Involvement of Sialic Acid on Endothelial Cells in Organ-Specific Lymphocyte Recirculation

Abstract. *Mouse lymphocytes incubated on cryostat-cut sections of lymphoid organs (lymph nodes and Peyer's patches) specifically adhere to the endothelium of high endothelial venules (HEV), the specialized blood vessels to which recirculating lymphocytes attach as they migrate from the blood into the parenchyma of the lymphoid organs. Treatment of sections with sialidase eliminated the binding of lymphocytes to peripheral lymph node HEV, had no effect on binding to Peyer's patch HEV, and had an intermediate effect on mesenteric lymph node HEV. These results suggest that sialic acid on endothelial cells may be an organ-specific recognition determinant for lymphocyte attachment.*

An important component of immune surveillance is the dissemination of immunocompetent lymphocytes throughout the body. This is accomplished by lymphocyte recirculation, a process by which mature lymphocytes move continuously from the bloodstream into lymphoid organs and back to the blood again (*1*). Blood-borne lymphocytes are able to enter certain lymphoid organs including lymph nodes and Peyer's patches (gut-associated lymphoid organs) by binding to and crossing specialized venules referred to as high endothelial venules (HEV) because of their cuboidal (or high) endothelial cells (*2*). The lymphocyte-HEV interaction can be studied by means of a rigorously validated in vitro assay developed for the rodent (*3–7*). In this assay, lymphocytes overlaid onto frozen sections of lymph nodes or Peyer's patches adhere selectively to the morphologically distinct HEV exposed in the sections (Fig. 1, A and C).

That the attachment of lymphocytes to HEV is highly specific has been verified in vivo and also with the frozen section assay. In particular, T lymphocytes show distinct binding preferences for peripheral lymph node HEV, and B lymphocytes preferably bind to Peyer's patch HEV (*7*). Furthermore, certain lymphoma lines exhibit nearly absolute binding specificities for either peripheral node HEV or Peyer's patch HEV (*6*). Specificity of binding of lymphocyte subsets and lymphoma lines to mesenteric lymph node HEV is intermediate between that to peripheral lymph node and Peyer's patch HEV. These results indicate that the lymphocyte subpopulations and high endothelial cells of different lymphoid organs must display surface adhesion molecules of varying specificity. The adhesive specificities dictated by these molecules are believed to underlie the distinctive recirculatory patterns of T and B cells through mucosal as opposed to nonmucosal lymphoid organs and may be involved in determining the dissemination sites of lymphoid malignancies.

The identification of the adhesion molecules that mediate lymphocyte adherence to HEV has recently received a great deal of attention. Cell surface antigens that appear to be involved in lymphocyte adherence to peripheral node HEV have been identified on murine lymphocytes by immunological methods (*8, 9*). However, these studies provide no information on the physical nature of the lymphocyte-HEV interaction or on the identity of the HEV attachment sites.

We previously prevented evidence that lymphocytes possess a cell-surface carbohydrate-binding (lectin-like) molecule that is involved in the binding of lymphocytes to peripheral node HEV in rat and mouse (*10*). We therefore examined the HEV for the presence of carbohydrates that serve as recognition sites for the putative adhesive lectin on lymphocytes. Preliminary support for the existence of such carbohydrate recognition determinants was based on the observation that periodate treatment of frozen sections (under conditions selective for carbohydrate oxidation) prevents binding of lymphocytes to both Peyer's patch HEV and peripheral node HEV (*11*). We now report that treatment of frozen sections of lymphoid organs with sialidase (*12*) affects lymphocyte binding to HEV in an organ-specific manner.

Sections of peripheral lymph nodes, mesenteric lymph nodes, and Peyer's patches from mice were cut on a cryostat, mounted on glass slides and fixed with paraformaldehyde (legend to Fig. 2). Treatment of the sections with 2 units of *Vibrio cholera* sialidase (Gibco) completely prevented lymphocyte binding to peripheral node HEV but had no effect on binding to Peyer's patch HEV (Fig. 2). Inclusion of sialic acid in the digestion mixture (0.1*M*) abolished the enzyme's activity. The *Vibrio* sialidase was active on peripheral node HEV at concentrations as low as 0.05 unit but had no effect on binding to Peyer's patch HEV at concentrations up to 50 units (Figs. 1 and 3). Sialidase had an intermediate effect on lymphocyte attachment to mesenteric node HEV, producing a maximal reduction of 50 to 60 percent (Fig. 3).

