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Influence of tolrestat on the defective leukocyte–endothelial interaction in experimental diabetes

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Abstract

One of the most devastating secondary complications of diabetes is the blunted inflammatory response that becomes evident even in the very early stages of poorly controlled diabetes mellitus. While the etiology of this diminished response is not clearly understood, it has been linked to a decrease in the respiratory burst of neutrophils, as well as a decrease in microvessel response to inflammatory mediators and defective leukocyte–endothelial interactions. Using video microscopy to visualize vessels of the internal spermatic fascia, we have characterized leukocyte–endothelial interactions in alloxan-induced diabetic and in galactosemic rats by quantitating the number of leukocytes rolling along the venular endothelium and the number of leukocytes sticking to the vascular wall after topical application of zymosan-activated plasma or leukotriene B_4 (1 ng/ml), as well as after the application of a local irritant stimulus (carrageenan, 100 μg). We observed that while 33 days of alloxan-induced diabetes or 7 days of galactosemia had no effect on total or differential leukocyte counts and on the wall shear rate, both treatments significantly ($P < 0.001$) reduced the number of leukocytes rolling along the venular endothelium by about 70% and the number of adhered leukocytes in postcapillary venules by 60%. These effects were not observed in diabetic and galactosemic animals treated with an aldose reductase inhibitor. The results suggest that impaired leukocyte–endothelial cell interactions are a consequence of an enhanced flux through the polyol pathway.

Keywords: Tolrestat; Leukocyte–endothelial interaction; Diabetes mellitus; Polyol pathway

1. Introduction

The long-term hyperglycemia associated with both type 1 (insulin-dependent) and type 2 (non-insulin dependent) diabetes mellitus results in the slow development of multiple secondary complications, which include a reduced inflammatory response, as well as neuropathy, nephropathy, retinopathy and cataract formation. The mechanisms through which altered glucose metabolism leads to the development of secondary complications are incompletely understood. Increased polyol pathway activity, *myo*-inositol depletion and protein glycation (Frank, 1991; Cameron and Cotter, 1993; Tomlinson, 1993; Bucala et al., 1995; Stevens et al., 1995) have all been implicated.

Quantitative alterations of inflammatory events are noticeable even in the early stages of poorly controlled diabetes mellitus (Garcia-Leme, 1989; Garcia-Leme and Farsky, 1993). Neutrophils are an essential and integral part of the inflammatory response and their function is reduced in diabetic patients and animals (Movat and Baum, 1971; Bagdade et al., 1974; Mohandes et al., 1982; Pereira et al., 1987; Wilson et al., 1987; Sannomiya et al., 1990; Fortes et al., 1991). Using an in vitro assay of neutrophil killing of *Escherichia coli*, Boland et al. (1993) reported that this neutrophil function was restored in diabetic patients treated with the aldose reductase inhibitor ponalrestat. Similar observations have been reported for diabetic animals (Wilson et al., 1987, 1989; Lockington et al., 1987), supporting the suggestion that excess glucose metabolism by aldose or aldehyde reductase may decrease the availability of the reduced form of the enzyme cofactor nicotinamide adenine dinucleotide phosphate (NADPH) and block the activity of NADPH oxidase (Kamura et al., 1995; Fukase et al., 1996), limiting the respiratory burst

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and bacterial killing. Defects in the inflammatory response are not limited to reduced respiratory bursts and bacterial killing but also include an impaired microvascular response to inflammatory mediators such as histamine and bradykinin (Fortes et al., 1983a,b, 1984, 1989), as well as impaired chemotactile responses between endothelial cells and leukocytes (Fortes et al., 1991). Utilizing videomicroscopy, we have previously demonstrated that treatment of diabetic animals with the aldose reductase inhibitor tolrestat corrected the reduced vasodilation of mesenteric terminal arteriole and the venule paired with it to the inflammatory mediators histamine and bradykinin topically applied (Fortes et al., 1996). All of these studies together provide evidence that the increased flux of glucose through the polyol pathway in diabetic animal may interfere with the inflammatory response at several different levels. Since leukocytes are an essential part of the inflammatory response, a defective leukocyte–endothelial interaction may interfere with the response to injury.

We have now extended our previous observations of the internal spermatic fascia and report that endothelial–leukocyte interactions are also reduced in diabetic and galactosemic animals and that these effects are normalized by aldose reductase inhibitor treatment.

2. Methods

2.1. Animals

Male Wistar rats weighing between 150 and 180 g obtained from our breeding colony at the Institute were randomized into six groups that were age- and weight-matched, with at least eight animals per group. The groups consisted of the following: (i) alloxan-diabetic rats; (ii) non-diabetic controls; (iii) alloxan-diabetic rats treated with tolrestat (5 mg/kg, daily) for 30 days; (iv) non-diabetic animals treated with tolrestat for 30 days; (v) galactosemic rats; and (vi) galactosemic rats treated with tolrestat (5 mg/kg, daily) for 7 days. In all of the treated groups, tolrestat was suspended in saline with 2% Tween 80 and was administered by gavage (at a daily dose of 5 mg/kg body weight). Diabetic and non-diabetic control groups received the same volume of vehicle alone, about 1 ml. The experimental protocols were approved and performed in accordance with the guidelines of the Institute of Biomedical Sciences Committee.

2.2. Induction of diabetes and galactosemia

Diabetes mellitus was induced with an i.v. injection of alloxan, 40 mg/kg, dissolved in physiological saline. Control rats were injected with physiological saline alone. After administration of alloxan, animals were allowed free access to food and water. After 33 days the presence of diabetes was verified by blood glucose concentrations > 11.2 mM determined with a blood glucose monitor (Biobras type TA80GL) in samples obtained from the cut tip of the tail according to Jarrett et al. (1970). Tolrestat treatment was initiated 3 days after the alloxan injection.

