A disaccharide that inhibits tumor necrosis factor α is formed from the extracellular matrix by the enzyme heparanase

(cytokines/extracellular matrix/heparan sulfate/delayed-type hypersensitivity)

OFER LIDER*, LIORA CAHALON*, DALIA GILAT*, RAMI HERSHKOVIZ*, DANIEL SIEGEL†, RAANAN MARGALIT*, ODED SHOSEYOV†, AND IRUN R. COHEN*‡

*Department of Cell Biology, The Weizmann Institute of Science, Rehovot 76100, Israel; and †The Kennedy–Leigh Center for Horticultural Research, Faculty of Agriculture, The Hebrew University of Jerusalem, Rehovot 76100, Israel

ABSTRACT   The activation of T cells by antigens or mitogens leads to the secretion of cytokines and enzymes that shape the inflammatory response. Among these molecular mediators of inflammation is a heparanase enzyme that degrades the heparan sulfate scaffold of the extracellular matrix (ECM). Activated T cells use heparanase to penetrate the ECM and gain access to the tissues. We now report that among the breakdown products of the ECM generated by heparanase is a trisulfated disaccharide that can inhibit delayed-type hypersensitivity (DTH) in mice. This inhibition of T-cell mediated inflammation *in vivo* was associated with an inhibitory effect of the disaccharide on the production of biologically active tumor necrosis factor α (TNF-α) by activated T cells *in vitro*; the trisulfated disaccharide did not affect T-cell viability or responsiveness generally. Both the *in vivo* and *in vitro* effects of the disaccharide manifested a bell-shaped dose–response curve. The inhibitory effects of the trisulfated disaccharide were lost if the sulfate groups were removed. Thus, the disaccharide, which may be a natural product of inflammation, can regulate the functional nature of the response by the T cell to activation. Such a feedback control mechanism could enable the T cell to assess the extent of tissue degradation and adjust its behavior accordingly.

Some years ago, we reported that activated T cells secreted heparanase, an enzyme that degrades the heparan-sulfate component of the extracellular matrix (ECM; refs. 1 and 2). The expression of heparanase by T cells, unlike that by tumor cells, was found to be tightly regulated by contact with specific antigen or mitogen. Naive T cells responded to activation by synthesizing heparanase *de novo* but memory T cells were able to release heparanase from preformed stores within minutes of contact with antigen (2).

In addition to the positive induction of heparanase expression by T-cell activators, heparanase expression was also found to be inhibitable by heparin (3–6). Heparin is very similar chemically to heparan sulfate, the natural substrate of heparanase, and it was proposed that heparin, by occupying the binding site of the enzyme, may act as a competitive inhibitor of heparanase activity (3, 5, 6). However, we found that low doses of heparin appeared to act directly on T cells to inhibit their release of heparanase (4, 5). Heparin is composed of different sulfated sugar molecules, and the saccharide moieties that inhibit T-cell heparanase release differ from those that inhibit blood coagulation (4). We reasoned that the T-cell inhibitory molecules in heparin might actually mimic molecules produced by the action of heparanase on its natural substrate heparan sulfate. We report here the isolation of a trisulfated disaccharide generated by the action of heparanase on the ECM (ECM disaccharide). Administration of nano-

gram amounts of ECM disaccharide suppresses T-cell mediated reactivity *in vivo*. Incubation of T cells with the ECM disaccharide inhibits T-cell production of the active form of tumor necrosis factor (TNF)-α, a major inflammatory cytokine.

MATERIALS AND METHODS

Heparanase. Mammalian heparanases have not yet been cloned; because T-cell heparanase was not available in quantity, we used heparanase obtained from human placentas. The heparanase enzyme was prepared and purified to >3.4 × 10^5-fold by ammonium sulfate precipitation followed by sequential chromatographies on carboxymethyl-, heparin-, and Con A-Sepharose columns (RAD Chemicals, Ness Ziona, Israel) by modifying the technique of Oosta et al. (7). The specific activity of the heparanase was determined, as described, by its ability to release $^{35}SO_4$-labeled fragments of heparan sulfate from the ECM (3, 4).

