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Contribution of Dermal Macrophage Trafficking in the Sensitization Phase of Contact Hypersensitivity

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We investigated cellular trafficking of dermal macrophages that express a macrophage calcium-type lectin (MMGL) during the sensitization of delayed-type hypersensitivity. In skin, dermal macrophages, but not epidermal Langerhans cells, have been shown to express MMGL. Epicutaneous sensitization by FITC produced a transient increase in MMGL-positive cells in regional lymph nodes. To directly investigate whether the increase was due to cell migration from dermis, MMGL-positive cells purified from skin were intradermally injected into syngeneic mice after labeling with a fluorescent cell tracer, followed by epicutaneous sensitization over the site of injection. MMGL-positive cells containing the tracer were found in the regional lymph nodes after sensitization. The majority of the MMGL-positive cell migrants were negative for FITC fluorescence despite the presence of FITC-labeled cells that included Langerhans cell migrants. Because the extent of MMGL-positive cell migration was greatly influenced by the selection of vehicles to dissolve FITC, the efficiency of sensitization was compared using the ear swelling test. Migration of both Langerhans cells (FITC-labeled cells) and MMGL-positive cells contributed positively to the efficiency of sensitization. Importantly, MMGL-positive cell migration was induced by vehicle alone, even in the absence of FITC. These results suggest that migration of dermal MMGL-positive cells accounts for the adjuvant effects of vehicles at least in part.

In the sensitization phase of a delayed-type hypersensitivity (DTH) reaction such as contact hypersensitivity, it has been generally recognized that epidermal Langerhans cells sequester Ag in the regional lymph nodes where Ag presentation to naive T cells takes place (1, 2). As a cellular mechanism for this process, lymphatic trafficking of Langerhans cells has been directly demonstrated by experiments using epicutaneous application of a fluorescent hapten, FITC (3). In addition to the Langerhans cells, the roles of dermal macrophages (and possibly dermal dendritic cells) during induction of hapten-specific contact hypersensitivity have also been documented in certain artificial experimental systems (4–6). However, mechanisms underlying the ability of dermal macrophages to induce contact hypersensitivity have not been well characterized. For example, it is not known whether dermal macrophages can sequester Ags from dermal sites to the regional lymph nodes as Langerhans cells do during the epicutaneous sensitization process. Additionally, the contribution of macrophage trafficking, if it exists, to the sensitization phase of contact sensitivity has not been determined. This is partly because of the lack of good markers for dermal macrophages distinguishable from epidermal Langerhans cells whose migration has been directly investigated by labeling with fluorescent haptens applied epikutaneously.

We have been studying the biological roles of a macrophage endogenous C-type lectin, MMGL. MMGL is a 42,000 Mₐ type II transmembrane glycoprotein containing a single calcium-dependent carbohydrate recognition domain with specificity for galactose/N-acetylgalactosamine at its carboxyl terminus (7, 8). MMGL was originally detected on tumoricidal peritoneal macrophages (7), and involvement in the tumor cell recognition and tumoricidal activity of macrophages has been reported (9–11). In addition, MMGL was found on tumor-infiltrated macrophages within the lung metastatic lesions produced by experimental metastasis of mouse ovarian tumor cells (12). Using a specific mAb produced in our laboratory (13), we revealed that MMGL-positive cells were widely distributed, but that the distribution was restricted to connective tissue (12, 14). In skin, dermal macrophages strongly express MMGL, whereas epidermal Langerhans cells are devoid of its expression (14). Therefore, we thought that MMGL could be used as an ideal marker to distinguish dermal macrophages from Langerhans cells in tissue environments.

In the present study we investigated whether dermal macrophages contribute to the induction of contact hypersensitivity using unmanipulated mice. We attempted to directly investigate lymphatic cell trafficking of dermal macrophages during the sensitization phase. We used an anti-MMGL mAb to distinguish Langerhans cell migrants and dermal macrophage migrants in regional lymph nodes. Using various conditions for epicutaneous sensitization, we investigated the relationship between efficiency of sensitization and MMGL-positive cell migration.

