A Distinct Endothelial Cell Recognition System That Controls Lymphocyte Traffic into Inflamed Synovium

S. JALKANEN, A. C. STEERE, R. I. FOX, E. C. BUTCHER

Lymphocytes are essential mediators of normal tissue inflammatory reactions and of pathologic tissue damage in, for example, rheumatoid arthritis and other autoimmune diseases. In a study of the mechanisms controlling lymphocyte entry into sites of inflammation from the blood, the function and specificity of lymphocyte-endothelial interactions were examined in inflamed joint tissue (synovium) from patients with rheumatoid arthritis. Synovial high endothelial venules (HEV) supported the binding of normal peripheral blood lymphocytes in vitro. The characteristics of this binding, which were similar to those of lymphocyte-HEV interactions controlling lymphocyte migration into organized lymphoid tissues, included a requirement for calcium ions, a dependence on metabolic activity, and a preferential adherence of circulating lymphocytes as opposed to immature thymocytes. However, the binding of lymphocytes to synovial HEV was not inhibited by a monoclonal antibody to lymphocyte receptors for lymph node HEV, and synovial HEV failed to bind either lymph node HEV–specific or mucosal HEV–specific B lymphoblastoid cells. The results suggest that a lymphocyte-endothelial cell recognition system that is distinct from such systems in organized lymphoid tissues directs the extravasation of normal lymphocytes as well as pathologically important effector cells into inflamed synovium.

LYMPHOCYTES PLAY AN IMPORTANT role as regulatory and effector cells within tissue sites of inflammation. An understanding of the mechanisms that control lymphocyte localization in inflamed tissues might lead to specific means of manipulating such responses, for example in putative autoimmune diseases. The exit of circulating lymphocytes from the blood into organized lymphoid tissues, such as lymph nodes, is controlled by interactions with specialized endothelial cells lining lymphoid organ venules (*1*). The lymphocyte-endothelial cell recognition system used in lymphocyte homing to peripheral lymph nodes is different from that in mucosal lymphoid tissues. Monoclonal antibodies have been described that selectively block either the binding of murine lymphocytes to high endothelial venules (HEV) in peripheral lymph nodes (*2, 3*) or their interaction with mucosal HEV (*4*). Furthermore, certain murine lymphomas and human lymphoid cell lines have been identified that bind selectively to either peripheral lymph node HEV or to mucosal HEV (*5, 6*). Although similar lymphocyte-endothelial cell interactions are likely to regulate lymphocyte traffic into chronically inflamed tissues, there are no previous functional studies of lymphocyte-endothelial cell recognition mechanisms in extranodal sites of inflammation. In rheumatoid arthritis and other inflammatory joint diseases, affected synovium shows villous hypertrophy, lymphoplasmacytic infiltrates, sometimes with formation of organized lymphoid follicles, and extensive vascular proliferation (*7, 8*). Many vessels, particularly in areas of heavy lymphocytic infiltration, are lined by plump endothelial cells and have the histologic appearance of HEV (*9*). To understand the role of the vascular endothelium in the homing of lymphocytes to a specific site of chronic inflammation, we studied the function and specificity of lymphocyte-endothelial cell interactions in rheumatoid synovium.

Of eight synovectomy specimens from patients with rheumatoid arthritis, four samples with marked lymphocytic infiltration were chosen for study. Staining of endothelium with antibody to factor VIII revealed a spectrum of vessel types in the synovium, including arterioles and venules (Fig. 1A); venules lined by high endothelial cells (Fig. 1B), typical of HEV in lymph nodes, were readily apparent. To examine the functional capacity of these HEV to interact with lymphocytes, we used an assay of lymphocyte binding to HEV in frozen sections (*10, 11*). Binding experiments on each of the four synovial specimens were repeated three times. Since the results of all the experiments were similar, the mean results ± standard errors (SE) are presented here. As shown in Figs. 1C and 2, normal human peripheral blood lymphocytes from four different donors bound well to the HEV of all four synovial specimens, and this binding was highly specific.

