

T lymphocytes inhibit the vascular response to injury

(artery/atherosclerosis/immunodeficiency/tissue repair/interferon)

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ABSTRACT The proliferation of vascular smooth muscle cells is controlled by specific growth factors and cytokines acting in paracrine networks. Macrophage products such as the platelet-derived growth factor and interleukin 1 promote smooth muscle proliferation and are released in the arterial wall during atherosclerosis and repair processes. T lymphocytes are also present in vascular tissue, but their role in vascular growth control *in vivo* has been unclear. We now demonstrate that rats in which T lymphocytes have been eliminated by a monoclonal antibody develop larger proliferative arterial lesions after balloon-catheter injury. Larger lesions also develop in athymic *rnu/rnu* rats that lack T lymphocytes, when compared with *rnu/+* littermates with normal T-cell levels. Finally, injection of the lymphokine interferon γ inhibits smooth muscle proliferation and results in smaller lesions compared with controls injected with buffer alone. These results indicate that T lymphocytes modulate smooth muscle proliferation during vascular repair. We propose that T lymphocytes may play an important, immunologically nonspecific role in tissue repair processes.

The proliferation of vascular smooth muscle cells (SMC) is controlled by specific growth factors and cytokines acting in paracrine networks (1–4). It was previously thought that the major growth-regulating factors were released by platelets adhering to deendothelialized arterial surfaces, but inflammatory cells may be equally important as growth regulators for SMC (2–4).

Macrophage products, such as the platelet-derived growth factor and interleukin 1, promote SMC proliferation in cell culture (1, 2, 5, 6) and are expressed in the arterial wall during atherosclerosis and repair processes after experimental injury (7–10). T lymphocytes are also present in vascular tissue during atherosclerosis and vascular repair (3, 11–14), but their role in vascular growth control *in vivo* has been unclear. We have previously found that the T-lymphocyte product interferon γ (IFN- γ) inhibits SMC proliferation (15, 16); this finding would point toward a growth-inhibitory role of T lymphocytes in vascular repair. It was, however, also found that the immunosuppressive drug cyclosporin A inhibits development of a proliferative lesion after injury to rat arteries, which could imply a growth-promoting effect of T cells (17). Whether T cells promote or inhibit SMC proliferation *in vivo* was, therefore, unclear. We have now attempted to determine the role of T lymphocytes in the vascular response to injury *in vivo* by using T-cell-deficient rats.

Proliferation of SMC can be studied *in vivo* by using a catheter to remove the endothelial lining of a carotid artery segment in the rat. This procedure results in SMC migration from the medial to intimal layer of the artery, SMC proliferation in the intima, and formation of an intimal lesion that

reduces the size of the arterial lumen (18). The lesions are dominated by SMC, but a few monocyte-derived macrophages and T lymphocytes are also present (13).

We have induced vascular injury in two different immunodeficient models: the athymic nude RNU rat, which does not produce T lymphocytes (19) and normal Sprague–Dawley rats that were T-cell depleted with a cytolytic antibody. In both cases, T-cell-deficient rats developed larger intimal thickenings than controls. In addition, injection of the T-cell product IFN- γ inhibited lesion formation. Together, these data suggest that T lymphocytes modulate smooth muscle proliferation during vascular repair processes by release of growth inhibitor(s).

MATERIALS AND METHODS

Animals. Five-month-old male Sprague–Dawley rats were obtained from Alab, Stockholm. They were fed standard rat pellets and kept in the animal house for 2 weeks before use. No signs of infection or other diseases were observed during this period. Immunodeficient rats were kept under aseptic conditions, including microfiltration of air and sterilization of food and water. *rnu/rnu* and *rnu/+* rats were maintained in the Central Institute for Laboratory Animal Breeding.