Galactosemia was induced by feeding animals with a diet containing 50% galactose for 7 days. Tolrestat treatment (5 mg/kg per day for 7 days) of galactosemic rats started concomitantly with the induction of galactosemia.

2.3. Leukocyte counts

Leukocyte counts were performed on blood samples collected at the time of sacrifice. Total leukocyte counts were made in a Neubauer chamber. Stained blood films were used for differential leukocyte counts (Kaplow, 1965). Blood samples for these measurements were collected from the abdominal aorta, collected while the rats were under anesthesia. The presence of ketone bodies was qualitatively assessed in plasma with the aid of reagent strips used according to the manufacturer's instructions (Miles Int., Cali, Colombia).

2.4. Surgical preparation

The animals were anesthetized with an i.p. injection of sodium pentobarbital, 40 mg/kg, additional anesthetic was

Table 1
Characteristics of saline- and tolrestat-treated alloxan (33 days) diabetic rats and their respective controls
Values are means ± S.E.M.; 10 animals were used in each group. +, Increase; −, decrease.

Group	Body weight change (g)	Plasma glucose (mmol/l)	Blood leukocyte counts (cells/mm^3)
Saline-treated			
Control	$+63.4 \pm 9.5$	5.7 ± 0.1	$18{,}430 \pm 632$
Diabetic	-12.9 ± 1.6[a]	33.7 ± 2.6[a]	$18{,}895 \pm 567$
Tolrestat-treated			
Control	$+63.7 \pm 6.1$	5.6 ± 0.2	$18{,}255 \pm 727$
Diabetic	-13.6 ± 2.6[a]	32.0 ± 1.8[a]	$18{,}105 \pm 585$

[a] $P < 0.05$ vs. controls.

Table 2
Blood leukocyte counts in saline- and tolrestat-treated alloxan (33 days) diabetic rats and their respective controls
Values are means ± S.E.M.; 10 animals were used in each group.

Group	Cells/mm^3			
	Lymphocytes	Monocytes	Neutrophils	Eosinophils
Saline-treated				
Control	11,058 ± 488	2820 ± 246	4188 ± 364	368 ± 49
Diabetic	11,054 ± 470	2513 ± 155	4912 ± 247	416 ± 57
Tolrestat-treated				
Control	11,354 ± 574	2734 ± 177	3882 ± 212	255 ± 72
Diabetic	10,519 ± 435	2770 ± 195	4508 ± 466	308 ± 70

given as required to maintain the same depth of anesthesia, usually not more than 10% of the required dose. The internal spermatic fascia of the wall of the scrotal chamber was exteriorized for microscopic examination in situ. This was done through a longitudinal incision of the skin and dartos muscle in the midline over the ventral aspect of the scrotum and opening of the cremaster muscle to expose the internal fascia. This procedure does not require extensive surgical manipulation for the observation of the vascular network and provides a valuable means for transilluminating a tissue for quantitative studies of the microcirculation. In addition, the preparation is not affected by respiratory movements of the animals, and its microcirculatory characteristics remain basically invariant throughout the course of the experiment. The animals were maintained on a special board thermostatically controlled at 37°C, which included a transparent platform on which the tissue to be transilluminated was placed. The preparation was kept moist and warmed by irrigating the tissue with warmed (37°C) Ringer Locke's solution, pH 7.20–7.40, containing 1% gelatin. The composition of the solution was (in mM) 154 NaCl; 5.6 KCl; 2 $CaCl_2.2H_2O$; 6 $NaHCO_3$ and 5 glucose. The rate of outflow of the solution onto the exposed tissue was controlled to maintain the preparation in continuous contact with a film of the liquid. A 500-line television camera was incorporated onto a triocular Zeiss microscope to facilitate observation of the enlarged image (×3400) on the video screen. Images were recorded on a video recorder with a ×40 long distance objective with a 0.65 numerical aperture. An image-splitting micrometer was adjusted to the phototube of the microscope as described by Baez (1969). The image splitter sheared the optical image into two separate images and displaced one with respect to the other. By rotating the image splitter in the phototube, the shearing was maintained in a direction at right angles to the axis of the vessel. The displacement of one image from the other allowed measurement of the vessel diameter. Vessels selected for study were third-order venules, defined according to their branch-order location within the microvascular network (Gore and Bohlen, 1977; Rhodin, 1986). These vessels corresponded to postcapillary venules, with diameters ranging from 12 to 16 μm.

The left carotid artery of each anesthetized (sodium pentobarbital, 40 mg/kg, i.p.) rat was catheterized and mean arterial blood pressure and heart rate were measured. The catheter was filled with heparinized saline (20 IU/ml). Direct blood pressure recordings were obtained by connecting the arterial cannula to a physiograph (MK-III, Narco Bio System, Houston, TX). Indirect heart rate recordings were obtained by counting waveforms generated on the physiograph tracings. Centerline red blood cell velocity was measured using an optical Doppler velocimeter (Microcirculation Research Institute, Texas A&M University, College Station, USA) that was calibrated against a rotating glass disk coated with red blood cells. Venular blood flow was calculated from the product of mean red

Table 3
Characteristics of saline-treated and tolrestat-treated 7 days galactosemic rats and their respective controls
Values are mean ± S.E.M.; eight animals were used in each group. +, Increase; −, decrease.