We then studied the general characteristics of this binding to see if they were similar to those of lymphocyte binding to HEV in organized lymphoid tissues. Lymphocyte adherence to lymph node HEV is a metabolically active, calcium-dependent process that can be inhibited by sodium azide or EDTA (*12*). We therefore incubated peripheral blood lymphocytes with 5 m*M* EDTA or 5 m*M* sodium azide at 4°C for 30 minutes, and then performed the HEV assay in the continued presence of the inhibitors. EDTA decreased the binding to synovial

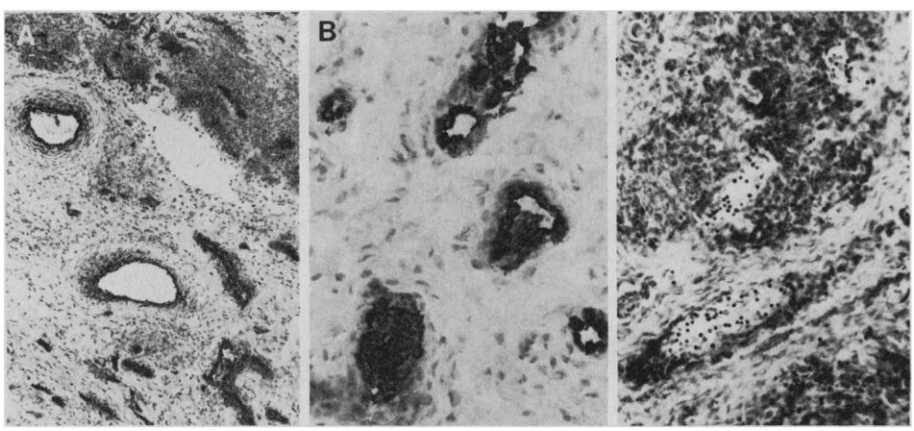

Fig. 1. Endothelial cells in synovium. (A) At low power, a spectrum of vessels is revealed after staining of endothelial cells in synovial frozen sections with antibody to factor VIII. Acetone-fixed frozen sections (6 μm) were subjected to immunoperoxidase staining by incubation with rabbit antiserum to factor VIII (1:400, Dako, Copenhagen), biotinylated goat antiserum to rabbit IgG (1:100, Vector Laboratories), and then peroxidase-avidin (1:100, Vector). The substrate was 5 mg of diaminobenzidine tetrahydrochloride in 10 ml of 0.5*M* tris-HCl (*p*H 7.6) with 4 μl of 30% H_2O_2. (B) Higher power, showing several venules lined by high endothelium. (C) Specific binding of peripheral blood lymphocytes (PBL) (dark, round cells) to synovial HEV in the in vitro binding assay. Lymphocytes were incubated on 12-μm-thick fresh frozen sections for 30 minutes at 7°C with mild agitation as described previously (*13*). After incubation, the bound cells were fixed to the section in 1% glutaraldehyde and stained with Thionine. No significant, reproducible binding of lymphocytes was observed to synovial cells or other nonvascular structures.

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HEV by 98% and sodium azide by 79%. Immature nonmigrating cells, such as the bulk of thymocytes, bind poorly to HEV in lymphoid tissues, but mature, migrating cell types bind well (*10, 11, 13*). Similarly, in this study, thymocytes bound only one-fourth as well as peripheral blood lymphocytes both to synovial (25 ± 6%, mean ± SE) and peripheral lymph node (25 ± 7%) HEV. Thus, the binding of peripheral blood lymphocytes to synovial HEV is a metabolically active, calcium-dependent, developmentally regulated process, similar in these respects to lymphocyte-HEV interactions in lymph nodes.

As already mentioned, HEV in lymph nodes and in mucosal lymphoid tissues express distinct, organ-specific determinants for lymphocyte recognition. To determine the specificity of synovial endothelium, we did blocking experiments using the monoclonal antibody MEL-14 (*3*), and also assessed the capacity of synovial HEV to bind lymph node HEV– and mucosal HEV–specific B lymphoblastoid cell lines. MEL-14 is directed against mouse lymphocyte homing receptor for lymph nodes; and it selectively blocks mouse lymphocyte binding to lymph node HEV without affecting adherence to mucosal (Peyer's patch) HEV (*3*). The function and specificity of lymphocyte-HEV interactions have been conserved during mammalian evolution (*14*). Thus MEL-14 also blocks mouse lymphocyte binding to human lymph node HEV but not to mucosal (appendix) HEV, and we were able to use this property to determine whether synovial HEV are of the lymph node type. MEL-14 cross-reacts weakly with human homing receptors as well, and partially inhibits binding of human lymphocytes to lymph node HEV when the antibody is present throughout the assay (*15*). Since the immune response in synovium more closely resembles that of nonmucosal as opposed to mucosal tissues [for example, immunoglobulin G (IgG) secretion predominates over IgA secretion (*16*) and T cells predominate over small B cells (*7*)], we predicted that MEL-14 would also inhibit lymphocyte binding to synovial HEV. However, as shown in Fig. 2, MEL-14 inhibited both mouse and human lymphocyte binding to peripheral lymph node, but not to mucosal lymphoid organ or to synovial HEV. In further studies with human lymphoid cell lines, neither LB-25, a B lymphoblastoid line that binds selectively to peripheral lymph node HEV, nor KCA, a line that binds to mucosal lymphoid HEV, bound well to HEV in the inflamed synovium (Fig. 3). Thus, the interaction of lymphocytes with synovial HEV is functionally different from those in either lymph nodes or mucosal lymphoid tissues.

