Thrombospondin Stimulates Motility of Human Neutrophils

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Abstract. Polymorphonuclear leukocytes (PMNs) migrate to sites of inflammation or injury in response to chemoattractants released at those sites. The presence of extracellular matrix (ECM) proteins at these sites may influence PMN accumulation at blood vessel walls and enhance their ability to move through tissue. Thrombospondin (TSP), a 450-kD ECM protein whose major proteolytic fragments are a COOH-terminal 140-kD fragment and an NH_2-terminal heparin-binding domain (HBD), is secreted by platelets, endothelial cells, and smooth muscle cells. TSP binds specifically to PMN surface receptors and has been shown, in other cell types, to promote directed movement. TSP in solution at low concentrations (30–50 nM) "primed" PMNs for f-Met-Leu-Phe (fMLP)-mediated chemotaxis, increasing the response two- to fourfold. A monoclonal antibody against the HBD of TSP totally abolished this priming effect suggesting that the priming activity resides in the HBD of TSP. Purified HBD retains the priming activity of TSP thereby corroborating the antibody data. TSP alone, in solution at high concentrations (0.5–3.0 μM), stimulated chemotaxis of PMNs and required both the HBD and the 140-kD fragment of TSP. In contrast to TSP in solution, TSP bound to nitrocellulose filters in the range of 20–70 pmol stimulated random locomotion of PMNs. The number of PMNs migrating in response to bound TSP was approximately two orders of magnitude greater than the number of cells that exhibited chemotaxis in response to soluble TSP or fMLP. Monoclonal antibody C6.7, which recognizes an epitope near the carboxyl terminus of TSP, blocked migration stimulated by bound TSP, suggesting that the activity resides in this domain. Using proteolytic fragments, we demonstrated that bound 140-kD fragment, but not HBD, promoted migration of PMNs. Therefore, TSP released at injury sites, alone or in synergy with chemotactic peptides like fMLP, could play a role in directing PMN movement.

POLYMORPHONUCLEAR leukocytes (PMNs)[1] migrate, in response to chemoattractants, to sites of infection or injury where they may phagocytose and destroy invading cells. Chemotaxis, the directional movement of cells along a soluble chemical gradient, is a receptor-mediated event that has been studied extensively in PMNs and other cell types (for reviews, see Zigmond, 1978; Schiffmann, 1982; Devreotes and Zigmond, 1988). In contrast, locomotion induced by a gradient of substrate-bound attractant is termed haptotaxis (Carter, 1967; McCarthy et al., 1983) and has not been as extensively investigated.

Thrombospondin (TSP), a multifunctional homotrimeric glycoprotein of approximately 450,000 M_r, is an extracellular matrix (ECM) protein that may be secreted at sites of injury or inflammation by platelets, endothelial cells, epithelial cells, smooth muscle cells, fibroblasts, monocytes, or PMNs (Mosher et al., 1982; Raugi et al., 1982; Jaffe et al., 1983; Sage et al., 1983; Wikner et al., 1987; Jaffe et al., 1985; Kreis et al., 1989). Functional studies demonstrate that TSP can mediate attachment and spreading, chemotaxis, and haptotaxis in several different cell types. Specifically, TSP promotes the adhesion and/or spreading of human keratinocytes, carcinoma cells, melanoma cells, endothelial cells, smooth muscle cells, and CHO cells (Varani et al., 1986; Roberts et al., 1987; Tuszynski et al., 1987; Varani et al., 1988; Lawler et al., 1988; Kaesberg et al., 1989), the motility of human keratinocytes (Nickoloff et al., 1988), and chemotaxis and haptotaxis of human carcinoma and melanoma cell lines (Taraboletti et al., 1987).

Although monocytes and PMNs synthesize and secrete TSP (Jaffe et al., 1985; Kreis et al., 1989), its function in these cells remains unresolved. We have recently demonstrated that PMNs have specific receptors for TSP and that TSP can "prime" PMNs for *N*-formyl methionyl-leucyl-phenylalanine (fMLP)-mediated superoxide generation (Suchard, S. J., L. A. Boxer, and V. M. Dixit, submitted for publication); i.e., TSP increases the amount of O_2^- produced by PMNs in response to fMLP without itself stimulating O_2^- production.

PMNs may encounter TSP in solution, bound to endothelial cells, or bound to the subendothelial basement mem-

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1. *Abbreviations used in this paper*: ECM, extracellular matrix; fMLP, *N*-formyl methionyl-leucyl-phenylalanine; HBD, heparin-binding domain; PMN, polymorphonuclear leukocyte; TSP, thrombospondin.

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brane at sites of injury, with TSP being derived from several possible sources: proliferating endothelial cells (Mumby et al., 1984), activated platelets (Murphy-Ullrich and Mosher, 1987), or smooth muscle cells stimulated by platelet-derived growth factor (Majack et al., 1985). TSP at these sites may attract PMNs, promote their adhesion, and direct their migration through basement membrane and interstitial matrices, requisite steps for diapedesis. Consistent with this hypothesis are the observations that TSP promotes in vitro attachment, spreading, chemotaxis, and haptotaxis of melanoma and carcinoma cells (Roberts et al., 1987; Taraboletti et al., 1987; Varani et al., 1986).

Other ECM proteins, particularly fibronectin and laminin, have been shown to stimulate or enhance PMN functions such as adhesion, degranulation, chemotaxis, phagocytosis, and superoxide generation (Pommier et al., 1984; Terranova et al., 1986; Bryant et al., 1987; Wachtfogel et al., 1988; Pike et al., 1989; Nathan, 1987, 1989). In human PMNs, soluble laminin primes for fMLP-mediated superoxide production and increases fMLP receptor expression (Pike et al., 1989). In addition, substrate-bound laminin, fibronectin, and vitronectin prime PMNs for a massive respiratory burst in response to fMLP or several cytokines (Nathan, 1987, 1989).

In addition to the effects of intact ECM proteins on PMNs, proteolytic fragments of ECM components may promote PMN function. These proteolytic fragments may be generated by proteases released by activated PMNs or other inflammatory cells found at sites of inflammation and may stimulate responses not observed with the intact molecule. For example, proteolytic fragments of fibronectin, but not intact fibronectin, are chemotactic for monocytes and stimulate degranulation in PMNs (Norris et al., 1982; Clark et al., 1988).