Group	Body weight change (g)	Plasma glucose (mmol/l)	Blood leukocyte counts (cells/mm^3)
Saline-treated			
Control	+27.6 ± 2.2	6.8 ± 0.09	18,636 ± 644
Galactosemic	−7.2 ± 0.8[a]	4.0 ± 0.23	18,715 ± 571
Tolrestat-treated			
Control	+26.1 ± 1.8	5.2 ± 0.05	18,106 ± 595
Galactosemic	−6.6 ± 0.9[a]	4.1 ± 0.10	18,100 ± 712

[a] $P < 0.05$ vs. controls.

Table 4
Blood leukocyte counts in saline- and tolrestat-treated galactosemic rats and their respective controls
Values are means ± S.E.M.; eight animals were used in each group.

Group	Cells/mm^3			
	Lymphocytes	Monocytes	Neutrophils	Eosinophils
Saline-treated				
Control	11,275 ± 564	2492 ± 259	4519 ± 309	350 ± 101
Galactosemic	11,463 ± 530	2339 ± 242	4608 ± 258	305 ± 91
Tolrestat-treated				
Control	11,565 ± 596	1870 ± 90	4503 ± 376	159 ± 62
Galactosemic	10,475 ± 588	2715 ± 415	4480 ± 294	430 ± 77

blood cell velocity (V_{mean} = centerline velocity/1.6) and microvascular cross-sectional area, with cylindrical geometry assumed. Venular wall shear rate (γ) was calculated from the Newtonian definition: $\gamma = 8(V_{mean}/D_{v,}$ where D_v = vessel diameter) (Davis,1987; Panés et al., 1996).

2.5. Experimental protocols

2.5.1. Leukocyte rolling

In a series of experiments, interaction of leukocytes with the luminal surface of the venular endothelium was studied in a segment of the vessel. Rolling leukocytes (''rollers'') were defined as those white blood cells that moved at a velocity less than that of erythrocytes in the same stream. The number of rolling leukocytes (''rollers'') was determined in 10-min periods. These leukocytes moved sufficiently slowly to be individually visible and were counted as they rolled past a 100-μm length venule (Fortes et al., 1991).

2.5.2. Chemoattractant-induced leukocyte adhesion

Leukocytes adhering to the endothelium were quantified following the application of irritant stimuli such as leukotriene B_4 or zymosan-activated plasma. A leukocyte was considered to be adherent to the venular endothelium if it remained stationary for > 30 s (Granger et al., 1989). Adherent cells (''stickers'') were expressed as the number per 100-μm length of venule. Adhesion was investigated under two conditions. In one, the internal spermatic fascia, after a suitable control period of normal circulation, was exposed to 0.1 ml of a solution containing 10% zymosan-activated homologous plasma in physiological saline. To obtain activated plasma, zymosan *a* from *Saccharomices cerevisae* was incubated (1 mg/ml) with plasma from normal animals for 1 h at 37°C. Following centrifugation at $1600 \times g$ for 10 min, the supernatant fraction, the zymosan-activated plasma, was collected and diluted 1:10 with physiological saline and topically added to the preparation. Adhesion of leukocytes was assessed after 10 min of addition of zymosan. Plasma treated identically, except for the addition of zymosan, was used as a control. Leukocyte adhesion was also quantitated using the same protocol in animals given a local application of leukotriene B_4 (1 ng/ml–0.1 ml). Each section of the vascular bed was tested only once, and no more than two determinations were performed on a single animal. The two measurements were averaged for each animal.

2.5.3. Carrageenan-induced leukocyte transmigration

In another series of experiments, the number of leukocytes that accumulated in a 2000 μm^2 standard area of connective tissue adjacent to a postcapillary venule was determined after the induction of a local inflammatory response. Cells were counted on the recorded image. Five different fields were evaluated for each animal to avoid variability based on sampling. Data were then averaged for each animal. The inflammatory reaction was evoked by injecting 100 μg of carrageenan in 0.1 ml of saline into the scrotum of the animals and the number of migrated cells was counted after 2 h of carrageenan injection.

Table 5
The effect of tolrestat treatment on carbohydrate and polyol levels (in nmol/mg protein) in the internal spermatic fascia of diabetic rats
Values are means ± S.E.M. n = number of animals used.

Group	Glucose	*myo*-Inositol	Sorbitol	Fructose
Untreated control, $n = 10$	51.5 ± 5.1	4.1 ± 0.4	0.18 ± 0.04	0.25 ± 0.03
Diabetic, $n = 14$	232.2 ± 18.2[a]	8.1 ± 0.9	0.40 ± 0.04[b]	1.76 ± 0.24[b]
Treated control, $n = 11$	38.3 ± 3.7	5.9 ± 1.2	0.12 ± 0.05	0.28 ± 0.03
Diabetic, $n = 11$	153.7 ± 9.9[a]	8.8 ± 1.8	0.22 ± 0.01	0.87 ± 0.1[a]

[a] $P < 0.001$ vs. controls.
[b] $P < 0.01$ vs. controls.

Table 6
The effect of tolrestat treatment on carbohydrate and polyol levels (in nmol/mg protein) in the internal spermatic fascia of galactosemic rats
Values are mean ± S.E.M. of eight animals in each group and are expressed as nmol/mg protein.

Group	Glucose	*myo*-Inositol	Sorbitol	Fructose	Galactose	Galactitol
Untreated						
Control	49.0 ± 6.4	8.9 ± 1.3	0.21 ± 0.04	0.56 ± 0.08	6.5 ± 2.9	0.7 ± 0.2
Galactosemic	42.5 ± 4.6	16.4 ± 4.2	0.18 ± 0.05	0.76 ± 0.15	41.3 ± 11.7[a]	53.1 ± 20.2[b]
Treated						
Control	45.7 ± 5.4	7.1 ± 1.1	0.12 ± 0.03	0.50 ± 0.06	4.4 ± 1.4	0.45 ± 0.12
Galactosemic	41.2 ± 3.9	10.0 ± 2.0	0.13 ± 0.02	0.65 ± 0.08	57.6 ± 19.4[a]	5.0 ± 1.2

[a] $P < 0.05$ vs. controls.
[b] $P < 0.001$ vs. controls.