Preparation of ECM Disaccharide. ECM-coated plates were prepared from bovine corneal endothelial cells as described (5). Each ECM-coated plate was incubated for 48 h at 37°C with 20 μl of mammalian heparanase (0.5 mg/ml) in 1 ml of phosphate citrate buffer (18 mM citric acid/64 mM Na_2HPO_4/50 mM NaCl/1 mM $CaCl_2$/1 mM dithiothreitol, pH 6.2). The culture medium containing the ECM heparan sulfate degradation products was then collected and applied to a Sepharose 4B column (0.7 × 35 cm). The mobile phase was phosphate citrate buffer at a flow rate of 5 ml/h. Fractions of 1.6 ml were collected and monitored at 206 nm to detect oligosaccharides (Fig. 1A) or at 232 nm to detect unsaturated disaccharides. Quantitation of the uronic acid containing disaccharides was done by using the carbazole assay (8). The lower molecular weight fractions, fractions 5 and 6, of the Sepharose 4B peak were combined and freeze-dried, and the powder was resuspended in 10% of the initial volume. The disaccharide was then isolated from the elution profile by using HPLC gel-filtration chromatography followed by HPLC ion-exchange chromatography. Samples of 0.1 ml were injected into an HPLC column [Toyo Soda (Tokyo) TSK-Gel G3000 SW; 7.5 mm × 50 cm; and G2000 SW; 7.5 mm × 50 cm; in series with a 7.5 mm × 10 cm guard column from Phenomenex (Belmont, CA)]. The mobile phase was 0.5 M NaCl at a flow rate of 1 ml/min. Fractions (1 ml) were collected and monitored at 206 and 232 nm. The column was calibrated with heparin-oligosaccharide standards (Seikagaku Kogyo, Tokyo). The peak labeled p1 in Fig. 1B, having the retention time of a disaccharide, was collected from nine identical runs. The substantially homogeneous fractions were combined and freeze-dried.

Abbreviations: DTH, delayed-type hypersensitivity; ECM, extracellular matrix; TNF-α, tumor necrosis factor α; PHA, phytohemagglutinin.
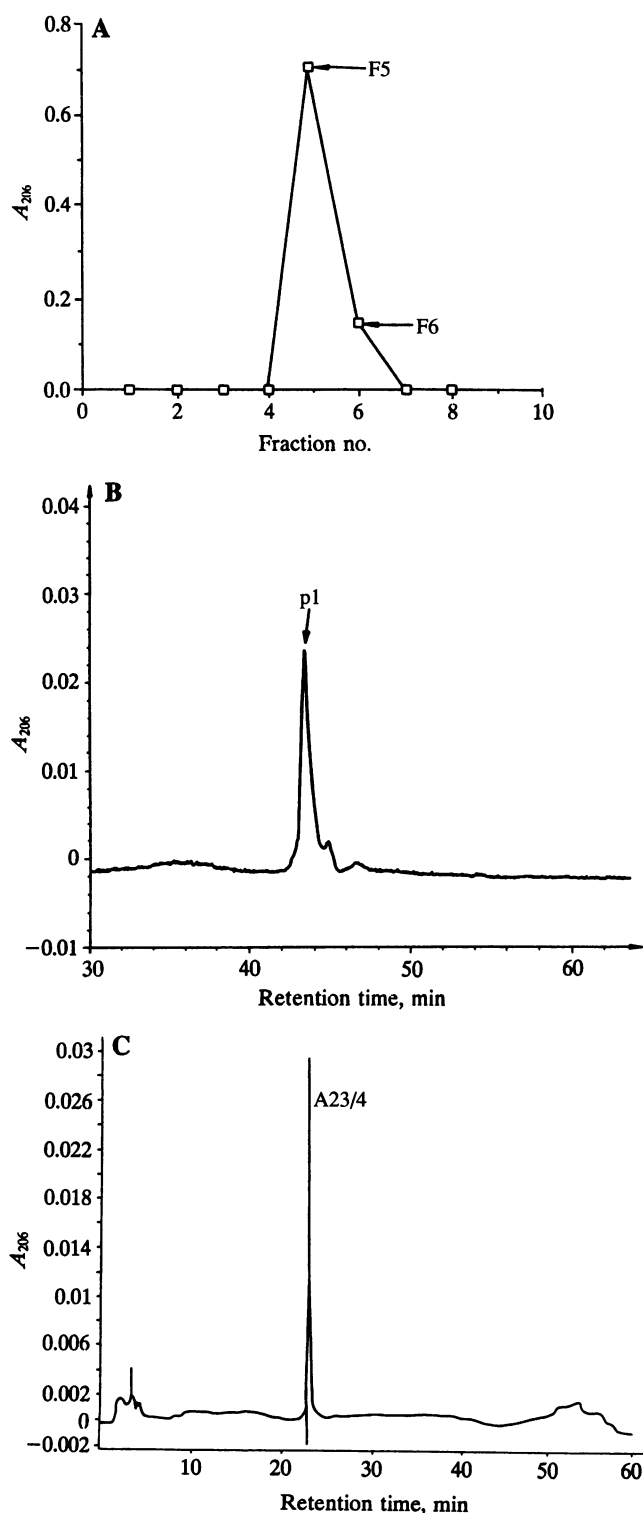‡To whom reprint requests should be addressed.

