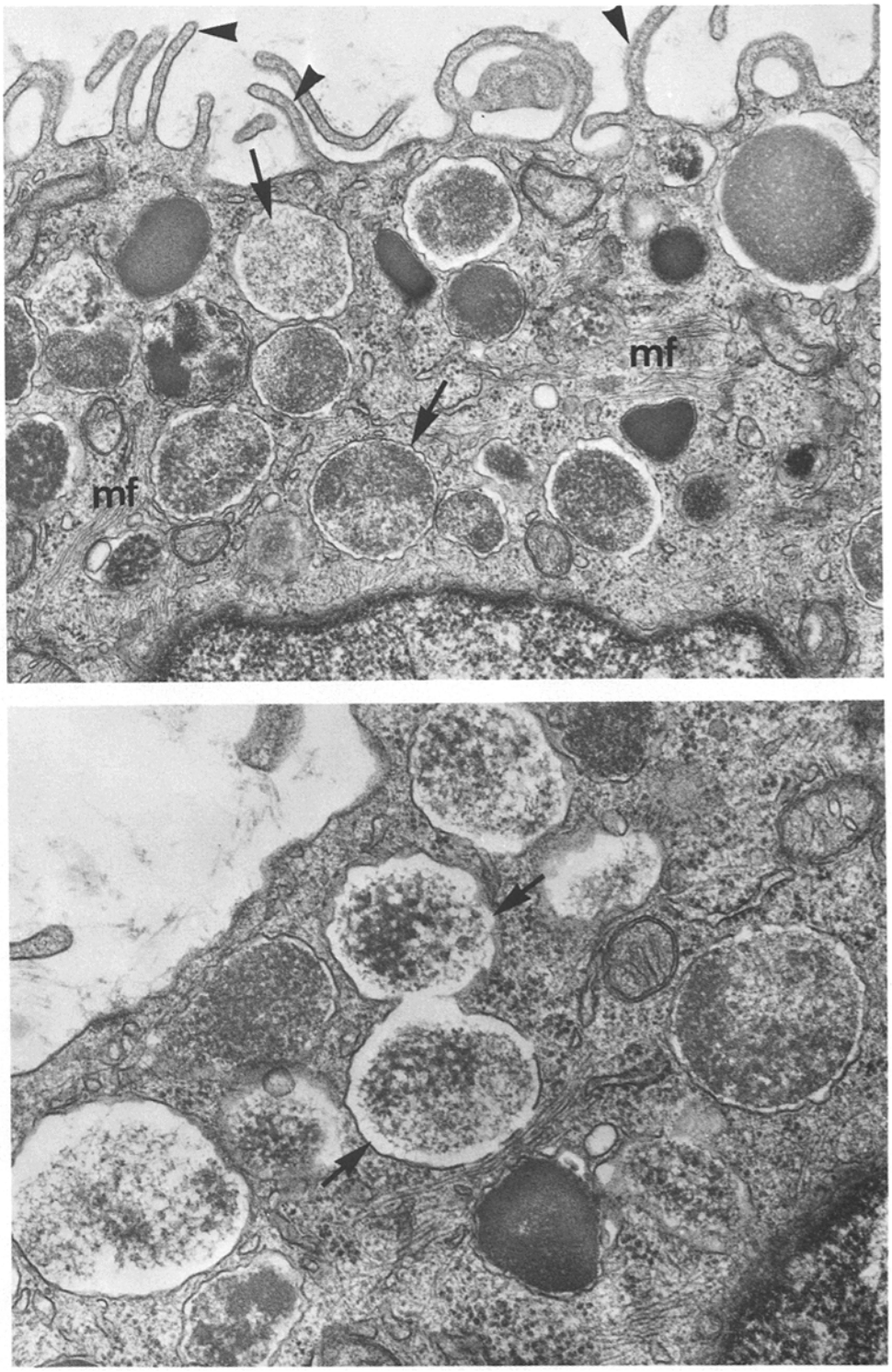

FIG. 4. Electron micrographs of portions of the cytoplasm of mast cells from the dermis of the skin of immunized mice obtained 18 h after challenge with SRBC. (a) Several granules have lost the homogeneous electron density of the cores (arrows). Erection of surface filopodia (arrowheads) is still evident, and the plasma membrane surrounding the cell remains intact. Numerous microfilaments (mf) and vesicles are present in the cytosol. × 28,800. (b) Two granules (arrows), in the cytoplasm of the same cell shown in (a) have fused with one another. Dissolution of the matrix of these granules is pronounced. × 45,000.

