Repetitive Fluctuations in Blood Glucose Enhance Monocyte Adhesion to the Endothelium of Rat Thoracic Aorta

Kosuke Azuma, Ryuzo Kawamori, Yukiko Toyofuku, Yoshio Kitahara, Fumihiko Sato, Tomoaki Shimizu, Kyoko Miura, Tomoyuki Mine, Yasushi Tanaka, Masako Mitsumata, Hirotaka Watada

Background—The aim of this study was to elucidate the effect of repetitive fluctuations in blood glucose concentrations on monocyte adhesion to the aortic endothelium.

Methods and Results—Nonobese type 2 diabetes, Goto–Kakizaki (GK) rats were fed twice daily to induce repetitive postprandial glucose spikes. Then, we compared the number of monocytes adherent to the endothelium of thoracic aorta in these rats with that in rats fed ad libitum. To suppress the glucose spikes, rats were injected with an inhibitor of sodium–glucose transporter, phloridzin, just before each meal for 12 weeks. GK rats fed twice daily showed significantly lower HbA1c than GK rats fed ad libitum. However, the former group showed markedly higher number of monocytes adherent to the endothelium than the latter, together with increased arterial intimal thickening. Phloridzin significantly reduced the number of adherent monocytes in GK rats fed twice daily.

Conclusion—Our data demonstrated that repetitive postprandial fluctuation in glucose concentration evokes monocyte adhesion to endothelial cells that was worse than that induced by stable hyperglycemia in vivo. Suppression of such fluctuations efficiently suppressed monocyte adhesion to the aortic endothelium. (Arterioscler Thromb Vasc Biol. 2006; 26:2275-2280.)

Key Words: monocyte–endothelial interaction ■ postprandial hyperglycemia ■ cardiovascular disease ■ NEMOes ■ glucose spike

Patients with diabetes are at increased risk of cardiovascular disease. Even when adjusted for other conventional risk factors, diabetic individuals exhibit 2- to 4-fold increased risk in comparison with nondiabetic subjects. Although glycemic exposure is clearly relevant to the progression of atherosclerosis, HbA1c, which reflects the level of glycemic exposure to the vascular wall, showed only a weak relation to the onset of cardiovascular disease. Instead, recent epidemiological data have suggested postprandial hyperglycemia as a risk factor for atherosclerotic disease. Patients showing postprandial hyperglycemia often have accompanying postprandial dyslipidemia, insulin resistance, and hyperinsulinemia. Thus, it is difficult to assess the importance of the postprandial rise in blood glucose by itself on the progression of atherosclerosis.

Atherosclerosis is a complex disease that is associated with functional change of the vascular endothelial layer. An increasing large body of evidence points to a critical role of monocyte–endothelial cell interactions in atherosclerotic plaque formation. The adhesion of circulating monocytes to the intimal endothelial cells is thought to be among the earliest events in naturally occurring experimental models of atherosclerosis. To quantitate the adhesion of monocytes to the endothelium in vivo, we recently established a NEMOes (New En face Method for Optimal observation of endothelial surface). This method allowed us to observe the entire surface of the endothelium with a clear focused image and thus to quantify the number of monocytes adhering in every region of the rat thoracic aorta after immunostaining of the monocyte/macrophage specific protein, CD68.

The Goto–Kakizaki (GK) rat is a genetic nonobese type 2 diabetes model produced by selective breeding with high glucose levels on the oral glucose tolerance test (OGTT), starting from a nondiabetic Wistar rat colony. Unlike several other rodent models of type 2 diabetes, GK rats show impaired insulin secretion and insulin resistance without showing obesity, marked hyperlipidemia, or marked hypertension. Compared with other type 2 diabetes models, GK rats appear to be suitable for investigating the effect of glucose with fewer confounding effects of various factors associated with obesity.