FIG. 1. Purification profiles of ECM disaccharide. (A) Sepharose-4B peak. (B) HPLC gel-filtration peak. (C) Sax-HPLC ion-exchange peak. See the text for details of ECM disaccharide purification.

The purified material was resuspended in 2 ml of double deionized water, desalted on a Sephadex G-10 column (26 × 150 mm), and eluted at 1.6 ml/min with double deionized water. Fractions (1 ml) were collected, monitored at 206 nm to detect carbohydrate molecules, and tested for conductivity to determine NaCl content. Desalted fractions were combined, freeze-dried, and resuspended in 1 ml of double deionized H₂O. A 1 ml sample, prepared by combining 100 μl of the resuspended solution and 900 μl of 0.2 M NaCl at pH 3.5, was

injected into an analytical Sax-HPLC column (4.6 × 250 mm; packed with Spherisorb; Phase Separation, Inc., Norwalk, CT; 5-μm particle size). The column flow rate was 1.5 ml/min and an NaCl linear gradient program was employed as shown in Table 1.

The column eluent was monitored at 206 nm (Fig. 1C) and the peak labeled A23/4 was collected and quantified by the carbazole assay. To characterize the isolated disaccharide molecule, we compared its retention time to various known heparin disaccharide standards with different levels of sulfation (Sigma) that were injected into the Sax-HPLC column under identical conditions. The retention time of these standards indicated that the ECM disaccharide was probably trisulfated. A trisulfated disaccharide standard (α-ΔUA-2S-1→4-GlcNS, 6S; Sigma; H 9267) had a very similar retention time to that obtained for peak A23/4: 23.07 min and 23.10 min, respectively. We were able to isolate a radiolabeled trisulfated disaccharide when the heparanase was used to degrade an ECM metabolically labeled with Na₂³⁵SO₄ (ref. 2; data not shown). This indicates that the isolated disaccharide did in fact originate from the ECM.

Fast atom bombardment-mass spectrometry (FAB-MS) of methylated, partially desulfated A23/4 was consistent with a molecular weight of about 530 for a methylated derivative. The proton NMR spectrum of the trisulfated disaccharide was recorded in ²H₂O at 500 MHz. Typical sugar signals were evident between 3 and 5.5 ppm. A doublet signal for the anomeric proton was found at 5.39 ppm having a coupling factor of 3 Hz, suggesting the presence of a glucosamine sugar unit in an α configuration. The chemical shift of the anomeric proton, together with what is believed to be the β-glucuronide specificity of placental heparanase, leads to the tentative conclusion that the ECM disaccharide has a glucosamine at the nonreducing end in an α configuration attached 1→4 to a glucuronide acid residue at the reducing end. The complete identification and the organic synthesis of the proposed molecule will be published elsewhere.

A desulfated disaccharide was obtained by incubating some of the disaccharide of peak A23/4 for 4 days at room temperature at pH 3.5. The nonsulfated disaccharide was isolated by its shorter retention time on the Sax-HPLC column, which was very similar to the retention time of a monosulfated disaccharide standard (α-ΔUA-2S-[1→4]-GlcNAc; Sigma; H 8767).

