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# Activated T lymphocytes produce a matrix-degrading heparan sulphate endoglycosidase

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**We have previously found that lines of activated T lymphocytes specifically autosensitized to the basic protein of myelin (BP), on intravenous inoculation into syngeneic rats, were able to penetrate blood vessels, accumulate in the nervous system and cause experimental autoimmune encephalomyelitis (EAE)[1]. An important question is how effector T cells reach such targets outside the walls of blood vessels. To investigate this we have studied *in vitro* the interaction of anti-BP effector T lymphocytes with the basement membrane-like extracellular matrix produced by vascular endothelial cells. We now report that activated but not resting T lymphocytes produce an endoglycosidase capable of degrading heparan sulphate side chains of the proteoglycan scaffold of the extracellular matrix. Moreover, the anti-BP T lymphocytes respond to BP presented by extracellular matrix by markedly enhanced elaboration of the endoglycosidase. These results suggest that tissue-specific antigens on blood vessel walls could direct lymphocyte homing by activating enzymes that facilitate penetration of the subendothelial basal lamina. They also suggest that effector T lymphocytes can recognize antigen which is not associated with a major histocompatibility complex signal.**

The experimental system described here features two elements: anti-BP T lymphocytes and endothelial cell cultures producing extracellular matrix (ECM). Our method for raising antigen-specific lines of helper/delayed hypersensitivity lymphocytes has been described in detail[2]. The lines were maintained by culture in growth medium lacking antigen but supplemented with a supernatant of cultures of lymphocytes stimulated by the mitogen concanavalin A (Con A) as a source of crude T-cell growth factor. Intravenous inoculation of Lewis rats with anti-BP line cells kept in growth medium had no discernible effect on the rats. However, after reactivation of the cells with BP in the presence of antigen-presenting cells syngeneic at the major histocompatibility complex (MHC), they proliferated vigorously and were able to enter and accumulate in the thymus and central nervous system, and induce EAE (refs 1–3). Activated anti-BP T lymphocytes blocked nerve conduction *in vitro*[4]. Moreover, after attenuation, activated anti-BP T lymphocytes could be used to vaccinate rats against active induction of EAE (refs 5, 6).

The bovine aortic endothelial cell system has been described elsewhere and shown to mimic the vascular endothelium *in vivo*[7,8]. At confluence the cells form a monolayer composed of non-overlapping and closely apposed cells which express differentiated functions such as a non-thrombogenic apical surface and production of prostacyclin and anti-factor VIII antigen[7,8], but most pertinent here is a massive secretion of an underlying extracellular matrix in a polar fashion and similar in organization and supramolecular composition (types III, IV and V collagen, heparan sulphate, laminin, fibronectin) to naturally occurring basal laminas[9,10]. The results of previous studies using high metastatic and low metastatic sublines of tumour cells[11–13] showed that higher amounts of heparan sulphate fragments released from the extracellular matrix by tumour cells correlated with greater potential to penetrate blood vessels and metastasize[11,12].

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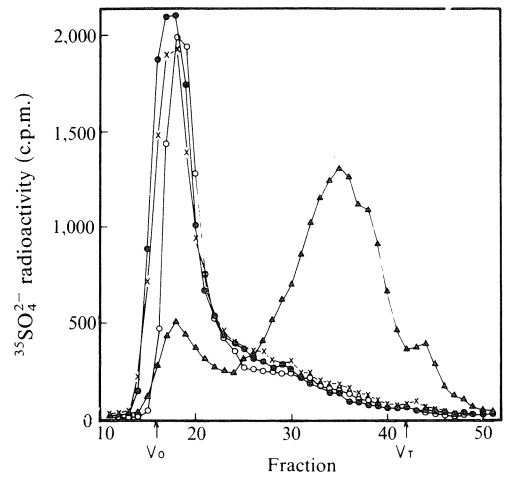


