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Stochastic Regulation of Cell Migration from the Efferent Lymph to Oxazolone-Stimulated Skin

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The systemic immune response is a dynamic process involving the trafficking of lymphocytes from the Ag-stimulated lymph node to the peripheral tissue. Studies in sheep have demonstrated several phases of cell output in the efferent lymph after Ag stimulation. When skin contact sensitizers are used as Ag, the efferent lymph cell output peaks ~96 h after Ag stimulation and is temporally associated with the recruitment of cells into the skin. To investigate the relative contribution of this high-output phase of effector lymphocytes to lymphocytic inflammation in the skin, we used a common contact sensitizer 2-phenyl-4-ethoxymethylene-5-oxazolone (oxazolone) to stimulate the skin and draining prescapular lymph node of adult sheep. The efferent lymph ducts draining the Ag-stimulated and contralateral control lymph nodes were cannulated throughout the experimental period. The lymphocytes leaving the lymph nodes during the 72-h period before maximum infiltration were differentially labeled with fluorescent tracers, reinjected into the arterial circulation, and tracked to the site of Ag stimulation. Quantitative tissue cytometry of the skin at the conclusion of the injection period (96 h after Ag stimulation) demonstrated more migratory cells derived from the Ag-stimulated lymph node than the contralateral control (median 18.5 vs 15.5 per field; p < 0.05). However, when corrected for total cell output of the lymph node, the Ag-stimulated migratory cells were 3.8-fold more prevalent in the skin than the contralateral control cells. These results suggest that the in situ immune response generally mirrors the frequency of recruitable lymphocytes in the peripheral blood. The Journal of Immunology, 2001, 166: 1517–1523.

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Materials and Methods

Animals

Randomly bred sheep, ranging in weight from 25 to 35 kg, were used in these studies. Sheep were excluded from the analysis if there was any gross or microscopic evidence of dermatitis. The sheep were given free access to

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† Abbreviations used in this paper: oxazolone, 2-phenyl-4-ethoxymethylene-5-oxazolone; CMFDA, 5-chloromethylfluorescein diacetate; CMTMR, 5-((4-chloromethyl)benzoyl)amino) tetramethylrhodamine; H&E, hematoxylin and eosin; DAPI, 4',6'-diamidino-2-phenylindole.
food and water. The care of the animals was consistent with guidelines of the American Association for Accreditation of Laboratory Animal Care (Bethesda, MD).

Ag stimulation

The sheep ear and neck was sheared bilaterally, and the lanolin was removed with an equal mixture of ether (J.T. Baker,Phillipsburg,NJ) and ethanol (AAPER,Shelbyville,KY).The Ag, a 5 or 7% solution of oxazolone (Sigma,St. Louis, MO) (20), was sprayed onto the ear and a localized region of the neck as a 4:1 oxazolone:olive oil mixture using a syringe and 23-gauge needle (21). A vehicle-only control was applied to the contralateral skin.

Lymph duct cannulation

In these experiments, the prescapular lymph node was used for all efferent lymph duct cannulations (22). After general endotracheal anesthesia and sterile surgical preparation, an incision was placed in the jugular furrow 5 cm cephalad to the suprasternal notch (23, 24). The efferent lymph duct was cannulated with a heparin-bonded polyurethane catheter (Solo-Cath, CBAS-C35; Setters Life Sciences, San Antonio, TX). The cannula was passed through a 5-cm s.c. tunnel and secured at the skin. The lymph was collected in 50-ml sterile centrifuge tubes (Falcon,Franklin Lakes,NJ) or 250-ml sterile plastic bags (Abbott,North Chicago,IL). Each collection bag contained 200 IU heparin,2000 IU penicillin (Cellgro;Mediatech, Herndon, VA), and 200 μg streptomycin (Cellgro;Mediatech).

Arterial cannulation

The common carotid artery was exposed using an incision in the jugular furrow 7 cm cephalad to the suprasternal notch. A 5-0 monofilament (Prolene;Ethicon,Somerville,NJ) purse string suture was placed in the carotid adventitia. A heparin-bonded polyurethane catheter (Solo-cath, CBAS-C35; Setters Life Sciences) was passed through a 14-gauge catheter (Insyte i.v. catheter;BD Infusion Therapy Systems, Sandy, UT) into the carotid artery. The catheter was secured using the purse string suture and surgical glue. The catheter was tunneled through the s.c. tissue to the dorsum of the neck and secured. The catheter was fitted with a sub-nose adapter and flushed with heparinized saline (100 U/ml)(Elkins-Sinn, Cherry Hill, NJ).