Delayed-Type Hypersensitivity (DTH) Assay. Groups of 10 female inbred BALB/c mice (The Jackson Laboratory) were sensitized on the shaved abdominal skin with 100 μl of 2% oxazalone dissolved in acetone/olive oil [4:1 (vol/vol)] applied topically (5). DTH sensitivity was elicited 5 days later by challenging the mice with 20 μl of 0.5% oxazalone in acetone/olive oil, 10 μl administered topically to each side of the ear. A constant area of the ear was measured immediately before challenge and 24 h after challenge with a Mitutoyo engineer's

Table 1. Elution of ECM disaccharide from Sax-HPLC column

| Time, min | Eluent composition, % | | |
	0.2 M NaCl (pH 3.5)	1.5 M NaCl (pH 3.5)	H₂O
0	100	0	0
2	100	0	0
35	38	62	0
40	38	62	0
45	0	100	0
47	0	100	0
50	0	0	100
55	0	0	100
58	100	0	0
60	100	0	0

micrometer. The individual measuring ear swelling was un-aware of the identity of the groups of mice. The DTH reaction is presented as the increment of ear swelling after challenge expressed as the mean ± SEM in units of 10^{-2} mm. The ECM disaccharide or other test material was administered 1 day before the initial sensitization. Percent inhibition of DTH is calculated as follows:

% inhibition =

$$\left[1 - \left(\frac{\text{treated} - \text{negative control}}{\text{positive control} - \text{negative control}} \right) \right] \times 100.$$

The positive control is the DTH reaction to oxazalone elicited in immunized mice in the absence of treatment. The negative control is the background swelling produced by the oxazalone antigen in naive, nonimmunized mice.

TNF-α Assays. CD4$^+$ T cells were isolated from peripheral blood mononuclear leukocytes obtained from healthy human donors, as described (9). The effects of the ECM disaccharide and other test materials on the secretion of TNF-α by human CD4$^+$ T cells were assayed both by biological activity and by an anti-TNF-α antibody that detected the TNF-α molecule immunologically rather than functionally. To detect TNF-α biological activities, 2×10^5 CD4$^+$ T cells were preincubated with 150 μl of ECM degradation products or purified disaccharides at various concentrations for 1.5 h at 37°C in 7% CO_2/93% air. Then, 100 μl of phytohemagglutinin (PHA) (Wellcome; 1 μg/ml) were added for 3 h of incubation in flat-bottom 96-well plates (Costar), 3–6 wells in each experimental group. The results were not affected by washing the T cells to remove the disaccharide from the culture medium before the addition of PHA. Subsequently, the contents of the wells were collected and centrifuged, and the media were assayed for the presence of biologically active TNF-α by using indicator cells sensitive to TNF-α (10). Assaying the amount of TNF-α secreted by the test CD4$^+$ T cells was done by using a monoclonal antibody assay kit (Biokine TNF kit, T-Cell Sciences, Cambridge, MA). This assay kit measures TNF-α protein and not TNF-α activity.

RESULTS

ECM Disaccharide Treatment Inhibits DTH Reactivity. We used the DTH reaction as a semi-quantitative measure of T-cell-mediated inflammation *in vivo*. Table 2 shows the results of 2 of >12 experiments in which the DTH response to oxazalone was measured in mice that had been treated with various saccharide substances 1 day before their initial skin sensitization. The DTH response was measured by challenging the ears with oxazalone 5 days after primary sensitization and 6 days after saccharide treatment. Doses of heparin between 10 and 1000 ng had no inhibitory effect on the DTH reaction (Table 2, Exp. A). The unpurified material released from the ECM by heparanase degradation (crude ECM) also had no inhibitory effect at a similar range of doses. In contrast, the purified ECM disaccharide (A23/4; see Fig. 1C) was strongly inhibitory; a single dose of 3.3 ng inhibited the DTH response by 77%. Note that the dose–response curve was bell shaped; doses higher, as well as lower, than the optimal dose of the disaccharide were less inhibitory.

In a second experiment (Table 2, Exp. B), the ECM disaccharide (A23/4) was tested along with its desulfated product. The ECM disaccharide isolated by using an HPLC gel filtration column (see peak p1 in Fig. 1B) was also tested for its dose–response characteristics. It can be seen that the desulfated ECM disaccharide lost its inhibitory effect compared with that of the parent trisulfated ECM disaccharide A23/4. Note that the trisulfated ECM disaccharide isolated by HPLC gel filtration (p1), showed a bell-shaped dose–response curve similar to that manifested by the ECM disaccharide isolated by ion-exchange chromatography (A23/4; Table 2, Exp. A). Thus, the bell-shaped dose–response curve is not an artifact of the manner in which the active ECM disaccharide was isolated.