In order to provide further assurance of the specificity of the enzyme effects, affinity-purified sialidase from *Clostridium perfringens* was employed. Again, enzyme treatment of the sections resulted in the elimination of lymphocyte binding to peripheral node HEV, a partial (50 percent) reduction of binding to mesenteric node HEV, and no effect on binding to Peyer's patch HEV (Fig. 4). The effects on peripheral node HEV and mesenteric node HEV were prevented if the enzyme was combined with sialyl-lactose, a substrate for sialidase.

These findings suggest that sialic acid residues on peripheral node HEV are required for lymphocyte attachment. Our results indicate that the target of the enzymes was sialic acid since (i) elimination of lymphocyte binding to HEV was observed with two different sialidases, one of which was affinity-purified; and (ii) the activity of the enzymes could be prevented either by product inhibition (*V. cholera* sialidase) or substrate inhibition (*C. perfringens* sialidase). These

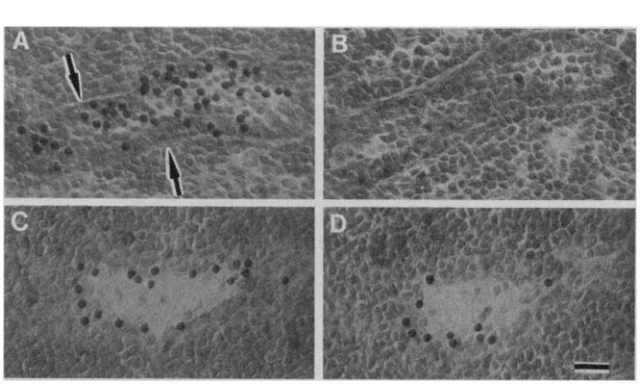


Fig. 1. Effect of sialidase treatment of peripheral node and Peyer's patch sections on lymphocyte attachment. Peripheral node and Peyer's patch sections were treated with control buffer (A and C) or 50 Gibco units of *Vibrio* sialidase (B and D) for 30 minutes at 37°C and tested for lymphocyte binding as described in Fig. 2. Binding of exogenous lymphocytes to HEV was easily distinguished by the characteristic appearance of densely bound, darkly staining lymphocytes overlying the histologically distinct HEV. (A and B) Adjacent sections of peripheral node showing the same HEV, arrowheads denote the boundary of HEV. (C and D) Adjacent sections of Peyer's patch with the same HEV. Scale bar, 20 μm.

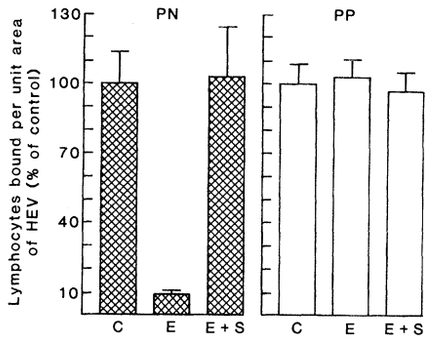
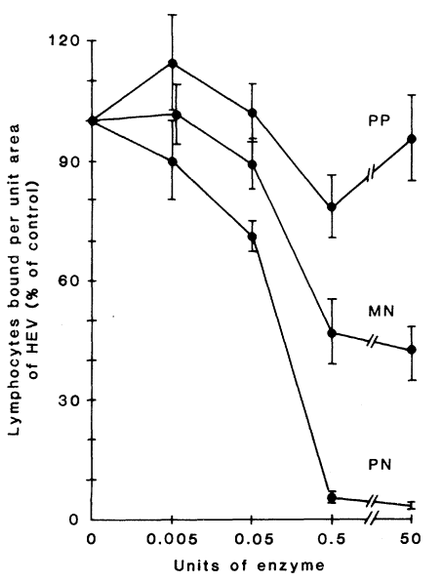