Antibodies. OX19 (20, 21), W3/13 (22), W3/25 (22), OX8 (23), and OX6 (24) mouse anti-rat monoclonal antibodies (mAbs) were obtained from Sera-Lab, (Crawley-Down, Sussex, U.K.). Fluorescein isothiocyanate-labeled rabbit anti-mouse IgG purchased from Dakopatts, (Glostrup, Denmark) was preabsorbed by incubation with 5% normal rat serum for 30 min at 37°C before use. The myeloma protein MOPC21 (25) was obtained from Sigma. All antibodies are listed in Table 1.

Antibody Purification. OX19 mAb was purified from ascites fluid by chromatography over protein G-Sepharose (Pharmacia). One milliliter of ascites fluid was mixed with 1 ml of 20 mM sodium phosphate buffer, pH 7.3, filtered through a 0.22- μ m Millipore filter and chromatographed on 5 ml of protein G-Sepharose 4 Fast Flow. mAbs were eluted with 0.1 M glycine-HCl buffer, pH 2.7, immediately neutralized with Tris, and dialyzed against phosphate buffer. Purity was determined by agarose electrophoresis and immunodiffusion, concentration was determined by spectrophotometry at A_{280} , and specificity was determined both by flow cytometric analysis on rat blood and spleen cells and immunohistochemical staining of frozen spleen sections. mAb OX19 was kept in frozen aliquots, which were diluted in phosphate-buffered saline (PBS) before use. mAb MOPC21 was obtained as chromatographically purified IgG and dialyzed and analyzed in the same way as mAb OX19.

Abbreviations: SMC, smooth muscle cell(s); IFN- γ , interferon γ ; mAb, monoclonal antibody.

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Table 1. mAbs used in study

mAb	Antigen	Cellular distribution	Ref.
OX19	CD5 equiv.	T cells (+ some B cells)	20, 21
W3/13	Leukosialin	T + PMN	22
W3/25	CD4 equiv.	T _H	22
OX8	CD8 equiv.	T _C	23
OX6	RT1B	MHC class II ⁺ cells	24
MOPC21	Not known	None	25

equiv., equivalent; MHC, major histocompatibility; PMN, polymorphonuclear leukocytes; T_H, MHC class II-restricted T cells; T_C, class I-restricted T cells.

T-Cell Depletion. Five-month-old Sprague-Dawley rats were injected i.p. with 1 mg of mAb OX19, 1 mg of mAb MOPC21, or an identical volume of PBS. These rats were subjected to surgery the next day (see below). Blood was drawn through the tail vein for flow cytometric analysis 5 and 14 days after injection. The rats were kept under aseptic conditions from injection to sacrifice.

IFN- γ Treatment. Five-month-old Sprague-Dawley rats were injected i.p. with recombinant rat IFN- γ at 200,000 units [ref. 26; obtained from Holland Biotechnology (Leiden, The Netherlands)]. Injections were repeated daily for 1 week starting on the day of surgery. Biological activity of the recombinant IFN- γ was assessed by its capacity to induce the class II histocompatibility protein RT1B (I-A) on cultured rat aortic SMC (15).

Catheter Surgery. Arterial injury was inflicted in the common carotid artery with a Fogarty 2F balloon catheter as described (13, 18). The filament catheter was used to deendothelialize the common carotid artery of *rnu/rnu* and *rnu/+* rats as described (27). Rats were anesthetized with flunisolium and fentanyl (Hypnorm, Janssen Pharmaceutical) before surgery.

Morphology. Anesthetized rats were killed by perfusion with 4% (wt/vol) paraformaldehyde in phosphate buffer, pH 7.2. The common carotid artery and spleen were removed, snap-frozen in liquid nitrogen, and sectioned in a cryostat. The point-sampling method was used for morphometric quantitation of lesions in hematoxylin/eosin-stained sections (28).

Immunohistochemistry. Frozen sections were fixed in absolute ethanol and preincubated with 2% normal goat serum in PBS/1% fat-free dry milk. The sections were incubated with the specific mouse anti-rat mAbs described above at optimal dilutions in PBS/dry milk, rinsed in PBS, incubated with biotinylated goat anti-mouse IgG, rinsed, incubated with peroxidase-conjugated streptavidin, rinsed, incubated with 1 mM 3-amino-9-ethylcarbazole in 20 mM acetate buffer, pH 5.2, rinsed, and counterstained with hematoxylin. Optimal concentrations of antibodies were determined by checkerboard titrations on rat spleen sections.

