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Chronic rejection of rat aortic allograft

II. Administration of cyclosporin induces accelerated allograft arteriosclerosis

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Abstract. Rat aortic allografts immunosuppressed with cyclosporin – but not with azathioprine or steroids – develop an early inflammatory lesion in the subendothelial space. This "endothelialitis" is followed by an influx of proliferating smooth muscle cells into the intima, resulting in intimal thickening and accelerated arteriosclerosis. Administration of azathioprine and steroids largely ameliorates the development of the accelerated lesion. Similar endothelialitis and accelerated arteriosclerosis have been observed previously in the autopsy material of cardiac transplant recipients. Our results confirm the suggestion that the development of accelerated allograft arteriosclerosis is most likely linked to cyclosporin administration.

Key words: Arteriosclerosis, immunosuppression – Immunosuppression, arteriosclerosis – Endothelialitis, experimental

Since the introduction of cyclosporin (CyA), several reports [10, 13, 14] have documented an early transplant arteriopathy affecting the first and second order epicardial and intramural branches of the coronary arteries of the cardiac transplant. These vascular changes include a marked luminal narrowing, caused by intimal thickening. This lesion is strikingly different from ordinary acute rejection, characterized by perivascular cellular infiltrate, edema, infiltration of the inflammatory leukocytes to the myocardium, and myocardial damage. A similar type of arteriopathy has also been described in renal [15] and hepatic transplants treated with CyA either alone or in combination with other immunosuppressive drugs [6].

All of these investigators suggest that at least the accelerated form of this lesion is linked to the administration of CyA. As these observations have been performed in human allografts, serial monitoring of the development of this lesion has not been possible. In the absence of an experimental model, the pathogenesis of the development of this lesion is unknown.

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We have developed a rat model to investigate transplant arteriosclerosis of chronic rejection, using aorta transplants between inbred rat strains. With this model we now demonstrate that the early, intimal, proliferative response in immunosuppressed rats, prior to generation of the chronic lesion, is associated with the use of CyA as an immunosuppressive drug. Furthermore, we show that an early "endothelialitis"-type reaction invariably precedes the influx of proliferating myocytes into the intimal space.

Materials and methods

Experimental animals

Rat strains WF (AG-B2, RT1^v) and DA (AG-B4, RT1^a) were used for the transplantations. All animals were purchased from the Zentralinstitut für Versuchstierzucht GmbH (Hannover, FRG). Male rats weighing 200–300 g and 1–3 months of age were used as donors and recipients.

Aorta transplantations

A segment of descending thoracic aorta of approximately 1.5 cm was excised, perfused with saline, and used as a transplant [12]. The experimental animals were anesthesized with intraperitoneal chloral hydrate (6 ml/kg). The graft was transplanted into a heterotopic position below renal arteries and above bifurcation. The cranial suture line was made as close as technically possible to the renal arteries to minimize the difference in diameter. End-to-end anastomosis was performed using 9-0 continuous nylon suture. The strain combination DA to WF strain was used for allografts. Syngeneic controls were made from DA to DA strain. The grafts were removed at 10 days, 20 days, 1 month, 3 months, and 6 months post-transplantation and were processed for histology.

Medication

The experimental animals were divided into seven groups, each containing at least five animals. The first three groups included allogeneic animals with a single medication: oral CyA, azathioprine (Aza), or methylprednisolone (MP). The fourth and the fifth groups in-