2.6. Tissue collection and biochemical analyses

After anesthesia of the animals, the left internal spermatic fascia was rapidly removed, frozen and stored at −80°C for subsequent carbohydrate analysis. Tissue galactose, galactitol, glucose, sorbitol, fructose and *myo*-inositol levels were quantitated using gas chromatography. For this procedure the carbohydrates were derivatized as previously described (Guerrant and Mass, 1984). In brief, this procedure involves the conversion of aldoses to their corresponding aldonitrile acetates and polyols to their corresponding acetates. These derivatives were separated and quantitated with a Hewlett Packard gas chromatograph (Model 5890, Piscataway, NJ), equipped with a capillary column with a cross-linked methyl silicone phase and a flame ionization detector. This procedure results in a linear instrument response for polyol concentrations ranging from 0.5 to 300 μg and has a limit of detection of 0.04 nmol/mg tissue. Values were expressed as nmol/mg of protein.

2.7. Reagents

The following reagents were used: alloxan hydrate, galactose, leukotriene B_4, zymosan (all from Sigma, MO, USA). Carrageenan sodium salt, a 60,000- to 100,000-Mr polysaccharide composed of sulphated galactose units, was from Marine Colloids (Springfield, NJ) and tolrestat (kindly supplied by Wyeth, São Paulo, Brazil).

2.8. Statistical analysis

Data are given as mean ± S.E.M. One-way analysis of variance followed by Tukey–Kramer multiple comparisons test and Student's *t*-test were used, when appropriate. The minimum acceptable level of significance was P at a value less than or equal to 0.05.

3. Results

3.1. Effects of diabetes, galactosemia and tolrestat-treatment on body weight, plasma glucose, blood leukocyte counts and tissue polyol levels

Body weight gain in the alloxan-treated animals 33 days after the induction of diabetes was significantly less ($P <$

Table 7
Diameter, mean arterial blood pressure levels (Map), heart rate (HR) and venular flow velocity, obtained in diabetic rats (33 days), galactosemic rats (7 days) and their respective controls
Values are mean ± S.E.M.; n = number of animals used in each group.

Group	Diameter (μm)	Map (mm Hg)	HR (bpm)	Flow velocity (mm/s)	Shear rate (s^{-1})	*n*
Untreated						
Control	15.5 ± 0.6	101.1 ± 5.4	360.0 ± 15.5	2.1 ± 0.1	1086.0 ± 64.2	6
Diabetic	14.8 ± 0.4	89.3 ± 9.5	337.5 ± 15.7	1.9 ± 0.2	958.4 ± 114.1	7
Control	16.2 ± 0.3	110.6 ± 3.1	370.0 ± 10.0	1.8 ± 0.0	867.3 ± 30.6	6
Galactosemic	16.1 ± 0.4	107.8 ± 3.5	367.5 ± 13.6	1.8 ± 0.1	892.1 ± 57.4	8
Treated						
Control	15.5 ± 0.6	97.5 ± 4.6	360.0 ± 15.5	2.0 ± 0.1	958.3 ± 69.0	6
Diabetic	15.7 ± 0.4	96.0 ± 5.6	360.0 ± 13.0	1.9 ± 0.1	977.9 ± 60.8	7
Control	16.3 ± 0.4	100.3 ± 6.7	360.0 ± 15.5	1.8 ± 0.1	915.2 ± 44.4	6
Galactosemic	16.1 ± 0.5	100.0 ± 4.5	352.5 ± 7.5	1.7 ± 0.1	846.6 ± 41.0	8

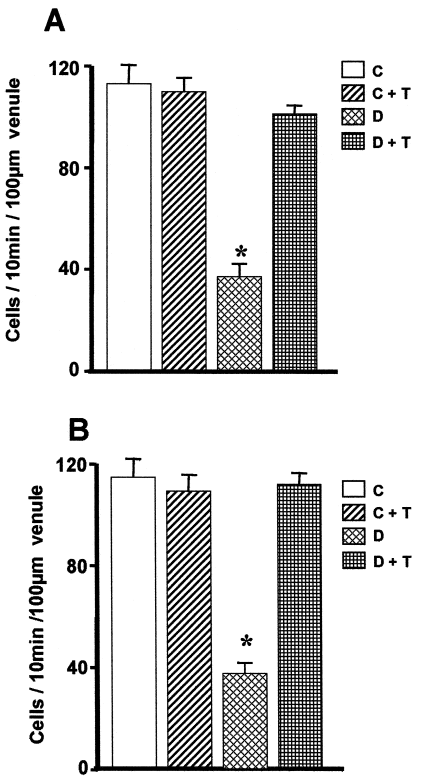

Fig. 1. Bar graphs showing number of rolling leukocytes (''rollers''/10 min/100 μm venule) in postcapillary venules of internal spermatic fascia studied using in vivo video microscopy. (A) Saline-treated (D) and tolrestat-treated alloxan diabetic (D+T) and their respective control rats (C and C+T, respectively). Ten animals were used in each group. (B) Saline-treated (G) and tolrestat-treated galactosemic (G+T) and their respective control rats (C and C+T, respectively). Eight animals were used in each group. * Significantly different from controls treated with saline or tolrestat and tolrestat-treated diabetic rats, $P < 0.001$.