Materials and Methods

Mice

Female, specific pathogen-free CD-1 (ICR) and BALB/c mice were purchased from SLC Japan (Shizuoka, Japan).
Reagents
Biotin-conjugated mAb mouse anti-rat κ and λ light chains (anti-κ/λ), Triton-X-100, FITC, streptomyrin, aprotinin, peptatin A, leupeptin, poly-L-lysine, and PMSF were purchased from Sigma (St. Louis, MO); DMEM was obtained from Nissui Pharmaceutical (Tokyo, Japan); FCS was purchased from BioWhittaker (Walkersville, MD); the bichinonic acid protein assay kit was obtained from Pierce (Rockford, IL); SDS-PAGE protein reference standards (phosphorylase b, BSA, aldolase, and carbonic anhydrase) were obtained from Daiichi Pure Chemicals (Tokyo, Japan); BSA fraction V was purchased from Seikagaku (Tokyo, Japan); aceton, dimethylformamide (DMF), DMSO, ethanol, ethyl acetate, dibutyl phthalate, olive oil, penicillin, and collagenase (Clostridium histolyticum) were obtained from Wako Pure Chemical (Tokyo, Japan); horseradish peroxidase-conjugated goat anti-rat IgG(H+L) and alkaline phosphatase-conjugated streptavidin were purchased from Zymed (South San Francisco, CA); His- tomark Red was obtained from Kirkegaard & Perry (Gaithersburg, MD); DNase I (grade II, bovine pancreas), polyclonal sheep anti-digoxigenin Fab fragments, alkaline phosphatase-labeled anti-digoxigenin Fab fragments, and digoxigenin-3-O-methyl-carbonyl-e-aminoacrylic acid-N-hydroxysuccinimide ester were obtained from Boehringer Mannheim (Mannheim, Germany); 5 (and 6)-(4-(chloromethyl)benzyo)narninonitramethylodihodamine (CMTMR) was obtained from Molecular Probes (Eugene, OR); SDS, parafomaldehyde, and glutaraldehyde were purchased from Nacalai Tesque (Kyoto, Japan); biotin-conjugated anti-mouse Thy 1.2 was obtained from Becton Dickinson (San Jose, CA); Cy5-conjugated mouse anti-rat IgG(H+L) was purchased from Jackson Immunoresearch (West Grove, PA); FluoroLink-Aby Cy3.5 labeling kit was obtained from Amersham (Aylesbury, U.K.); and mouse monoclonal anti-rat anti-IgG(H+L) was obtained from Bergisch Gladbach, Germany). Preparation of culture supernatant of rat hybridoma cells producing mAb against MMGL (mAb LOM-14; IgG2b) and mAb LOM-8.7; IgG(2a) was purchased from BioWhittaker (Walkersville, MD). The method of contact sensitization was based on those of earlier studies (15, 16) with modifications. Mouse forelimbs were shaved using a small animal clipper, and 80 μl DMSO) in 0.1 M sodium borate (pH 8.8) for 3 h at 25°C. Labeling of Abs Sheep anti-digoxigenin Fab fragments (1 mg) were conjugated with Cy3.5 using the FluoroLink-Aby Cy3.5 labeling kit according to the manufacturer’s instructions. Digoxigenin labeling of mAb LOM-14 was conducted by incubation of the purified mAb (1 mg) with digoxigenin-3-O-methyl-carbonyl-e-aminoacrylic acid-N-hydroxysuccinimide ester (44 μg dissolved in 22 μl DMSO) in 0.1 M sodium borate (pH 8.8) for 3 h at 25°C.

Sensitization and elicitation of contact hypersensitivity reaction
The method of contact sensitization was based on those of earlier studies (15, 16). With mice, forelimbs were shaved using a small animal clipper, and 80 μl of FITC solution (0.5%, w/v) dissolved in a solvent was epicutanous applied to the shaved forelimbs. The solvents used for sensitization are as follows: aceton/dinityl phthalate (AD; 1/1), 100% ethanol, aceton/olive oil (4/1), 30% SDS in water, ethyl acetate, DMF, and DMSO. On day 6, the baseline ear thickness (0 h) of each animal was measured using a dial thickness gauge. Mice were challenged by applying 20 μl of 0.5% FITC solution, in AD on the outer surface of the left ear. Ear thickening was defined as follows: (ear thickness of the right ear at 0 h) – (ear thickness of the left ear at 0 h) = (ear thickness of the left ear at 24 h) – (ear thickness of the right ear at 0 h). Detection of MMGL in lymph node extracts
Brachial lymph nodes were removed from anesthetized mice and homogenized in Dulbecco’s modified PBS (containing 0.91 mM CaCl2, 0.49 mM MgCl2, containing 1% Triton X-100, 0.025% NaN3, 0.1 μM aprotinin, 1 μM pepstatin A, 1 μM leupeptin, and 1 mM PMSF (lysis buffer) using a Potter-Elvehjem homogenizer. They were then extracted for 1 h on ice (1 ml of lysis buffer/100 mg organ wet weight). The homogenates were centrifuged at 100,000 × g for 30 min, and the supernatants were collected. The protein concentration in the organ lysates was assessed using a bichinonic acid protein assay kit. Proteins in the lysate were separated by SDS-PAGE (10% gel) under nonreducing conditions and transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA) using a Milli Blot-SDE system (Millipore). The membrane was treated in 10 nM sodium phosphate and 0.15 M NaCl (pH 7.2; PBS) that contained 2% normal goat serum and 3% BSA for 18 h at 4°C to block nonselective Ab binding. The membrane was subsequently incubated with mAb LOM-14 (1/10 dilution of culture supernatant in PBS containing 0.2% Tween-20) for 90 min at room temperature, followed by incubation with horseradish peroxidase-conjugated goat anti-rat IgG(H+L) diluted at 1/1000 in PBS/0.2% Tween-20 for 90 min at room temperature. The binding of mAb was visualized using ECL Western blotting detection reagent and Hyperfilm ECL (Amersham, Arlington Heights, IL).

Immunohistochemistry for light microscopy
MMGL-positive cells were immunohistochemically detected on frozen sections of lymph nodes with mAb LOM-14 (1/10), biotinylated mAb mouse anti-rat α/β (1/100), and alkaline phosphatase-streptavidin (1/100) as described previously (12). In some experiments, biotin-conjugated antibody Thy 1.2 (1/100) and alkaline phosphatase-streptavidin were used to detect T lymphocytes. The Ab binding was histochemically detected using HistoMark Red, and the cell nucleus was counterstained in Mayer’s hematoxylin. The sections were observed under a microscope and photographed (Olympus, Tokyo, Japan). As a negative control, normal rat serum (10%) was used instead of mAb. In immunoreactions, Brachial lymph nodes were obtained from ICR mice that had been epicutaneously treated with FITC on the forelimb skin 24 h earlier. Single cell suspensions of lymph nodes from individual mice were prepared by cutting tissue using needless in PBS containing 0.1% BSA and 0.1% NaN3. Cells were washed and resuspended in the same buffer at 107 cells/ml, and they were analyzed on an EPICS Elite flow cytometer (Coulter, Miami, FL) using gates of forward and side light scatter to collect signals of cell-associated fluorescence. Cells with signals of >200 channels (linear scale) were arbitrarily assigned to the bright fluorescence population. A total of 106 cells were analyzed.