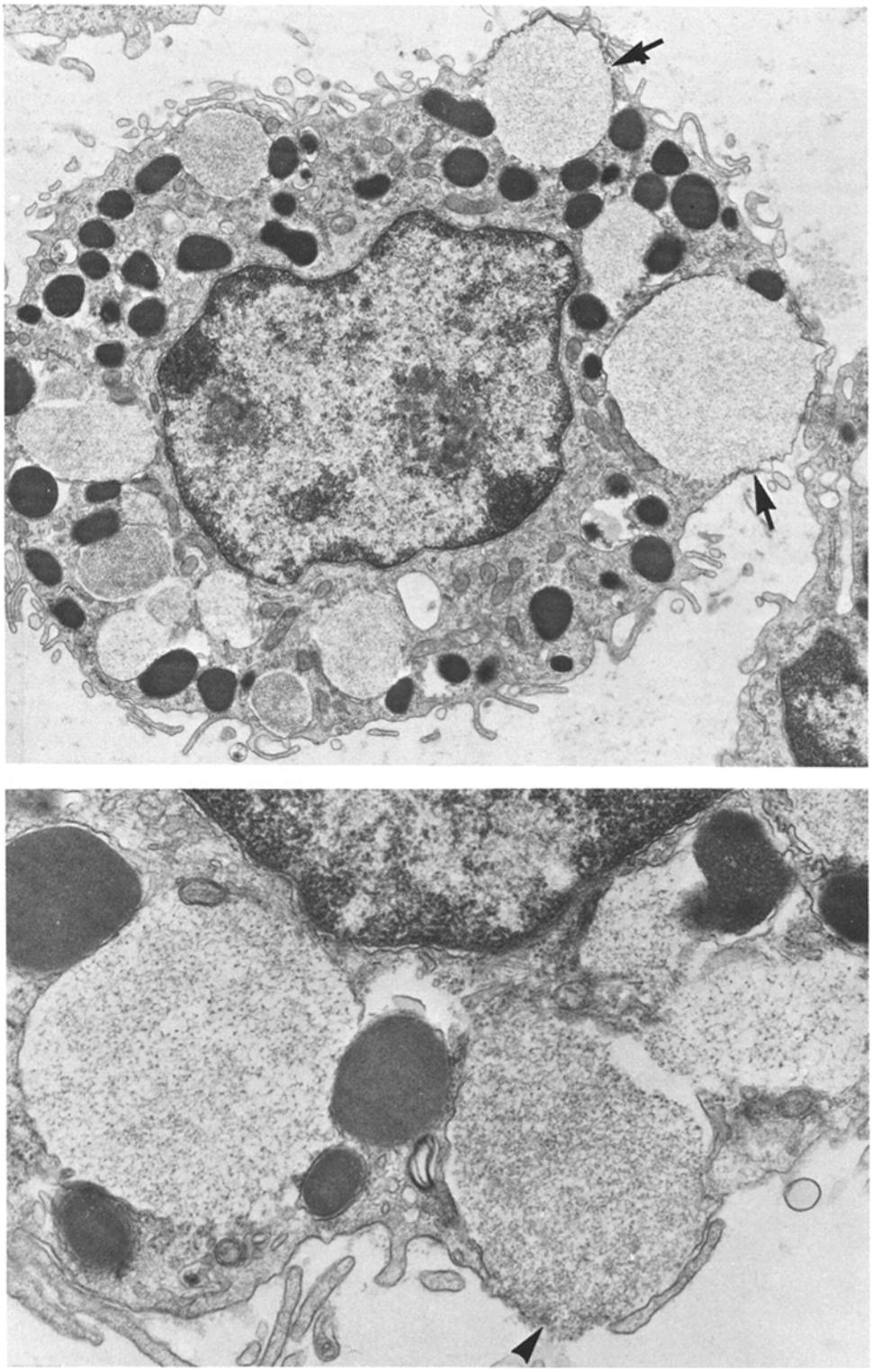

FIG. 5. Electron micrographs of mast cells from the dermis of the footpad skin of immunized mice obtained 18 h after challenge with SRBC. (a) Fusion of granules has produced enlarged cavities. The matrix of these granules shows advanced dissolution. Only some of the granules are affected; however, there is no restriction of the process to any given region of the cell. The largest cavities (arrows) tend to occur near the plasma membrane. Extension of surface filopodia is again notable. × 11,340. (b) Apparent exocytotic fusion of a mast cell granules with the plasma membrane (arrowhead). × 28,800.

granules did not seem to occupy a fixed position in the cell. Such granules were found at the periphery as well as toward the center of the cell. Even in occasional mast cells that had undergone nearly complete degranulation, the nucleus and cytoplasmic membrane remained intact and the cell retained its original shape, sugesting that this was degranulation by exocytosis, rather than cytolysis; but a few mast cells were noted to be disrupted by a cytolytic process. Actual extrusion of altered granules into the extracellular space was infrequently observed in DTH.