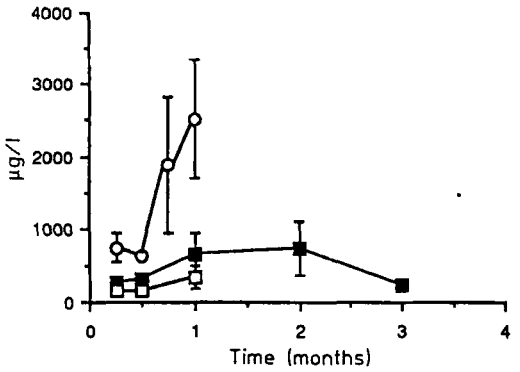

Fig. 1. Morning CyA blood levels (µg/l) in three groups of rats immunosuppressed with oral CyA. ■ 5 mg/kg per day CyA as single drug; ○ an escalating dose of 10–20 mg/kg per day CyA in combination with 2 mg/kg per day Aza and 0.5 mg/kg per day MP; □ 5 mg/kg per day CyA in combination with Aza and MP. *Bars* indicate SD

cluded animals with triple treatment, where CyA, Aza, and MP were given together. The CyA dose in these two groups differed, aiming to "high" (±2500 µg/l) or "low" (±150 µg/l) CyA blood concentrations. The last two groups were control groups, lacking any immunosuppressive treatment, and included an allogeneic and a syngeneic group.

The drugs were mixed in food (CyA) or drinking water (Aza, MP). The medication was continued up to the moment of sacrifice. The drugs were given orally, from the day of transplantation, in the following doses: 5 mg/kg per day (low dose) or 10–20 mg/kg per day (high dose) CyA (Sandoz Pharmaceuticals, Basel, Switzerland), 2 mg/kg per day Aza (Imuran; Leiras Pharmaceuticals, Turku, Finland), or 0.5 mg/kg per day MP (Upjohn, Kalamazoo, Mich., USA). In addition, 5 mg/kg CyA was injected subcutaneously on the day of transplantation to those rats receiving the oral CyA treatment.

The CyA blood levels of the recipient rats receiving oral CyA are illustrated in Fig. 1. At a dose of 5 mg/kg per day as a single drug, the morning CyA blood levels, up to 600–700 µg/l, were observed 1–2 months after transplantation; later, the levels declined (without alteration in medication dose) to 150–300 µg/l. At the same dose, when combined with Aza and MP in triple therapy, the CyA blood levels remained lower, with a morning CyA blood level of 330 µg/l at 1 month post-transplantation. Thus, either Aza or, more likely, MP had a reducing effect on CyA blood concentration. Therefore, a second triple therapy group was created that received CyA in combination with Aza and MP in an escalating dose from 10 to 20 mg/kg per day, resulting in blood levels up to 2000 µg/l or more.

Histological specimens and staining

For evaluation of morphological changes, paraffin sections were stained with Mayer's hematoxylin and eosin (H&E) and orcein for elastic fibers. In rat aortas, longitudinal sections were prepared where the vessel wall on both the graft and host side of the suture line could be observed; cross-sections were also prepared for evaluation of circular changes in the graft. Quantitative histology (morphometry) was always done from the middle (one-third) section of the transplant. These specimens were compared to the thoracic aorta of the recipient as normal control. The cross-sections were prepared from the center of the graft to avoid any effects of the suture line.

Immunohistochemistry

For immunohistochemistry, frozen sections, 3–4 µm thick, were stained by immunoperoxidase (IP) technique using monoclonal anti-rat antibodies to leukocyte common antigen (LCA), certain lymphocyte subset-specific antigens, and α-smooth muscle actin. Anti-LCA (OX-1), anti-B cell LCA (OX-33), anti-interleukin-2 receptor (OX-39), and anti-pan-T cell (W3/13) antibodies were obtained from Sera Lab (Sussex, UK), and anti-smooth muscle actin from Bio-Makor (Rehovot, Israel). The cryosections were stained using a 2-layer indirect IP technique described in detail elsewhere [8]. Briefly, the sections were incubated with appropriately diluted monoclonal antibody (usually 1:20), washed, and incubated consecutively with peroxidase-conjugated rabbit anti-mouse Ig and goat anti-rabbit Ig (Dako Immunoglobulins, Glostrup, Denmark), followed by treatment with the substrate solution containing the chromogen 3-amino-9-ethylcarbazole (AEC). Hydrogen peroxide was added to the AEC solution immediately prior to use. The samples were counterstained with Mayer's hemalum solution and mounted.