Flow Cytometry. Blood was drawn from the tail vein, red cells were lysed using Becton Dickinson lysing solution, and leukocytes were stained for 30 min on ice with mAbs at optimal dilutions in PBS/1% bovine serum albumin (Sigma)/0.1% sodium azide. Leukocytes were subsequently washed with PBS, stained with fluorescein-labeled anti-mouse IgG, washed again, fixed in 4% paraformaldehyde, and analyzed in a FACScan flow cytometer (Becton Dickinson) by using an argon laser emitting at 488 nm and fluorescein filters. Five thousand leukocytes were analyzed in each sample, and the frequency of antibody-positive mononuclear cells was determined in an analytical light scatter gate. All antibodies were used at optimal concentrations determined by titrations on rat peripheral blood leukocytes.

DNA Analysis. Five-millimeter segments of carotid arteries were used for determination of DNA content, according to

Labarca and Paigen (29). Intimal DNA content was calculated as described by Clowes *et al.* (30).

RESULTS

We studied the role of T lymphocytes in the response to vascular injury in two different T-cell-deficient models. In the first one, T cells were eliminated by mAb OX19. In the second, injury was induced in the congenitally T-cell-deficient RNU strain of rats.

T-Cell-Depleted Rats Develop Larger Lesions After Vascular Injury. For T-cell elimination, 5-mo-old Sprague-Dawley rats (six per group) were injected with the mouse anti-rat T-cell mAb OX19. It reacts with the rat CD5 antigen cluster of differentiation, which is present on all T cells, and a minor population of B cells (20, 21). Flow cytometric analysis of peripheral blood T cells showed total elimination with recurrence of negligible amounts of T cells up to 14 days after injection of 1 mg of OX19 protein (Figs. 1 and 2). Similar results were obtained by staining with the pan-T-cell mAbs OX19 and W3/13. Analysis using the subset-specific antibodies W3/25 (CD4 equivalent) and OX8 (CD8 equivalent) confirmed that both major subsets were eliminated.

Control rats were injected with either PBS or the mouse monoclonal IgG MOPC21. The latter protein is of the same isotype as mAb OX19 but does not react with any known epitope in mammals (25). Injection of either MOPC21 or PBS did not eliminate T cells from the circulation (Figs. 1 and 2). The slight relative reduction in T-cell levels after surgery was probably due to the posttraumatic reaction with an increase in neutrophils and monocytes.

Vascular injury was induced in all three groups with the use of a Fogarty balloon catheter. It was introduced in the external carotid artery and inflated in the common carotid artery. This standardized trauma to the artery generates a proliferative SMC response that results in intimal thickening (18). The response to injury was analyzed 14 days later; arteries were fixed by paraformaldehyde perfusion, sectioned, and analyzed by morphometry. The cross-sectional surface area of the intima was used as an indicator of lesion size because it is a good estimate of tissue volume of the intimal lesion (18).

The intima of mAb OX19-treated, T-cell-deficient rats was significantly larger than that of control rats injected with either mAb MOPC21 or PBS (Fig. 3). Similar results were obtained when the intima/media ratio was analyzed in the three groups (data not shown). Lesions of mAb MOPC21-treated rats appeared somewhat larger than those of the PBS group, but the difference was not significant (Fig. 3).