Each chain of the homotrimeric TSP molecule contains a heparin-binding domain (HBD) at the NH_2 terminus and a COOH-terminal 140-kD fragment (Galvin et al., 1985). The 140-kD fragment can be further proteolyzed to 120- or 70-kD fragments. In melanoma cells, the HBD promotes chemotaxes, whereas the 140-kD fragment promotes haptotaxis (Taraboletti et al., 1987). The haptotactic activity of the 140-kD fragment resides in the 18-kD COOH-terminal portion of the fragment, as indicated by a loss in haptotactic activity when the 140-kD fragment is further proteolyzed to the 120-kD fragment. These data indicate that different regions of TSP may be required for chemotaxis versus haptotaxis.

Using a Boyden chamber assay, we found that soluble TSP at low concentrations primed PMNs for fMLP-mediated chemotaxis. In addition, soluble TSP at much higher concentrations was itself chemotactic for PMNs. Finally, we determined that bound TSP stimulated the nondirectional migration of PMNs. In general, our studies indicate that the effect of TSP on PMN function can be dictated by both its conformation (either soluble or bound to a substrate) and the presence of other chemoattractants. Therefore, TSP working alone or in synergy with chemotactic peptides may play a role in directing PMNs to a site of injury.

Materials and Methods

Cells

Human PMNs were isolated from human peripheral blood as previously described (Curnutte and Babior, 1974). Briefly, fresh whole blood was obtained by venipuncture from healthy volunteers and immediately added to acid citrate dextrose. The PMNs were purified by dextran sedimentation followed by hypotonic lysis to remove the majority of erythrocytes and then centrifuged through Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) to remove contaminating mononuclear cells. PMNs were resuspended at 10^6/ml in MEM (Whittaker Bioproducts, Inc., Walkersville, MD) with 0.1% BSA (Sigma Chemical Co., St. Louis, MO), and gradually warmed to room temperature over a period of 1 h before migration assays were initiated.

Materials

Human TSP was isolated from the supernatant of thrombin-activated platelets by affinity chromatography over gelatin-Sepharose and heparin-Sepharose (Pharmacia Fine Chemicals) followed by gel filtration (Bio-Gel A 0.5m; Bio-Rad Laboratories, Richmond, CA) as outlined in Dixit et al. (1984). TSP was concentrated in an ultrafiltration cell (Amicon Corp., Danvers, MA), and stored at −70°C.

TSP fragments were generated by digestion with 16 U/mg thrombin for 8 h at 37°C, after which digestion was stopped with 10 μg/ml aprotinin and 50 U hirudin. Fragments were isolated by passing the digest over a 2-ml heparin-Sepharose column. The 140-kD fragment was not retained by the column and was collected in the void volume. The 25-kD HBD was eluted from the heparin-Sepharose column with 0.6 M NaCl. Fragments were concentrated by ultrafiltration (Amicon Corp.). The 140-kD fragment was further cleaved to the 120-kD fragment using chymotrypsin (1:100, wt/wt) for 1.5 h at 37°C. The reaction was terminated with 1 mM PMSF. The 70-kD TSP fragment was produced by chymotrypsin cleavage of TSP (1:100, wt/wt), for 0.5 h at 20°C in the presence of 5 mM EDTA (Galvin et al., 1985). Intact TSP was also chymotrypsinized (1:100, wt/wt) for 40 min at 37°C and used without further fractionation. The extent of digestion and purity of TSP fragments were confirmed by SDS-PAGE using 5-20% mini-slab gels and the discontinuous buffer system of Laemmli (1970).

Polyclonal antibodies to TSP and monoclonal antibodies A2.5, D4.6, A6.1, and C6.7 were produced as previously described (Dixit et al., 1985*a*,*b*; 1986). Fab fragments were generated as follows: 1 mg rabbit anti-TSP IgG was incubated with 50 μl of a 50% slurry of immobilized papain beads (Pierce Chemical Co., Rockford, IL) in 1.5 ml buffer for 5 h at 37°C. Fc fragments and intact IgG were removed using Protein A Affi-Gel (Bio-Rad Laboratories). The flow through fraction containing Fabs was dialyzed against PBS and concentrated using a Centricon 30 (Amicon Corp.). A neutralizing antibody against human platelet-derived growth factor (anti-PDGF) was obtained from R & D Systems, Inc. (Minneapolis, MN).

Iodination of TSP and Fragments

TSP was iodinated using the iodogen method (Fraker and Speck, 1978) essentially as follows: 100-200 μCi of carrier-free $Na^{125}I$ was added to 300 μl of 2 mg/ml TSP in an iodogen tube. After a 40-min incubation at room temperature, unbound iodine was removed by passing the material over a Sephadex G25 column. HBD and the 140-kD fragment were iodinated using Bolton-Hunter reagent (New England Nuclear, Boston, MA).

Cell Migration Assays

Chemotaxis. Chemotaxis assays were conducted in modified Boyden chambers (manufactured by Guy Duremberg, Pasadena, CA; Boyden, 1962), using 3-μm-pore polyvinylpyrrolidone-free polycarbonate filters (Nuclepore, Pleasanton, CA). Filters were soaked in MEM with 0.1% BSA for a minimum of 2 h before assays. Attractant (TSP or fMLP) was diluted in MEM with 0.1% BSA and placed in the lower wells of the Boyden chambers. An equimolar amount of HBD or 140-kD fragment (with respect to intact TSP) was added to Boyden chambers where indicated. Each upper well contained 3.3×10^5 PMNs in 0.3 ml MEM with 0.1% BSA. Chambers were incubated for 30 min at 37°C in a humidified incubator with 5% CO_2. Following each experiment, filters were fixed with propanol, stained with hematoxylin, cleared with xylene, and mounted on slides. Three replicate filters were used for each treatment, and five replicate fields were scored for each filter. Filters were scored by counting the number of cells that migrated through the filter in each high power field. All results were expressed as a migration index ± SEM; the index being the score seen with a particular treatment (TSP or other attractant) divided by the score of the control (no attractant).

Checkerboard assays were used to distinguish between chemotaxis (directed movement) and chemokinesis (random movement) (Zigmond and

Hirsch, 1973; Wilkinson and Allan, 1978). For this assay, varying concentrations of TSP were placed in upper wells, lower wells, or both upper and lower wells of Boyden chambers to determine whether PMN migration was greater with a positive (chemotaxis) or negative (chemokinesis) gradient to TSP. As a negative control, TSP was denatured by boiling for 30 min before addition to the wells.