0.05) than that of the control animals. Blood glucose concentrations were significantly elevated ($P < 0.05$) about 6-fold in samples from diabetic animals collected at the terminal time point. Ketone bodies were present in samples collected from 80% of the diabetic animals (data not shown). None of these parameters were altered by tolrestat treatment (Table 1). Total and differential leukocyte counts were unaltered by diabetes or tolrestat treatment (Tables 1 and 2).

Body weight gain in the animals fed a 50% galactose diet for 7 days was significantly less ($P < 0.05$) than that of the control animals. Neither galactose feeding nor tolrestat treatment of galactosemic rats had an effect on plasma glucose levels or leukocyte counts (Tables 3 and 4).

Glucose levels were increased more than 4-fold and sorbitol levels were increased 2-fold in the internal spermatic fascia of diabetic rats. While tolrestat treatment prevented the accumulation of sorbitol, fructose levels in the diabetic animals were only reduced by 60% with tolrestat treatment (Table 5). Galactose feeding produced a 76-fold increase in tissue galactitol levels that were reduced by about 90% with tolrestat treatment (Table 6). Tissue *myo*-inositol levels were not significantly altered by diabetes, galactosemia or tolrestat treatment (Tables 5 and 6).

3.2. Influence of diabetes and tolrestat treatment on blood pressure, heart rate, flow velocity and wall shear rate

Under baseline conditions, diabetic rats had similar mean arterial blood pressure and heart rate relative to control rats. Tolrestat treatment did not interfere with these parameters (Table 7). Mesenteric venules of diabetic rats had a similar centerline red blood cell velocity and wall shear rate relative to control rats. Tolrestat treatment did not interfere with these parameters (Table 7). Similarly to that observed in diabetic rats, there were no differences in mean arterial blood pressure, heart rate, flow velocity and wall shear rate in galactosemic and control rats. Tolrestat treatment did not interfere with these parameters (Table 7).

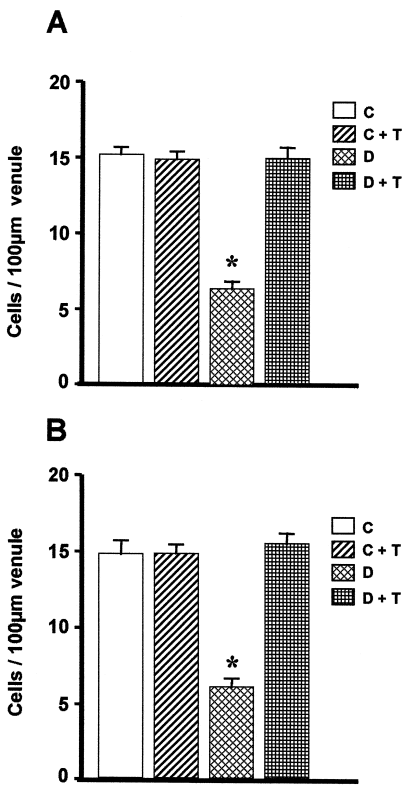

Fig. 2. Bar graphs showing the number of adhered leukocytes (''stickers''/100 μm venule) in postcapillary venules of internal spermatic fascia 10 min after application of zymosan-activated plasma using in vivo video microscopy. (A) Saline-treated (D) and tolrestat-treated alloxan diabetic (D+T) and their respective control rats (C and C+T, respectively). Ten animals were used in each groups. (B) Saline-treated (G) and tolrestat-treated galactosemic (G+T) and their respective control rats (C and C+T, respectively). Eight animals were used in each group. * Significantly different from controls treated with saline or tolrestat and tolrestat-treated diabetic rats, $P < 0.001$.

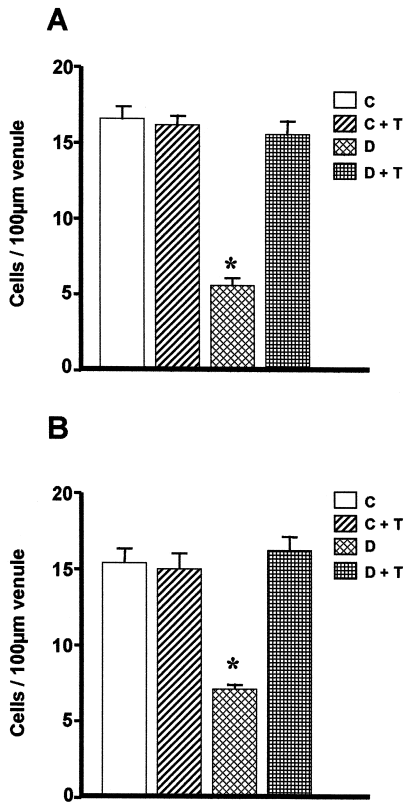

Fig. 3. Bar graphs showing the number of adhered leukocytes (''stickers''/100 μm venule) in postcapillary venules of internal spermatic fascia 10 min after application of leukotriene B_4 studied using in vivo video microscopy. (A) Saline-treated (D) and tolrestat-treated alloxan diabetic (D+T) and their respective control rats (C and C+T, respectively). Ten animals were used in each group. (B) Saline-treated (G) and tolrestat-treated galactosemic (G+T) and their respective control rats (C and C+T, respectively). Eight animals were used in each group. * Significantly different from controls treated with saline or tolrestat and tolrestat-treated diabetic rats, $P < 0.001$.