**Fig. 1** Activated T lymphocytes degrade subendothelial extracellular matrix. Lewis rat anti-BP T lymphocytes were activated 72 h earlier by incubation with BP in the presence of irradiated syngeneic thymus accessory cells, or used without activation as described previously[1]. $2 \times 10^6$ activated (▲) or $4 \times 10^6$ non-activated (○) lymphocytes suspended in RPMI medium containing 10% fetal calf serum were then seeded on top of the labelled extracellular matrix. Labelled extracellular matrix was also incubated with medium containing irradiated thymus accessory cells and BP without lymphocytes (×) or with activated lymphocytes in the presence of 10 μg ml$^{-1}$ heparin (●). The medium was collected after 24 h at 37 °C and 0.5–0.7-ml aliquots of the centrifuged medium applied for gel filtration on Sepharose 6B columns (0.7 × 35 cm in most experiments and 1.1 × 70 cm in some experiments) as described previously[11]. The excluded volume, $V_o$ was marked by blue dextran, and the total included volume $V_t$, by phenol red. Similar elution profiles ($K_{av}$ values) and recoveries were obtained whether the centrifuged media were subjected to gel filtration in dissociation conditions (4 M guanidine-HCL in 0.1 M sodium acetate, *p*H 5.5) or eluted with phosphate-buffered saline (PBS). Each experiment was performed at least four times and the variation in elution positions ($K_{av}$ values) was less than 10%. Removal of any residual irradiated accessory cells by Ficoll–Hypaque density purification did not affect the results. The sulphate-containing moieties of glycosaminoglycans in the extracellular matrix were metabolically labelled by incubating subconfluent cultures of endothelial cells with radioactive $^{35}S$ as $Na_2[^{35}S]O_4$ which was taken up by the cells and incorporated into sulphated proteoglycans. The endothelial cells were then removed entirely by treatment with 0.5% Triton X-100, leaving the underlying, labelled extracellular matrix intact and firmly attached to the tissue culture plastic as described elsewhere[9,10]. The activity of endoglycosidases in cells or media was assayed by incubating the labelled extracellular matrix with test material and measuring the release into the culture medium of cetylpyridinium chloride-precipitable, $^{35}S$-labelled material eluted in the low-molecular weight ($K_{av} = 0.63$) region when analysed by Sepharose 6B gel filtration[11]. This material contained mostly heparan sulphate sequences smaller than intact heparan sulphate side chains ($K_{av} = 0.32$) released by treating the extracellular matrix with papain or alkaline borohydride[10].

Our strategy in the present study was to incubate labelled ECM with anti-BP T lymphocytes and assay the low-molecular weight heparan sulphate-containing fragments released into the culture medium as a measure of the degradation of the extracellular matrix by specific endoglycosidase. Figure 1 shows that non-activated anti-BP T lymphocytes maintained in growth medium had little or no enzyme activity detected during 24 h of incubation. Populations of irradiated accessory thymocytes from naive Lewis rats that included normal thymus lymphocytes and antigen-presenting cells also showed a very low specific enzyme activity. In contrast, anti-BP T lymphocytes that had been activated 72 h earlier by incubation with BP and irradiated (1,500 R) syngeneic thymus accessory cells as a source of antigen-presenting cells showed high endoglycosidase activity as indicated by the release of low-molecular weight ($K_{av} = 0.63$, $M_r$ $10^4$) labelled degradation products. The elaboration of endo-

glycosidase activity was not limited to T lymphocytes reactive to BP and similar results were obtained with T lymphocyte lines reactive to ovalbumen or to the purified protein derivative of *Mycobacterium tuberculosis* (PPD). The anti-PPD line expressed endoglycosidase activity on incubation with PPD but not with BP (not shown).

To rule out the possible contribution of residual accessory cells surviving irradiation, Ficoll–Hypaque-purified activated lymphocytes were suspended in fresh medium, both in the absence or presence of 10% fetal calf serum and a similar degradation profile was obtained (not shown). To examine whether endoglycosidase was secreted, the activated lymphocytes were resuspended in serum-free medium, incubated (48 h, 37 °C) in growth conditions and 1 ml of the conditioned medium incubated with the $Na_2[^{35}S]O_4$-labelled ECM. More than 80% of the released radioactivity was in the form of heparan sulphate degradation products eluted as peak II, reflecting the presence of a soluble endoglycosidase similar to that of intact activated cells (not shown). The fact that this activity was obtained in the absence of serum indicates that the cells secreted the enzyme and did not merely activate an enzyme present in serum. That this activity was specific for heparan sulphate was suggested by its inhibition in the presence of 10 $\mu g\ ml^{-1}$ heparin (Fig. 1). Previous studies have shown that more than 70% of the $^{35}SO_4^{2-}$ radioactivity in the labelled extracellular matrix is incorporated into heparan sulphate[11,12]. Up to 85% of this radioactivity was released in the form of degraded heparan sulphate side chains on incubation of the extracellular matrix with activated anti-BP cells. The low-molecular weight degradation products were confirmed to be fragments of heparan sulphate side chains by their sensitivity to deamination with nitrous acid[14], precipitation with 0.05% cetylpyridinium chloride in 0.6 M NaCl (ref. 14), and resistance to digestion with papain and chondroitinase ABC. Thus, activation of anti-BP T lymphocytes was associated with induction of specific heparan sulphate endoglycosidase activity.