Quantitation of histology in rat aorta transplants

The morphological changes were quantitated according to standard morphometric principles [1] and expressed as point score units (psu), i.e., mean number of points falling over a given anatomical

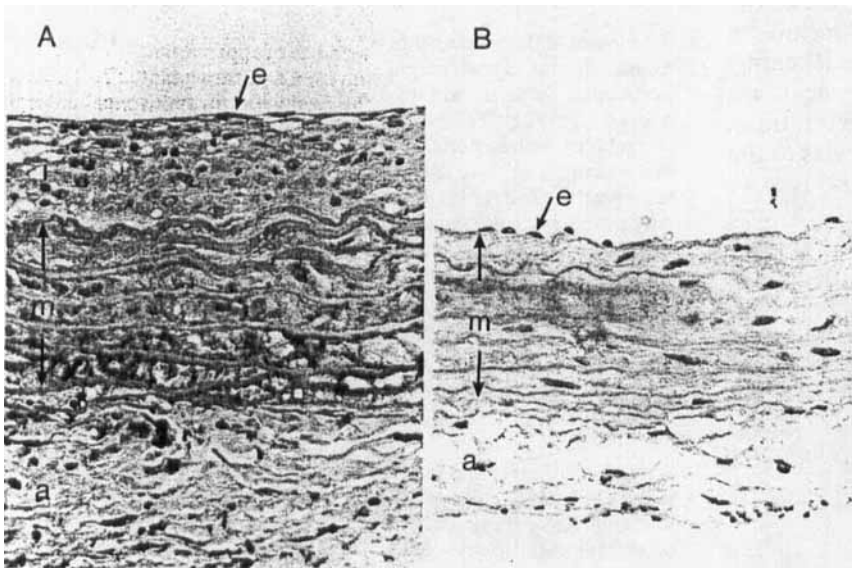

Fig. 2 A, B. Morphology of nonimmunosuppressed: **A** DA to WF aortic allograft and **B** DA to DA syngeneic graft 2 months post-transplantation. *e* Endothelium; *i* intima; *m* media separated by internal and external elastic laminae *(arrows); a* adventitia. Note a low grade inflammation in allograft adventitia, loss of nuclei in the media, and the significant intimal thickening in the allograft, lacking in the syngeneic graft. H & E, ×300