3.3. Influence of diabetes mellitus and galactosemia on leukocyte–endothelial interaction and the effect of tolrestat

3.3.1. Leukocyte rolling

Postcapillary venules of the internal spermatic fascia chosen for microscopic observations had resting diameters ranging between 12 and 16 μm. Leukocytes that have been displaced from the axial zone of the cell column roll along the vessel wall and eventually bind to and interact with endothelial cells during an inflammatory response. Relative to non-diabetic animals, we observed a 70% decrease in the number of rolling cells (''rollers'') in diabetic animals (Fig. 1A). Comparable results were obtained in ketotic and non-ketotic diabetic animals (data not shown). The decrease in the number of rolling cells was not dependent on the number of circulating leukocytes since total and differential leukocyte counts in the peripheral blood were not different between non-diabetic and diabetic animals (Tables 1 and 2). Treatment of diabetic rats with tolrestat prevented the decrease in the number of rolling leukocytes. In contrast, treatment of control rats with tolrestat was without effect (Fig. 1A). Similar results were observed in galactosemic animals (Fig. 1B).

3.3.2. Chemoattractant-induced leukocyte firm adhesion

In diabetic and galactosemic animals, following the topical administration of zymosan activated plasma or leukotriene B_4, the number of leukocytes adhering to the vessel (''stickers'') was significantly reduced ($P < 0.001$) compared to non-diabetic controls (Figs. 2 and 3). Chronic administration of tolrestat prevented this impaired response in diabetic and galactosemic rats, but produced no effect in non-diabetic or non-galactosemic controls (Figs. 2 and 3). No leukocyte adhesion was observed following application of non-activated plasma to the preparations (data not shown).

3.3.3. Carrageenan-induced leukocyte transmigration

When the animals were injected with an irritant (100 μg carrageenan) into the scrotum to induce a local inflam-

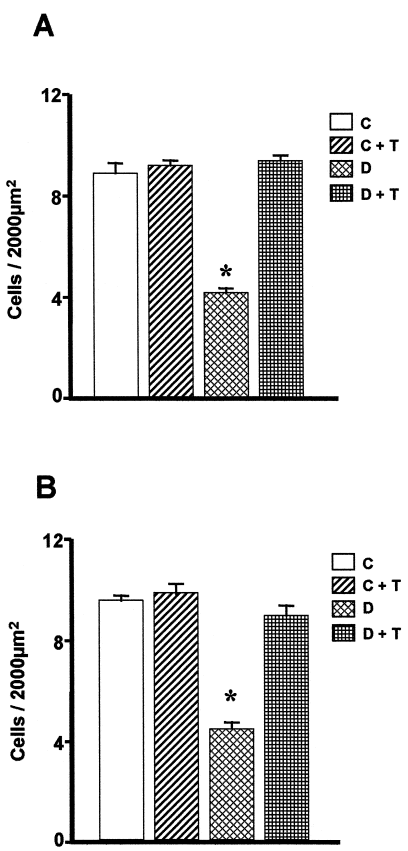

Fig. 4. Bar graphs showing the number of migrated leukocytes (migrated cells/2000 μm^2) in postcapillary venules of internal spermatic fascia 2 h after local stimuli of carrageenan. (A) Saline-treated (D) and tolrestat-treated alloxan diabetic (D+T) and their respective control rats (C and C+T, respectively). Ten animals were used in each group. (B) Saline-treated (G) and tolrestat-treated galactosemic (G+T) and their respective control rats (C and C+T, respectively). Eight animals were used in each groups. * Significantly different from controls treated with saline or tolrestat and tolrestat-treated diabetic rats, $P < 0.001$.

matory response, marked differences between the number of adherent cells in control, diabetic and galactosemic rats were observed. In the former group, the number of cells rolling along the vessel was reduced (data not shown), and leukocytes accumulated in the connective tissue adjacent to the venule in a pattern characteristic of the inflammatory reaction. In contrast, in diabetic and galactosemic animals, the number of rolling leukocytes remained practically unaltered by the irritant, and only a few cells were found in an equivalent area in the perivascular tissue (Fig. 4). Chronic administration of tolrestat prevented these impaired responses in both diabetic and galactosemic rats (Fig. 4).

4. Discussion

The precise mechanism by which chronic hyperglycemia leads to the development of the long-term complications of diabetes (Reichard et al., 1993; The Diabetes Control and Complication Trial Research Group, 1993) is not completely understood. Accumulating evidence has suggested that the hyperglycemia-induced acceleration of polyol pathway activity mediates a number of metabolic derangements that result in the cellular dysfunction that characterizes diabetic complications. We now suggest that this pathway may also be involved in leukocyte dysfunction in diabetes mellitus.

For the present study, postcapillary venules were chosen for observations on leukocyte–endothelial interactions in particular because they are considered to be the major site for leukocyte adhesion to the vascular wall in response to noxious stimuli (Garcia-Leme, 1989).

The initial low affinity interaction between leukocyte and venular endothelium is manifested as a rolling behavior. Rolling leukocytes can then become firmly adherent (stationary) on the vessel wall where the process of transendothelial migration can occur if a chemotactic signal is generated in the perivascular compartment (Panés et al., 1999). In the present study we studied rolling behaviour without any stimulus except the ''exposure trauma''. Although some adhesion might occur under these conditions, it is not readily apparent.

A large number of mediators have been implicated in the initiation of leukocyte–endothelial cells adhesion during inflammation. The experimental strategy we employed to assess the contribution of specific mediators to this facet of the inflammatory response involved exposure of non-inflamed venules to exogenous sources of mediators such as LTB_4 and zymosan-activated plasma that generates LTB_4, C_{3a} and C_{5a}. These mediators are highly chemotactic for neutrophils (Björk et al., 1982; McMillan and Foster, 1988; Fretland et al., 1991). Therefore, in this case, neutrophils are the most likely candidates responsible for the leukocyte response seen in our study.