Athymic Nude Rats Develop Larger Lesions After Vascular Injury. The observation that vascular lesions were larger in T-cell-depleted rats suggested that T cells may inhibit lesion

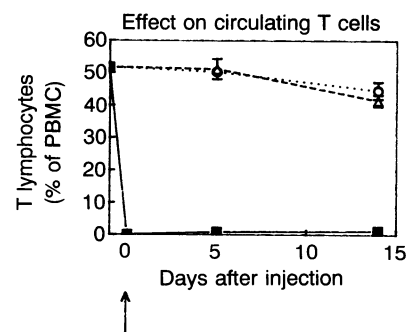


FIG. 1. *In vivo* administration of mAb OX19 (■) to Sprague-Dawley rats leads to persistent T-cell depletion (—). Control mAb MOPC21 (---) or PBS (· · ·) do not affect T-cell levels in peripheral blood (means \pm SEM, $n = 6$). Arrow indicates time of mAb injection.

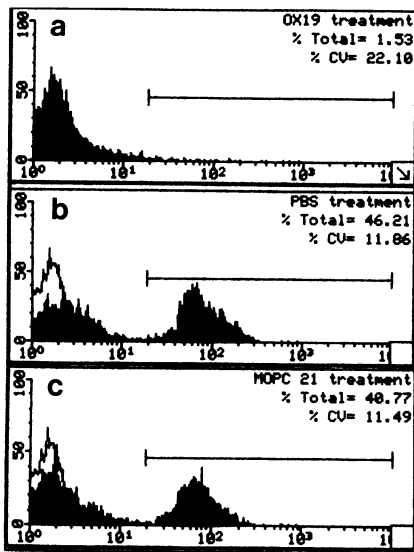


FIG. 2. Flow cytometric analysis of peripheral blood mononuclear cells shows elimination of T cells 14 days after *in vivo* mAb OX19 treatment. Histograms show fluorescein isothiocyanate fluorescence (logarithmic amplification) after staining with mAb OX19 followed by fluorescein isothiocyanate anti-mouse IgG in OX19-treated (a), PBS-treated (b), and mAb MOPC21-treated (c) rats. Horizontal bars indicate populations with significant mAb OX19 binding. Unshaded peaks at left show control samples in which mAb was omitted during staining.

formation. It was, however, also possible that the cytolytic procedure could affect the response to injury. We, therefore, studied vascular injury responses in a totally different model of T-cell deficiency, the nude rat. The inbred RNU strain carries the recessive *rnu* gene, which in the homozygote *rnu/rnu* individual results in thymic aplasia, lack of circulating T lymphocytes, and hairlessness (19, 31). The *rnu/+* heterozygote, in contrast, has a thymus, hair, and normal peripheral T cells (19, 31).

We first analyzed circulating T cells in the RNU strain by flow cytometry. *rnu/rnu* rats completely lacked immunodetectable T cells at birth, and T-cell values in 160-g *rnu/rnu* rats were <2% of those in *rnu/+* heterozygotes. T cells began to appear at later stages of development, however, and T-cell levels in 450-g *rnu/rnu* rats were 30% of *rnu/+* individuals. Studies on the role of T cells in vascular injury responses, therefore, had to be done in 160-g rats. It was not possible to carry out balloon-catheter experiments in such small animals,

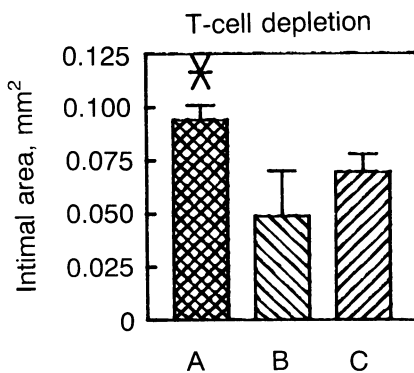


FIG. 3. T-cell depletion leads to development of larger arterial lesions after balloon injury to the carotid artery. Lesions of mAb OX19-injected rats (bar A) show significantly larger intimal cross-sectional area than those of controls treated with either PBS (bar B) or mAb MOPC21 (bar C) (mean \pm SEM, $n = 6$). *, Significantly different from either control group ($P < 0.05$, Student's *t* test).