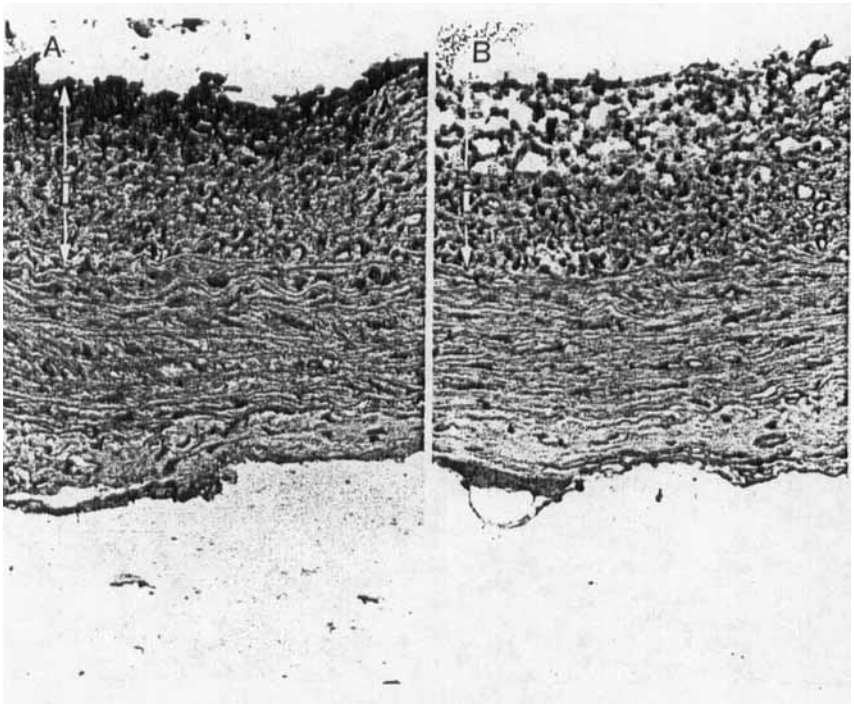

Fig. 3 A–C. Morphology of DA to WF aortic allograft immunosuppressed with: **A** 5 mg/kg per day CyA, **B** 2 mg/kg per day Aza, or **C** 0.5 mg/kg per day MP 1 month post-transplantation. Note the presence of "round" inflammatory cells in the luminal part of intima (**A**). Abbreviations as in Fig. 2. H & E, × 300

area using straight, cross-sectional lines and a 0.02 mm grid. The following variables were evaluated: the number of nuclei and the thickness of the different layers of aorta, i.e., the adventitia, media, and intima, separated from each other by the intimal and external elastic lamina. A minimum of five technically successful transplantations were made for each point in time if not otherwise noted, and their means were used as final scores.

Autoradiography

Some rats received 250 μCi of ^{3}H-thymidine (^{3}H-TdR, NEN, Boston, Mass., USA) by i.v. injection 30 min before sacrifice. The histology was processed from paraffin sections, stripping film autoradiography (Kodak AR-10) was performed, and the labeling of the nuclei in the transplanted aorta wall was compared to the labeling index in the recipient aorta.

Results

A model for allograft arteriosclerosis

Aortic allografts were exchanged between inbred rat strains, from DA to WF strain (allografts) or from DA to DA strain (syngeneic controls). The grafts were removed at various time intervals post-transplantation, and the changes in the graft adventitia, media, and intima were quantitated using standard morphometric principles.

Following transplantation to nonimmunosuppressed recipients (controls), there was a prolonged inflammatory episode of significant intensity in the allograft adventitia that was less intense in syngeneic grafts. In the media, there was a gradual loss of cellularity in the allograft that was lacking in the syngeneic graft. The most prominent alterations were recorded in the allograft intima. During the observation period of 6 months, a gradual increase in the cellularity and in the thickness of the intima was observed. This resulted in a strikingly arteriosclerotic-like thickening, presumably due to the proliferation of myocytes, which migrated from the media across the internal elastic lamina to the subendothelial space. These changes were not present in the syngeneic graft. The morphology of the slowly proceeding vascular wall response in the aortic allograft of nonimmunosuppressed recipients is illustrated in Fig. 2.

Taken together, we consider these slowly proceeding alterations in the allograft intima as representing the gradual arteriosclerotic process observed previously under chronic rejection in different human allografts and as most likely being due to the histoincompatibility and to the persistence of inflammation in the vicinity of the allograft vasculature.

Effect of single drug immunosuppression on the development of allograft arteriosclerosis

Groups of WF recipients were transplanted with DA allografts and immunosuppressed with 5 mg/kg per day CyA, 2 mg/kg per day Aza, or 0.5 mg/kg per day MP. The allografts were removed at various time intervals thereafter, and paraffin and frozen sections were performed and stained.

Aortic wall alterations were observed in all types of transplants, regardless of the kind of immunosuppression the rats were given. However, the responses with the CyA-immunosuppressed transplants were clearly different from those observed in the allografts immunosuppressed with Aza or with steroids (Fig. 3).