Isolation of MMGL-positive cells from skin
BALB/c mouse shaved skin from the abdomen, fore- and hindlimbs, and dorsum were cut by scissors. Single cell suspensions of lymph nodes from individual mice were prepared by cutting tissue using needless in PBS containing 0.1% BSA and 0.1% NaN3. Cells were washed and resuspended in the same buffer at 107 cells/ml, and then they were analyzed on an EPICS Elite flow cytometer (Coulter, Miami, FL) using gates of forward and side light scatter to collect signals of cell-associated fluorescence. Cells with signals of >200 channels (linear scale) were arbitrarily assigned to the bright fluorescence population. A total of 106 cells were analyzed.

Flow cytometry
Brachial lymph nodes were obtained from ICR mice that had been epicutaneously treated with FITC on the forelimb skin 24 h earlier. Single cell suspensions of lymph nodes from individual mice were prepared by cutting tissue using needless in PBS containing 0.1% BSA and 0.1% NaN3. Cells were washed and resuspended in the same buffer at 107 cells/ml, and they were analyzed on an EPICS Elite flow cytometer (Coulter, Miami, FL) using gates of forward and side light scatter to collect signals of cell-associated fluorescence. Cells with signals of >200 channels (linear scale) were arbitrarily assigned to the bright fluorescence population. A total of 106 cells were analyzed.

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Cell tracer experiments
MMGL-positive cells (1–5 × 107 cells/ml) isolated from skin were suspended in 1 ml of DMEM/Ham’s F-12 medium-10% FCS, and 6 μl of Cy5-labeled anti-MMGL mAb LOM-8.7 (1/250 dilution in 3% BSA/DPBS) was added. After incubation for 30 min at 4°C, the cells were washed twice in 0.1% BSA/DPBS and suspended in 0.8 ml of 0.1% BSA/DPBS, and then 200 μl of goat anti-rat IgG(H+L) microbeads were added. The suspension was incubated for 15 min at 8°C, and then positive selection was conducted using an RS² column with a magnetic cell sorter I (Miltenyi). Cells retained in the column were recovered by washing the column outside the magnetic field. An aliquot of cell suspension was subjected to cytofluorographing and was immunohistochemically stained using digoxigenin-conjugated mAb LOM-14 plus alkaline phosphatase anti-digoxigenin Ab (1/100 dilution). Positive reaction was detected using HistoMark Red as described for frozen sections.

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MMGL-positive cells (1–5 × 107 cells/ml) isolated from skin were suspended in 1 ml of DMEM/Ham’s F-12 medium-10% FCS, and 6 μl of Cy5-labeled anti-MMGL mAb LOM-8.7 (1/250 dilution in 3% BSA/DPBS) was added. After incubation for 30 min at 4°C, the cells were washed and resuspended in PBS at 5 × 107 cells/ml. An aliquot of the labeled cells (106 cells in 20 μl) was intradurally injected...
into the shaved skin of a forelimb of a BALB/c mouse. After 16 h, mice were epicutaneously treated on the forelimb skin over the site of cell injection with 80 μl of FITC solution (0.5%, w/v) in AD or AD alone, or they received no treatment. After 22 h, brachial lymph nodes were collected, and frozen sections of the lymph nodes were immunohistochemically examined using mAb LOM-14 and Cy5-conjugated anti-rat IgG followed by examination under a confocal microscope as described above (‘Immunohistochemistry for light microscopy’ section). Tissue sections prepared from the forelimb skin, where CMTMR-labeled cells were injected, were examined under a fluorescence microscope (Olympus).

Statistical analyses

Student’s t test, the z test for proportions, Spearman’s rank correlation test, and Kendall’s rank correlation test were used. The methods used for specific analyses are specified in the figure legends.

Results

FITC as an immunogen in contact sensitization

To determine the specificity of FITC as an Ag in our experimental conditions, mice were sensitized by epicutaneous application on forelimb skin. After 6 days, mice were challenged by epicutaneous application on ear, and then ear swelling was measured at 24 h (Fig. 1). Mice sensitized with FITC dissolved in AD followed by challenge with FITC in AD developed substantial ear swelling. By contrast, mice sensitized with FITC in AD followed by challenge with AD alone and mice sensitized with AD alone followed by treatment with FITC in AD produced only marginal responses. Mice sensitized with AD alone followed by challenge with AD did not produce any response compared with untreated mice. These results demonstrate that FITC plays a major role as an antigenic substance in these experimental conditions. A time-course study revealed that ear swelling had reached at 16–24 h after challenge. There was a transient peak of the ear swelling response (50–60% of the maximal swelling) at 2–4 h. At 48 h, the ear swelling was decreased by 80% relative to the amount found at 24 h. Since FITC was applied on only one side of the auricle, swelling was only evident on the treated half of the auricle by microscopic observation. At this site, extensive vasodilatation was observed (data not shown).