For "priming" experiments, TSP or equimolar amounts of purified HBD was added to cells in the upper well at the beginning of the assay with fMLP (Sigma Chemical Co.) in the lower well. To test specificity of priming and determine the active molecular domain, TSP was preincubated with 100 μg/ml of anti-TSP monoclonal antibodies or heparin for 1 h at 37°C. In some experiments, TSP was incubated for 30 min at 37°C with 10 μg/ml of rabbit anti-PDGF IgG and then tested for chemotactic and priming activity.

Haptotaxis. Initially for haptotaxis experiments, 3-μm-pore nitrocellulose filters (Millipore Continental Water Systems, Bedford, MA) were assembled into modified Boyden chambers using the same conditions as the chemotaxis assays, but in the absence of cells, and incubated for 3 h at 37°C. The filters were then removed, rinsed five times with PBS, one time with MEM containing 0.1% BSA, blotted to remove excess fluid, and immediately returned to the chambers. Since TSP and TSP fragments diffuse through and bind to these filters without reaching saturation, we were fairly confident that a gradient was established within the filters under these conditions. Subsequently, filters were either floated on or submerged in attractant diluted in PBS with 1 mM calcium for 2 h at 37°C or overnight at 4°C (McCarthy et al., 1983). Filters incubated in this manner gave the same results as filters precoated in the chambers, and because of ease of preparation, were used in the majority of experiments. TSP and TSP fragments were incubated with filters at equimolar concentrations. Filters were put in the chambers with the high concentration side down, representing a presumed positive gradient, or up, representing a presumed negative gradient. Filters coated on both sides contained no gradient and did not require specific orientation. This method allowed us to distinguish between directed and random movement of PMNs. Lower wells contained only MEM with 0.1% BSA. Each upper well contained 3.3×10^5 PMNs at 10^6 cells/ml. Chambers were incubated for 2.5 h at 37°C in a humidified incubator with 5% CO_2. Filters were fixed, stained, and mounted as outlined above. Three replicate filters were used for each treatment, and five replicate fields were scored for each filter. Filters were scored both by the leading front method (Zigmond and Hirsch, 1973) and by counting the number of cells in each microscope field that had traveled 50 μm into the filter. These two scoring methods gave similar results.[2] In this paper, we have reported the number of cells traveling 50 μm into the filter since this method reflects the behavior of the entire PMN population. All results were expressed as a migration index ± SEM, the index being the score seen with a particular treatment (TSP or other attractant) divided by the score of the control (no attractant).

As a negative control, TSP was denatured by boiling 30 min in 10% SDS. After incubation with denatured TSP, filters were rinsed 10 times to remove excess SDS. For assays in which anti-TSP antibodies were used, chambers were assembled with TSP-coated filters, high concentration side up, antibodies added to the upper wells, and filters incubated for 1 h at 37°C. After antibody preincubation, cells were added to a final concentration of 10^6/ml (3.3×10^5 PMNs in each well), and incubated for 2.5 h at 37°C. To rule out the participation of small quantities of TSP-associated PDGF in these assays, TSP-coated filters were incubated with rabbit anti-PDGF IgG (10 μg/ml) as outlined above.

Protein Diffusion and Binding Assays

^{125}I-labeled TSP, ^{125}I-labeled 140-kD fragment, and ^{125}I-labeled HBD were placed in the lower wells of Boyden chambers to measure binding to and diffusion through nitrocellulose and polycarbonate filters. Labeled proteins were mixed with unlabeled proteins so that the specific activity remained constant at 5 μCi/mg. Chambers were assembled with filters and incubated in the absence of cells. Nitrocellulose filters were also incubated with ^{125}I-labeled TSP by floating or submerging filters to determine the amount of TSP bound under those conditions. Additionally, filters with bound ^{125}I-TSP were incubated in Boyden chambers in the presence of cells to determine whether TSP was released from the filters during the experimental time course. The amount of radioactivity bound to filters or present in wells was determined by counting samples (filters or media) on a Multigamma gamma counter (1261; LKB Instruments, Inc., Bromma, Sweden).

2. The number of cells at 50 μm was an appropriate scoring method because in no case during this study did the majority of cells travel beyond that distance. Results obtained by the scoring combination of leading front and number of cells at 50 μm were similar to counting all cells at 10- or 20-μm intervals (locomotion index; Maderazo and Woronick, 1978) when tested for several filters.

Results

Diffusion of TSP in Boyden Chambers

Initially we conducted experiments to demonstrate that we could establish gradients of TSP in Boyden chambers and to determine the interaction of TSP with nitrocellulose and polycarbonate filters under the conditions of our assay system. ^{125}I-labeled TSP, HBD, and 140-kD fragment were used to quantify the diffusion of these molecules through nitrocellulose or polycarbonate filters. Diffusion of TSP from the lower to upper wells of Boyden chambers was dose-dependent and relatively linear over the time course of our assays (Fig. 1). When using nitrocellulose filters, after 3 h ~3% of the TSP present in the lower wells had diffused to the upper wells. TSP diffused more readily through polycarbonate filters, with ~2% diffusing from lower to upper wells during a typical 30-min incubation. Thus, when using polycarbonate filters, 1 μM TSP added to the lower wells of Boyden chambers resulted in 20 nM TSP being present in the upper wells after a 30-min incubation (data not shown). Additionally, the HBD and 140-kD fragment diffused through both nitrocellulose and polycarbonate filters more quickly than intact TSP; >20% diffused to the upper well after 3 h (data not shown). Diffusion of each fragment was dose- and time-dependent, and, in contrast to native TSP, was similar for both filter types.