After immunosuppression with CyA for 1–2 months, the intima was significantly thicker and the number of nu-

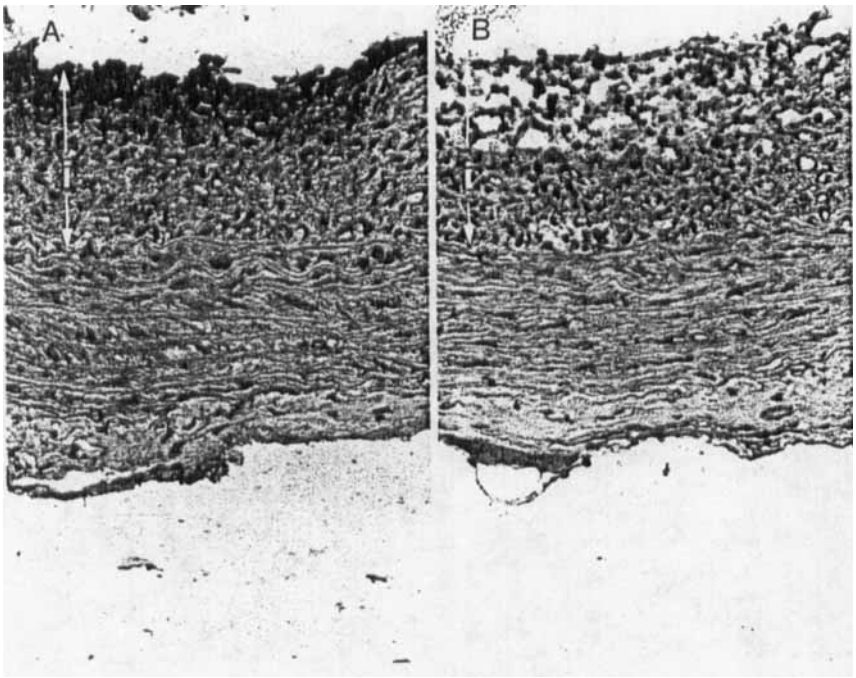

Fig. 4 A, B. Immunohistochemistry (IP staining) of DA to WF aortic allografts immunosuppressed with 5 mg/kg per day CyA: **A** staining with anti-LCA antibody, demonstrating a positive reaction at the luminal part of the intima *(i)*; **B** staining with anti-smooth muscle actin antibody, demonstrating a positive reaction in the abluminal part of intima *(i)* and some reactivity in the media. Mayer's hemalum counterstaining, × 300

clei in the intima was higher. Most interestingly, there was a subendothelial inflammatory response in the allograft intima, affecting the luminal part in particular, and lasting for a period of 2 months post-transplantation. In the media, the nuclei were well preserved and there was practically no inflammation in the allograft adventitia. Immunosuppression with Aza or steroids revealed a more prominent inflammatory response in the allograft adventitia and a more extensive loss of nuclei in the media; the intimal response was smaller and nearly devoid of any inflammation with leukocytes.

Immunohistochemical examination of the allografts confirmed the morphology and demonstrated that the "round" cells in the luminal part of the intima were, indeed, anti-LCA-reactive leukocytes. Most of them were resting lymphocytes of T-cell lineage, not expressing any activation markers, such as as interleukin-2 receptor (not shown). Instead, the elongated cells in the abluminal part of the intima were anti-α-actin-reactive smooth muscle cells (Fig. 4). In Aza- and MP-immunosuppressed allografts, as in nonimmunosuppressed controls, practically all cells in the intima were smooth muscle cells (not shown).

In autoradiography, ^{3}H-TdR-incorporating cells were seen in the media and intima; most labeled nuclei clearly represented nuclei of the proliferating smooth muscle cells (Fig. 5).

Fig. 5. Autoradiography of DA to WF aortic allograft immunosuppressed with 5 mg/kg per day CyA 2 months post-transplantation. Note the ^{3}H-TdR-incorporating nuclei, which obviously represent proliferating smooth muscle cells. Mayer's hemalum counterstaining, × 750

Time course of alterations

Adventitia. Most of the nuclei in adventitia were nuclei of inflammatory cells. The inflammatory response in allograft adventitia was strongest in the Aza-treated group and not significantly different from the intensity of inflammation observed in nonimmunosuppressed controls. In the groups immunosuppressed with CyA or steroids, the periarterial inflammation was significantly ($P < 0.01$–0.001) reduced at 1–2 months post-transplantation, confirming that Aza, unlike CyA or steroids, was not a particularly good immunosuppressive drug in the rat (Fig. 6).

