Brief Definitive Report

Rapid G Protein–regulated Activation Event Involved in Lymphocyte Binding to High Endothelial Venules

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Summary

The homing of blood borne lymphocytes into lymph nodes and Peyer's patches is mediated in part by recognition and binding to specialized high endothelial venules (HEV). Here we demonstrate that a rapid pertussis toxin–sensitive lymphocyte activation event can participate in lymphocyte recognition of HEV. In situ video microscopic analyses of lymphocyte interactions with HEV in exteriorized mouse Peyer's patches reveal that pertussis toxin has no effect on an initial "rolling" displayed by many lymphocytes, but inhibits an activation-dependent "sticking" event required for lymphocyte arrest. This is the first demonstration that physiologic lymphocyte–endothelial interactions can involve sequential rolling, activation, and activation-dependent arrest, previously shown only for neutrophils. The inhibitory effect of the toxin is dependent on its G protein–modifying ADP-ribosyltransferase activity and can be reversed by phorbol myristic acetate, which bypasses cell surface receptors to trigger activation-dependent adhesion. Lymphocyte sticking can occur within 1–3 s after initiation of rolling. We conclude that a rapid receptor-mediated activation event involving G protein signaling can trigger stable lymphocyte attachment to HEV in vivo, and may play a critical role in regulating lymphocyte homing.

The traffic of lymphocyte subsets is directed by binding to specialized venules, especially the high endothelial venules (HEV) in lymphoid tissues and in sites of inflammation (1, 2). These interactions display tissue selectivity that has been explained in part by the description of lymphocyte homing receptors capable of binding to tissue-selective endothelial cell adhesion receptors, the vascular addressins (3). Additional complexity in the regulation of lymphocyte–endothelial cell (EC) recognition is implied, however, by the observation that adhesion pathways used by lymphocytes can also be used in other physiologic settings, for example, by neutrophils interacting with acutely inflamed endothelium. Recently, we and others have proposed a model of leukocyte–EC interaction as an active, multistep process in which stable binding to the endothelium involves at least three sequential events (4, 5). In this model, interaction is initiated by primary, activation-independent but unstable adhesion that serves to slow the passage of the leukocyte allowing sampling of the EC surface and of any local soluble factors. If activating factors are present to which the leukocyte can respond, activation (the second step) occurs, leading to triggering of activation-dependent adhesion receptors. Binding of activation-dependent adhesion receptors to their EC ligands results in stable binding, resistant to physiologic shear, thus completing the process of "recognition." Additionally, the recruitment of the bound leukocyte from the vessel wall into the surrounding tissues may be regulated independently of leukocyte–EC recognition mechanisms, for example by tissue-derived chemoattractant or haptotactic signals. The requirement for sequential engagement of primary adhesion, activation, and secondary adhesion pathways allows the possibility of regulating leukocyte–EC recognition at each of the three steps involved.

However, the central features of the three-step model have been confirmed experimentally only in the case of neutrophil interactions with acutely inflamed venules in the rabbit, in which a primary "rolling" interaction involving the leukocyte selectin is followed by an activation-dependent "sticking" and arrest mediated by $\beta 2$ (CD18) integrins (6–8). Intravital microscopic studies, as well as in vitro models of neutrophil interactions with vascular adhesive ligands, demonstrate that under physiologic shear, each of the three steps is required for completion of the recognition process and for stable arrest of neutrophils (9, 10). We have suggested that a similar process might be used by other leukocytes, including lymphocytes (4). However, although in vitro studies demonstrate that lymphocytes can be triggered by activation to upregulate adhesion molecules, including integrins, the kinetics of such in vitro activation-dependent adhesion seem too slow (occurring over minutes) to explain the extremely rapid interactions of circulating lymphocytes with HEV in vivo, which occur within seconds (4, 11).