Ultrastructure of Blood Vessels in Control Mice. Postcapillary venules in the dermis and hypodermis of the footpad skin of challenged normal mice, or in immunized but not challenged mice, contained high endothelial walls held together by tight junctions (Fig. 6). A basal lamina, 50–60 nm thick, adhered to the endothelial wall and formed a continuous layer around the endothelium, which split to enclose the pericyte but separated the venule from the surrounding connective tissue. Occasionally lymphocytes, leukocytes, or erythrocytes were observed in the lumen of postcapillary venules in control animals, but these cells were not observed outside of the vessel.

Vascular Changes after Challenge of Immune Mice. Observations of postcapillary venules from the dermis and hypodermis of footpads of animals that were immunized and then challenged with SRBC 4 d later showed definite changes in endothelial cells.

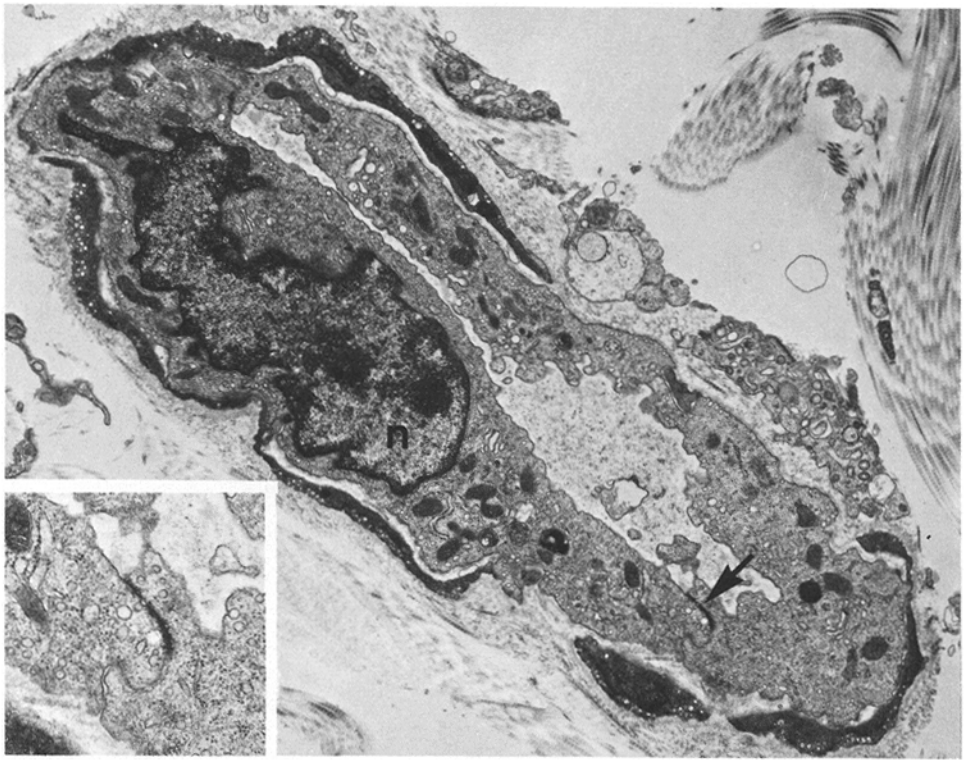

FIG. 6. Postcapillary venule from the dermis of the footpad skin of an immunized but unchallenged mouse. Tight junctions (arrow; see inset) appear in the region of contact between adjacent cells. The nucleus (n) is flattened and makes the cell surface bulge slightly into the lumen. Processes of pericytes are in close proximity to the endothelium and are surrounded by the basement membrane of the venule. × 7,600. Inset × 15,600.

These changes were quite prominent 18 h after testing. Favorable planes of section revealed separations in the intercellular junctions of endothelial cells of postcapillary venules (Figs. 7 and 9). This resulted in wide gaps (0.1–1.0 μm) between cells. At times, endothelial cell cytoplasm was reduced to a thin membrane, resembling that of the endothelia of fenestrated capillaries. In a fortunate plane of section, leukocytes were seen to be migrating by diapedesis through these endothelial gaps (Fig. 9).