ECM Disaccharide Inhibits Secretion of Active TNF-α. Many factors are involved in the biological effects mediated by activated T cells; however, since the cytokine TNF-α is a major element in the inflammatory response, we investigated the effect of the ECM disaccharide on the production of active TNF-α. Table 3 shows the optimal concentrations of various fractions of ECM required to inhibit the secretion of active TNF-α by human CD4$^+$ T cells incubated with PHA. The

Table 2. Treatment of mice with isolated ECM disaccharide inhibits DTH reactivity

Experiment	Treatment	Dose, ng per mouse	DTH response, $\times 10^{-2}$ mm	% inhibition
A	None; negative control	—	1.5 ± 0.2	—
	None; positive control	—	18.1 ± 1.1	—
	Heparin	10–1000	16.9 ± 1 to 19.1 ± 1.3	0
	Crude ECM	10–1000	17.2 ± 1.4 to 18.5 ± 1.6	0
	ECM disaccharide (A23/4)	0.2	19.7 ± 1.7	0
		0.8	18.1 ± 1.2	0
		3.3	4.7 ± 0.9	77*
		12.5	11 ± 1.4	40*
		50	18.2 ± 0.9	0
B	None; negative control	—	1.4 ± 0.3	—
	None; positive control	—	18.9 ± 1.7	—
	ECM disaccharide (A23/4)	3.3	3 ± 1	83*
	Desulfated ECM disaccharide	3.3	18.5 ± 1.1	0
	ECM disaccharide (p1)	0.8	15.5 ± 0.9	15
		3.3	7.7 ± 1.3	60*
		12.5	10.1 ± 1.6	45*
		50	18.2 ± 0.9	0

Groups of BALB/c mice were injected subcutaneously with heparin, the medium of ECM that had been degraded by incubation with heparanase (crude ECM), or trisulfated disaccharide isolated from the crude ECM (A23/4 or p1; Fig. 1). A desulfated isolate of A23/4 was also tested. Negative control, the degree of ear swelling caused by challenging naive mice; positive control, the DTH response of sensitized, untreated mice.
*$P < 0.01$, treated vs. untreated (positive control) mice.

Table 3. ECM disaccharide inhibits T-cell secretion of active TNF-α

T-cell treatment	Optimal concentration, pg/ml	TNF-α activity, % killing	% inhibition of TNF-α activity
None	—	45 ± 5	0
Crude ECM	10^6	21 ± 4*	53
Sepharose 4B	100	22 ± 5*	50
HPLC (p1)	10	9.9 ± 2*	78
Sax-HPLC (A23/4)	16	10.8 ± 3*	76

Purified human CD4$^+$ T cells were incubated for 1.5 h with various concentrations of crude. ECM (ECM medium collected after heparanase digestion); Sepharose 4B (low molecular weight fraction of crude ECM purified on a Sepharose 4B column; see Fig. 1A); HPLC (disaccharide purified by HPLC; see Fig. 1B); or Sax-HPLC (disaccharide) purified by Sax-HPLC; see Fig. 1C). The T cells were then activated with PHA and the medium was collected and assayed for TNF-α activity by its ability to induce the killing of TNF-α-sensitive CL7 cells.
*P < 0.01, comparing the treated groups to the control (none) group.

crude ECM fraction obtained after digestion of the ECM with heparanase was least active: a concentration of 10^6 pg/ml was optimum. Purification of the low molecular weight fraction by using Sepharose 4B (see Fig. 1A) led to optimal activity at 100 pg/ml. ECM disaccharide isolated by HPLC gel filtration (HPLC, see Fig. 1B) or by HPLC ion exchange (Sax-HPLC A23/4; see Fig. 1C) produced maximal inhibition of TNF-α activity by concentrations of 10–16 pg/ml. Similar to the inhibition of the DTH reaction in mice *in vivo*, inhibition of secretion of active TNF-α *in vitro* showed a bell-shaped curve of activity, shown for the Sax-HPLC fraction in Fig. 2.