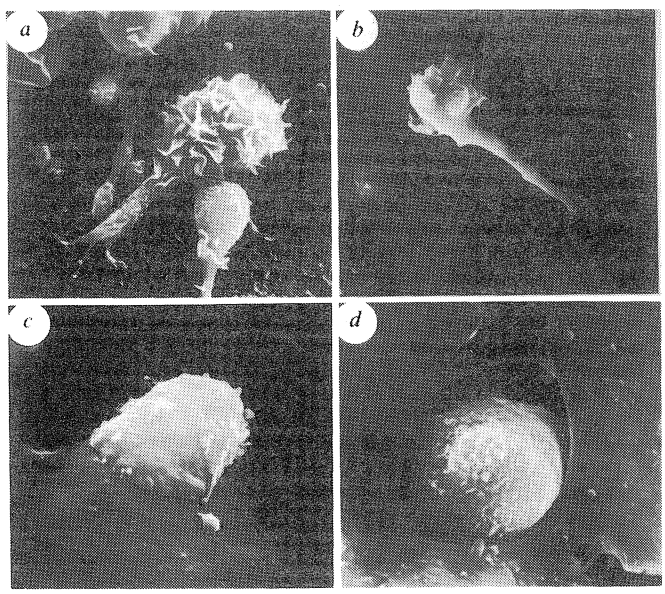


**Fig. 2** Activated T lymphocytes invade cultured vascular endothelium. Activated anti-BP T lymphocytes[1] were separated by Ficoll density centrifugation from any contaminating accessory cells and incubated (2–6 h; $2\times10^6$ cells per 35-mm culture dish) on top of denuded extracellular matrix (*a*) or intact confluent monolayers of bovine vascular endothelial cells (*b–d*). The cultures were then washed to remove non-adhering cells, fixed (2.5% glutaraldehyde in PBS) and processed for scanning electron microscopy as described elsewhere[13]. *a*, Adherence and extension of pseudopods at 12 h incubation in direct contact with the extracellular matrix (×1,920). About 30% of the activated lymphocytes adhering to endothelial cells manifested within 3 h initial invasion by means of extension of pseudopods (*b*, ×1,560) or penetration of the endothelial cell monolayers as seen after 6 h of incubation (*c*, ×3,600; *d*, ×3,000).

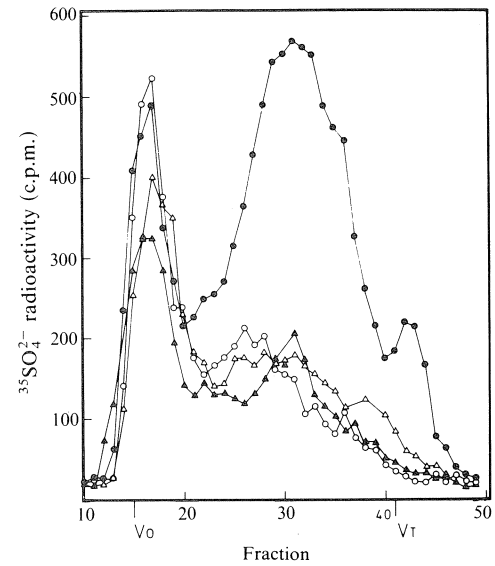


**Fig. 3** Activated anti-BP T lymphocytes respond to BP-pulsed extracellular matrix by augmented endoglycosidase activity. Ficoll-purified activated (●, ○) or unactivated (▲, △) anti-BP T lymphocytes ($4\times10^6$) were incubated for 12 h (activated lymphocytes) or 48 h (non-activated lymphocytes) with labelled extracellular matrix. Some of the extracellular matrix-coated dishes had been pulsed with BP (250 $\mu g\ ml^{-1}$ for 2 h at 37 °C) and extensively washed with PBS before the addition of cells (●, ▲) and others were used without BP pulsing (○, △). The medium was then collected and analysed for specific endoglycosidase activity measured by the amount of radioactivity eluted as peak II as described in the legend to Fig. 1 and ref. 11.

Figure 2 shows that activated anti-BP T lymphocytes could invade the vascular endothelium, primarily between adjacent endothelial cells adherent to the extracellular matrix. Invasion began by an extension of a cytoplasmic process (Fig. 2*b*) in about 30% of the cells, in a manner similar to that observed with metastatic lymphoma cells[13]. Non-activated line cells showed a significantly lower (< 10%) rate of attachment and invasion through the endothelial cell monolayer. Hence, the production of extracellular matrix-degrading heparan sulphate endoglycosidase by anti-BP T lymphocytes and their ability to invade the vascular endothelium *in vitro* correlated with the capacity of the activated lymphocytes to accumulate in the central nervous system and induce EAE *in vivo*[1].