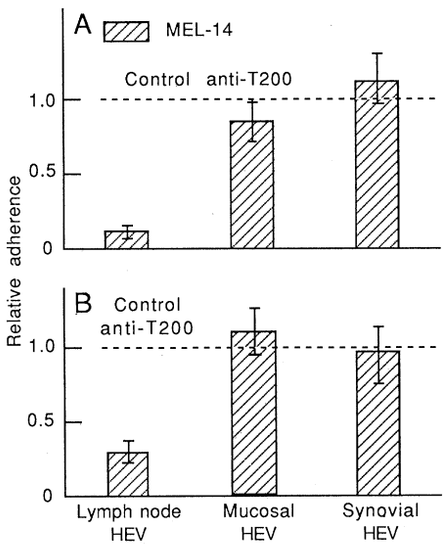

Fig. 2. Binding of human or mouse lymphocytes to synovial HEV is not inhibited by MEL-14. Mouse mesenteric lymph node cells (A) or human PBL (B) were incubated for 30 minutes on ice with purified MEL-14 or with class-matched negative control antibody [30G12, anti-mouse T200 (*26*)] (180 μg of each antibody with 1.8×10^7 human cells in 600 μl of medium or 18 μg of antibody with mouse cells). When human cells were used, the HEV assay was done in the presence of the antibodies. When mouse cells were used, the antibody-coated cells were washed once through fetal calf serum before the assay. The number of cells bound per HEV was determined under dark-field illumination for each sample, and the mean number of cells per HEV was calculated with its standard error. (At least 120 HEV were assessed for each sample.) These values were normalized by dividing by the mean number of control antibody-treated PBL per HEV [in (B), approximately four cells per HEV] or mouse lymphocytes per HEV [in (A), approximately two cells per HEV]. The binding of control lymphocytes thus defines a relative adherence ratio (RAR) of unity in each experiment. RAR are reported with their standard errors, calculated by the delta method. Data reduction, statistics, and details of the assay system have been described (*13*).

Our results imply that a distinct lymphocyte-endothelial recognition system regulates lymphocyte traffic into inflamed synovium. We do not know whether this recognition system is unique to synovium (or even to a particular disease of synovium), or instead is associated with sites of inflammation in many tissues. The latter possibility seems less likely on teleologic grounds, since there seems to be no reason to link inflammatory responses in unrelated tissues such as, for example, joints and brain (*17*). Indeed, it now seems possible that endothelial cells in many organs express tissue-specific determinants for lymphocyte recognition. Although we do not know which particular lymphocyte subsets are capable of interacting with synovial HEV, we have previously

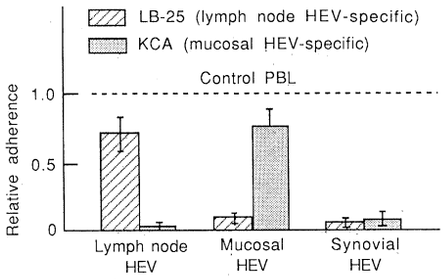

Fig. 3. Lymphoblastoid cell lines discriminate between lymphoid organ and synovial HEV. For each sample, human PBL were mixed with B lymphoblastoid cells, and the mixture was incubated on the indicated frozen sections. Using a dark-field microscope, lymphocytes adherent to HEV were scored as either lymphoblastoid cells or PBL (they were easily distinguishable because of their size difference). The RAR were calculated as the ratio of sample (KCA or LB-25) cells to PBL on HEV, divided by the input ratio. The binding of PBL defines an RAR of unity. Statistics were calculated as described (*13*), according to the delta method. The results of three independent experiments with synovial tissues from four different patients were not statistically different; the data here are pooled and presented as means ± SE.