TNF-α Bioactivity and TNF-α Protein Concentration. It has been reported that TNF-α may be produced in a biologically inactive form (11). We therefore tested whether inhibition of TNF-α activity by the ECM disaccharide could be ascribed to the production of TNF-α in its inactive rather than in its active form. TNF-α was measured by both the bioassay, which reflects TNF-α activity, and the antibody assay, which reflects the concentration of TNF-α protein independent of its activity (Table 4). It can be seen that incubating T cells with ECM disaccharide led to a 70% decrease in the biological activity of TNF-α secreted by the T cells in response to activation by
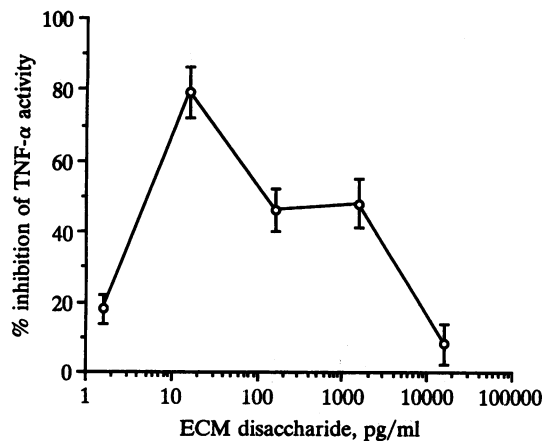

FIG. 2. Inhibition of the secretion of active TNF-α by the ECM disaccharide. Purified human CD4$^+$ T cells were incubated with the indicated concentrations of the ECM disaccharide (A23/4) purified on an anion-exchange column. After 1.5 h, the T cells were activated with PHA and the media were collected and assayed for TNF-α activity by the ability of the media to induce the killing of indicator CL7 cells. The percent inhibition of TNF-α activity by the ECM disaccharide was computed by using 60% killing of the indicator cells as the reference activity. Data are presented as the mean ± SD.

Table 4. ECM disaccharide induces secretion of biologically inactive TNF-α

ECM fraction	TNF-α bioassay		TNF-α protein assay	
	Target cell killing, % ± SD	Inhibition, %	TNF-α, pg/ml	Inhibition, %
None	45 ± 6	—	274 ± 18	—
HPLC	14 ± 3	70*	232 ± 22	15

The effect of ECM disaccharide purified by HPLC ion-exchange chromatography on TNF-α produced by CD4$^+$ human T cells was tested both by bioassay, as described in the legend of Table 3, and by antibody assay of the concentration of TNF-α protein.
*P < 0.01, comparing the treated vs. untreated groups.

PHA. In contrast, the concentration of TNF-α protein measured by the antibody assay was decreased by only 15%. Hence, the magnitude of the decrease in TNF-α activity cannot be attributed to inhibition of release of TNF-α protein but to a decreased activity of the released TNF-α. Therefore, it appears that treatment with the ECM disaccharide leads the T cell to primarily secrete the biologically inactive form of TNF-α, although it also causes a mild decrease in TNF-α protein concentration.

The decrease in TNF-α activity was not accompanied by an inhibition in T-cell responsiveness generally. T cells incubated with concentrations of ECM disaccharide optimal for inhibition of their TNF-α activity incorporated thymidine and underwent blast transformation in response to mitogen or antigen (data not shown). Thus, the effect of the ECM disaccharide on T cells is relatively selective.

DISCUSSION

The interactions between ECM-associated heparan sulfate proteoglycan and adjacent glycoproteins of the ECM stabilize the matrix structure and help to maintain its integrity (3). Therefore, cleavage of heparan sulfate by heparanase results in the disassembly of the ECM barrier, enabling leukocyte migration into inflamed tissues. The results presented here indicate that heparan sulfate not only can serve as a structural element but also can provide regulatory signals cleaved from its own substance.

The heparanase secreted by T cells has not been cloned and was not available in quantities sufficient to be used for our studies. However, it seems that T-cell heparanase and placental heparanase are the same in terms of molecular weight, pH dependency, and substrate specificity; moreover, they both digest the ECM to generate similar breakdown products (3, 12). Indeed, preliminary experiments indicate that activated CD4$^+$ T cells can cause the release of the trisulfated disaccharide when incubated with ECM *in vitro* (unpublished data). Our working hypothesis, therefore, is that T-cell heparanase, like placental heparanase, is capable of producing the inhibitory trisulfated disaccharide from ECM heparan sulfate. In view of the present results, we propose the scheme illustrated in Fig. 3. Among the products of activated T cells are cytokines such as TNF-α and enzymes such as heparanase. Both types of molecules participate in the inflammatory response. However, the effects of these molecules can have opposite influences on T-cell reactivity: TNF-α participates in a chain reaction that can upregulate the presentation of antigen and activation signals that further activate T cells; heparanase, in contrast, can generate ECM disaccharide that feeds back to downregulate the production of biologically active TNF-α. Thus, we propose that the same heparanase that enables activated T cells to penetrate the tissues produces a disaccharide signal from the ECM debris that directs the T cells to secrete an inactive form of TNF-α. In this way, negative feedback is inherent in the inflammatory response. The results of prelim-