Increase in number of MMGL-positive cells in draining lymph nodes after sensitization

We investigated whether epicutaneous sensitization with Ag affects the number and distribution of MMGL-positive cells in draining lymph nodes (Fig. 2). In normal mouse lymph nodes, MMGL-positive cells were mainly detected in the medulla, subcapsular sinus, and interfollicular sinus (17). A scattered distribution of MMGL-positive cells with dendritic morphology was occasionally seen in the T cell area, especially at the border between T and B cell areas (Fig. 2d). Twenty-four hours after epicutaneous application of FITC dissolved in AD on forelimb skin, a marked increase in the number of MMGL-positive cells was observed within the T cell area of brachial lymph nodes, especially around the border between T and B cell areas (Fig. 2d). The localization of MMGL-positive cells was also recognized by comparative observation of serial sections stained with anti-Thy 1.2 mAb, which revealed localization of T lymphocytes (Fig. 2c). This is also confirmed by comparing their position relative to that of high endothelial venules, which are present in the T cell area (Fig. 2h). Unexpectedly, solvent alone (AD) produced an effect similar to that of AD containing FITC, indicating that the solvent makes an important contribution to the increase in MMGL-positive cells in the T cell area (Fig. 2e).

MMGL in lymph node lysates after sensitization

To confirm the effect of epicutaneous sensitization on the molecular level, we investigated whether the amount of MMGL in lymph node lysates changes upon sensitization. It has been demonstrated that MMGL can be detected in lymph node detergent extracts by SDS-PAGE and immunoblot analyses using mAb LOM-14 (14). Draining lymph node lysates of known protein amount were compared for the signals representing MMGL with or without epicutaneous sensitization (Fig. 3). Signals representing MMGL were markedly increased upon sensitization not only with FITC in AD but also with AD alone. These results are consistent with the immunohistochemical observations of lymph nodes. The apparent m.w. of MMGL in lymph node lysates was consistent with that determined in our previous studies (13, 14).

Appearance of FITC-presenting cells in draining lymph nodes

In the initiation phase of DTH, Langerhans cells are known to migrate to the T cell area of draining lymph nodes to exert Ag presentation activity (3). After epicutaneous application of FITC, such migrants are visible as fluorescent cells in lymph nodes. To investigate whether FITC was incorporated within MMGL-positive cells that were transiently increased in lymph nodes upon sensitization, frozen sections of brachial lymph nodes obtained 24 h after sensitization were stained using mAb LOM-14 and were observed using a confocal microscope for two-color fluorescence (Fig. 4, a and b). The cell-associated fluorescein signals were detected in the T cell area. MMGL-positive cells were also detected in the vicinity of the fluorescein-positive cells; however, the majority of MMGL-positive cells were devoid of the fluorescein label. A minor population of MMGL-positive cells (7 ± 2%) was positive for fluorescein (Table I). The fluorescein signals that...
appeared not to be associated with cells were also observed around the interfollicular sinus (Fig. 4a). These signals may be due to molecular transport of FITC-labeled substances along reticular fibers.

Flow cytometric analysis of lymph node cell suspension
The migration of Langerhans cells was also demonstrable by flow cytometric analysis. Single cell suspension prepared from brachial lymph nodes 24 h after epicutaneous application of FITC/AD on the forelimb skin (Fig. 5a) was compared with that from untreated lymph nodes (Fig. 5c). The number of events representing cells that contained significant FITC label (as defined in Materials and Methods) was 223 of $10^6$ cells analyzed for the FITC/AD condition compared with 39 events/$10^6$ cells for untreated lymph nodes. The latter number presumably reflects background signals due to noise. These data also provide evidence, in addition to the confocal microscopic observation, of significant migration of FITC-labeled cells into the draining lymph nodes upon epicutaneous application of FITC/AD. The results of flow cytometric analyses are summarized in Table I.

Adjuvant effects of solvents and their effects on MMGL-positive cell increase in draining lymph nodes
AD has been used as an optimal solvent for sensitization with FITC (3, 15, 16). Since AD did not work as an immunogen by itself (Fig. 1), it is conceivable that AD has some adjuvant effects on sensitization. Since AD by itself had activity inducing a transient increase in MMGL-positive cells in the lymph node T cell area (Fig. 2), this activity could be related to the adjuvant effects. To investigate this possibility, FITC was dissolved in a variety of solvents, and the degree of sensitization as well as the effect of increasing MMGL-positive cells in lymph nodes were compared upon sensitization. The degree of sensitization monitored by ear swelling was variable when solvents were changed (Table I). When both APCs (FITC-positive cells) and MMGL-positive cells were abundant in lymph nodes (AD), the maximal response was obtained. Even though substantial numbers of FITC-presenting cells or non-cell-associated FITC-modified molecules were seen in the T cell area, when MMGL-positive cells were not substantially
increased in the T cell area (acetone/olive oil), or when they decreased in the medulla of the lymph nodes (ethyl acetate and DMF), the degree of sensitization was at relatively low levels.

When SDS was used as the vehicle, FITC-presenting cells were seldom seen in lymph nodes (Table I and Fig. 5b), whereas a significant increase in MMGL-positive cells was observed in lymph nodes. Despite the apparent lack of the migration of cells containing FITC, significant sensitization was obtained. When DMSO was used as a solvent, sensitization to FITC did not take place. In this case, neither a migration of FITC-positive cells nor an increase in MMGL-positive cells in the lymph nodes was observed.