TSP-induced Chemotaxis

Since diffusion of TSP in Boyden chambers was facilitated by using polycarbonate filters, these filters were used for all of the chemotaxis assays. TSP present in the lower wells of

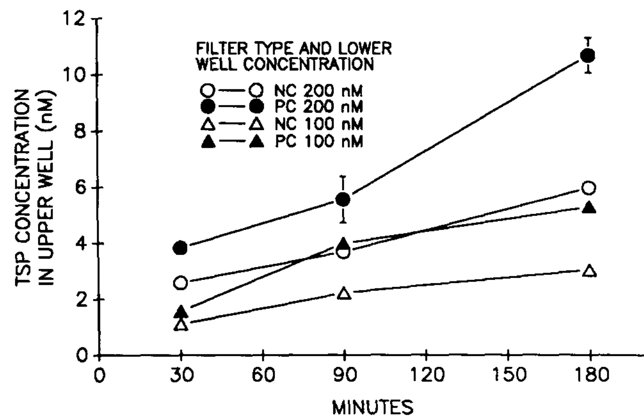

Figure 1. Time course of diffusion of TSP from lower to upper wells of Boyden chambers. 100 (△, ▲) or 200 (○, ●) nM of ^{125}I-TSP was placed in the lower wells of Boyden chambers fitted with either nitrocellulose (*NC*) or polycarbonate (*PC*) filters. The chambers were incubated at 37°C for 180 min, and the upper wells assayed for radioactivity at 30, 90, and 180 min. The curves represent the concentration of TSP that diffused from lower to upper wells during the time course of the experiment. Values represent the mean ± SEM for triplicate samples. Representative of three experiments.

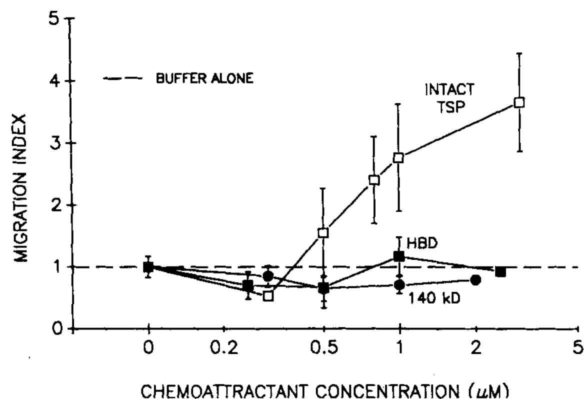

Figure 2. PMN chemotaxis to TSP and TSP proteolytic fragments. TSP (□), 140-kD fragment (●), or HBD (■) were added to the lower wells of Boyden chambers and polycarbonate filters were used to separate upper from lower wells. PMNs (3.3×10^5) were added to the upper wells and the chambers placed in a humidified incubator at 37°C with 5% CO_2 for 30 min. 10 nM fMLP was used as a positive control for chemotaxis. Data are expressed as mean migration index (number of PMNs migrating in response to attractant divided by the number of PMNs migrating in response to buffer) ± SEM. The control value for buffer alone is defined as 1.0 and is represented by the dashed line. The migration index for fMLP was 7.78 ± 1.11. Values represent the mean ± SEM for triplicate samples. Representative of three experiments.

Boyden chambers stimulated migration of PMNs (Fig. 2). The PMN response was dose-dependent with a maximal effect (3.5 times the control) observed at 3 μM. Since large quantities of TSP are difficult to obtain, only selected combinations of concentrations were included in the checkerboard analysis used to determine the random versus directed nature of PMN movement in response to TSP (Table I). The increasing values below the diagonal indicate that PMNs migrated in response to a positive gradient of TSP (i.e., chemotaxis). No increase in the values along and above the diagonal, from left to right, indicate that TSP was not chemokinetic for PMNs. These results verified that TSP was chemotactic and not chemokinetic for PMNs. Chemotaxis was specific for native TSP since heat denatured TSP did not promote cell migration (Table II). Interestingly, a chymotrypsinized preparation of TSP containing both the HBD and 140-kD fragment was about five times more potent than intact TSP in stimulating chemotaxis (Table II). Neither purified HBD nor the 140-kD fragment of TSP, in the same concentration range, were chemotactic for PMNs (Table II, Fig. 2). However, the addition of both purified fragments provoked a chemotactic response similar to that of chymotrypsinized TSP (Table II). These data indicated that binding of both TSP fragments to the PMN surface was required for chemotaxis.

Table I. Checkerboard Assay of PMN Chemotaxis to TSP

TSP in lower well (*nM*)	TSP in upper well (*nM*) 0	100	1,000
0	1.00 ± 0.38	0.45 ± 0.40	0.74 ± 0.19
100	3.85 ± 1.77	1.28 ± 0.22	–
1,000	4.56 ± 1.12	–	0.91 ± 0.20

Polycarbonate membranes were used in modified Boyden chambers. Results are expressed as a migration index that is defined as the number of cells migrating in response to TSP divided by number of cells migrating in response to buffer. Values represent the mean ± SEM for triplicate samples. For 10 nM fMLP, the migration index = 8.77 ± 1.03.

Table II. Chemotaxis of PMNs to Intact, Proteolyzed, and Denatured TSP and Isolated Proteolytic Fragments

Intact TSP	2.76 ± 0.86
Chymotrypsinized TSP	17.51 ± 7.43
HBD	0.94 ± 0.22
140-kD fragment	0.93 ± 0.22
HBD + 140-kD fragment	11.68 ± 2.69
TSP (100°C)	0.83 ± 0.04

Results are expressed as a migration index defined as the number of cells migrating in response to TSP divided by the number of cells migrating in response to buffer. Values represent the mean ± SEM for triplicate samples. TSP and fragments were present at 1 μM in lower wells of Boyden chambers.

TSP Priming of fMLP-mediated Chemotaxis

Having previously observed that TSP primed PMNs for fMLP-mediated superoxide production (Suchard, S. J., L. A. Boxer, and V. M. Dixit, submitted for publication), we wanted to determine whether TSP would also prime cells for fMLP-mediated chemotaxis. Chemotaxis in response to fMLP was significantly increased by the addition of 30–50 nM TSP to the upper wells of Boyden chambers (Fig. 3). The effect was greatest at 10 nM fMLP where TSP increased the response by more than a factor of 4. In general, this enhancement ranged between two- to fourfold reflecting the variation observed among normal donors (compare the response in Fig. 3 with that in Table III). The effect of TSP was considered a priming response since TSP alone, at these concentrations, had no effect on PMN motility. The priming of TSP for fMLP-mediated chemotaxis was inhibited by heparin and anti-TSP antibodies (Table III). Both polyclonal anti-TSP antibody and heparin reduced chemotaxis to the control levels observed with fMLP alone. Monoclonal antibody A2.5, which recognizes the HBD of TSP, inhibited the TSP-induced enhancement of chemotaxis by ~75%. mAbs C6.7 (recognizing an epitope near the carboxy terminus of TSP), D4.6 (recognizing the 50-kD fragment containing the fibrinogen-binding domain), and A6.1 (recognizing the trypsin-resistant 70-kD core) did not significantly affect TSP-induced priming (Table III). The ability of the isolated HBD alone to prime for fMLP-mediated chemotaxis confirmed the results obtained with the anti-TSP monoclonal antibodies (Table III) indicating that the priming activity of TSP resides in this portion of the molecule.