In the present study, we assessed the effect of postprandial hyperglycemia on monocyte adhesion to endothelium using...
Abbott Park, Ill). The remaining RD rats were divided into 2 groups euthanized under anesthesia induced by intraperitoneal injection of the age of 10 weeks, 11 rats each in the RD and AL groups were acclimatized to laboratory conditions for approximately 2 weeks. At /H11005 rats (n=35) were fed ad libitum (AL group). The animals were provided by oral gavage, and phloridzin was injected subcutaneously twice daily just before each meal. Food intake was measured weekly as an oxidative stress marker.

**Animals**

The study protocol was reviewed and approved by the Animal Care and Use Committee of Ajinomoto Co Inc. Male GK rats (n=94) and male Wistar rats (n=5) were obtained from Charles River Japan (Yokohama, Japan) at the age of 6 weeks and housed in a polycarbonate cage with a wooden chip mat on the floor. Water was available ad libitum for all rats. Standard chow (22.6% protein, 53.8% carbohydrate, 5.6% fat, 6.6% mineral and vitamin mixture, and 3.3% fiber; total: 356 kcal/100 g) (CRF-1, Charles River Japan, Yokohama, Japan) was used for this study. The animal room was kept on a 12-hour reverse light/dark cycle (dark, 7:00 AM to 7:00 PM; light, 7:00 PM to 7:00 AM), at a constant temperature (22±1°C) and a relative humidity of 55±5% throughout the experimental period.

**Diet Restriction and Drug Administration**

Some GK rats (n=59) were trained to consume the diet chow in 1 hour, which was provided twice a day during the dark period (9:00 AM to 10:00 AM and 3:00 PM to 4:00 PM). We designated this group as the GK rats with restricted diet (RD group). Another group of GK rats (n=35) were fed ad libitum (AL group). The animals were acclimatized to laboratory conditions for approximately 2 weeks. At the age of 10 weeks, 11 rats each in the RD and AL groups were euthanized under anesthesia induced by intraperitoneal injection of sodium pentobarbital (50 mg/kg; Nembutal, Abbott Laboratories, Abbott Park, Ill). The remaining RD rats were divided into 2 groups and treated with either vehicle alone (0.5% methylcellulose, n=24; VC group) or an inhibitor of the sodium–glucose cotransporter phloridzin (100 mg/kg, n=24; PZ group). Methylcellulose was provided by oral gavage, and phloridzin was injected subcutaneously twice daily just before each meal. Food intake was measured weekly and body weight was measured at the age of 10, 16, and 22 weeks in each group. In addition to 10-week-old rats, 16-week-old rats (n=5 respectively), and 22-week-old rats (n=19 respectively) were also euthanized under anesthesia (supplemental Figure I, available online at http://atvb.ahajournals.org).

**Laboratory Data**

The plasma glucose level was measured by the glucose oxidase method using an autoanalyzer (model Dri-Chem 5500, Fuji, Tokyo, Japan). Plasma insulin was measured using an insulin ELISA kit (Morinaga, Takamatsu, Japan). Glycohemoglobin values were measured by ion-exchange high-performance liquid chromatography (23GHilV; TOSO, Japan). Total cholesterol (TC), nonesterified fatty acid (NEFA), triglycerides, and urine creatinine were measured by SRL Co (Tachikawa, Japan). Urine 8-hydroxydeoxyguanosine (8-OHdG) was measured by ELISA using anti–8-OHdG monoclonal antibody (8-OHdG Check; Japan Institute for the Control of Aging, Shizuoka, Japan). Urine 8-OHdG divided by urine creatinine was used as an oxidative stress marker.

**Oral Glucose Tolerance Test**

OGTT was performed in the RD group (before randomization), the AL group, and Wistar rats at the age of 10 weeks (n=5, respectively). Briefly, after fasting for 5 hours, 1 g/kg of glucose was administered by oral gavage. Blood samples were taken from the tail vein at 0, 15, 30, 60, 120, and 180 minutes for the measurement of blood glucose and serum insulin.