Topology of the Vasculature As Envisaged by Fluoresceinated Dextrans. To examine the potential effect of mast cell-released mediators on the local vasculature, mice were injected intravenously with fluoresceinated dextran. This permitted viewing by fluorescence microscopy of the entire skin of the ear under various experimental conditions, with the vascular tree intact. This tracer was found particularly suitable for examining possible vascular leaks, because the dextran particles are small enough not to be held back by the filtering effect of the endothelial basal lamina, and thus extravascular accumulation outside of the blood vessels was made evident by diffuse green fluorescence.

Fig. 8a shows the tracer confined to the blood vessels in mice that were contact sensitized with DNFB but not challenged in the ear skin with DNFB. The fluoresceinated dextran outlined the lumena of veins, venules, postcapillary venules, and capillaries, but was not seen outside of blood vessels. A variety of blood cellular elements were noted to permeate the vascular lumen. A similar result was obtained 18

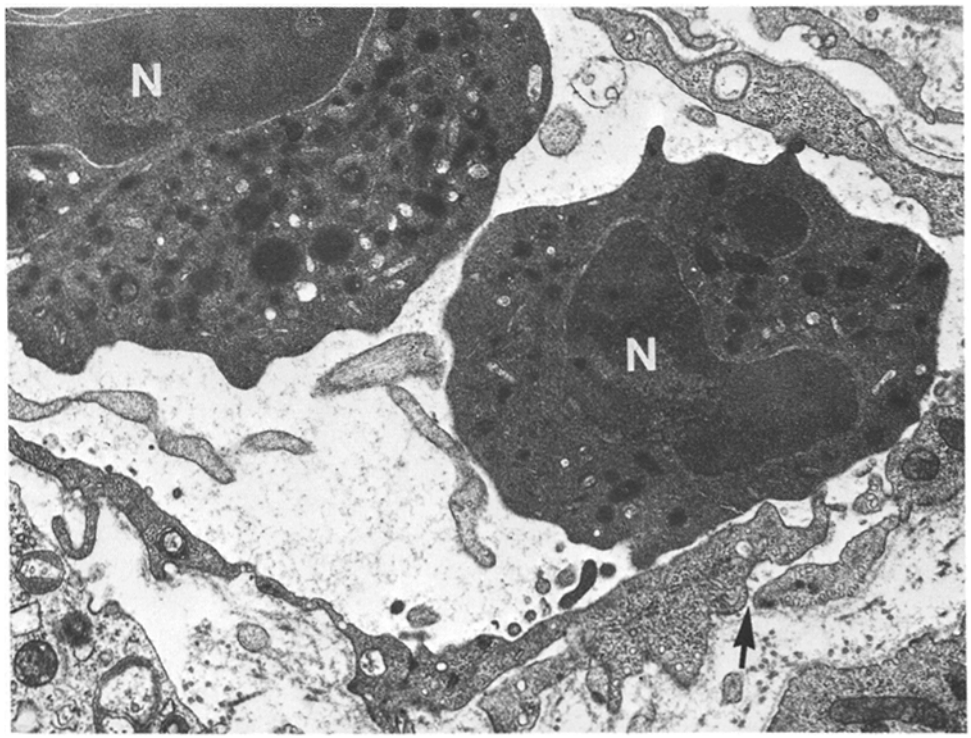

FIG. 7. Two neutrophils (N) occupy the lumen of a postcapillary venule in the dermis of the footpad skin of a mouse that was immunized and killed 18 h after challenge with SRBC. One of the neutrophils extends pseudopods toward the endothelial wall. Separation of the junction between two endothelial cells can be seen (arrow). × 22,500.