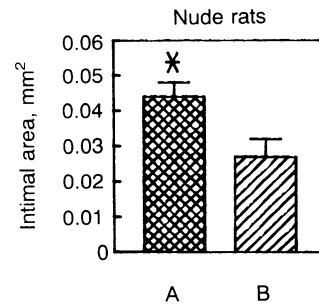


FIG. 4. Eight-week-old athymic *rnu/rnu* rats (bar A) develop significantly larger arterial lesions after mechanical injury compared with immunocompetent, heterozygous *rnu/+* littermates (bar B) (mean \pm SEM, $n = 5$). *, Significantly different from *rnu/+* ($P < 0.05$).

and, instead, a nylon filament was used to denude the carotid artery surface (27).

Nylon-filament denudation of the common carotid artery in *rnu/rnu* rats resulted in lesions significantly larger than those of *rnu/+* littermates (Fig. 4). DNA analyses of the injured arteries were done to confirm that the difference was due to a larger cell mass in the *rnu/rnu* rats. The DNA concentration per 5-mm arterial segment was significantly higher in *rnu/rnu* rats compared with *rnu/+* ones, indicating that the larger intimal thickenings were caused by an increase in cell number (Table 2). Immunohistochemical analysis showed significantly fewer W3/13-positive intimal cells in *rnu/rnu* compared with *rnu/+* rats (<0.1% vs. 1.5%).

IFN- γ -Treated Rats Develop Smaller Lesions After Vascular Injury. Taken together, our findings in the nude and the T-cell-depleted rats suggest that the presence of T cells reduces the development of intimal proliferative lesions after injury. The low frequency and scattered distribution of T cells in the intima (13, 15, 17) make it likely that the growth-inhibitory effect is exerted via soluble lymphokines rather than cell-cell contacts. We, therefore, studied the effect of one of the major lymphokines, IFN- γ , on the vascular response to balloon-catheter injury in Sprague-Dawley rats. These rats were injected with recombinant rat IFN- γ at 200,000 units i.m. once daily for 7 days starting from the day of surgery. The response to injury in carotid arteries was evaluated morphometrically 14 days after surgery. Fig. 5 shows that the intimal lesions in IFN- γ -treated rats were significantly smaller than those in PBS-injected controls.

Histocompatibility Gene Expression in Vascular Lesions Reflects the Response to IFN- γ and Is Inversely Correlated to Lesion Size. IFN- γ not only inhibits proliferation but also induces expression of class II histocompatibility genes in SMC, which do not normally express these genes (15-16). We therefore used SMC expression of the class II histocompatibility gene *RT1B* (I-A equivalent) as an indicator of a tissue response to injected IFN- γ . Immunohistochemical staining for RT1B (Fig. 6) indicated that lesions of IFN- γ -treated rats contained large numbers of RT1B⁺ SMC, whereas PBS controls contained only 5-10% RT1B⁺ SMC in the intima. mAb OX19-treated rats, finally, lacked RT1B⁺ SMC in the intima, indicating that the increase in lesion size

Table 2. Intimal cross-sectional area and DNA content of carotid arteries in *rnu/rnu* and *rnu/+* rats

	Intimal area, mm ²	DNA content, μ g/5-mm vessel
<i>rnu/rnu</i>	0.044 \pm 0.004*	0.67 \pm 0.06*
<i>rnu/+</i>	0.027 \pm 0.005	0.46 \pm 0.04

Data represent means \pm SEM ($n = 5$ per group). *Significant difference from *rnu/+* at $P < 0.05$.

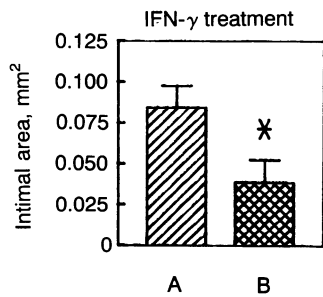


FIG. 5. Treatment with recombinant rat IFN- γ inhibits development of intimal thickenings after arterial injury. Rats were treated with IFN- γ at 200,000 units daily for 7 days (bar B), and controls were injected with PBS (bar A) (means \pm SEM; $n = 5$). *, Significantly different from PBS controls ($P < 0.05$).

was associated with a lack of IFN- γ stimulation (Fig. 6). The number of mAb W3/13-positive cells was also reduced in mAb OX19-treated rats (<1%) compared with PBS- and mAb MOPC21-treated controls (which contained 1.6% and 2.3% W3/13⁺ cells, respectively). The few W3/13⁺ cells that remained in mAb OX19-treated rats could be either T lymphocytes that survived treatment or granulocytes, which also express the W3/13 antigen.