To compare the relationship between the efficiency of sensitization and the appearance of MMGL-positive cells in lymph nodes, the data in Table I were rescored as follows: 0 for ±, 1 for +, 2 for ++, and 3 for ++++. Then the differences between experimental and control conditions were shown as an index in Table II. For example, Table I indicates that MMGL-positive cell distribution in the medulla was observed as ++++ for the FITC/AD condition and ++ for the control. The index is 3–2 = 1. Because the increase in the number of cells in the deep T cell area was less dramatic (Table I), only the values for T cell area (border), subcapsular sinus, and medulla were taken into account, and the sum of these values was expressed as an overall index (Table II). Table II also includes the rank order of the overall index and the rank order of the DTH response (ear swelling). Based on these calculations, the correlation between the MMGL-positive cell increase in the lymph nodes and the DTH response was demonstrated in Fig. 6. These results indicated that DTH responses were proportional to the increase in MMGL-positive cells in lymph nodes. Statistical significance of the correlation was confirmed by either Spearman rank correlation analysis (p < 0.05) or Kendall rank correlation analysis (p < 0.02).

**Isolation of MMGL-positive cells from mouse skin**

Although the experiments demonstrated a transient increase in MMGL-positive cells in the draining lymph nodes and the apparent contribution of this increase to the sensitization process, there

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Table I. Effects of vehicle (solvents) on the efficiency of sensitization and on the appearance of MMGL-positive cells in the draining lymph nodes

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Ear Swellinga (%)</th>
<th>Cell-associated FITC (cells/10⁶)</th>
<th>Molecular transport</th>
<th>FITC Bright Cells (cells/10⁶)</th>
<th>MMGL-Positive Cell Distributionb</th>
<th>Coexpression, c FITC+/MMGL-cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FITC/AD</td>
<td>100</td>
<td>+</td>
<td>+</td>
<td>T cell area (deep)</td>
<td>++</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>FITC/EtOH</td>
<td>54 ± 8</td>
<td>+</td>
<td>+++</td>
<td>Subcapsular sinus</td>
<td>++</td>
<td>11 ± 4</td>
</tr>
<tr>
<td>FITC/AO</td>
<td>44 ± 10</td>
<td>+</td>
<td>+++</td>
<td>Medulla</td>
<td>++</td>
<td>9 ± 3</td>
</tr>
<tr>
<td>FITC/SDS</td>
<td>41 ± 15</td>
<td>-</td>
<td>++</td>
<td></td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>FITC/EtOAc</td>
<td>37 ± 15</td>
<td>+</td>
<td>++</td>
<td></td>
<td></td>
<td>12 ± 3</td>
</tr>
<tr>
<td>FITC/DMF</td>
<td>27 ± 10</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>3 ± 2</td>
</tr>
<tr>
<td>FITC/DMSO</td>
<td>−3 ± 9</td>
<td>±</td>
<td>±</td>
<td></td>
<td></td>
<td>42 ± 9</td>
</tr>
<tr>
<td>Control</td>
<td>6 ± 9</td>
<td>−</td>
<td>−</td>
<td></td>
<td></td>
<td>ND</td>
</tr>
</tbody>
</table>

a Abbreviation for solvents to dissolve FITC is described in Materials and Methods. Control denotes unsensitized mice.

b The values were normalized as percent of reaction produced by sensitization with FITC/AD. The maximal response (100%) corresponded to 75.7 kDa, BSA (66 kDa), aldolase (42 kDa), and carbonic anhydrase (30 kDa).

c Immunohistochemical analysis of lymph node frozen sections.

d +++, continuous distribution of positive cells along subcapsular sinus; +, a scattered distribution of positive cells.

e 5–20% were stained; +, 5–10% were stained; ±, < 3 cells with scattered intracellular fluorescein signals per field; −, virtually no FITC-positive cell in the T cell area.

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**FIGURE 3.** Immunoblot analyses for the expression of MMGL in the draining lymph nodes after epicutaneous application of contact sensitizer on mouse forelimb skin. Lysates of brachial lymph nodes from untreated mice (lanes 1 and 2), from mice treated with a solvent (AD) alone (lanes 3 and 4), and from mice treated with FITC-dissolved in the solvent (lanes 5 and 6) were subjected to SDS-PAGE (10% gel) using 200 μg protein/lane (lanes 1, 3, and 5) or 50 μg protein/lane (lanes 2, 4, and 6) under nonreducing conditions. The electrophoretically separated proteins were transferred to a polyvinylidene difluoride membrane, stained with rat anti-MMGL mAb LOM-14 plus peroxidase-labeled goat anti-rat IgG (H+L), and detected using the ECL system (Amersham). The positions and molecular mass (in kilodaltons) of standard proteins are shown on the right. The standards are phosphorylase b (97 kDa), BSA (66 kDa), aldolase (42 kDa), and carbonic anhydrase (30 kDa). df, dye front.
was no evidence that MMGL-positive cells migrated from the dermis. The epicutaneous labeling with FITC did not provide evidence supporting this hypothesis (Fig. 4, a and b). Therefore, we tried to isolate MMGL-positive cells from mouse skin to address this question directly. BALB/c mouse skin was digested by collagenase, and MMGL-positive cells were isolated by a magnetic cell sorter using the anti-MMGL mAb LOM-8.7. Cells were immunohistochemically analyzed using digoxigenin-conjugated anti-MMGL Ab. Representative cells with intracellular FITC label (green) are marked by small arrows. FITC-modified molecules that appeared to be transported along reticular fibers are seen in the medulla and in the interfollicular sinus (a, long arrow). MMGL-positive cells are seen as red fluorescence. Most of MMGL-positive cells were devoid of FITC fluorescence. c, Cells isolated from BALB/c mouse skin by a magnetic cell sorter using anti-MMGL mAb LOM-8.7 were collected by cytocentrifugation. Cells were stained using digoxigenin-conjugated mAb LOM-14, directed to an independent epitope on MMGL, and alkaline phosphatase anti-digoxigenin (arrows). d–i, MMGL-positive skin cells from BALB/c mice were labeled with a fluorescent cell tracer, CMTMR, and then intradermally injected into forelimb skin of BALB/c mice. Twenty-four hours after epicutaneous application of FITC/AD (d–g) or AD alone (h and i), frozen sections of the draining lymph nodes were stained using mAb-LOM-14 plus Cy5-conjugated anti-rat IgG and visualized with a confocal microscope. Fluorescence signals of FITC (d), CMTMR (e and h), and Cy5 (f and i) are shown individually, and the signals of d, e, and f are combined in g, in which FITC, CMTMR, and Cy5 signals are shown by green, red, and blue, respectively. CMTMR-positive cells (arrowheads) were also positive for MMGL but were devoid of FITC, resulting in the images with pink color in g. FITC-positive cells are marked by arrows in the d and g. Some cells were MMGL positive but CMTMR negative and are marked by small arrows in i. Scale bars = 100 μm (a), 20 μm (b and d–i), and 25 μm (c).