Immunology: Lider *et al*

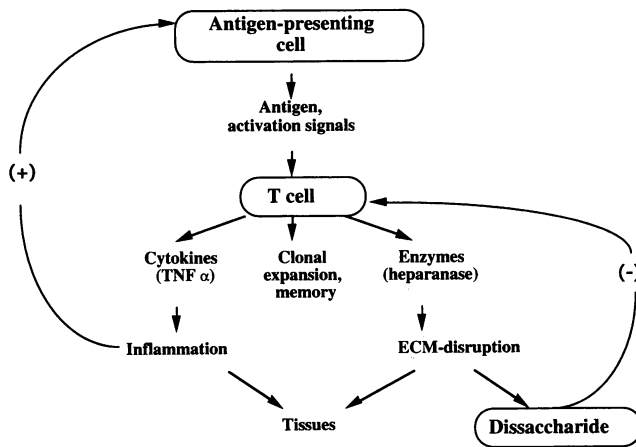*Proc. Natl. Acad. Sci. USA* 92 (1995)   5041

FIG. 3.   Feedback control of T-cell mediated inflammation. T cells are activated by processed peptide antigen and other activation costimulatory signals presented by antigen-presenting cells. The activated T cells proliferate and generate memory T cells. The T cells also produce effector molecules, such as the inflammatory cytokine TNF-α and the enzyme heparanase. The inflammatory cytokines can upregulate T-cell activation. The activity of the heparanase enzyme results in the disruption of the ECM, allowing cells to penetrate the tissues. But heparanase degradation of the ECM also generates the ECM disaccharide that feeds back to downregulate the inflammatory activity of the T cells, causing the replacement of active TNF-α with inactive TNF-α.

inary studies suggest that the ECM disaccharide may influence the behavior of endothelial cells and macrophages, as well as T cells, during inflammation. Thus, the inhibition of the DTH reaction by the ECM disaccharide *in vivo* may involve more than an effect on T cells. In any case, the inhibitory effect of the ECM disaccharide seems to be dependent on its optimal concentration, too little or too much of the ECM disaccharide is not inhibitory. We do not yet know the mechanism responsible for the bell-shaped dose–response curve, but in general, we may say that T cells, at least, are sensitive to the concentration of ECM disaccharide. Perhaps, the concentration of disaccharide can inform the T cells about the nature of the tissue damage they have wrought. Thus, T cells can measure the quality of their performance. Such performance information is essential to all cognitive systems, including the immune system (13).

The enzymatic activity of heparanase has been shown to cause the release of various growth factors and cytokines bound to heparan sulfate (12, 14). These factors mediate angiogenesis, the proliferation of smooth muscle cells and fibroblasts, and other processes that follow in the wake of the inflammatory response (3). Recently, we have found that the functions of heparanase are not limited to its enzymatic activity; mammalian heparanase at pH 7.2 is not active enzymatically but acts as an adhesion molecule binding T cells and

probably other leukocytes to the ECM (15). The heparanase molecule becomes enzymatically active only when the local hydrogen ion concentration falls below pH 6.8, a phenomenon associated with inflammation. Thus, the heparanase molecule is beginning to reveal its protean role in regulating the defense, degradation, and repair of tissues.

The precise structure of the proposed chemical model of the ECM disaccharide suggested by the proton NMR and fast atom bombardment-MS spectra is not known; however, the present results may be appreciated even before final chemical characterization of the ECM disaccharide. This trisulfated disaccharide heralds a class of carbohydrate molecules capable of regulating inflammatory reactions on the basis of natural feedback signals that are generated by the immune response itself. By administering such signal molecules therapeutically, we might succeed in communicating with the immune system in its own chemical language (16).

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