TSP Binding to Filters

Before conducting haptotaxis assays that rely on substrate bound attractant, we evaluated binding of TSP and its fragments to nitrocellulose and polycarbonate filters. ^{125}I-labeled TSP, HBD, and 140-kD fragment were used to quantify protein binding to filters under the same conditions that were used to prepare them for haptotaxis assays. The amount of

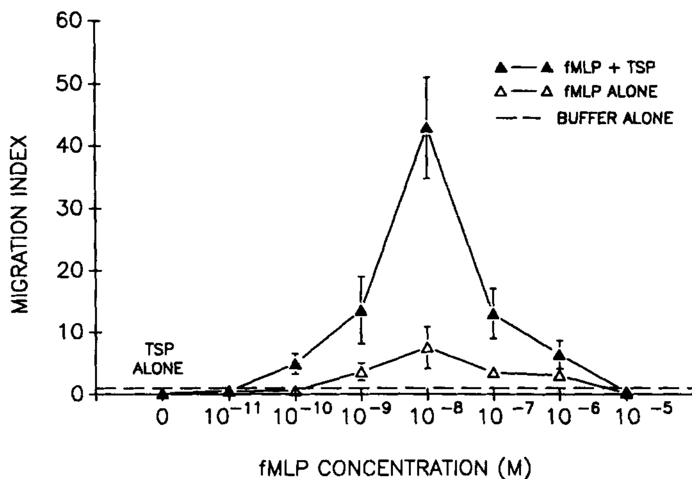

Figure 3. TSP priming for fMLP-mediated chemotaxis in PMNs. Various concentrations of fMLP were present in the lower wells of Boyden chambers and polycarbonate filters were used to separate the lower from upper wells. 50 nM TSP (▲) or buffer control (△) were added to PMNs in MEM + 0.1% BSA immediately before their addition to the upper wells at the onset of the chemotaxis assay. Chambers were placed in a humidified incubator at 37°C with 5% CO_2 for 30 min. Data are expressed as mean migration index ± SEM. See Fig. 2 for an explanation of the migration index. Values represent the mean ± SEM for triplicate samples. Representative of three experiments.

TSP bound to nitrocellulose filters was three to four times greater than the binding of either of its fragments (Fig. 4). Additionally, nitrocellulose filters bound ~10 times as much TSP as polycarbonate filters (Fig. 4), and binding was dose-dependent (data not shown). Binding of TSP to nitrocellulose filters did not saturate at concentrations up to 1 μM (data not shown). The release of bound TSP from nitrocellulose filters was minimal during a 3-h incubation with cells; <2% of the total TSP bound was released. Furthermore, SDS-PAGE analysis demonstrated that TSP was not degraded during filter preparation or incubation (data not shown). The combined effect of TSP binding and diffusion (Fig. 1) through nitrocellulose filters made it likely that a protein gradient had been established within these filters. The same results were obtained whether filters were prepared in modified Boyden chambers or by floating on protein solutions.

Migration of PMNs Stimulated by Bound TSP

We investigated the ability of bound TSP to induce PMN motility for two reasons: (*a*) TSP stimulates haptotaxis in melanoma cells and keratinocytes (Taraboletti et al., 1987; Nickoloff et al., 1988), and (*b*) TSP promoted chemotaxis in PMNs. We determined that 20–70 pmol TSP bound to nitrocellulose filters stimulated significant movement of PMNs with a maximal effect observed at 50 pmol (Fig. 5). A checkerboard assay for haptotaxis was performed using nitrocellulose filters that had either been floated on TSP solutions or submerged. The values below the diagonal reflect a presumed positive gradient while those above the diagonal reflect a presumed negative gradient. The values along the diagonal indicate random motility to a uniform concentration of TSP bound to the filter. The results from this assay indicated that TSP stimulated random locomotion rather than directional haptotaxis (Table IV). Filters with 3–100 pmol bound that were uniformly coated or coated on one side and placed with the coated surface up stimulated significant movement of cells, while filters coated on one side and placed with the coated surface down only caused significant movement of cells at 100 pmol bound. Although migration was not directional, the number of cells migrating in response to 30–50

Table III. Inhibition of TSP Priming for fMLP-Mediated Chemotaxis by Heparin, Polyclonal Anti-TSP Antibodies, and Monoclonal Anti-TSP Antibodies

fMLP alone	1.00 ± 0.37
fMLP + TSP	2.08 ± 0.24
fMLP + HBD	2.29 ± 0.19
Heparin	0.91 ± 0.01
Polyclonal anti-TSP	0.93 ± 0.05
Monoclonal antibodies	
A2.5	1.24 ± 0.11
D4.6	1.89 ± 0.18
A6.1	2.26 ± 0.24
C6.7	2.32 ± 0.21

Values represent the mean ± SEM for triplicate samples. The fMLP control is expressed as a value of 1.0, and the number of cells migrating within each treatment is divided by the number of cells migrating to fMLP alone. 50 nM TSP was preincubated with 100 μg/ml heparin or antibody for 60 min at 37°C and placed in the upper wells of Boyden chambers with PMNs at the onset of the chemotaxis assay. For some experiments, 50 nM HBD was substituted for TSP in the upper wells. 10 nM fMLP was present in the lower wells.

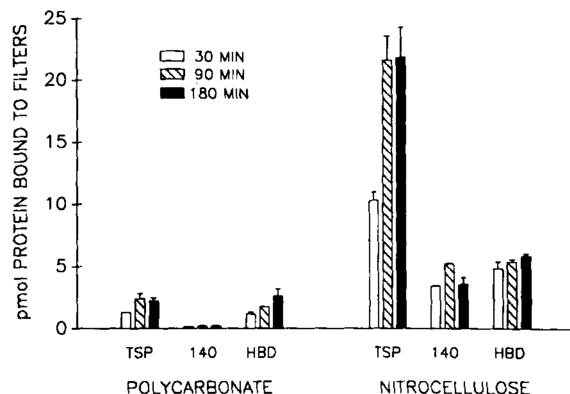

Figure 4. Binding of TSP or TSP proteolytic fragments to nitrocellulose and polycarbonate filters. Data are expressed as pmol ^{125}I-TSP, ^{125}I-140-kD fragment, or ^{125}I-HBD bound to filters. Filters were incubated with 200 nM labeled protein at 37°C for 30, 90, or 180 min. After incubation, filters were rinsed 5 times with PBS, excess fluid removed by blotting, and radioactivity determined by gamma counting. Values represent the mean ± SEM for triplicate samples. Representative of three experiments.