The present study was designed to explore directly the rel-

evance of the model to lymphocytes. To this end, we assessed the involvement of lymphocyte activation in lymphocyte–HEV interactions, using pertussis toxin (PTX) as a tool. The rationale for this approach was based on two considerations. First, many chemoattractant factors that are capable of triggering activation-dependent adhesion of leukocytes act via PTX-sensitive G protein–mediated intracellular signaling pathways. Second, Huang et al. (12) demonstrated that PTX-treated lymphocytes home poorly into lymph nodes from the blood in spite of retaining a capacity to bind in in vitro assays to lymph node HEV via the (activation-independent) lymph node homing receptor, the L-selectin. These considerations led us to hypothesize that a PTX-sensitive signal transduction pathway might play an essential role in lymphocyte–HEV recognition and binding in vivo.

Materials and Methods

Sample Lymphocyte Preparation. Peripheral and mesenteric lymph node lymphocytes from young adult BALB/c mice (Simonsen Labs, Gilroy, CA) were suspended at a concentration of 5×10^6/ml in DMEM (without sodium bicarbonate [Sigma Chemical Co., St. Louis, MO] supplemented with 20 mM Hepes, pH 7.0 (Research Organics, Cleveland, OH)], and containing 1 or 4% iron-supplemented calf serum (HyClone Labs, Logan, UT), and were incubated at 37°C with or without PTX (100 ng/ml; List Biological Labs, Campbell, CA) for 2 h. In some experiments, a mutant pertussis holotoxin (PT9K 129G) was used. This mutant PTX retains the agglutinating, hemolytic, and antigenic properties of native PTX, and gave indistinguishable subunit banding patterns with native PTX (13, 14) when compared by SDS-PAGE and Coomassie staining. During the last 30 min of incubation, the cells were labeled with either FITC at 60 μg/ml in DMEM, 4% calf serum as described (15), or with 5-carboxyfluorescein diacetate (CFDA-AM; Molecular Probes, Eugene, OR) used at a 1:4,000 dilution from a 1-mg/ml stock in DMSO in DMEM, 1% serum. Alternatively, after 2 h at 37°C, cells were labeled for 20 min with tetramethylrhodamine isothiocyanate (TRITC; Sigma Chemical Co.) at 1.7 μg/ml in 4% serum at room temperature. Labeled cells were washed through a calf serum cushion and resuspended at $\sim 5 \times 10^7$/ml in DMEM for intravenous injection. In some instances, PMA (Sigma Chemical Co.) was added to a 100-ng/ml final concentration, and cells were incubated an additional 5 min at 37°C immediately before injection. This brief treatment does not down-regulate L-selectin homing receptor expression.

In Situ Video Microscopic Analyses of Lymphocyte–HEV Interactions. Syngeneic recipients were anesthetized, and an aseptic abdominal incision was made to exteriorize the small intestine and Peyer's patches. A selected patch was embedded in silicon high-vacuum gel (Dow Corning, Midland, MI) to provide a matrix allowing application of a coverslip and positioning for epifluorescence microscopy and video recording using a silicon-intensified target video camera (Cohu, San Diego, CA). The small intestine was bathed in freshly made Dulbecco's PBS at 37°C. The recipient was maintained at 37°C by a stage-mounted strip heater regulated via a temperature probe beneath the mouse.

Approximately 2.5×10^7 labeled cells in 0.5 ml DMEM were injected intravenously into the recipients' lateral tail vein. Injected cells were then observed and recorded entering and interacting with Peyer's patch HEV for the duration of each experiment. In many experiments both the experimental and control cells of different colors were injected, observed, and interactions recorded in the same Peyer's patch HEV. Because there is a transient increase in blood flow immediately after injection, and because the Peyer's patch often requires minor repositioning to focus on HEV, data analyses were routinely initiated ~30 s after infusion of sample cells and continued during the next 2.5 min. Evaluation of the interactions of individual cells was carried out by replaying the video recording on a four-head VCR with shuttle wheel single frame forward and reverse control (JVC, Elwood Park, NJ). Initial control experiments showed no significant differences in the in vivo behavior of cells labeled with TRITC, FITC, or CFDA.