We also investigated whether the presence of specific antigen bound to the extracellular matrix influenced endoglycosidase activity. After incubation of extracellular matrix with BP, unattached BP was washed off, and then endoglycosidase activity of anti-BP T lymphocytes incubated on the BP–extracellular matrix complex was measured (Fig. 3). We found that activated anti-BP T lymphocytes responded to the BP–extracellular matrix complex by a markedly increased endoglycosidase activity relative to that observed when incubated with matrix alone for only 12 h, too short a time to detect appreciable enzyme activity produced by activated lymphocytes on untreated matrix. Non-activated anti-BP T lymphocytes showed little or no specific enzyme activity over 48 h of incubation whether or not the extracellular matrix was pulsed with BP (Fig. 3). In a series of four experiments, we estimated that the BP–extracellular matrix complex elevated the endoglycosidase activity expressed by the anti-BP lymphocytes by about fivefold. Thus, the presence of BP antigen bound to the ECM triggered augmented expression of the enzyme. Activated lines of T lymphocytes specific for purified protein derivative did not respond to the BP–extracellular matrix complex (not shown). Measurement of incorporation of $^3$H-thymidine into DNA demonstrated that the BP–

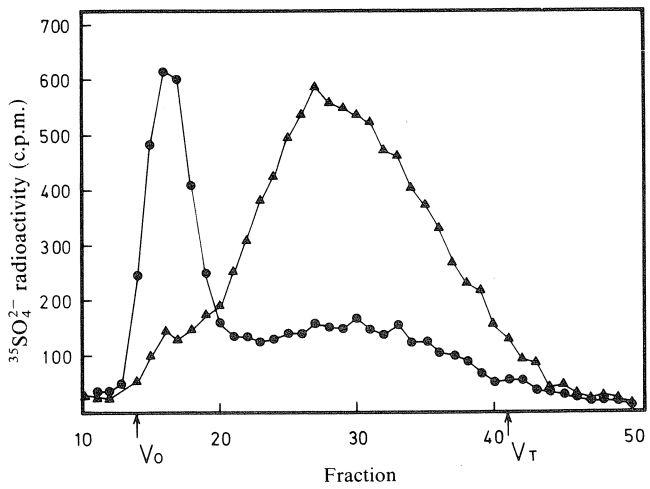


**Fig. 4** Non-activated T lymphocytes respond to Con A-pulsed extracellular matrix by augmented endoglycosidase activity. Ficoll-purified non-activated anti-BP T lymphocytes ($1 \times 10^6$) were incubated (48 h, 37 °C) with untreated labelled extracellular matrix (●) or with labelled matrix that had been pulsed with 250 μg $ml^{-1}$ Con A (Miles Yeda) for 2 h at 37 °C, followed by five washes with PBS (▲). The medium was then centrifuged and analysed by gel filtration as described for Fig. 1.

extracellular matrix complex did not induce proliferation of anti-BP T lymphocytes (not shown).

Figure 4 shows that specific enzyme activity could be induced by incubating for 48 h non-activated anti-BP line lymphocytes with extracellular matrix that had been treated with the T lymphocyte mitogen Con A. Activated T lymphocytes responded to Con A-treated extracellular matrix within 12 h (not shown). The induction of enzyme activity by the Con A–extracellular matrix complex was not accompanied by proliferation of the line cells (not shown). We do not as yet know why BP–extracellular matrix induced augmented endoglycosidase activity only in activated anti-BP T lymphocytes, while Con A–extracellular matrix induced a high enzyme activity in either activated or non-activated anti-BP T lymphocytes. In any case, the absence of lymphocyte proliferation in the presence of BP or Con A was functional evidence of the absence of accessory cells that could have presented BP or Con A.

The present finding that rat T lymphocytes of the helper/delayed hypersensitivity class recognized antigen or mitogen associated with extracellular matrix of bovine origin in the absence of syngeneic MHC accessory cells indicates that association of antigen with MHC may not be a universal requirement for recognition by T lymphocytes, and implies that T lymphocytes might have receptors capable of recognizing and responding to antigen alone (see ref. 15). But it is clear that activated T lymphocytes, like metastatic tumour cells[12,13], have molecular machinery for penetrating the vascular basal lamina and that specific antigen leaking from the target and sequestered by the extracellular matrix may direct the expression of this potential.

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