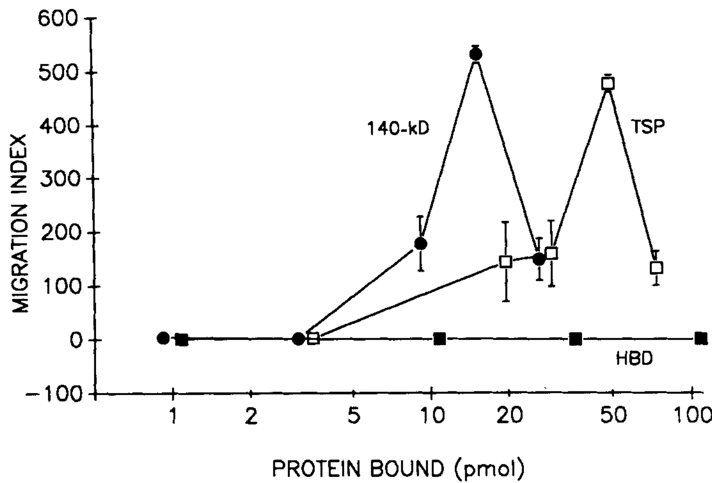

Figure 5. PMN migration in response to TSP or TSP proteolytic fragments bound to nitrocellulose filters. Nitrocellulose filters were floated for 2 h at 37°C in various concentrations of TSP (□), 140-kD fragment (●), or HBD (■), rinsed five times in PBS, and placed coated-surface up in Boyden chambers. Lower wells contained buffer, and upper wells contained PMNs (3.3×10^5) in buffer. Chambers were incubated for 2.5 h at 37°C in a humidified incubator with 5% CO_2. Protein bound (in pmol) was determined as described in Fig. 4. 10 nM fMLP in the lower well, in the absence of a TSP-coated filter, was used as a positive control for cell movement. See Fig. 2 for an explanation of the migration index. The migration index for the buffer control was 1.0, and for fMLP was 4.67 ± 0.83. Values represent the mean ± SEM for triplicate samples. Representative of three experiments.

pmol bound TSP was approximately two orders of magnitude greater than the number of cells that chemotaxed in response to soluble TSP or fMLP (Table IV and Fig. 5). The locomotory response was specific for native TSP since denatured TSP did not support cell migration (data not shown). The migration index was 2.6 for bound denatured TSP (not significantly different from buffer controls) as compared to 46.0 for bound native TSP.

Anti-TSP polyclonal antibody and its Fab fragments (100 μg/ml), but not control IgG or anti-human serum albumin Fab fragments, significantly inhibited the movement of PMNs stimulated by bound TSP (>80% inhibition; Table V). Antibodies against human PDGF, in quantities sufficient to inhibit PDGF-mediated PMN chemotaxis, did not affect the activity of TSP in this or the other functional assays. These data demonstrate that the locomotory response is specific for TSP. Of the four monoclonal antibodies tested, mAb C6.7, which recognizes an epitope near the carboxy terminus of TSP, inhibited TSP-induced migration to the greatest extent (Table V). Monoclonal antibody A2.5, which recognizes an epitope on the HBD, also inhibited migration but to a lesser extent. However, heparin did not inhibit TSP-induced movement (data not shown) suggesting that mAb A2.5 may have caused conformational changes in the TSP molecule that influenced function, rather than directly blocking the involvement of the HBD. mAbs A6.1 and D4.6, which recognize the central core domain of TSP, were not effective in inhibiting PMN movement. Control mouse IgG also did not inhibit cell movement (Table V).

The results obtained with mAbs led us to use proteolytic

Table IV. Checkerboard Assay of PMN Migration Stimulated by TSP Bound to Nitrocellulose Filters

TSP at bottom of filter (*pmol*)	TSP at top of filter (*pmol*) 0	1	3	10	30	100
0	1.00 ± 0.14	1.50 ± 0.13	4.20 ± 0.59	32.50 ± 8.06	123.30 ± 33.40	39.80 ± 2.97
1	1.71 ± 0.08	1.54 ± 0.23	– –	– –	– –	– –
3	1.62 ± 0.38	– –	2.50 ± 1.52	– –	– –	– –
10	1.42 ± 0.48	– –	– –	5.29 ± 1.94	– –	– –
30	1.46 ± 0.42	– –	– –	– –	106.10 ± 24.30	– –
100	42.80 ± 1.98	– –	– –	– –	– –	39.50 ± 3.77

Results are expressed as a migration index that is defined as the number of cells traveling 50 μm in response to TSP divided by the number of cells traveling 50 μm in response to buffer. Values represent the mean ± SEM for triplicate samples. The concentration of TSP (pmol) indicated in the checkerboard represents the amount of protein bound to filters, determined as described in Fig. 4. For comparison, 10 nM fMLP has a chemotactic migration index of 6.58 ± 2.06, and 3 μM soluble TSP has a chemotactic migration index of 3.64 ± 0.78.

Table V. Inhibition of PMN Migration to Bound TSP by Anti-TSP Polyclonal and Monoclonal Antibodies

Treatment	Antibody concentration (*μg/ml*)	Control (%)
	Polyclonals	
TSP, no antibody		100 ± 40.0
Polyclonal anti-TSP	100	1 ± 0.0
Fab anti-TSP	100	20 ± 13.0
Nonspecific rabbit IgG	100	86 ± 15.0
Fab anti-HSA	100	78 ± 5.4
	Monoclonals	
TSP, no antibody		100 ± 16.0
C6.7	20	25 ± 2.7
A2.5	20	57 ± 8.5
D4.6	20	79 ± 2.6
A6.1	20	69 ± 6.1
Nonspecific mouse IgG	20	116 ± 22.0

Nitrocellulose filters were soaked in 225 μg/ml TSP, and then exposed to various antibodies. No other attractant was present in Boyden chambers. Filters were scored by counting number of cells traveling 50 μm. The response of PMNs to TSP alone is defined as 100%. Values represent the mean ± SEM for triplicate samples.

fragments of TSP to establish which domain of TSP was responsible for the observed locomotory activity. Migration was promoted by the bound 140-kD fragment, but not the HBD (Fig. 5). Comparing activity of bound 140-kD fragment versus bound TSP on a mole:mole basis, the 140-kD fragment was found to be more effective at stimulating PMN movement than intact TSP (Fig. 5). Random migration in response to bound 140-kD fragment peaked at ~20 pmol while the response to intact TSP peaked at ~50 pmol.