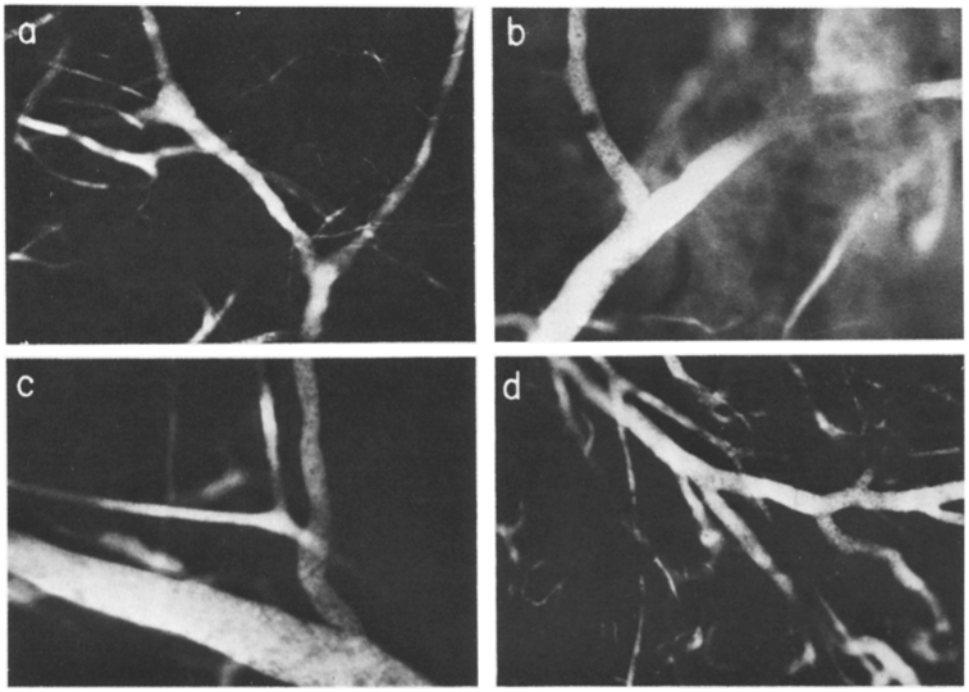

FIG. 8. Fluorescence micrographs from the dermis of the ear skin of mice contact sensitized with DNFB 5 d previously and injected intravenously with fluoresceinated dextran 17 min before killing. (b, c, and d) Micrographs from animals challenged 18 h previously by contact painting with DNFB, whereas (a) is from an unchallenged mouse. (c and d) Micrographs from animals treated with reserpine and cyproheptadine, respectively. Extravasation of the vascular tracer into the extravascular space is noted in the immunized and challenged animal (b), whereas the tracer is strictly confined within the vessels in immunized, but unchallenged animals (a), and also in immunized and challenged animals that were treated with reserpine (c) or cyproheptadine (d). All × 80.

h after DNFB challenge to the ears of nonimmune mice. However, when tracer was injected into mice that were immunized and their ears skin tested with DNFB 5 d after sensitization, a large amount of extravasation of fluoresceinated dextran was evident outside of the blood vessels (Fig. 8 b). The extravascular appearance of the tracer was seen as early as 1 min after the injection of tracer, which was performed 18 h after challenge. At some points, permeation of extracellular spaces with tracer was so severe as to obscure visualization of the neighboring blood vessels (Fig. 8 b). When tracer was injected into mice that were similarly sensitized and tested with DNFB, but which were injected with the monoamine-depleting drug reserpine or with the 5-HT antagonist cyproheptadine, there was no extravasation of tracer (Fig. 8 c and d). Thus, treatment of mice with reserpine or with cyproheptadine resulted in prevention of blood vessel leaks in contact hypersensitivity.

Ultrastructural Identification of Leaky Blood Vessels. To confirm that vascular leaks resulted from separation of endothelial cells, colloidal carbon was employed as a tracer that was visible at the ultrastructural level. Carbon particles were noted entirely confined to the lumen of postcapillary venules in immunized, but unchallenged, mice. In contrast, many carbon particles were found outside the endothelial lumen of postcapillary venules in footpad skin taken from immunized mice that were killed 5

min after colloidal carbon injection and 18 h after SRBC challenge (Fig. 9). In fact, many more particles were outside the endothelium than inside the vessel lumen. Diapedesis of cells through the open gaps were also seen at sites of penetration of tracer into the tissue (Fig. 9). The pericytes and the basal lamina served as a partial barrier to the further diffusion of carbon particles beyond the endothelial wall.

T Cell Dependence of Mast Cell Activation and Endothelial Gap Formation in DTH. Mast cell activation and endothelial cell changes seen in actively sensitized animals were also demonstrated in recipients of spleen cells from immunized donors (Table II). In further studies, cells used for transfers were fractionated with nylon-wool columns. The fraction of nonadherent non-immunoglobulin-bearing lymphocytes that transferred DTH reactions was also found capable of transferring to recipients the capacity for mast cell activation that included filopodial extension and degranulation (Fig. 10); and the characteristic gaps in the endothelium of postcapillary venules (Table II). It was concluded that the mechanism by which these ultrastructural changes occurred was transferable by T cells. To further rule out involvement of B cells in these changes, similar ultrastructural studies were undertaken of DTH reactions elicited in mice immunized with high doses of SRBC (0.5 ml of 50% SRBC; 1×10^9 cells) and pretreated with B cell-depleting doses of cyclophosphamide (200 mg/kg)