Analyses of Lymphocyte Behavior. The behavior traces are plots of distance moved along an HEV from the site of initial interaction vs. time in seconds after the initial interacting contact. The position of each selected cell was determined by analysis of the video image at 0.33-s intervals from the time and site of initial interaction, until the cell either released to the circulation ("passed," with the cell trace exiting the plot vertically), or became stably attached at a given site on the HEV wall ("arrested," with the trace exiting the plot horizontally). Note that a typical noninteracting cell would pass completely through an HEV in $\leqslant 0.66$ s.

For frequency plots summarizing population behaviors, a rapid procedure for quantifying the motion of cells was used. Individual cells were analyzed on a second-by-second basis for 5 s after initiation of interaction or until the cell passed from the HEV. Each 1-s period of interaction was assigned a value of 0 for sticking behavior, defined arbitrarily as static binding to the HEV wall during at least two consecutive seconds; or of 1, indicating predominant rolling interaction including "stop-and-go" behavior with static binding of <1 s. If during an interaction sequence a cell "skipped," releasing transiently from the vessel wall, only the time interval during which rolling or sticking took place was scored. For each cell, the values assigned to each second of interaction were averaged, yielding a time-averaged indicator of the cells' mean behavior. Inherent in this scoring procedure is a bias toward more interactive behaviors, so that the very brief <1-s initial rolling displayed by many PMA-preactivated lymphocytes (illustrated in Fig. 1 *e*) is not reflected in the mean score. In most experiments, at least 20 lymphocytes interacting with two to three or more independent distinct HEV were scored. The histograms present the relative distribution of behaviors among normal, PTX-treated, and PTX plus PMA–treated lymphocyte populations from three to five separate animals for each treatment type.

Results and Discussion

Lymphocyte interactions with HEV, specialized vessels that support lymphocyte extravasation into lymphoid tissues, can be visualized in the mouse by intravital microscopy of exteriorized Peyer's patches (11). Fluorescence-labeled lymphocytes are injected intravenously, and their interaction with Peyer's patch HEV is observed and recorded by video epifluorescence microscopy. We analyzed the in situ behavior of lymph node lymphocytes between ~30 s and 3 min after intravenous injection.

Individual lymphocytes displayed wide variability in behavior. Many interacted transiently with the HEV, then released only to interact again further downstream, as described by Bjerknes et al. (11). During periods of interaction, some lymphocytes "rolled" along the venule without stopping, others (the majority) rolled a variable distance before