To evaluate the inflammatory response as a whole, we used an irritant agent widely used to study the inflammatory process, carrageenan (tested 2 h after application) (Pereira et al., 1987; Fortes et al., 1991; Tomlinson et al., 1994). After 1–2 h of carrageenan stimulus we and others have described an influx of neutrophils (Petrone et al., 1980; Pereira et al., 1987; Fortes et al., 1991). The absence of this response in diabetic and galactosemic animals suggests that neutrophils are the main leukocyte subtype altered by diabetes mellitus (Fortes et al., 1991). Corroborating this, histological studies have established that the phenotype of adhering and migrating cells are neutrophils in the early event (first 8 h) and monocytes and lymphocytes at later times (longer than 8 h) (Kubes and Granger, 1996). As pointed out by these authors, although it is tempting to conclude that the rolling cells follow the same time frame, it remains impossible to determine the phenotype of the rolling population of leukocytes. Therefore, we have used the rather vague term ''leukocyte'' to describe the rolling cells as suggested by Kubes and Granger (1996) in a recent review.

It has been well established that leukocyte infiltration is a multi-step mechanism that requires that leukocytes moving at very high speeds in the mainstream of blood make initial transient contact with endothelial cells lining the vessel wall and roll along at a greatly reduced velocity relative to red blood cells. Once cells begin to roll, they can then firmly adhere and finally migrate out of the vasculature. It should be noted that this is an interdependent series of events, in as much as inhibiting leukocyte rolling prevents subsequent leukocyte adhesion and ultimately leukocyte emigration out of the vasculature (Kubes, 1997). Our observations demonstrate that leukocytes rolling along the venular endothelium and sticking to the venular wall, as well as migrating into the perivascular tissue under the influence of irritant stimuli, are impaired in the diabetic state. While it is conceivable that all the stages of leukocyte recruitment area are affected by the diabetic state, decreased rolling behaviour alone might explain decreased adhesion and migration since the initial rolling interaction is a precondition for firm adhesion to occur in vivo at physiological blood flow (Lindbom et al., 1992). The reduced number of rollers, adhered and migrated cells in diabetic rats was not observed in diabetic animals treated with tolrestat. In contrast, no quantitative changes were observed in control animals treated with tolrestat. These observations demonstrate a positive association between polyol pathway activation and leukocyte dysfunction in experimental diabetes mellitus.

Besides increased polyol pathway flux, the major metabolic changes caused by hyperglycemia are elevated oxygen free radical formation, decreased resistance to oxidative stress and advanced glycosylation. All of these factors could trigger the development of diabetic complications.

Evidence suggests that the diabetic condition is associated with increased free radical damage. In diabetic patients, serum thiobarbituric acid-reactive material, which is

taken as an index of increased lipid peroxidation, was elevated (Nishigaki et al., 1978; Sato et al., 1979). The increased peroxidation and oxidative stress in diabetes has been attributed to the increased plasma glucose levels since Hunt and Wolff (1991) and Hunt et al. (1988) convincingly demonstrated that glucose undergo autoxidation under physiological conditions, thus becoming a source of free radical generation. In some tissues such as peripheral nerve, reduced levels of glutathione, the major source of protection against oxidative stress, are decreased in diabetic animals (Obrosova et al., 1999). Preliminary evidence suggests that tissue levels of reduced glutathione (GSH) in diabetic animals are restored with ARI treatment. Restoration of GSH levels may be mediated by ARI effects on the availability of NADPH (Hohman et al., 1997). Together these observations suggest that antioxidant treatment could also be particularly important in the prevention and treatment of diabetic complications such as decreased leukocyte migration. In support of this hypothesis, we have previously demonstrated that vitamin C and probucol, antioxidant agents, corrected the reduced cell migration in alloxan-diabetic rats (Zanardo et al., 1998).

An important advance in our understanding of the pathogenesis of diabetes is the observation that glucose or its analogues interact with proteins. In animal models, the end products of this non-enzymatically catalyzed reaction have been linked to the development of the long-term complications of diabetes (Reiser, 1991). Non-enzymatic glycation of proteins may also interfere with leukocyte behaviour. Masuda et al. (1990) have demonstrated that glycosylated protein separated from the serum of diabetic rats is capable of decreasing membrane fluidity of control leukocyte which may alter leukocyte function such as leukocyte migration. Corroborating this hypothesis, we recently demonstrated that aminoguanidine, an inhibitor of advanced glycation end products formation, prevented the decreased leukocyte rolling and migration in alloxan-diabetic rats (Sannomiya et al., 1997).

Together these findings indicate that the three mechanisms, polyol pathway activity, oxidative stress and non-enzymatic glycation, although distinct, all contribute to the alteration of leukocyte behaviour observed in diabetes mellitus.

The protective effect of tolrestat was unrelated to the number of circulating leukocytes; total and differential leukocyte counts in the peripheral blood were unaltered by diabetes or tolrestat treatment. Tolrestat treatment did not interfere with plasma glucose levels, leading us to exclude an influence of the drug on hyperglycemia per se or hyperosmolality secondary to hyperglycemia. Additionally, the reduction in body weight gain was essentially of the same magnitude in diabetic animals treated or not treated with the drug.

We used galactosemic rats to examine the effects of increased polyol pathway activity without the confounding effects of hyperglycemia and insulin deficiency seen in the chemical-induced diabetic rat (Forster et al., 1996). Galactosemic rats also exhibited an impaired leukocyte–endothelial interaction that was preserved by tolrestat treatment. We interpret the similarity of the findings in both diabetic and galactosemic rats and the association in both conditions with the increased formation and accumulation of polyols (galactitol and sorbitol in galactose-fed and diabetic rats, respectively) as evidence supporting the hypothesis that increased metabolism by the polyol pathway plays a role in the altered leukocyte function in diabetic rats. The present results are in accord with other observations in galactose-fed and diabetic rats in which impaired microvascular reactivity could be obviated by treatment with an aldose reductase inhibitor (Cameron et al., 1996; Forster et al., 1996; Fortes et al., 1996).