Media. The nuclei of the media layer were mostly nuclei of smooth muscle cells. In all allografts, the nuclei of the media gradually disappeared, indicating a slowly proceeding media necrosis. The pace of disappearance of nuclei from the allograft media was similar in nonimmunosuppressed control allografts, but slightly faster in groups receiving Aza or steroids. There was, for the first 2 months, a better preservation of nuclei in the allograft media immunosuppressed with CyA (Fig. 7).

Intima. The most drastic differences were observed in the allograft intima. The increase in intimal cellularity and

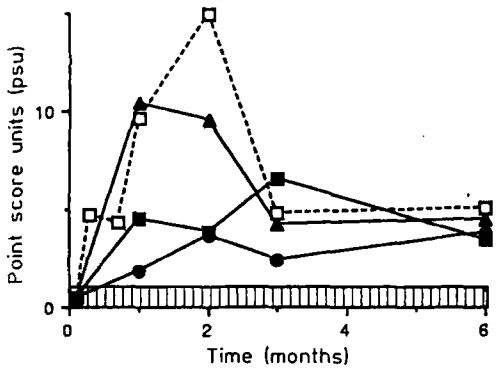

Fig. 6. Adventitial nuclear density in DA to WF aortic allografts during a follow-up of 6 months. *Solid lines* represent experimental groups, *broken lines* nonimmunosuppressed controls. ▭ Normal control aorta ± SD; □ DA-WF aortic allograft; ▲ azathioprine; ● cyclosporin A; ■ methyl prednisolone

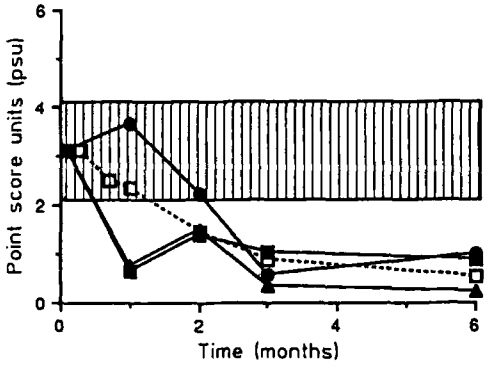

Fig. 7. Media nuclear density in DA to WF aortic alografts. *Solid lines* represent experimental groups, *broken lines*, nonimmunosuppressed controls. Symbols as in Fig. 6

thickness (Fig. 8 A, B) in the grafts immunosuppressed with Aza and MP was similar to that in the control allografts. In rats immunosuppressed with CyA, there was a very prominent early cellular infiltrate (particularly in the luminal part of allograft intima), more than double in intensity to any one of the controls. This coincided with an early increase in the thickness of the intimal layer. Later, the differences, compared to nonimmunosuppressed controls or to rats immunosuppressed with Aza and MP, were reduced. No differences were recorded between the four groups of grafts 3–4 months post-transplantation.

Triple treatment

In order to investigate whether and to what extent the CyA effect on the allografts was modified by Aza and MP treatment, two additional experimental groups were established employing either a high or a low CyA dosage in combination with Aza and MP. These allografts were evaluated 1 month post-transplantation (Table 1), when the differences between CyA-immunosuppressed grafts and the controls were most prominent.

Even though the CyA blood concentration in the two triple-treated rat groups differed by a factor of 10 (Fig. 1), the histological changes in both groups were similar: the allografts of both groups revealed some degree of adventitial inflammation (Table 1), higher than in CyA monotherapy, but not to the extent seen in allografts treated with Aza alone or without immunosuppression. The media preserved its nuclei better than with Aza or with steroids. Most importantly, the intimal changes were far less dramatic than when CyA was used as monotherapy. The histological picture resembled that observed in ordinary control allografts at the corresponding points in time (Fig. 9).