Fig. 2. Effect of *Vibrio cholera* sialidase treatment of peripheral lymph nodes (PN) and Peyer's patch (PP) sections on lymphocyte binding to HEV. Peripheral nodes (cervical, axial, and brachial nodes) and Peyer's patches from BALB/c mice (females, 5 to 8 weeks old) were frozen in a common block and sections, 10 μm thick, were cut. The sections were mounted in wells on glass slides, air dried, and fixed with paraformaldehyde (*10*). C denotes treatment of sections with control buffer (100 μl of 50 m*M* sodium acetate, 100 m*M* NaCl, 4 m*M* $CaCl_2$, *p*H 5.5); E denotes treatment with 2 Gibco units of *Vibrio* enzyme in the same buffer; and E + S denotes treatment with 2 units of enzyme plus 100 m*M* sialic acid. Treatments were for 30 minutes at 37°C in a humidified chamber and were followed by washing in Dulbecco's phosphate-buffered saline at 4°C. Lymphocyte attachment to the frozen sections was assayed (*10*) with mesenteric node lymphocytes (1.2×10^7 cells per milliliter) suspended in a physiological buffer—no supplementary NaCl—with a 30 minute gyration period at 7° to 10°C. The density of exogenous lymphocyte binding was at least 50 times greater to HEV than to nonspecific sites (Fig. 1). The density of binding was quantified with a Bioquant II digitizing morphometry system (R&M Biometrics) at a magnification of ×200 by counting the number of lymphocytes (at least 200 in each control section) bound to HEV (7 to 20 segments of HEV per section) and dividing by the total area of counted HEV (computed in units of 10^4 μm^2) in the section. Binding was computed as the percent of binding in the controls not exposed to enzyme. Means and standard errors were computed from five replicate sections.

specificity controls indicate that sialidase, and not contaminating enzymes, was responsible for the biological effects. Furthermore, we established that the *Clostridium* sialidase had no other detectable glycosidic activities or protease activity (*13*).

Sialidase had no effect on the ability of Peyer's patch HEV to bind lymphocytes, even when the concentration of the enzyme was 100 times that required to eliminate binding to peripheral node HEV. The resistance of Peyer's patch HEV to sialidase cannot be attributed to diffusible inhibitors emanating from the sections, since the various tissues were exposed to the enzyme in common wells. Therefore, the attachment sites on Peyer's patches probably do not require sialic acid (*14*). Nonetheless, the periodate sensitivity of these sites suggests that their activity depends on carbohydrate moieties. It seems plausible that the organ-specific binding of lymphocytes to HEV may be based on carbohydrate determinants that vary with the anatomical site of the HEV. The mesenteric node HEV attachment sites show partial susceptibility to sialidase. This finding is consistent with the conclusions reached by Stevens *et al.* (*7*) in studies of cell binding—namely, that mesenteric node HEV expresses both peripheral node– and Peyer's patch–like recognition determinants.

Our earlier results indicated that a cell-surface lectin on lymphocytes has a role in the binding of lymphocytes to peripheral node HEV. Sugar competition studies indicated that this lectin binds D-mannose-6-phosphate and to a lesser extent L-fucose and D-mannose. Sialic acid did not specifically inhibit lymphocyte binding to the HEV even though removal of sialic acid prevented lymphocyte binding to peripheral node HEV (*15*). One possible explanation of these findings is that sialic acid on a peripheral node HEV constituent affects the degree of exposure or activity of adjacent attachment sites. Such a modulation activity of sialic acid—whether it is a specific regulatory function or a nonspecific steric effect—would have to be organ specific in view of the complete resistance of Peyer's patch HEV and the partial resistance of mesenteric node HEV to sialidase treatment. Another explanation of our observations is that the attachment site on peripheral node HEV consists of sialic acid as part of a specific oligosaccharide structure. Sendai virus, for example, binds to host cells by recognizing certain gangliosides but not others (*16*). The number and linkages of the sialic acid moieties as well as the associated oligosaccharides are critical for this interaction. Perhaps, the adhesive lectin on lymphocytes recognizes a branched oligosaccharide on peripheral node HEV consisting of L-fucose or D-mannose (two structurally related sugars) on one terminus and sialic acid on another terminus (*17*). The critical contribution of sialic acid to this postulated sialyloligosaccharide may be to impart a negative charge to the structure. Evidence that charge is important to lymphocyte binding to peripheral node HEV is indicated by our findings that (i) high ionic strength partially inhibits this interaction and augments the inhibitory activities of L-fucose and D-mannose (*10*); and (ii) as inhibitors, charged monosaccharides and