It is well known that during the inflammatory process leukocytes are rapidly transported via the circulatory system to areas of tissue injury where they adhere to the endothelium and emigrate to the perivascular space (Grant, 1973). These events depend upon the interaction between hemodynamic parameters (flow and resistance) which affect the transport of leukocytes to an injury site as well as a balance of forces between leukocyte–endothelium adhesion forces and the wall shear stress which tend to sweep them away (Atherton and Born, 1972; Schmid-Schoenbein et al., 1975; Mayrovitz et al., 1977). To evaluate the possible interference of hemodynamic changes on the leukocyte behaviour (rolling, sticking and migration) studied, we measured arterial blood pressure (to estimate vascular resistance) and blood flow velocity (to estimate blood volume). We also calculated the wall shear rate since the dependence of leukocyte adhesion on shear rate has been demonstrated in vivo (Granger et al., 1989; Ley and Gaehtgens, 1991; Ley et al., 1991; Perry and Granger, 1991) and in vitro (Lawrence and Springer, 1991; Lawrence et al., 1990). Low shear rates promote leukocyte adherence to microvascular endothelium in postcapillary venules (Perry and Granger, 1991). We observed no differences between the blood pressure levels, venular flow velocity and shear rates of diabetic and control rats. Since tolrestat treatment did not modify either blood pressure, flow velocity or shear rates, we can exclude hemodynamic changes as the cause of the prevention effects of tolrestat on leukocyte alterations in diabetic rats. Our data contrast with those obtained by Panés et al. (1996). These authors demonstrated an exacerbated inflammatory response to ischemia–reperfusion in diabetic rats due to an exaggerated leukocyte–endothelial adhesion and albumin response. However, the lower shear rates observed by these investigators in diabetic compared with control animals may have contributed to their observation of increased adhesion in diabetic rats. Organ specificity of vascular alterations in diabetes may also contribute to the discrepancy between our findings and those of Panés et al. (1996).

Diabetes mellitus is associated with disturbances of neutrophil function such as abnormal adherence and

chemotaxis (Rayfield et al., 1982; Wilson, 1986; Pereira et al., 1987). The mechanisms underlying leukocyte accumulation in a tissue depend on the interaction between the cells and the vascular endothelium. During the development of inflammatory responses, leukocytes roll along the lining endothelium of postcapillary venules and eventually become firmly attached to the vascular wall before migrating into tissues. The expression of adhesion molecules might be reduced in diabetes mellitus and this could be responsible for the decreased rolling behaviour. Specific adhesion glycoproteins expressed on the surface of leukocytes and endothelial cells play a relevant role in the adhesion phenomenon (Perry and Granger, 1991; Albelda et al., 1994; Granger and Kubes, 1994; Malik and Lo, 1996). Leukocyte rolling is dependent on the selectin family of adhesion molecules. P-selectin (induced in minutes) and E-selectin (4–6 h for maximum induction) expressed on activated endothelium each contribute significantly to the rolling events (Bevilacqua et al., 1987; Geng et al., 1990; Abassi et al., 1993; Jones et al., 1993). Since neither adhesion molecule is thought to be expressed constitutively, a stimulus such as surgical manipulation associated with intravital microscopy is required for the expression of either adhesion molecule (Kubes, 1997).

Glycoproteins of the CD11/CD18 complex (β_2 integrins) expressed on leukocytes interact with ligands such as intercellular adhesion molecule-1 (ICAM-1) on endothelial cells to mediate leukocyte adhesion and emigration (Von Adrian et al., 1991, 1992). Blockade of cell adhesion molecules either on leukocytes or endothelial cells or both can effectively inhibit leukocyte adhesion. One possible explanation for the abnormal leukocyte function in diabetes mellitus might be a down regulation of adhesion molecules involved in leukocyte recruitment such as selectin or ICAM-1. This latter adhesion molecule is constitutively expressed on vascular endothelium and shows marked upregulation on most tissues during the development of acute and chronic inflammatory diseases (Dustin et al., 1986; Adams et al., 1989; Cosimi et al., 1990; Wegner et al., 1990; Norris et al., 1991; Crockett-Torabi, 1998). Further, a reduced expression of ICAM-1 has been linked to the appearance of a protein plasma factor in alloxan-diabetic rats (Sannomiya et al., 1996). Recently, we have observed a low level of immunoreactivity to anti-ICAM-1 in venules of the internal spermatic fascia from diabetic rats (unpublished observations). Though oxidants are critical for ICAM-1 transcription in endothelial cells in response to some proinflammatory mediators, like TNF-α and interferon γ (Rahman et al., 1998), and enhanced free radical generation has been demonstrated in diabetes, we could not find an increased expression of this adhesion molecule in our diabetic rats (Sannomiya et al., 1996, unpublished observations). Alteration of the expression of other adhesion molecules on leukocytes and/or endothelial cells might occur in diabetes. Studies are in progress to address this issue.

Based on our present findings, we hypothesize that the accelerated formation of sorbitol in diabetic animals may increase the intracellular osmolarity or decrease the availability of the enzyme cofactor NADPH leading to disturbance of endothelial cell functions that might alter leukocyte–endothelial interactions. Regardless of the mechanism, inhibition of the polyol pathway corrected the defective leukocyte–endothelial interaction found in experimental diabetes and may have a similar effect in diabetic patients.

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