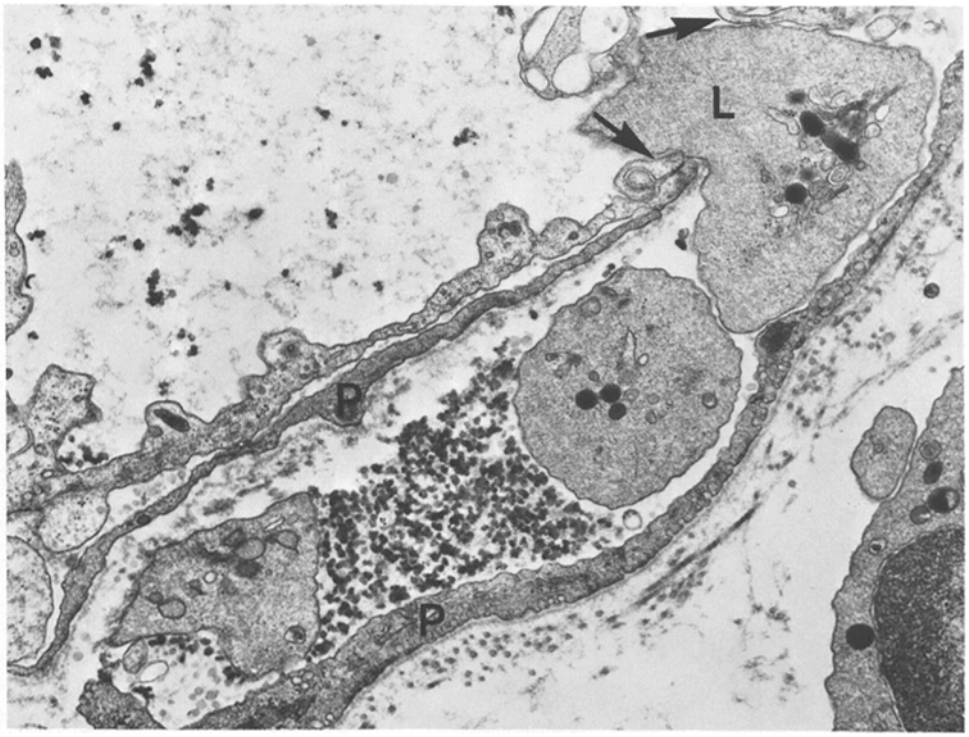

FIG. 9. A postcapillary venule from the footpad skin of an immunized mouse killed 18 h after challenge, and 5 min after injection of colloidal carbon. Parts of the foot process of a leukocyte (L) (probably a neutrophil) are seen leaving the vascular lumen through a gap that has opened between endothelial cells (arrows). Note that many of the carbon particles have entered the gap between endothelial cells; however, the carbon remains confined (probably by the basal lamina) and can be seen between the processes of pericytes (P). × 16,000.

TABLE II

T Cell Dependence of Mast Cell and Endothelial Cell Changes in Murine DTH

Group	Immunization	Footpad swelling at 18 h	Mast cell degranulation	Gaps at endothelial cell junctions
		%		
A	0.2 ml of 0.01 % SRBC intravenously (2×10^5 SRBC)	35	+	+
B	Recipients of unfractionated immune spleen cells	34	+	+
C	Recipients of unfractionated normal spleen cells	12	−	−
D	Recipients of nylon nonadherent sensitized spleen cells	25	+	+
E	0.5 ml of 50% SRBC (1×10^9 SRBC)	9	−	−
F	0.5 ml of 50% SRBC + cyclophosphamide 200 mg/kg at day −2	26	+	+
G	Nonimmune controls	10	−	−

Summary table of three separate experiments in which 6-wk-old BDF_1 male mice were actively sensitized (groups A, E, and F) by intravenous injection of SRBC at day 0 and were footpad tested with SRBC on day 4, or received spleen cells from actively sensitized (groups B and D) or normal donors (group C) (all transfers were at a ratio of one donor:one recipient) on day 4 and were footpad tested with SRBC on day 5. In each experiment there were five to six mice/group, and electron microscopy was performed on the footpad skin of three or four mice/group.