To determine whether the PMN response to bound 140-kD fragment was directional, filters were coated with 140-kD fragment on one side, and the filters mounted in Boyden chambers with either the high concentration up or the high concentration down. PMN migration was greatest for a presumed negative gradient of the 140-kD fragment (data not shown). Thus, as was seen with intact TSP, directional migration (haptotaxis) was not stimulated by the 140-kD fragment. The 140-kD fragment was further proteolyzed to 120/18 or 70-kD fragments, the fragments bound to filters and evaluated for their ability to stimulate PMN motility. Filters were submerged in a 200 nM solution of each protein and the results normalized for the amount of protein bound to each filter. The HBD was used as a negative control in these assays. TSP, the 140-kD fragment, and the 120/18-kD fragment stimulated migration to a similar extent (Fig. 6). This stimulatory activity was lost following further proteolysis to the 70-kD fragment indicating that the migration promoting activity of TSP resides in the COOH terminus distal to the 70-kD core domain.

Discussion

Unlike other ECM proteins, TSP is normally at low levels in the plasma and may only be a transitory component of the extracellular matrix (Lawler, 1986; Murphy-Ullrich and Mosher, 1985). However, when platelets are activated the concentration of TSP in the plasma can be elevated to levels within the nanomolar range (Lawler, 1986). In addition, once TSP has been released at a specific site it may be rapidly removed and degraded by surrounding cells through receptor-mediated endocytosis (Murphy-Ullrich and Mosher, 1987; McKeown-Longo et al., 1984), thereby making it a transient component of an inflammatory response. Consistent with this hypothesis is the observation that TSP is present at injury sites within the first 2 d of wounding but disappears as wound healing progresses (Raugi et al., 1987). Additionally, platelets in the damaged area release factors other than TSP, such as PDGF, that may be involved in the initial recruitment of PMNs to the damage site (Deuel et al., 1982). Henry (1965) observed that in vivo PMNs accumulate in clots that are 4–13 h old. In situ PMNs are recruited to blood clots early and appear to be involved in the removal of both TSP and fibrin from these clots (Murphy-Ullrich and Mosher, 1985). As shown in the present study, TSP may be an important mediator of PMN function at sites of inflammation or tissue injury. The potency of TSP in stimulating PMN motility appears to depend on the presence or absence of other chemoattractants and whether it is in solution or bound to a substrate.

We have demonstrated that PMNs incubated with TSP, at concentrations that do not alone stimulate locomotion (30–50 nM), have a two- to fourfold greater chemotactic response to fMLP. This priming effect is not restricted to fMLP-mediated chemotaxis since we have observed a similar priming effect of TSP on fMLP-generated O_2^- (Suchard, S. J., L. A. Boxer, and V. M. Dixit, submitted for publication). Our ability to abolish the priming effect of TSP for chemotaxis with either heparin or an mAb against the HBD, and to generate a priming response with purified HBD indicates that this response is mediated through the HBD of TSP. Recently, we have shown that there are specific receptors on PMNs that recognize the HBD of TSP and that the binding of TSP to these receptors is required for the priming of fMLP-mediated O_2^- production (Suchard, S. J., L. A. Boxer, and V. M. Dixit, submitted for publication).

Laminin (50–75 μg/ml) also primes for fMLP-mediated superoxide production in human PMNs but requires a 30–45 min preincubation (Pike et al., 1989), in contrast to the 3 min preincubation required for TSP priming of fMLP-

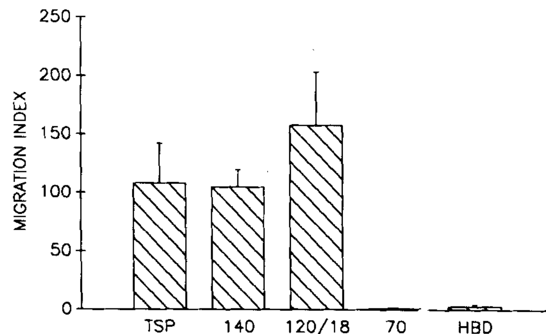

Figure 6. PMN migration stimulated by TSP or TSP proteolytic fragments bound to nitrocellulose filters. Methods were identical to those for Fig. 5. Filters were submerged in 200 nM TSP or TSP fragments for 2 h at 37°C. The results were normalized for the amount of protein bound to each filter. See Fig. 2 for explanation of migration index. The migration index for the buffer control was 1.0. Values represent the mean ± SEM for triplicate samples. Representative of three experiments.

mediated O_2^- production (Suchard, S. J., L. A. Boxer, and V. M. Dixit, submitted for publication). The requirement for a long preincubation period observed with laminin may reflect the time required for increased fMLP receptor expression, an event coupled to the enhanced fMLP response (Pike et al., 1989). Fibronectin both enhances the respiratory burst in monocytes (Kuroiwa et al., 1988) and stimulates phagocytosis in PMNs first activated with fMLP or C5a (Pommier et al., 1984). These studies, along with the present study, suggest that ECM proteins may be instrumental in modulating PMN functional responses to other agonists.