Discussion

We have found in our rat aortic allograft model for chronic rejection that CyA, when administered alone, but not Aza or steroids, induces accelerated arteriosclerosis. Arteriosclerosis is a major manifestation of chronic rejection in all transplants [7, 9]. Morphologically, the accelerated arteriosclerosis with CyA closely resembles the more delayed, concentric, and generalized intimal proliferative response observed previously in chronic rejection in experimental models [2, 5] and in humans [3]. There is, however, one important difference. In the pathogenesis of accelerated arteriosclerosis, a subendothelial inflammatory response – "endothelialitis" – is a prominent feature at very early stages of development. Accelerated arteriosclerosis has previously been described in human heart transplants and been suspected of being related to CyA administration [10, 13, 14]. Upon retrospective screening of our own

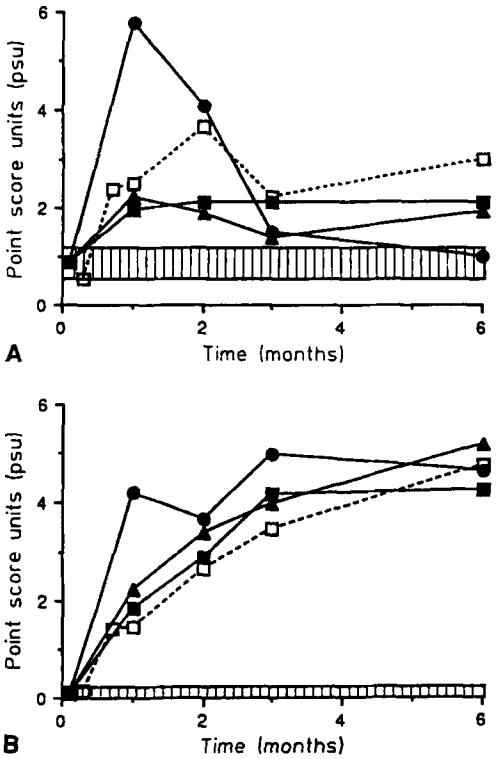

Fig. 8. **A** Intimal nuclear density and **B** thickness in DA to WF aortic allografts. *Solid lines* represent experimental groups, *broken lines*, nonimmunosuppressed controls. Symbols as in Fig. 6

Table 1. Responses of allograft adventitia, media, and intima to different immunosuppressive therapies

Strain combination Therapy (no. of animals)		DA-WF CyA (3)	DA-WF Aza (3)	DA-WF MP (5)	DA-WF Triple[a] (3)	DA-WF Triple[b] (4)	DA-WF (6)	DA-DA (5)
Adventitia	nuclei	1.8 ± 0.8	10.4 ± 3.4	4.5 ± 2.0	6.9 ± 5.3	5.1 ± 2.9	10.7 ± 5.2	0.5 ± 0.5
Media	nuclei	3.7 ± 0.4	0.8 ± 0.2	0.7 ± 0.6	3.3 ± 0.5	3.9 ± 1.6	2.4 ± 0.8	3.1 ± 1
Intima	nuclei	5.8 ± 2.1	2.2 ± 0.2	1.9 ± 0.7	3.0 ± 0.7	2.9 ± 0.5	2.5 ± 1.5	0.9 ± 0.2
	thickness	4.2 ± 1.9	2.2 ± 0.7	1.8 ± 0.4	0.8 ± 0.6	1.3 ± 0.4	1.4 ± 1.0	0.1 ± 0.1

[a] Low B-CyA concentration
[b] High B-CyA concentration

autopsy material of 11 cardiac transplant recipients (unpublished), accelerated coronary arteriosclerosis was, on two occasions, the cause of death, and one of these two cases clearly demonstrated the endothelialitis documented in this communication.