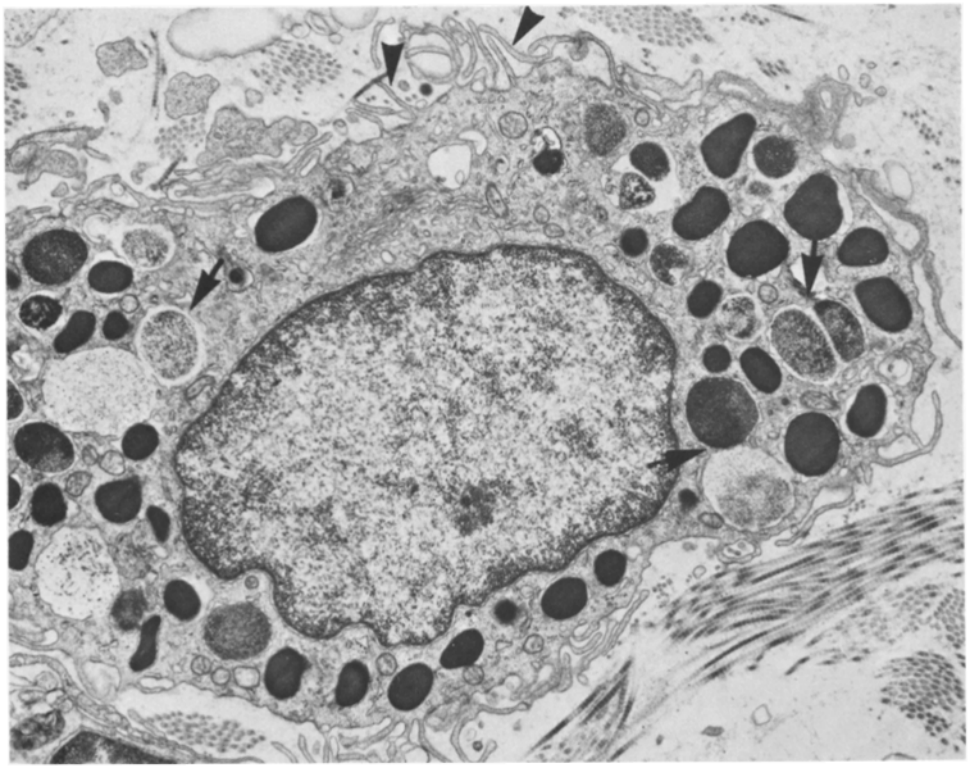

FIG. 10. A mast cell from the dermis of the footpad skin of a normal mouse that received an intravenous transfer of nylon-nonadherent splenic lymphocytes from mice immunized with SRBC. The animal was killed 18 h after challenge with SRBC. The cell shows erected surface filopodia (arrowheads). Dissolution and fusion of granules (arrows) are also evident. × 10,000.

(5). Similar to the DTH response of animals noted above, DTH responses of these animals (which had no detectable antibody or B cells) showed mast cell surface activation and degranulation and, also, the gaps between endothelial cells of postcapillary venules (Table II). Thus, it was concluded that these changes were T cell, and not B cell, dependent in DTH.

Discussion

Three independent pharmacological treatments employed in previous studies and discussed earlier have indicated that local release of 5-HT is required to elicit DTH in mice (1–3). This evidence indicated that endogenous release of 5-HT probably occurs in DTH, and that the site of action of released 5-HT may be the local vasculature. It seemed likely that the action of 5-HT is to alter local endothelial cells to permit diapedesis of bone marrow-derived leukocytes.

The current study has demonstrated that local mast cells are activated during the evolution of DTH responses and that this activation is accompanied by exocytosis and consequent release of 5-HT. Thus, the surfaces of the mast cells exhibit extension of filopodia and their granules can be seen to fuse with one another and with the plasma membrane during DTH to create tortuous channels within the cells. These channels are open to extracellular fluid, and release of granular content is made apparent by dissolution of granular matrices. Although mast cells that were degranulating by exocytosis exhibited surface activation of filopodia, as previously noted in anaphylactic antibody-mediated degranulation of basophils (8, 9), massive extrusion of granules from the cells, as occurs in anaphylactic degranulation of basophils or in rat mast cells (10), was rarely observed.

Radioautographic experiments with [^{3}H]5-HT showed that 5-HT is depleted from skin mast cells in DTH, confirming its release during exocytosis (Fig. 1). Moreover, there is also an alteration of postcapillary venules during DTH. This change, involving the formation of gaps between junctions of endothelial cells, is identical to the previously described action of 5-HT in this species (11). Ultrastructural studies revealed that colloidal carbon tracer and leukocytes moved out of the vessels through these endothelial gaps (Fig. 9). Studies with fluorescein-conjugated dextran demonstrated that these vascular changes are indeed dependent on release of 5-HT. Vascular permeability in DTH was blocked by pretreatment with reserpine (to deplete 5-HT), or by cotreatment with cyproheptadine (to antagonize the interaction of 5-HT with endothelial receptors) (Fig. 8). Because mast cell and endothelial cell alterations were also noted in recipients of an enriched T cell fraction of sensitized cells and were also noted in actively sensitized animals that were severely depleted of B cells, these changes are probably T cell dependent (Table II). Ultrastructural identification of mast cell degranulation and endothelial cell activation in DTH of humans (12) suggest that an analogous process may be involved in cell-mediated reactions of this species.