FIGURE 4. Macrophage migration from skin to draining lymph nodes upon Ag application. a and b, FITC/AD was applied to the skin of ICR mice, and frozen sections of the draining lymph nodes were stained using digoxigenin-conjugated anti-MMGL mAb LOM-14 plus Cy3.5-conjugated anti-digoxigenin Ab. Representative cells with intracellular FITC label (green) are marked by small arrows. FITC-modified molecules that appeared to be transported along reticular fibers are seen in the medulla and in the interfollicular sinus (a, long arrow). MMGL-positive cells are seen as red fluorescence. Most of MMGL-positive cells were devoid of FITC fluorescence. c, Cells isolated from BALB/c mouse skin by a magnetic cell sorter using anti-MMGL mAb LOM-8.7 were collected by cytocentrifugation. Cells were stained using digoxigenin-conjugated mAb LOM-14, directed to an independent epitope on MMGL, and alkaline phosphatase anti-digoxigenin (arrows). d–i, MMGL-positive skin cells from BALB/c mice were labeled with a fluorescent cell tracer, CMTMR, and then intradermally injected into forelimb skin of BALB/c mice. Twenty-four hours after epicutaneous application of FITC/AD (d–g) or AD alone (h and i), frozen sections of the draining lymph nodes were stained using mAb-LOM-14 plus Cy5-conjugated anti-rat IgG and visualized with a confocal microscope. Fluorescence signals of FITC (d), CMTMR (e and h), and Cy5 (f and i) are shown individually, and the signals of d, e, and f are combined in g, in which FITC, CMTMR, and Cy5 signals are shown by green, red, and blue, respectively. CMTMR-positive cells (arrowheads) were also positive for MMGL but were devoid of FITC, resulting in the images with pink color in g. FITC-positive cells are marked by arrows in the d and g. Some cells were MMGL positive but CMTMR negative and are marked by small arrows in i. Scale bars = 100 μm (a), 20 μm (b and d–i), and 25 μm (c).
recovered per mouse, and 2–3% of them were MMGL positive. After magnetic cell sorting, 0.75–1.25 \times 10^6 cells were obtained per mouse, and 90% of them were MMGL positive (Fig. 4c).

Migration of MMGL-positive cells into lymph nodes upon sensitization

MMGL-positive cells obtained from BALB/c mouse skin were labeled with a cell tracer (CMTMR) and then they were intradermally injected into recipient BALB/c mouse forelimb skin. Twenty-four hours after treatment with FITC/AD (Fig. 4, d–g) or with AD alone (Fig. 4, h and i) at the site of injection, CMTMR-positive cells were found in the T cell area of the brachial lymph nodes (Fig. 4, e, g, and h). These cells were also positive for MMGL.

**Table II.** Statistical analysis for the correlation between the efficiency of sensitization and the appearance of MMGL-positive cells in the draining lymph nodes

<table>
<thead>
<tr>
<th>Conditions</th>
<th>T cell area (border)</th>
<th>Subcapsular sinus</th>
<th>Medulla</th>
<th>Overall Index</th>
<th>Rank for Overall Index</th>
<th>Ear Response</th>
<th>Rank for Ear Response</th>
</tr>
</thead>
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<tr>
<td>FITC/AD</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>5</td>
<td>1</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>FITC/EtOH</td>
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<td>0</td>
<td>0</td>
<td>2</td>
<td>3</td>
<td>54</td>
<td>2</td>
</tr>
<tr>
<td>FITC/AD</td>
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<td>0</td>
<td>0</td>
<td>1</td>
<td>4.5</td>
<td>44</td>
<td>3</td>
</tr>
<tr>
<td>FITC/SDS</td>
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<td>1</td>
<td>0</td>
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<td>2</td>
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<td>4</td>
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<tr>
<td>FITC/EtOAC</td>
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<td>−1</td>
<td>1</td>
<td>4.5</td>
<td>37</td>
<td>5</td>
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<tr>
<td>FITC/DMF</td>
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<td>−2</td>
<td>0</td>
<td>6</td>
<td>27</td>
<td>6</td>
</tr>
<tr>
<td>FITC/DMSO</td>
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<td>0</td>
<td>−1</td>
<td>−1</td>
<td>7</td>
<td>−3</td>
<td>7</td>
</tr>
</tbody>
</table>