In contrast to the priming effect, we have demonstrated that a 2–10-fold higher concentration of substrate-associated or soluble TSP can stimulate significant migration of PMNs. When checkerboard analyses were performed to determine whether or not the migration of PMNs was directional, it became clear that soluble TSP stimulated directed migration (chemotaxis) whereas bound TSP stimulated random migration. The concentration of TSP in the lower wells of Boyden chambers necessary to promote chemotaxis of PMNs was 0.5–3 μM (0.225–1.35 mg/ml). Chemotaxis in human melanoma cells is stimulated by somewhat lower concentrations of TSP, in the range of 100–160 nM (~45–70 μg/ml) initial TSP concentration (Taraboletti et al., 1987). Under the conditions of our assay, the concentration of soluble TSP actually reaching the cells in the upper wells was ~10–60 nM. TSP levels in plasma are generally 0.2–0.4 nM (equivalent to 100–160 ng/ml) rising to a maximum of 70 nM in serum after activation of platelets (Saglio and Slayter, 1982; Lawler, 1986). Therefore, it is possible that localized sites along blood vessel walls contain concentrations of TSP high enough to initiate chemotaxis of PMNs.

When proteolytic fragments of TSP were used to determine which portion of the molecule contained the chemotactic activity for PMNs, we found that neither the HBD nor the 140-kD fragment could stimulate chemotaxis. However, preparations containing both of these fragments stimulated chemotaxis to an even greater extent than intact TSP, increasing the response about fivefold. These results indicated that the binding of both the HBD and the 140-kD fragment were required for chemotaxis. This finding is different from that of Taraboletti et al. (1987) who have shown that the HBD of TSP is as potent a chemoattractant for melanoma cells as the intact molecule.

The observation that human PMNs display chemotactic behavior in response to TSP is interesting since other ECM proteins do not appear to stimulate chemotaxis in these cells. Laminin from 10 to 50 μg/ml stimulates chemotaxis in rabbit peritoneal PMNs (Terranova et al., 1986), but concentrations up to 100 μg/ml do not promote the same response in human peripheral PMNs (Basara et al., 1985). Vitronectin and fibronectin also give negative results at 100 μg/ml (Basara et al., 1985). In addition, the 120-kD fragment of fibronectin, which is chemotactic for monocytes, does not attract PMNs at concentrations up to 10 μM (Clark et al., 1988), although it does stimulate degranulation (Wachtfogel et al., 1988). It should, however, be noted that the concentrations of ECM proteins tested in these other studies were much lower than TSP concentrations required for activity in the present study. Therefore, we cannot rule out the possibility that higher concentrations of laminin, fibronectin, or vitronectin could stimulate chemotaxis in human PMNs.

As an alternative to chemotaxis, migration due to substrate-bound TSP could represent the movement of PMNs on blood vessel walls before diapedesis. Mumby et al. (1984) found that more TSP is produced by subconfluent cultures of endothelial cells than by confluent cultures. They speculated that subconfluent cultures might represent injured endothelium where cell–cell contacts are reduced. Thus, during and following injury, platelets, endothelial cells, and smooth muscle cells may secrete TSP which then binds to endothelial cells or denuded matrix where it may be encountered by PMNs and promote increased levels of PMN movement.

The accelerated random migration observed with bound TSP is similar to that observed for melanoma cells in response to bound laminin and fibronectin (McCarthy and Furcht, 1984). However, unlike melanoma cells, PMNs do not appear to exhibit an additional directional component to their movement in response to TSP. In contrast to chemotaxis, locomotion of PMNs in response to bound TSP was stimulated by relatively low concentrations (<100 pmol bound). Both the distance traveled and, to an even greater extent, the number of cells migrating were increased ~100-fold by TSP bound to filters as compared to either soluble TSP or fMLP. Haptotaxis in G361 melanoma cells (Taraboletti et al., 1987) appears to occur at lower concentrations of bound TSP than required for a PMN response. Polycarbonate filters soaked in as little as 20 nM TSP promote melanoma cell haptotaxis. However, it should be pointed out that these authors incubated filters in protein solution overnight at 37°C, significantly different conditions than those used in the present study.

An anti-TSP polyclonal antibody and its Fab fragments, but not rabbit IgG, inhibited TSP-mediated cell movement indicating that this response was specific for TSP. More importantly, using monoclonal antibodies to specific regions of the TSP molecule, we were able to determine which portion of the molecule was responsible for stimulating PMN motility. Monoclonal antibody C6.7 was the most effective inhibitor of cell movement, consistent with the hypothesis that the active cell-binding site is near the carboxy terminus, on the 18-kD fragment. While mAb A2.5, which recognizes the HBD, also slightly inhibited cell movement (to a lesser extent than mAb C6.7), this antibody has been shown to alter TSP conformation so that sites on the carboxy terminus are affected (Galvin et al., 1985). The antibody data indicated that, in contrast to TSP priming of fMLP-mediated chemotaxis (where activity was located in the HBD), PMN migration in response to bound TSP was stimulated by the 140-kD COOH terminal fragment.

As expected, bound 140-kD fragment promoted movement similar in magnitude to bound intact TSP confirming that the activity was localized to the COOH terminus of the molecule. Interestingly, bound 120/18-kD fragment was equipotent to the 140-kD fragment in stimulating migration. Since the 18-kD fragment retains the C6.7 antibody binding site and is disulfide-linked to the 120-kD fragment after chymotrypsin cleavage (Dixit et al., 1985*a*), it is not surprising that the 120/18-kD fragment retains activity. Similar to our results, Taraboletti et al. (1987) found that the 140-kD fragment is as effective as intact TSP in stimulating haptotaxis in melanoma cells. However, in their system the 120-kD fragment has no activity. Cleaving the 140-kD fragment to a 120/18-kD fragment also destroys the attachment and spreading activity of TSP for melanoma cells and keratino-

cytes (Roberts et al., 1987; Varani et al., 1988). The differences observed in the activity of the 120/18-kD between this study and others might reflect variations in fragment preparation leading to a slightly altered conformation of TSP proteolytic fragments. Alternatively, a different site on the TSP molecule may be involved in PMN migration than is required for melanoma cell haptotaxis or adhesion.

The effectiveness of the inflammatory response depends on the presence of substances that can direct PMNs to a specific site, mediate their adhesion to endothelial cells, and/or subendothelial matrix, and promote transendothelial cell migration. Our results demonstrate that TSP meets several of the criteria of an inflammatory mediator. TSP can enhance the chemotactic response of PMNs for formylated chemotactic peptides, TSP can itself stimulate PMN chemotaxis, and when bound to a substrate, TSP can increase cell movement at or within sites of injury or infection. Therefore, TSP secreted luminally by endothelial cells in response to injury or other inflammatory mediators may participate in the recruitment of PMNs to a site of infection. Future studies in our laboratory will focus on the elements of signal transduction that may be unique for TSP.

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