demonstrated that many "virgin" B and T lymphocytes (that have not encountered specific antigen) express receptors for both lymph node and mucosal HEV (*2, 18, 19*); thus they may express multiple homing receptors. This might explain the capacity of peripheral blood lymphocytes to interact with synovial HEV, as well as the presence of small B and T lymphocytes in the synovial infiltrate (*7, 8*). However, many memory and effector lymphocytes exhibit organ-specific migratory properties (*19*), and we have proposed (*19–21*) that they express receptors specific for the endothelial cells characteristic of their site of initial antigenic stimulation. The use of organ-specific homing mechanisms by effector and memory populations might not only serve to increase the efficiency of specific immune responses within related tissues but, equally important, might decrease opportunities for autoimmune cross-reactions by excluding, for example, effector cells arising in response to mucosal pathogens from entering relatively privileged sites such as joints.

The present findings may have clinical relevance. In rheumatoid arthritis, T lymphocytes in the synovium are thought to play a key pathogenetic role in the disease process, and suppression of lymphocyte function via medication, or depletion of lymphocytes via thoracic duct drainage (*22*) or irradiation (*23*), generally results in clinical improvement of the patient. Recognition of a specific receptor mediating the formation of synovial infiltrates raises the possibility of selectively blocking lymphocyte extravasation into synovium by monoclonal

antibody or pharmacologic agents. If feasible, such therapy might be more specific and less toxic than currently available modalities.

REFERENCES AND NOTES

1. J. L. Gowans and E. J. Knight, *Proc. R. Soc. London* **159**, 257 (1964).
2. W. M. Gallatin, I. L. Weissman, E. C. Butcher, *Nature (London)* **304**, 30 (1983).
3. R. A. Rasmussen, Y. H. Chin, J. J. Woodruff, T. G. Easton, *J. Immunol.* **135**, 19 (1985).
4. For results in the rat, see Y. H. Chin, R. A. Rasmussen, J. J. Woodruff, T. G. Easton, *ibid.* **136**, 1 (1986).
5. E. C. Butcher, R. G. Scollay, I. L. Weissman, *Eur. J. Immunol.* **10**, 556 (1980).
6. S. Jalkanen, R. Bargatze, L. Herron, E. C. Butcher, *ibid.*, in press.
7. C. L. Young, T. C. Adamson, J. H. Vaughan, R. I. Fox, *Arthritis Rheum.* **27**, 32 (1984).
8. D. G. Malone, S. M. Wahl, M. Tsokoo, H. Catell, J. L. Decker, R. L. Wilder, *J. Clin. Invest.* **74**, 1173 (1984).
9. A. J. Freemont, C. J. P. Jones, M. Bromley, P. Andrews, *Arthritis Rheum.* **26**, 1427 (1983); M. Ziff, *ibid.* **17**, 313 (1974).
10. H. B. Stamper and J. J. Woodruff, *J. Exp. Med.* **144**, 828 (1976).
11. E. C. Butcher, R. G. Scollay, I. L. Weissman, *J. Immunol.* **123**, 1996 (1979).
12. J. J. Woodruff, J. Katz, L. E. Lucas, H. B. Stamper, *ibid.* **119**, 1603 (1977).
13. A detailed description of the human lymphocyte HEV binding assay, and of standard data reduction techniques used, has been reported; S. Jalkanen and E. C. Butcher, *Blood* **66**, 577 (1985). The delta method, used to calculate the standard error of relative adherence ratios, is from C. R. Rao, *Linear Statistical Inference and Its Applications* (Wiley, New York, 1965), pp. 319–322.
14. N. Wu, S. Jalkanen and E. C. Butcher, unpublished results.
15. S. Jalkanen *et al.*, in preparation.
16. J. H. Herman, J. Bradley, M. Ziff, J. D. Smiley, *J. Clin. Invest.* **50**, 266 (1971).
17. In this context, it is relevant that Herman *et al.* (*16*) have presented evidence that circulating antigen-specific plasma cell precursors, induced by subcutaneous (and hence lymph node) immunization with tetanus toxoid, are excluded from synovium. By contrast, lymph node lymphocytes activated by peripheral immunization are quite effective at migrating to subcutaneous sites of inflammation, as well as at returning to lymph nodes (*24*, *25*). These studies in vivo suggest that synovial HEV determinants may indeed be joint-specific.
18. S. K. Stevens, I. L. Weissman, E. C. Butcher, *J. Immunol.* **128**, 844 (1982).
19. E. C. Butcher, *Curr. Top. Microbiol. Immunol.*, in press.
20. E. C. Butcher, and I. L. Weissman, in *Fundamental Immunology*, W. Paul, Ed. (Raven, New York, 1984), pp. 109–127.
21. For example, mouse gut intraepithelial lymphocytes, a mucosal effector cell population, bind exclusively to mucosal (Peyer's patch) HEV and not to lymph node HEV, thus explaining their specific localization to mucosal surfaces (M. Schmitz, D. Nunez, E. C. Butcher, in preparation). Human lamina propria blast cells exhibit a similar specificity for mucosal HEV (S. Jalkanen, R. MacDermott, J. Nash, E. C. Butcher, unpublished results).
22. C. M. Pearson, H. E. Paulus, H. I. Machleder, *Ann. N.Y. Acad. Sci.* **256**, 150 (1975).
23. S. Strober *et al.*, *Ann. Intern. Med.* **102**, 441 (1985).
24. W. Chin and J. B. Hay, *Gastroenterology* **79**, 1231 (1980).
25. M. L. Rose, D. M. V. Parrot, R. G. Bruce, *Immunology* **35**, 415 (1978).
26. J. A. Ledbetter and L. A. Herzenberg, *Immunol. Rev.* **47**, 63 (1979).