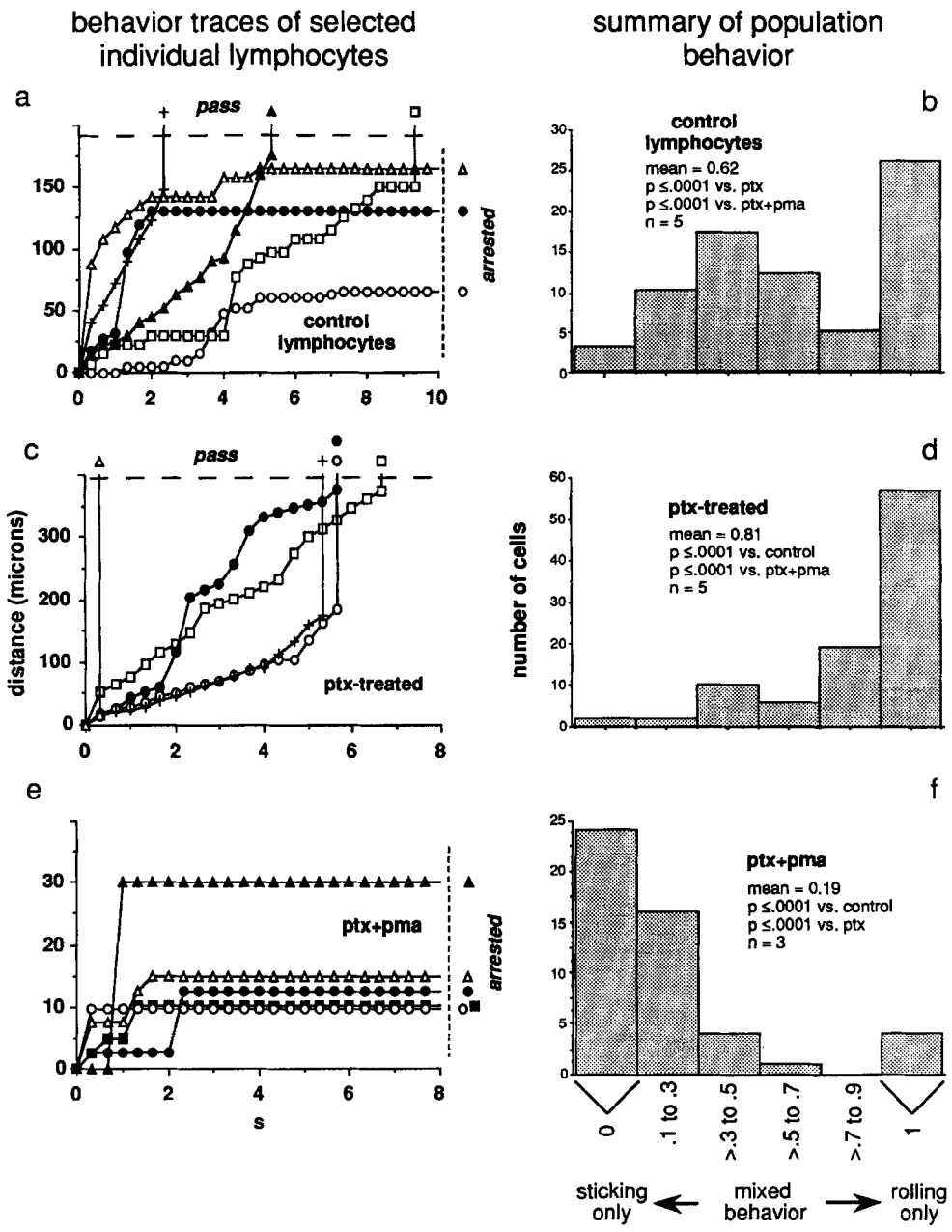

Figure 1. In situ behavior of injected normal, PTX-treated, and PTX plus PMA-treated lymphocytes during interaction with Peyer's patch HEV. Behavior traces of selected individual lymphocytes (*left*) and histograms of the frequency of time-averaged lymphocyte behaviors (*right*) illustrate the varied behavior of normal lymphocytes (*a* and *b*), the predominant rolling phenotype of PTX-treated lymphocytes (*b* and *c*), and the rapid sticking behavior of PTX plus PMA-treated cells (*e* and *f*). The histograms (*right*) present pooled analyses of three to five experiments for each treatment (see Materials and Methods). Lymphocytes were treated as described in the legend to Table 1 and in Materials and Methods.

stopping either briefly (<1 s, termed "stop-and-go behavior") or longer (>1 s, termed "sticking"). Overall, ~35% arrested stably, attaching statically to the vessel wall for ≥5 s (Table 1). The detailed behavior of several individual lymphocytes is displayed in Fig. 1 *a* as a plot of distance traveled from the site of initial interaction with the vessel wall vs. the elapsed time. A histogram summarizing the frequency of lymphocytes displaying predominant rolling, mixed (rolling and sticking), or predominant sticking behaviors is presented in Fig. 1 *b*. The data illustrate the variability of normal lymphocyte interactions.

To determine the effect of PTX, lymphocytes were incubated with PTX (100 ng/ml for 2 h at 37°C) before injection. PTX-treated lymphocytes interacted with the same frequency as normal cells, but demonstrated a striking reduction in stable arrest (Table 1). Most importantly, the character of interaction was altered dramatically, consisting almost exclusively of rolling with interspersed stop-and-go behavior (illustrated in Fig. 1 *c*), with static interactions rarely exceeding 1 s in duration. Fig. 1 *d* emphasizes the almost exclusive rolling behavior of PTX-treated lymphocytes. Thus, PTX treatment appears to uncouple physiologically a primary rolling phenotype from a PTX-sensitive sticking and arrest.