DISCUSSION

The present study demonstrates that (i) the absence of T cells results in larger vascular lesions in response to mechanical injury; and (ii) administration of the T-cell lymphokine IFN- γ inhibits lesion formation. Together, our findings suggest that T cells inhibit the development of the intimal thickening and that one mechanism by which this could be accomplished is secretion of IFN- γ .

Injection of the cytolytic anti-T-cell antibody OX19 resulted in larger intimal proliferative lesions after balloon-catheter injury compared with PBS-treated controls. Injection of the nonspecific isotype-matched mAb MOPC21 led to significantly smaller lesions than in mAb OX19-treated rats. There was, however, a tendency, although not significant in our small groups of rats, toward larger lesions in mAb MOPC21-treated rats compared with PBS-treated ones. This could, perhaps, be caused by immune complexes, which may affect repair processes in vascular tissues (3). The significant difference between T-cell-depleted rats and both types of controls indicates that either the lack of T cells or the cytolytic process affected the response to injury. To resolve

this question, we studied the vascular response to injury in an entirely different T-cell-deficient model, the nude rat.

The difference in lesion size between *rnu/rnu* rats and heterozygous *rnu/+* littermates, which only differ with regard to one gene, clearly suggests that the lack of functional T cells is accompanied by the disappearance of T-cell control of vascular SMC proliferation in response to injury. It is unlikely, although not strictly ruled out, that the *rnu/rnu* homozygote could differ from the *rnu/+* heterozygote with regard to a gene that controls SMC proliferation, in addition to the one that determines thymus development, or alternatively, that the *rnu* gene could be important also for SMC proliferative capacity. The similar findings in the T-cell-depleted and congenitally athymic rats strongly suggest, however, that the absence of T cells in itself leads to larger intimal lesions. Moreover, we have recently observed (32) that 6-mo-old *rnu/rnu* athymic rats, which have significant levels of (extrathymically differentiated) T cells (33), do not develop larger lesions than their heterozygous controls. This result also supports the idea that T cells inhibit SMC proliferation.

Our conclusions differ from those of Ferns *et al.* (34), who recently reported that intimal lesions are equally large in *rnu/rnu* and *rnu/+* rats. It should, however, be noticed that these studies were done in rats that were older and, therefore, probably contained circulating T cells. Thus, the findings of Ferns *et al.* concur with our observations in T-cell-containing, 6-mo-old *rnu/rnu* rats. In contrast, the present study shows that the truly T-cell-deficient, young *rnu/rnu* rat develops larger intimal lesions after injury compared with age-matched, heterozygous controls. Together, these data argue against the conclusions drawn by Ferns *et al.* (34) and, instead, support the idea that T cells modulate the response to injury.

The major growth inhibitor produced by T cells is the lymphokine IFN- γ . This protein has been shown to inhibit SMC proliferation in culture at ng/ml concentrations (15, 16). Similar doses also induced expression of the class II major histocompatibility protein RT1B on the surface of the SMC (15, 16). *In vivo* studies demonstrated that RT1B-expressing SMC in the postinjury intima did not replicate (15). These findings suggested that IFN- γ acts as an endogenous inhibitor of SMC proliferation during the response to injury (15).