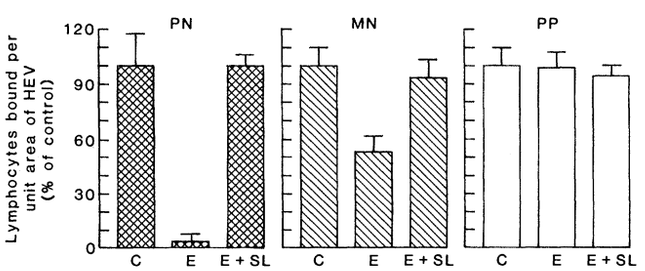


Fig. 3 (left). Dose-response curve for sialidase treatment. Peripheral node (PN), mesenteric node (MN), and Peyer's patch (PP) sections were exposed to varying amounts of *Vibrio* sialidase (Gibco) and tested for lymphocyte attachment as described in Fig. 2. Binding is expressed relative to the controls not exposed to enzyme. Means and standard errors were based on six independent replicate sections. Higher concentrations of sialidase (>5 units per section) while eliminating binding of lymphocytes to peripheral node HEV created new binding sites in subcapsular and medullary sinuses of the nodes. Contaminating erythrocytes in the lymphocyte suspension also bound to these sinus sites. Fig. 4 (right). Effect of *C. perfringens* sialidase treatment of peripheral node, mesenteric node, and Peyer's patch sections on lymphocyte binding. C denotes treatment of sections, prepared as in Fig. 2, with control buffer (50 m*M* sodium acetate and 100 m*M* NaCl, *p*H 5.0); E denotes treatment with 0.01 unit of enzyme (affinity-purified *Clostridium* sialidase (Sigma) in the same buffer; and E + SL denotes treatment with 0.01 unit of enzyme plus 2.5 m*M* sialyllactose (Sigma). The sections were otherwise treated as described in the legend to Fig. 2. Means and standard errors were based on five independent replicate sections.

polysaccharides are superior to their uncharged counterparts (*10*).

Determination of the exact role of sialic acid in the organ-specific adherence of lymphocytes to HEV awaits the isolation and characterization of the HEV attachment sites from the various lymphoid organs.