To rule out nonspecific toxicity, we next asked if we could reverse the inhibitory effect of PTX with PMA, which can directly stimulate protein kinase C (PKC) and trigger

Table 1. *Frequency and Outcome of Interactions between Entering Lymphocytes and HEV*

	Cell treatment		
	Control	PTX	PTX + PMA
Percent of cells entering HEV that interact	84 ± 3	88 ± 3	88 ± 4
Percent of interacting cells:			
That return to the blood (pass)	66 ± 4	95 ± 2	28 ± 6
That arrest (>5 s)	34 ± 4	5 ± 4	72 ± 4

Percentage of injected fluorescence-tagged lymphocytes entering HEV that interact with the vessel wall, and of interacting lymphocytes that return to the circulation (pass) or interact stably (>5 s). For the data, all cells interacting with selected HEV during some or all of the observation period were analyzed. Similar results were obtained in three to five independent animals for each cell treatment, and the data are pooled and expressed as the percent of cells displaying the indicated behavior, with the standard error. Lymph node cells were incubated with 100 ng/ml PTX for 2 h at 37°C immediately before injection. Control cells were incubated in parallel in medium for 2 h at 37°C. For PTX plus PMA treatment, PMA was added to a 100-ng/ml final concentration during the last 5 min of incubation with PTX.

activation-dependent lymphocyte adhesion (16). PTX-treated lymphocytes pulsed with PMA for 5 min just before injection demonstrated restored and even enhanced sticking and arrest (Table 1 and Fig. 1, *e* and *f*). Lymphocytes treated with PMA alone behaved similarly (not shown). Although PKC does not appear to play a critical role in physiologic lymphocyte–HEV interactions (12), the ability of PKC stimulation to mimic the effects of in vivo activation, and to circumvent the inhibition of sticking by PTX, suggests that PTX does not interfere with sticking mechanisms directly but rather may block an activation pathway that triggers these mechanisms.

PTX ADP-ribosylates sensitive G proteins, interfering with G protein–mediated signal transduction from cell surface receptors (17). A mutant pertussis holotoxin, specifically lacking ADP-ribosyltransferase activity by virtue of two amino acid substitutions in the S1 subunit (13, 14), had no effect on the in vivo behavior of lymphocytes (Fig. 2). The results suggest that lymphocyte sticking and arrest is triggered by an activation event mediated or regulated by PTX-sensitive G proteins.

Under physiologic conditions, this activation event may occur while lymphocytes are rolling along the endothelium. Consistent with this hypothesis, normal lymphocytes take longer to stick and arrest than PMA-preactivated cells (Fig. 1, *a* and *e*). Physiologic activation and triggering of shear-resistant adhesion receptors must nonetheless occur surprisingly rapidly. The mean interaction time before sticking of those normal lymphocytes that go on to arrest stably is only ~1–3 s (mean, 1.3 s; range, 0.2–4 s, n = 58 lymphocytes from five experiments). In keeping with such a rapid change in adhesive state, Bjerknes (11) reported a fairly abrupt increase between 1 and 3 s in the probability that an interacting cell would bind stably.

It should be emphasized that the conclusions here are drawn from the behavior displayed by the majority of injected lymph node lymphocytes. The fact that most lymphocytes interact with Peyer's patch HEV, and that a significant fraction of them adhere, is consistent with previous studies demonstrating that the major populations of B and T lymph node lymphocytes home well to organized lymphoid tissues, including the Peyer's patches (18). Less abundant subsets of lymphocytes, particularly memory and blast cell subsets, which can display highly tissue-selective homing properties, may behave differently (2). In particular, it will be important to determine if lymphoblasts returning to the circulation after local antigenic stimulation may (like PMA-preactivated cells) be able to stick and arrest on HEV independent of the receptor-mediated vessel-associated activating signals implied by the current studies. Indeed, a small subset of normal lympho-