We, therefore, postulated that the T-cell growth control of intimal SMC observed in the present study may be exerted by IFN- γ . This hypothesis was tested by injection of recombinant rat IFN- γ daily during 1 week after injury. Such a treatment significantly inhibited lesion formation and reduced the lesion size at 2 weeks by \approx 50%. Although a direct

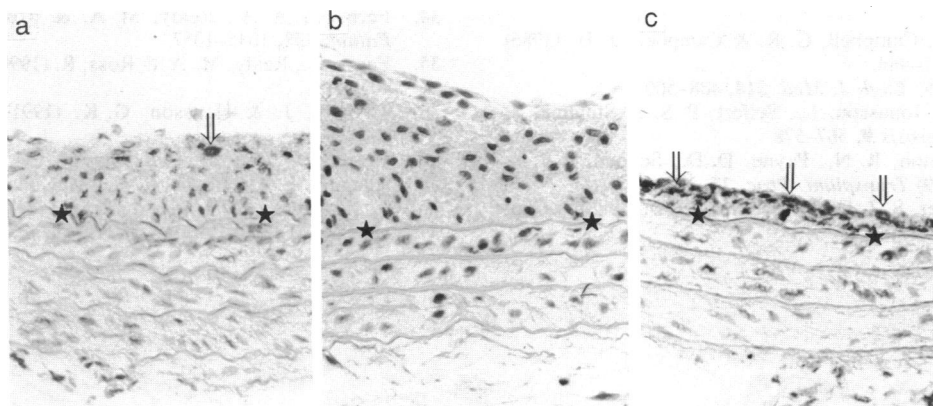


FIG. 6. Arterial lesions of rats injected with PBS (a), mAb OX19 (b), and IFN- γ (c). Lesions of T-cell-depleted OX19 rats (b) are larger than those of controls (a), which, in turn, are larger than those of IFN- γ -treated rats (c). Expression of the IFN- γ -inducible histocompatibility protein, RT1B (arrows), is high in c, moderate in a, and absent in b. Stars indicate position of the internal elastic lamina. RT1B was visualized by mAb OX6 and an avidin-biotin immunoperoxidase system. Sections were counterstained with hematoxylin. ($\times 195$.)

comparison between the T-cell-deficient models and the IFN- γ -treated one is not possible, the findings show that IFN- γ may be, at least, one of the T-cell factors that reduce vascular SMC proliferation. Interestingly, the magnitude of the effect of T-cell depletion on the tissue response appeared larger than the effect on the frequency of W3/13⁺ cells in the intima. This fact could suggest that endocrine effects of lymphokines may be at least as important as the paracrine ones.

The immunosuppressive drug cyclosporin A has been shown to inhibit the vascular response to injury (17). This inhibition could either be from a direct effect of cyclosporin on SMC proliferation or be mediated via its inhibitory effect on T-cell activation. The present data rule out the second possibility because the effects of pharmacologic, antibody-mediated and genetic immunodeficiency should be similar rather than opposite. Instead, the present findings support the alternative possibility that cyclosporin A directly inhibits SMC proliferation. This conclusion is also supported by another recent finding that cyclosporin A inhibits growth factor-induced SMC replication in culture (35, 36).

Our present observations demonstrate that cells of the immune system may not only initiate and propagate immunologically specific responses against foreign and self antigens but also regulate tissue responses to injury in a broader sense. The acute inflammatory response is followed by a repair process characterized by proliferation of vascular cells and fibroblasts. Our current data suggest that activated T cells can reduce or retard the development of this process. This hypothesis agrees with cell culture data showing that the T-cell product IFN- γ inhibits proliferation of SMC, endothelial cells, and fibroblasts, as well as development of contractile SMC filaments and collagen production (16, 37, 38, 39). Consequently, tissue repair processes would be inhibited during the phase of T-cell activation and immune responses after injury.

In conclusion, our findings indicate that T lymphocytes regulate the vascular response to injury by inhibiting SMC proliferation. Further studies are obviously needed to obtain a more comprehensive view of this growth regulatory network. It is, however, clear that T lymphocytes, in addition to their specific functions in activating the immune response, are involved in regulating repair processes of the vasculature.

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