Electronic cell volume

The volume profile of cells in suspension was analyzed by an electronic cell counter (25, 26) (Coulter Counter ZM Analyzer; aperture,100 μm, 1/current, 1/4;1/gain,1/1) connected to a pulse height analyzer (Coulter Channelizer Model 256; Beckman Coulter, Miami, FL). Calibration of the system to obtain absolute volumes was performed daily using 10-μm microparticles (Beckman Coulter). Channel numbers lower than 6 corresponded to cellular debris and electronic noise. The volume of normal lymphocytes was considered to be 250 μm³ (range 142–400 μm³) and large lymphocytes 400 μm³ (range 400-1150 μm³) based on published data (27). Forward light scatter by flow cytometry was used to confirm the relative cell volume distributions (28).

Cyttoplasmic fluorescent dyes

The green 5-chloromethyIrhodamineisocyanate (CMFDA) and red 5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine (CMTMR) fluorescent dyes were commercially available (Molecular Probes,Eugene, OR). The packaged dyes were dissolved in DMSSO to yield a 10-mM stock solution that was stored at −70°C before labeling. Cells to be labeled were resuspended at 5 × 10⁷ cells/ml in warmed DMEM (Sigma) containing 2.5–10 μM CMFDA or CMTMR dye. The cells from the efferent lymph were typically collected over a 4-h period. Equal numbers of cells, usually limited by the control lymph, were labeled with either the CMFDA or CMTMR dyes. The cells were incubated for 30 min in a 37°C water bath shaker then washed twice in prewarmed DMEM and prepared for injection. Before injection, aliquots of the labeled cells were taken for flow cytometry analysis to confirm adequate fluorescence labeling and equal numbers of CMFDA- and CMTMR-labeled cells. The total number of injected cells over the 72-h period ranged from 1.9 to 5.2 × 10⁹.

Hematoxylin and eosin (H&E) histology

After euthanasia, the tissues were harvested and immediately processed by quick freezing or aldehyde fixation. Quick frozen tissue was sliced into 4 × 4 × 4-mm blocks, coated with OCT embedding media (TissueTek,Elkhart,IL) and placed in 15-mm cryomolds. The cryomolds were placed in liquid nitrogen-cooled 2-methylbutane followed by immersion in liquid nitrogen. The tissue was stored at −86°C before processing. The slides were stained in Harris hematoxylin (Harris Modified SH26-F00D; Fisher, Pittsburgh, PA) for 2 min followed by sequential rinses including a brief acid rinse. The slides were counterstained with Eosin Y (0.5% eosin,50% ethanol; Fisher) for 20 s then rinsed in ethanol and xylene (Fisher) followed by mounting with Permunt (Fisher).

Fluorescence histology

Tissue sections demonstrating the fluorescent cells were obtained after aldehyde fixation. Tissue was sliced into 4 × 4 × 4-mm blocks, placed in a glass vial, and fixed with 10% Formalin (Fisher). All fixed tissues were washed overnight in 30% sucrose and quick-frozen before sectioning. The tissue was cut into 6-μm sections and immediately fixed in methanol (Fisher) and air-dried. For fluorescence microscopy, the aqueous mounting media with 4,6-diamidino-2-phenylindole (DAPI) (1.5 μg/ml) (Vectashield mounting medium; Vector Laboratories,Burlingame,CA) was used in most experiments.

Immunohistochemistry

Cryostat sections were obtained from organ specimens perfused with OCT compound and snap frozen. After warming the slide to 27°C, the sections were fixed for 10 min in acetone. The slides were washed with PBS buffer and blocked with 20% sheep serum in PBS. The slides were treated with mAb at saturating concentrations for 1 h at 27°C and washed twice. The biotinylated detection Ab was added at 5 μg/ml (Vectastain-ABC;Vector Laboratories) and incubated for 20 min at 27°C. The slides were washed twice and treated with ABC developing reagent (Vector Laboratories) in PBS. After incubation, the slides were washed twice, developed with the Vector diaminobenzidine substrate, and counterstained with Harris hematoxylin (Fisher). The sheep anti-CD4 mAb C5D (29) and the anti-CD8 mAb IL-A51 (30) have been previously described.