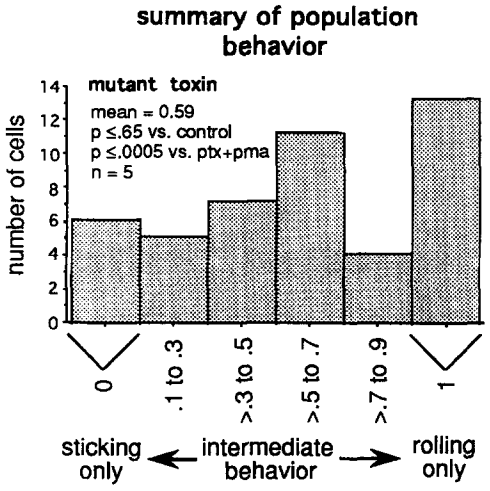

Figure 2. In situ behavior of lymphocytes pretreated with an enzymatically inactive pertussis holotoxin. The distribution of behaviors of lymphocytes treated with the mutant toxin is not significantly different from that of untreated control lymphocytes (Fig. 1 *b*). Procedures were as for Fig. 1, *c* and *d* (see Materials and Methods), except that a purified mutant holotoxin (PT9K 129G), specifically lacking ADP-ribosyltransferase activity, was used. Results are from a representative experiment of five performed with the mutant toxin.

cytes observed here bound almost immediately to HEV, especially during the initial 30-s period of stabilization after intravenous injection (not included in the quantitative studies presented). These may represent such preactivated lymphocytes among the mesenteric lymph node lymphocyte population.

G protein signaling pathways appear well suited to the very rapid alterations in cellular behavior required for lymphocyte sticking to HEV under physiologic flow conditions. Cellular activation through G protein–linked receptors can be extraordinarily rapid, with global effects on cellular activities. Examples include the rapid depolarization of retinal neurons by photo activation of rhodopsin; and perhaps more relevant, stimulation of the aggregation of neutrophils within seconds after triggering of the FMLP receptor. Lymphocytes are known to use PTX-sensitive G proteins to transduce signals from a number of cell surface receptors, notably including receptors for cytokines of the chemokine family (19–21), which have been hypothesized to play a role in regulation of lymphocyte–endothelial interactions (4). The hypothesized role of chemokines in lymphocyte–endothelial recognition has received additional support from the recent demonstration that members of the chemokine family can bind to and be displayed at the lumenal surface of venules involved in leukocyte or lymphocyte traffic (22, 23). Chemokines are also thought to be important in lymphocyte chemotaxis through tissues (19–21), and may be able to regulate diapedesis independently of the rapid activation of HEV binding studied here. Crosslinking of some lymphocyte surface receptors can also trigger activation-dependent lymphocyte adhesion mechanisms (reviewed in reference 5), and it will be important to determine if these activating pathways also involve G protein–mediating signaling.

In conclusion, our results demonstrate that a rapid activation-dependent adhesion event involving PTX-sensitive G proteins can participate in lymphocyte–HEV interactions, leading to stable arrest of lymphocytes under shear flow. For the major population of unactivated lymph node lymphocytes studied here, this activation-dependent sticking appears to follow a primary rolling interaction that may allow these lymphocytes to receive stimulating signals by initiating contact with the endothelium and slowing their passage. Our findings are compatible with in vitro studies suggesting that PTX can inhibit activation-dependent participation of the integrin LFA-1 in lymphocyte binding to lymph node HEV in frozen sections (24). The observations establish a strong parallel with similar cellular events involved in neutrophil interactions with inflamed endothelium (4, 6–10, 25), and support a general model of leukocyte–EC interaction as a multistep process (4, 5). In this model, the local factors responsible for lymphocyte activation may assume equal importance with lymphocyte homing receptors and vascular addressins in determining the specificity of lymphocyte–HEV interactions and lymphocyte homing in organized lymphoid tissues and sites of inflammation.

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