**En face Method for Optimal Observation of Endothelial Surface**

The rats in each group at the age of 10, 16, and 22 weeks (10-week-old rats: n=11 each for AL and RD; 16-week-old rats: n=5 each for AL, VC, and PZ; 22-week-old rats: n=11 each for AL, VC, PZ) were subjected to quantitation of monocyte adherence to the thoracic aorta by NEMOes, as described previously. For the detailed method used, please see the online data supplement.

**Real-time Quantitative RT-PCR**

Total RNA was extracted from abdominal aorta of each group (n=6 respectively) at the age of 22 weeks by using ISOGEN RNA extraction kit (Nippon gene) according to the instructions provided by the manufacturer. Then cDNAs were synthesized by using Superscript II RNase H Reverse Transcriptase and oligo-dT primers. The resulting cDNAs were amplified using SYBER Green PCR kit (Applied Biosystems, Foster City, Calif). Quantitative PCR was performed on an ABI PRISM 7700 sequence detection system (Perkin Elmer Life Sciences Inc). The relative abundance of mRNAs was calculated by the comparative cycle of threshold (Ct) method with β-actin mRNA as the invariant control. The primers used in this study are listed in supplemental Table I.

**Western Blotting Analysis**

Western blotting was performed using the tissue extracts from abdominal aorta of each group (n=4) and the antibodies used for NEMOes as described previously.

**Cross-Sectional Immunohistochemistry**

After recording the en face view, the specimens used for en face immunostaining were paraffin embedded and sectioned (4-μm thick) or embedded in Optical Cutting Temperature compound and sectioned (8-μm thickness) as described previously. Paraffin-embedded sections were used for immunostaining, as well as for staining with hematoxylin/eosin and phosphotungstic acid hematoxylin. Frozen sections were used for Oil Red O staining. For the detection of α-smooth muscle isoform of actin, peroxide staining was performed using anti-human smooth muscle actin (α-SMA) antibody (DAKO Japan, Tokyo, Japan) as described previously. For fluorescent staining, the sections were placed in citrate buffer in a microwave at 100°C for 3 minutes, and the sections were then blocked using 10% goat serum for 30 minutes at room temperature and incubated with monoclonal anti–8-OHdG antibody (Japan Institute for the Control of Aging, Shizuoka, Japan) at 37°C for 60 minutes. Then, the slides were incubated with goat anti-mouse IgG-conjugated with Alexa594 (Molecular Probes Inc, Eugene, Ore) for 30 minutes at room temperature. Nuclei were counterstained in 4’,6-diamidino-2-phenylindole (Vectashield Mounting Medium with DAPI; Vector Laboratories Inc, Burlingame, Calif). Fluorescence images of the slides were obtained using a Zeiss Axioskop 2 plus microscope (Carl Zeiss Japan Co, Tokyo, Japan) and were captured as digital images using Axiosvision 3.0 software.

**Morphometry**

To measure intimal areas of the thoracic aorta, 4 cross sections of each aorta spaced at ~4-mm intervals were stained with hematoxylin and eosin. The cross-sectional intimal areas of a lesion in a given photomicrograph were measured with an image analysis software (Image-Pro4.5; Planetron Co, Tokyo, Japan) by examiners blinded to the treatment regimen. The average intimal area was then calculated for each aorta. Aortas of 22-week-old rats in each group (n=11 respectively) were analyzed.