* Same as Table 1.
* Based on the data shown in Table 1.
* Indices for T cell area (border), subcapsular sinus, and medulla were added.
* Rank order for the overall index.
* Rank order for the ear swelling response.
Under these conditions, sensitization was successfully observed. In the artificial system, the epidermis was removed, and the Ag was distributed within the dermis (19). In contrast, the resident macrophages of the dermis were demonstrated using mice in which the epidermis was removed or untreated (control) with FITC/AD or AD alone on the forelimb skin. Twenty-four hours later, brachial lymph node frozen sections were observed using a confocal microscope, and the number of cells with CMTMR fluorescence was counted in randomly selected T cell areas (200 μm x 200 μm) prepared from four different lymph nodes. Each value represents the mean cell number ± SEM obtained from 10 different areas. Statistical significance compared with the control according to Student’s t test is shown.

which was detected by mAb LOM-14 plus Cy5-conjugated anti-rat IgG (Fig. 4, e–i). Most of the CMTMR-positive cells did not contain FITC fluorescence upon treatment with FITC/AD (Fig. 4, d, e, and g). The results indicated that the CMTMR-positive cells represent MMGL-positive cell migrants from the site of intradermal injection. In the vicinity of CMTMR-positive cells in the sections, MMGL-positive cells without CMTMR label were also observed (Fig. 4, h and i, small arrows). These cells presumably represent migrants of host origin. Normal rat serum plus Cy5-conjugated anti-rat IgG (cytochemical control) did not produce positive signals (data not shown). Skin samples containing the site of injection were also examined, and a good retention of the CMTMR fluorescence within the cells was observed. Quantitative comparison using frozen section samples revealed that similar numbers of CMTMR-positive cells were detected upon treatment with FITC/AD or with AD alone (Fig. 7). On the other hand, CMTMR-positive cells were not detected in untreated lymph nodes. These results indicated that the migration was induced by epicutaneous sensitization.

Discussion

The importance of trafficking of Ag-presenting Langerhans cells from dermis into the draining lymph nodes has been well established in experiments using mice receiving allogenic skin transplants and using FITC as an Ag (3). One piece of definitive evidence for the migration of Langerhans cells is the observation of FITC-labeled dendritic cells containing Birbeck granules (the hallmark of Langerhans cells) in the T cell area (3). Subsequent studies also demonstrated the migration of Langerhans cells when using skin grafts on nude mice (18) as well as when using a rat system (19). In contrast, the capabilities of dermal macrophages to induce contact sensitization were demonstrated using mice in which Ag was directly applied to tape-stripped skin (4). In this artificial system, the epidermis was removed, and the Ag was directly applied to the dermis or even onto the panniculus carnosus. Under these conditions, sensitization was successfully observed.

On the other hand, when Ag was applied through intact skin, cell migration from the epidermis (or from the upper dermis) appeared to be essential for the sensitization process (4). Thus, the roles of dermal macrophages in the sensitization process in unmanipulated animals have not been well characterized.

In the present study we demonstrated the contribution of dermal macrophages to the efficiency of contact sensitization using unmanipulated mice. Lymphatic trafficking of dermal macrophages was involved in the process. Our conclusion is based on the following observations. First, dermal macrophages express a macrophage lectin, MMGL, whereas epidermal Langerhans cells do not (14). Second, we found in the present study that the number of MMGL-positive cells in the draining lymph node T cell area increased transiently upon epicutaneous application of Ag (FITC). Because the majority of these cells were devoid of FITC fluorescence, the increased MMGL-positive population could be distinguished from Langerhans cell migrants. Third, the degree of the MMGL-positive cell increase in the lymph nodes was greatly influenced by the conditions of Ag application, especially by solvents used to dissolve the Ag. The efficiency of sensitization was also influenced by these conditions, and the increase in MMGL-positive cell number and the efficiency of sensitization were positively correlated. For optimal sensitization, the following requirements were recognized: 1) FITC-presenting cell (mainly of Langerhans-cell origin) migration, 2) MMGL-positive cell increase, and 3) lack of depletion of MMGL-positive cells from the medulla. Fourth, we successfully isolated MMGL-positive cells from mouse skin by positive selection using a mAb against MMGL, and we injected these cells into syngeneic recipient mice intradermally after labeling them with a fluorescent cell tracer. We observed the appearance of the labeled cells in the draining lymph nodes of the recipient mice upon epicutaneous sensitization.