Fluorescence microscopy

The tissue was imaged on a Nikon Optiphot-2 microscope (Melville,NY) equipped with an epiphotic fluorescence attachment. The microscope was equipped with 10× binocular eyepiece tubes and 20× and 60× plan apochromat objectives. The epifluorescent filter blocks were the blue filter UV-2A (400 nm DM), green filter B-1E (510 nm DM), and orange filters G-2A (580 nm DM) (Nikon). Additional filter sets included orange (560 nm DM) and combined (530 nm DM) filters (Omega Optical,Brattleboro, VT). The fluorescent images were recorded using a DC120 CCD camera (Kodak,Rochester,NY) with 24-bit color and 1280 × 960 picture resolution. For most fluorescent images, shutter speed (range 1/500 to 16 s) was 1.5–3 s with a 1 × zoom lens. Light exposure was minimized. Repeat images were routinely obtained in triplicate in order to control for fluorescence bleaching. The images were processed by the MDS 120 system software (Kodak) and recorded as digitized TIFF files. The archived images were processed using the MetaMorph Imaging System 4.0 software (Universal Imaging,Brandywine, PA).

Cell-count measurement

Cell counting was performed using digital images of fluorescence or H&E histology. The 24-bit color images were thresholded based on a red-green-blue (RGB) color space model. In H&E images, each color channel was optimized to threshold nuclei. After thresholding, a 30 × 60-μm grid overlay was used to define regions of interest. The grid overlaying the superficial venous plexus was used to define the 30 × 60-μm regions of interest. In fluorescence images, a similar grid overlay was used with a 400 × 200-μm region of interest. The MetaMorph Imaging System 4.0 (Universal Imaging) cell counting procedure with standard area estimates was used to count the number of cells in a cluster. The data was logged into Microsoft Excel (Redmond,WA) by dynamic data exchange.

Flow cytometry

The cellular fluorescence was assessed by flow cytometry using a Coulter Epics XL flow cytometer with Expo 2.0 software (Beckman Coulter). The flow cytometry data was collected at room temperature and exported to the Microsoft Excel spreadsheet for data analysis using WinList 3.0 (Verity, Topsham, ME). The flow cytometry experiments were calibrated daily using Sphero Rainbow Calibration Particles (SpheroTech, Libertyville, IL).

Statistical analysis

The migratory data was based on multiple comparisons of paired data by Student-Newman-Keuls or Mann-Whitney test for parametric ANOVA. The data is expressed as mean ± SD. The significance level for the sample distribution was defined as p < 0.05.
Results

Sheep ear and lymph node response to oxazolone

The inflammatory response to oxazolone was consistent with previous descriptions in rodents and sheep (31, 32). The ear became demonstrably thicker 24 h after application of the oxazolone. Although inflammatory cells such as polymorphonuclear leukocytes were prominent within the first 24–48 h, quantitative cytometry of the ear demonstrated a significant increase in the number of mononuclear cells infiltrating the superficial dermis at 96 h after Ag stimulation ($p < 0.05$) (Fig. 1). Immunohistochemistry demonstrated that most of the infiltrating cells were CD4$^+$ with fewer cells demonstrating a CD8$^+$ phenotype (Fig. 2). Similarly, stimulation of the skin with oxazolone resulted in a significant increase in the draining prescapular lymph node mass. Based on 26 sheep, the median mass of the Ag-stimulated lymph node was 8.64 g compared with 5.95 g for the contralateral control lymph node ($p < 0.001$) (Fig. 3).

Cell output in the efferent lymph

The cell output in the oxazolone-stimulated prescapular efferent lymph was qualitatively similar in 24 of the 26 sheep despite the absence of presensitization. The cell output in the efferent lymph demonstrated a marked increase in cell output beginning 72 h after Ag stimulation (Fig. 4). The peak in cell output was typically between 90 and 110 h after stimulation. Cell volumes were also assessed at each lymph collection period. “Blast cells” defined as large cells by Coulter Counter electronic sizing (33) increased in the efferent lymph as the total cell output increased; the peak of the large cells was observed between 90 and 120 h after stimulation (Fig. 5).