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References and Notes

1. J. L. Gowans, *J. Physiol. (London)* **146**, 54 (1959).
2. J. L. Gowans and E. J. Knight, *Proc. R. Soc. London Ser. B* **159**, 257 (1964).
3. H. B. Stamper, Jr., and J. J. Woodruff, *J. Exp. Med.* **144**, 828 (1976).
4. ———, *J. Immunol.* **119**, 772 (1977).
5. E. C. Butcher, R. G. Scollay, I. L. Weissman, *ibid.* **123**, 1996 (1979).
6. E. C. Butcher *et al.*, *Eur. J. Immunol.* **10**, 556 (1980).
7. S. K. Stevens, I. L. Weissman, E. C. Butcher, *J. Immunol.* **128**, 8 (1982).
8. Y.-H. Chin, G. D. Carey, J. J. Woodruff, *ibid.* **129**, 1911 (1982); Y.-H. Chin *et al.*, *ibid.* **131**, 1368 (1983).
9. W. M. Gallatin, I. L. Weissman, E. C. Butcher, *Nature (London)* **304**, 30 (1983).
10. L. M. Stoolman and S. D. Rosen, *J. Cell Biol.* **96**, 722 (1983); L. M. Stoolman, T. Tenforde, S. D. Rosen, *ibid.* **99**, 1535 (1984). Although these experiments are reported for the rat, the pattern of inhibition by saccharides and polysaccharides is essentially the same in the mouse system (T. A. Yednock, in preparation).
11. S. D. Rosen and L. M. Stoolman, in *Vertebrate Lectins*, K. Olden, Ed. (Van Nostrand, New York, in press). Periodate experiments, which were first carried out in the rat system, were extended to the mouse as follows. Sections of mouse (female, BALB/c) peripheral nodes and Peyer's patches, frozen in a common block, were cut, mounted in 1.4-cm-diameter wells in epoxy-coated glass slides (Carlson Scientific) and fixed with paraformaldehyde as described in (*10*). Experimental sections in individual wells were exposed to 100 μl of 50 m*M* sodium metaperiodate in 50 m*M* sodium acetate, 100 m*M* NaCl, *p*H 5.0 for 1 hour at 4°C in the dark. The same treatment was used for control sections, except that periodate was omitted. The sections were then washed extensively, and binding of mesenteric node lymphocytes to the sections was carried out by the standard procedure (*10*) (legend to Fig. 2) in a physiological buffer, except that 10 m*M* sodium borohydride was included in the lymphocyte suspension during the binding step. The sodium borohydride prevented nonspecific binding of lymphocytes to aldehyde groups generated by periodate oxidation of the sections. The periodate treatment completely eliminated binding to both peripheral node HEV and Peyer's patch HEV. At least ten HEV segments were counted per section. Control binding to peripheral node HEV (eight independent sections) was 46.9 ± 5.1 lymphocytes per unit area of HEV (10^4 μm^2); binding to periodate-oxidized peripheral node sections (four sections) was 0.0 ± 0. Control binding to Peyer's patch HEV (eight sections) was 41.9 ± 4.1; binding to periodate-oxidized Peyer's patch sections (four sections) was 0.0 ± 0. See legend to Fig. 2 for a description of the counting procedure.
12. In an earlier study, J. J. Woodruff *et al.* [*J. Immunol.* **119**, 1603 (1977)] investigated the effect of sialidase treatment of lymphocytes on the ability of rat lymphocytes to bind to peripheral node HEV in frozen sections and found slightly augmented binding relative to untreated lymphocytes.
13. Standard assays [Hall *et al.*, *Methods Enzymol.* **50**, 439 (1978)] were carried out for α- and β-galactosidase, *N*-acetyl-β-glucosaminidase, *N*-acetyl-β-galactosaminidase, α-fucosidase, and α-mannosidase. Briefly, 0.1 unit of *Clostridium* sialidase (Sigma) was combined with 2.4 nmol of the appropriate *p*-nitrophenyl substrate (0.9 nmol in the case of *p*-nitrophenyl β-D-*N*-acetylgalactosamine) in a final volume of 5 ml of 500 m*M* sodium acetate buffer, *p*H 5.0. After a 90-minute incubation at 37°C, the reaction was stopped by the addition of 1 ml of 50 m*M* NaOH. Absorbance at 400 nm was measured against a substrate blank. No activity was detected with any of the substrates. The limit of detectability was the hydrolysis of 0.8 nmol of substrate per 90 minutes. Proteolytic activity was measured with azocasein as a substrate. Briefly, 0.1 unit of sialidase was mixed with 7.5 mg of azocasein (Sigma) in a total volume of 0.5 ml of 100 m*M* tris-HCl, 5 m*M* $CaCl_2$, *p*H 7.8. After 50 minutes at 37°C, the reaction was ended by adding 2.5 ml of 3 percent trichloroacetic acid to each tube. The solutions were filtered and read at 366 nm in comparison with a substrate blank. With a limit of detectability of 6 ng of trypsin, no activity was detected in the sialidase.
14. A role for sialic acid is not ruled out in Peyer's patch attachment sites, since certain *O*-acetylated forms of sialic acid are resistant to the bacterial sialidases used in this study [R. Schauer, *Methods Enzymol.* **50**, 64 (1978)]. Also certain sialylated structures (for example, ganglioside G_{M1}) are sialidase-resistant (*17*).
15. At 25 m*M*, sialic acid has no effect on lymphocyte binding to peripheral node HEV in the frozen section assay (legend to Fig. 2). At higher concentrations, sialic acid is inhibitory, but these effects are attributable to the elevated ionic strength of the sugar solution. The activity of sialic acid never exceeds that of an equimolar solution of NaCl [(*10*) and unpublished observations].
16. M. A. Markwell *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 5406 (1981).
17. Branched oligosaccharides containing neighboring terminal L-fucose and sialic acid have been described [J. L. Magnani *et al.*, *J. Biol. Chem.* **257**, 14365 (1982); Y. Fukushi, E. Nudelman, S. B. Levery, S.-I. Hakomori, *ibid.* **259**, 10511 (1984).
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Stimulation of Bone Resorption in Vitro by Synthetic Transforming Growth Factor–Alpha