Accelerated transplant arteriosclerosis that occurred during administration of CyA to the rat was clearly different from the ordinary arteriosclerotic process in aortic allografts. It was obviously not linked to periarterial inflammation, which seems to be the driving force behind the slow arteriosclerotic process in nonimmunosuppressed allografts [12]. CyA effectively suppressed the periarterial inflammation far better than, for example, Aza. On the other hand, endothelialitis and early influx of proliferating smooth muscle cells to the intimal space was seen only in allografts immunosuppressed with CyA alone.

The data from the triple therapy experiments are more difficult to interpret, as the possible effects and interactions of these three drugs on the inflammation, on the generation of cytokines and lipid mediators, and on growth factor synthesis have not yet been worked out. In the rat, steroids seem to oppose the immunosuppressive effect of CyA [11]. This may also be the case here: CyA alone was more effective in suppressing the inflammatory reaction in the adventitia than when given in combination with Aza and steroids. Considering the pathogenesis of this process, it is more likely that accelerated arteriosclerosis is due to the direct effect of CyA on the vascular wall – either to endothelium or to smooth muscle cells or to both – than that it is regulated via the periarterial inflammation.

Bunchman and Brookshire [4] have recently demonstrated that when human vascular endothelial cells are incubated with CyA in vitro, the culture supernatant has an enhancing effect on the proliferation of human smooth muscle cell lines. Incubation of the supernatant with anti-endothelin antibody blocked the mitogeneic effect. As direct incubation of CyA with unconditioned culture medium induced no proliferative response of smooth muscle cells, a likely explanation is that endothelin (and possibly other growth factors produced by the endothelial cells) is responsible for the mitogenesis of smooth muscle cells. If these in vitro data can be applied to our in vivo model, we suggest that CyA, when administered alone, will induce endothelial cell damage of the allograft. This damage, in turn, may be responsible for the accumulation of inflammatory cells in the subendothelial space, possibly by

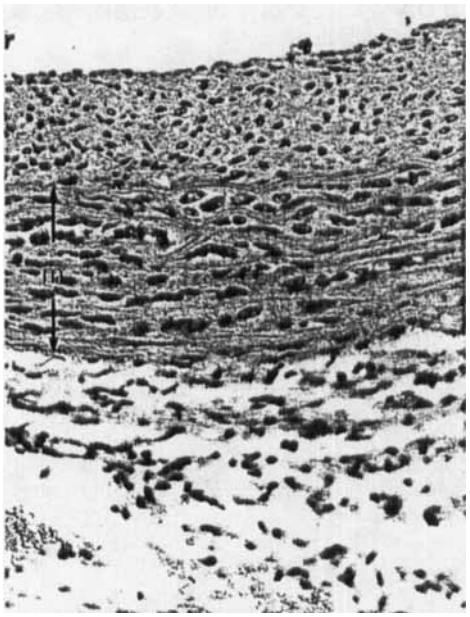

Fig. 9. Morphology of aortic allografts immunosuppressed with the combination of 10–20 mg/kg per day CyA, 2 mg/kg per day Aza, and 0.5 mg/kg per day MP. Note the low level of inflammation in the adventitia, preservation of nuclei in the media (*m*), and the absence of "endothelialitis" in the subendothelial area. H & E, × 300

short-acting cytokines. Finally, endothelin, and possibly other growth factors, may be responsible for the mobilization of the smooth muscle cells to proliferation and for their influx into the intima.

Histoincompatibility is another requirement in the pathogenesis of this disorder. No similar changes were seen in the recipients' own aorta or in syngeneic transplants (not shown). Furthermore, the intensity of immunosuppression also affected the generation of the lesion and/or the effect was modified by the anti-inflammatory effect of steroids: when all three immunosuppressive drugs were administered simultaneously, both endothelialitis and accelerated arteriosclerosis were suppressed. The molecular mechanisms behind this suppression have yet to be worked out.

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