One might argue that the CMTMR-positive cells in lymph nodes do not represent cellular trafficking from dermis but represent in situ uptake of fluorescent molecules that might be released from the labeled cells. However, such a possibility is unlikely for the following reasons. First, we used CMTMR as a cell tracer. CMTMR is trapped within cells in the form of a thiol-conjugated product by the activity of cellular glutathione S-transferase (20, 21). This label is much more resistant to lipophilic environments than widely used lipophilic cell tracers, such as DiI and PKH26. Actually, observation of skin samples containing the site of injection revealed a good retention of the fluorescence label within cells. Second, there was very low background for CMTMR fluorescence in lymph nodes. The background was lower than that produced by FITC. For example, a strong FITC fluorescence along reticular fibers was observed. Third, if macrophages incorporated CMTMR in situ, it is natural to expect macrophages also to have incorporated FITC, because FITC-labeled molecules were much more abundant in the lymph nodes after epicutaneous application of FITC. However, CMTMR-positive cells were not positive for FITC fluorescence (Fig. 4, d, e, and g). For these reasons, we concluded that CMTMR-labeled cells appearing in draining lymph nodes represent migrants from the dermal site of injection.

One might also argue that the main reason for the increase in MMGL-positive cells in the T cell area is not cell migration of dermal macrophages but up-regulation of MMGL on resident macrophages and/or dendritic cells. We do not exclude the possibility that the induction or up-regulation of MMGL expression in some types of cells may be responsible for MMGL-positive cell increase in part. However, it should be noted that our results clearly demonstrated the presence of lymphatic trafficking of MMGL-positive cells that was inducible by epicutaneous sensitization. It should also be emphasized that Langerhans cell migrants did not acquire...
MMGL expression in the lymph node environments immediately. Because the present study allows us to examine the nature of Langhans cell migrants that could be labeled with FITC, the questions remains as to whether MMGL expression can be induced on some resident dendritic cells in lymph nodes by signals mediated by soluble factors or by cell-cell interaction between macrophage migrants and the resident cells. It has been reported that occlusion of afferent lymphatic vessels by surgical manipulation severely decreases the number of subcapsular sinus macrophages in lymph nodes (22, 23). Furthermore, the occlusion of afferent lymphatic vessels is known to result in the change in localization of macrophages (detected by mAb MOMA-1) from the subcapsular sinus to the T cell area of the lymph nodes in an early stage after the occlusion (23). These results suggest the possibility that lymph node macrophages are continuously supplied by lymphatic cellular trafficking, changing localization within the lymph nodes, and that this then results in their turnover. Alternatively, it is also possible that lymph node macrophages are dependent on factors provided by afferent lymphatic vessels. Although the latter possibility has not been ruled out, our present results not only appeared to be compatible with the former possibility, but provided new insights into the origin of lymph node macrophages in relation to lymphatic cell trafficking.

One interesting point concerning MMGL-positive cell migration was that this phenomenon was not only greatly influenced by the vehicles used to dissolve Ag, but was produced by the vehicle alone (Figs. 2, 3, and 7 and Table I). TCRs are likely to recognize FITC determinants rather than substances included in a vehicle such as AD, because the presence of FITC was required to produce a DTH response in mice in this system (Fig. 1). Thus, vehicles such as AD can be regarded as adjuvants during sensitization, and one of the mechanisms of the adjuvant effects is considered to be the ability to induce dermal macrophage migration.

The majority of MMGL-positive cells in the draining lymph nodes were devoid of FITC fluorescence, suggesting that the majority of MMGL-positive migrants may not serve as APCs (Fig. 4). What are the roles of MMGL-positive cell migrators other than that of Ag presentation? One possibility is that MMGL-positive cells could cooperate with Ag-presenting dendritic cells through cytokine production in the lymph node environments. The contribution of cytokines that can be produced by macrophages, including TNF-α, IL-1β, and IL-12, to the sensitization phase has already been proposed (18, 24–26). Another possibility is that MMGL-positive macrophages could contribute to the maintenance of high endothelial venules (HEV) and their expression of ligands for L-selectin, which are required for lymphocyte recirculation into lymph nodes. In this case, MMGL-positive macrophages would contribute to the sensitization phase by enhancing the probability that circulating naive T lymphocytes will encounter the APCs in the lymph nodes. The transient accumulation of MMGL-positive cells in areas surrounding HEV (Fig. 2) may suggest such a possibility. Experiments by others have also demonstrated that occlusion of afferent lymphatic vessels results in the reduction of functional HEV ligands (GlyCAM-1 and CD34) and MECA-79 epitope (a determinant of peripheral lymph node addressin) expression on HEV as well as in changes in HEV morphology (22, 23, 27). The occlusion of afferent lymphatic vessels also decreased the number of lymph node macrophages, suggesting that macrophages supplied by afferent lymphatics may be responsible for the maintenance of HEV function (23). In an in vitro study, lymph node subcapsular sinus macrophages appeared to contribute to the maintenance of HEV adhesive function (28). However, the question of whether some uncharacterized soluble factors, rather than cellular components, might be responsible for the maintenance of HEV function is still controversial (23, 27).

A remaining question is whether MMGL molecules themselves are involved in the process of migration of dermal macrophages. It would be interesting to know whether administration of anti-MMGL mAbs, which efficiently interfere with the binding of carbohydrate ligands (13), could block the migration of dermal macrophages and whether such treatment could affect the sensitization phase of contact hypersensitivity. We are in the process of studying whether anti-MMGL mAbs can block part of the process that is responsible for the migration of dermal macrophages.

In conclusion, we observed that epicutaneous sensitization produced a transient increase in MMGL-positive cells in the T cell area of the regional lymph nodes. The extent of the increase was not only greatly influenced by the conditions of sensitization, especially by the selection of vehicles used to dissolve Ag, but was also positively correlated with the efficiency of sensitization. Finally, we provided direct evidence that epicutaneous sensitization produces trafficking of MMGL-positive cells into regional lymph nodes.

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References