Recirculation of labeled cells

Cells from the Ag-stimulated and control efferent lymph were collected, differentially labeled with cytoplasmic fluorescent dyes, and reinjected into the arterial circulation. The labeled cells were injected throughout one of three 72-h time period: 24–96, 48–120, and 72–144 h. The infiltration of fluorescent cells paralleled the recruitment of unlabeled cells with maximum accumulation at 96 h (not shown). Based on these observations, most experiments focused on the 24- to 96-h injection period. During the injection period, the efferent lymph was continuously monitored for the appearance of labeled cells. Approximately 12 h after the beginning of the intraarterial injection, fluorescently labeled cells appeared in the efferent lymph and plateaued at 48 h (Fig. 6). Similarly, equal numbers of Ag-stimulated and control lymph-derived cells accumulated in the skin, gut, and lung lymph nodes (Fig. 7).

Recruitment of lymphocytes into the skin

The oxazolone-treated skin was harvested, aldehyde fixed, and examined by fluorescence microscopy (Fig. 8). Quantitative tissue cytometry at 96 h ($n = 12$) demonstrated an increased prevalence of cells derived from the Ag-stimulated lymph. However, the absolute difference in the number of cells identified in the Ag-stimulated tissue was relatively small (median 18.5 vs 15.5 per field; $p < 0.05$) (Fig. 9). To obtain a measure of the total number of cells migrating into the Ag-stimulated skin from the experimental and control lymph nodes, the prevalence of cells in the Ag-stimulated skin was adjusted to reflect the total number of lymph cells leaving the lymph node. When the number of recruited cells for each of the four sheep shown in Fig. 9 was corrected for the relative cell output of each lymph node, the predicted number of cells recruited from the Ag-stimulated lymph node was substantially greater (mean 3.8-fold) (Fig. 10).

Discussion

In this report, we directly compared the skin migration of lymphocytes leaving the oxazolone-stimulated and contralateral control lymph nodes. The bilateral lymph duct cannulations permitted the tracking of the cells being released into the efferent lymph from both the Ag-stimulated and control lymph nodes. In contrast to labeling the entire lymph node, the collection and reinjection of efferent lymphocytes eliminated the contamination of nonmigratory B cells or other resident cells in the lymph nodes that might confound the analysis. The bilateral comparison also controlled for selective lymphatic recirculation. Most important, this study permitted the direct study of the migratory behavior of lymphocytes derived from the high-output phase of the Ag-stimulated lymph

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**FIGURE 1.** Time course of infiltrating mononuclear cells in the perivascular tissue near the superficial venous plexus of the sheep ear. The ear was stimulated with epicutaneous oxazolone at time 0 and harvested at 24-h intervals from 48 to 144 h. Each time point represents three to five sheep. The processed tissue was stained with H&E, and digital photomicrographs were obtained. The mononuclear cells infiltrating the tissue in the area of the superficial venous plexus were counted using a 30 × 60-μm grid overlay; each rectangular section of the grid constituted one microscope field. The number of cells were counted as described in Materials and Methods and expressed as the mean ± SD.

**FIGURE 2.** Immunohistochemistry staining of the lymphocytes infiltrating the sheep ear 96 h after Ag stimulation with oxazolone. The sheep ear is stained for (A) CD4 (17D) and (B) CD8 (IL-A51). The dorsal surface of the contralateral ear as well as the ventral surface of the ipsilateral ear showed scant staining (not shown).
The findings demonstrated the dominant contribution of the Ag-stimulated lymph node to the in situ immune response. This contribution was based not on a striking migratory preference, but the numerical advantage produced by the dramatic increase in cell output from the Ag-stimulated lymph node.