A unifying hypothesis to draw our findings together with other published findings is as follows: DTH responses are probably mediated by specifically sensitized T cells of the Ly1$^+$ phenotype (13) that normally enter the tissues from the recirculating pool in the blood. When these Ly1$^+$ T cells encounter antigen, they release a variety of lymphokine-like factors (14), among which those with chemotactic and migration-inhibiting activities may be most relevant to DTH. However, the elaboration of these

factors is necessary, but not sufficient, for the elicitation of DTH. In addition, there must be diapedesis of bone marrow-derived accessory leukocytes through endothelial gaps in response to the net chemotactic forces elaborated by the T cells. Our findings support the view that T cells in murine DTH interact with local mast cells, leading to mast cell activation and release of 5-HT, which in turn causes the separation of endothelial cells to allow diapedesis of effector cells. Thus, there is a 5-HT-dependent diapedesis in murine DTH.

It still remains an open question as to whether degranulation by exocytosis is required for the release of the 5-HT, which is important in the elicitation of DTH. It should be considered that release of sufficient 5-HT to elicit DTH may proceed via an additional release mechanism that might be accompanied by less prominent exocytosis. In this regard, we have recently demonstrated that murine mast cells can secrete 5-HT in the presence of inhibited histamine release and in the absence of overt degranulation (15).

The results of the current and previous studies make it highly likely that the degranulation and 5-HT release leading to endothelial changes is T cell dependent and is not mediated by an intermediary B cell product such as IgE antibody. In the actively sensitized animals challenged at 4 d, there is little evidence for B cell activation as measured by serum antibody (4), plaque-forming cells (16), or antigen-binding rosette-forming B cells (6). Moreover, mice that are treated with anti-mu sera and are rendered agammaglobulinemic and unable to make antibody responses have intact DTH (17, 18). The mast cell and endothelial changes we have noted in DTH are seen in recipients of an enriched T cell fraction of sensitized cells, and similar changes are seen in animals treated with a high dose of cyclophosphamide, which eliminates detectable B cells (Table II). However, it cannot be ruled out that a few B cells present in actively sensitized animals, and present in the contaminating Ig^+ cells of the nylon-nonadherent fraction, and resistent even to high doses of cyclophosphamide could produce small, but sufficient, quantities of IgE-type antibody to act as an intermediary and passively sensitize tissue mast cells (19). Thus the mast cell and endothelial changes we have observed could conceivably be T cell dependent rather than T cell mediated. Further study will be necessary to irrefutably rule out small numbers of IgE-secreting B cells in the mediation of these events.

The demonstration that the monoamine 5-HT is released from mast cells and plays a crucial role in DTH by acting on vascular endothelium does not imply that this is the only action of vasoactive amines in DTH. Because mouse mast cells contain histamine, it is probable that this diamine is also being released in DTH. Murine vessels are insensitive to histamine (2), but regulatory T cells are known to be activated through histamine-2 surface receptors (3). Thus, in mice there may be a functional split in the role of the two major vasoactive amines in DTH responses. The principal vasoactive amine of this species, 5-HT, may be released in small amounts early during the response, producing vascular changes, but also paralyzing receptors of endothelial cells so that they become resistant to further activation by 5-HT. Later, with more massive degranulation, significant amounts of histamine may also be released, which then signals regulatory T cells via histamine-2 receptors, leading to suppression and termination of the DTH response. In conclusion, it is clear from our results that 5-HT release from mast cells is a prominent factor in DTH responses and this supports the

hypothesis previously established with pharmacological data that 5-HT plays a major role in the production and evolution of DTH responses.

Summary

We have previously suggested that the release of serotonin (5-hydroxytryptamine) (5-HT) by local tissue mast cells is required for the elicitation of delayed-type hypersensitivity (DTH) in mice. In the current study, light microscopic radioautographs from animals treated with [^{3}H]5-HT indicated that local mast cells released 5-HT between 6 and 18 h during the evolution of DTH. Ultrastructural examination of mast cells revealed surface activation, indicated by extension of surface filopodia, and degranulation by fusion and exocytosis. Light and electron microscopic studies of the endothelium of postcapillary venules at sites of DTH revealed the development of gaps between adjacent cells. The development of gaps permitted extravasation of tracers that was abolished by depletion or antagonism of 5-HT. Thus mast cells degranulated and released 5-HT in DTH, and this 5-HT acted on local vessels. Recipients of nonadherent, non-immunoglobulin-bearing sensitized lymphocytes also demonstrated similar mast cell degranulation and the formation of endothelial gaps. This indicated that mast cell degranulation and 5-HT release in murine DTH were probably T cell dependent.

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