Abstract. *Experiments were conducted to test the hypothesis that tumor-derived transforming growth factor–alpha (TGF-α) is responsible for the increased bone resorption and hypercalcemia seen in some malignant diseases. Homogeneous synthetic TGF-α prepared by the solid-phase synthesis method stimulated bone resorption directly in vitro in a concentration-dependent manner. Incubation times of 72 hours or more were required to stimulate resorption, which is similar to the time course of bone resorption by epidermal growth factor.*

Transformed cells produce endogenous transforming growth factors (TGF's) that can reversibly induce the transformed phenotype in indicator cells in vitro. These polypeptide stimulators of cell growth and replication may represent examples of autocrine or paracrine secretion by tumor cells (*1*). One class of these factors, called TGF-α, competes for binding to the epidermal growth factor (EGF) receptor. Rat TGF-α has been sequenced and synthesized (*2*). We showed earlier that TGF-α activity is present in the partially purified material responsible for the bone-resorbing activity in tumors associated with hypercalcemia (*3*). We now report that synthetic TGF-α stimulates bone resorption directly in vitro. Increased bone resorption may be an endocrine effect of TGF's secreted by tumor cells and may be responsible for the bone destruction frequently associated with malignancy.

Synthetic rat-type TGF-α was prepared as described by Tam *et al.* (*2*). Purity of the protein was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, amino acid analysis, and reversed-phase high-performance liquid chromatography (*2*). The biological activity of TGF-α preparations and synthetic TGF-α was monitored by

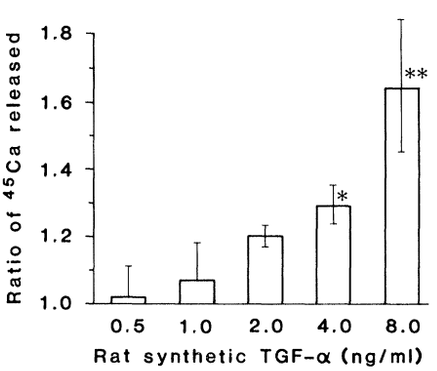

Fig. 1. Stimulation of bone resorption by synthetic TGF-α. Synthetic TGF-α was dissolved in 100 μl of 10 m*M* acetic acid containing bovine serum albumin (BSA) (fraction V; 5 mg/ml) (Sigma) and serially diluted into BGJb plus 5 percent fetal bovine serum. Doses are expressed as nanogram equivalents of EGF per milliliter of culture medium. Controls were treated with equivalent concentrations of 10 m*M* acetic acid containing BSA. Bone resorption was assessed by measuring the release of ^{45}Ca from previously labeled fetal rat long bones (*5*). Bones were cultured for 96 hours. Bone-resorbing activity is expressed as the treated-to-control ratio of ^{45}Ca release (± standard error of the mean of four bones per group). Statistical differences were calculated with Student's *t*-test for unpaired data (*$P < 0.05$; **$P < 0.025$).