The similar prevalence of labeled cells from the Ag-stimulated and control lymph nodes found in the oxazolone-treated skin has several important physiologic implications. First, the substantial numbers of recruited cells derived from the control efferent lymph suggests that the Ag-stimulated lymph node has a limited ability to direct lymphocyte migration to the site of Ag challenge. Although lymph node-acquired functional characteristics such as increased adhesivity or enhanced locomotion could still theoretically play a role in migration, the regulation of lymphocyte recruitment into oxazolone-stimulated inflammation was largely independent of the lymph node of origin. A corollary of this observation is that most of the selectivity of lymphocyte recruitment exists at the level of the local microcirculation and endothelial lining cells. Our data support the suggestion that local factors, such as the expression chemokines, chemokine receptors, or adhesion molecules, play a dominant role in regulating the recruitment of lymphocytes and the duration of the inflammatory process. Third, the observation that significant numbers of lymphocytes are not recruited until 72–96 h after Ag stimulation is consistent with adaptive changes in the microcirculation beyond the expression of regulatory membrane molecules. The 3- to 5-day time course suggests the possibility of significant structural changes in the microcirculation. Future experiments are being designed to assess these possibilities with direct observation of the microcirculation using intravital microscopy.

The biologic significance of the numerically small, but statistically significant, predominance of lymphocytes from the Ag-stimulated lymph node is uncertain. The finding was consistent irrespective of technical factors (sided stimulation and color of fluorescent dye) and was observed at all time points up to 144 h (data not shown). There are several possible interpretations of the slight predominance of lymphocytes from the Ag-stimulated lymph node. First, it remains possible that a subset of lymphocytes from the Ag-stimulated lymph node demonstrated preferential migration to the site of Ag stimulation. This migratory preference...
could be due to lymphocyte characteristics such as selective adhesivity or enhanced locomotion. Supportive evidence for this concept is the finding that the 10% difference in the number of cells infiltrating into the tissue is identical with the percentage of "blast" cells in the efferent lymph. Second, cell cycle analysis has shown that most of the large "blast" cells in the efferent lymph are undergoing active cell division (data not shown). Although equal numbers of cells were injected into the arterial circulation, the migration of these premitotic cells into the peripheral tissues may have resulted in cell division in situ. The cytoplasmic fluorescent dyes would be expected to segregate equally into the postmitotic cells and be detectable in the tissues. Third, the Ag-stimulated tissue might selectively retain the cells derived from the stimulated lymph node (34). This selective retention would most likely be the result of functional characteristics of the lymphocytes, rather than an effect of residual Ag. Studies using radiolabeled skin contact sensitizers have shown that most of the Ag is cleared within 24 h of oxazolone stimulation (35).

Studies defining the lymph node as the site of immunologic reactivity have frequently used epicutaneous Ags. Simple chemical compounds, often referred to as contact sensitizers, have several advantages for the study of the localized immune response (15, 20, 36–39). Foremost, contact sensitizers demonstrate a unique capacity to trigger an intense cellular immune response. Contact sensitizers trigger a selective T lymphocyte infiltration in the skin and paracortical hyperplasia in the draining lymph node (32). Recent
molecular studies have suggested that this unique “toxicity” is a result of a chemical modification of immunologically relevant proteins (40, 41). The selective T cell response may reflect hapten modification of class I-restricted peptides (42, 43). More practical advantages include the ability to easily control the dose and route of Ag administration. In addition, the Ag can be applied by “painting” the skin, without surgery or injections that could result in unpredictable lymphatic drainage.

A potential criticism of this study is that we did not formally demonstrate Ag-specific reactivity in vitro. It is possible that the absence of more dramatic recruitment from the Ag-stimulated lymph node simply reflects a failure to trigger an Ag-specific immune response. We believe this is unlikely because of the in vivo evidence of lymphocytic reactivity: 1) intense lymphocyte recruitment into the skin, 2) increases in size of the stimulated lymph node, 3) selective paracortical hyperplasia in the lymph node, 4) increased cell output in the Ag-stimulated lymph node, and 5) a “blast” response of lymphocytes in the effluent lymph. Furthermore, specific immune responses to oxazolone have been demonstrated in a variety of species including sheep, mice, and rats (21, 44–46).

The use of multicolored probes in tracking cell migration is an important application for fluorescent cell tracers. Particularly useful are fluorophores, such as CMFDA and CMTMR, with strong spectra. The signal isolation possible with these dyes is notably important for fluorescent cell tracers. Particularly useful have been multicolor studies that use CMFDA for lymphocytes and CMTMR for monocytes (40, 44).

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References