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Altered Regulation of Airway Epithelial Cell Chloride Channels in Cystic Fibrosis

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In many epithelial cells the chloride conductance of the apical membrane increases during the stimulation of electrolyte secretion. Single-channel recordings from human airway epithelial cells showed that β-adrenergic stimulation evoked apical membrane chloride channel activity, but this response was absent in cells from patients with cystic fibrosis (CF). However, when membrane patches were excised from CF cells into media containing sufficient free calcium (approximately 180 nanomolar), chloride channels were activated. The chloride channels of CF cells were similar to those of normal cells as judged by their current-voltage relations, ion selectivity, and kinetic behavior. These findings demonstrate the presence of chloride channels in the apical membranes of CF airway cells. Their regulation by calcium appears to be intact, but cyclic adenosine monophosphate (cAMP)–dependent control of their activity is defective.

EVIDENCE OF A DEFECT IN CHLORIDE transport across epithelial cells in patients with cystic fibrosis (CF) was published by Quinton (*1*) who used ion-replacement studies to show that the elevated transepithelial voltage across isolated, perfused CF sweat ducts was due to an abnormally low Cl^- permeability. Knowles *et al.* (*2*) found that a Cl^- diffusion potential could be generated across normal, but not CF, airway mucosa during superfusion with solutions of low Cl^- concentration. These findings were confirmed by Widdicombe, Welsh, and Finkbeiner (*3*) for primary cultures of tracheal cells. Monolayers from CF patients failed to show an increase in the current associated with Cl^- secretion when they were exposed to isoproterenol or prostaglandin E_2. Unlike the situation in normal cells, the apical membrane potential of CF cells was not depolarized by secretory stimuli that evoke an increase in apical membrane Cl^- conductance. Thus, impaired apical Cl^- conductance may limit water and electrolyte secretion and thereby contribute to the accumulation of mucus that compromises the pulmonary function of CF patients. Nevertheless, it was not apparent whether the reduced expression of airway cell Cl^- conductance resulted from an absence or impairment of the conductance mechanism per se, or from a defect in its regulation.

We applied patch-clamp techniques (*4*) for single-channel recording to upper airway cells in primary culture to determine whether the decreased Cl^- conductance of CF cells was due to alterations in the presence, properties, or regulation of single Cl^- channel activity. This approach required relatively little material, in contrast to isotopic flux determinations or transepithelial measurements. We used primary cell culture because it is unlikely that the secondary effects of inflammatory or immune responses would be expressed in culture. The culture methods (*5*) were similar to those described by Widdicombe and co-workers (*6*). We noted, as have others (*3*), no readily apparent differences in the morphologic appearance or growth characteristics of cells derived from CF airways.

During cell-attached recording from normal cells, the β-adrenergic stimulant epinephrine evoked Cl^- channel activity after a delay that was partly due to diffusion of this β-agonist from the site of addition to the recording site (Fig. 1). The average delay between agonist addition and initiation of channel activity was approximately 3 minutes. All of the membranes that we studied were electrically quiet before addition of agonist, so that epinephrine induced single-channel activity in a previously inactive membrane. Unitary events of two different conductance levels could be discerned during cell-attached stimulation by epinephrine (Fig. 1). Both conductance levels were observed frequently in the same membrane patch but were also present individually in separate recordings. Responses identical to that shown in Fig. 1 were obtained upon addition of an analog of cyclic

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