**Statistical Analysis**

All data were expressed as mean±SEM. All statistical analyses were performed with Stat-View version 4.5 (Abacus Concepts Inc, Berkeley, Calif). The unpaired Student’s t test and Fisher’s exact test were used to compare parametric data and nonparametric data, respectively, between 2 groups. When comparisons of more than 3 groups...
Results

Daily Blood Glucose Profile in Each Experimental Model
Supplemental Figure I shows the study design used in this study. Supplemental Figure II shows the results of OGTT after training of diet restriction for 2 weeks. Both the AL and RD groups developed mild fasting hyperglycemia and impaired glucose tolerance of similar severity. Next, to validate our experimental design, we checked the daily blood glucose and serum insulin profile in each group at the age of 10 and 22 weeks. At the age of 10 weeks, the AL group exhibited consistent hyperglycemia with consistent hyperinsulinemia. The VC group showed lower glucose level before feeding and a postprandial rise in blood glucose twice a day with the commensurate change of serum insulin level. Phloridzin almost completely suppressed the increase in blood glucose level after feeding with the decrease in insulin level (Figure 1). At the age of 22 weeks, the pattern of daily blood glucose level in each group was similar to that observed at the age of 10 weeks. However, the differences of daily insulin level among each group at the age of 22 weeks were smaller than that observed at the age of 10 weeks, probably because of the progression of insulin resistance and glucose toxicity in β cells (supplemental Figure III).

Metabolic Changes in Each Experimental Group
The amount of food intake in the AL group was significantly higher than that in the other groups (supplemental Figure IV). As a result, body weight gain in the AL group was significantly higher than in the VC and PZ groups (supplemental Figure IVB and IVC). The HbA1c level in the AL group was markedly higher than those in the other groups. The HbA1c level in the PZ group was the lowest among these groups (Table). We also measured urine 8-OHdG level as an index of systemic oxidative stress. Although we could not find significant differences among groups, mean 8-OHdG concentration in the AL group was the highest, whereas that in the PZ group was the lowest. The relative value of each group was almost comparable with that of HbA1c value (supplemental Figure V).

At the midpoint of this study (at the age of 16 weeks), we measured serum lipids in each group (Table). TC of the PZ group was significantly lower than other groups. NEFA was significantly higher in the AL group than in other groups.

Repetitive Fluctuation of Daily Blood Glucose Promotes Monocyte Adhesion to Endothelium
Next, we quantitated the number of monocytes adhering to the aortic endothelium of each group. As shown in Figure 2G and supplemental Figure VI, the number of monocytes increased with aging in all groups except for the PZ group. Strikingly, whereas the VC group showed lower HbA1c, the VC group exhibited a markedly elevated number of monocytes adherent to the endothelium than did the AL group in both branching site and nonbranching areas. Treatment with phloridzin markedly reduced the number of monocytes attached to the endothelium than did the AL group in both branching site and nonbranching areas. Treatment with phloridzin markedly reduced the number of monocytes attached to the endothelium and the number was less than that in the AL group (representative images are shown in Figure 2A through 2F). These results suggest that fluctuations in blood glucose concentrations provoke monocyte adhesion to the vascular endothelium. In addition, the number of adherent monocytes in the AL group was higher than the PZ group, whereas both the AL and PZ groups showed little fluctuation of daily blood glucose level. This result suggests that the degree of glucose exposure might be also 1 of the determinants of the number of monocytes adherent to the endothelium.

Increased Expression of CS-1 Fibronectin in Aorta Exposed to Repetitive Glucose Fluctuation
To search for the molecules involved in endothelial monocyte adhesion caused by fluctuation of blood glucose concentra-

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![Figure 1.](image-url) Daily blood glucose profile of each group. Mean blood glucose (A) and serum insulin (B) hourly profiles of the AL, VC, and PZ groups at 10 weeks of age (n=5 each).

<table>
<thead>
<tr>
<th>Age</th>
<th>AL</th>
<th>VC</th>
<th>PZ</th>
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<tbody>
<tr>
<td>HbA1c, %</td>
<td></td>
<td></td>
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<tr>
<td>10 wk</td>
<td>4.07±0.42</td>
<td>3.76±0.40†</td>
<td>3.73±0.32†</td>
</tr>
<tr>
<td>16 wk</td>
<td>5.85±0.17</td>
<td>3.90±0.03†</td>
<td>3.36±0.02†</td>
</tr>
<tr>
<td>22 wk</td>
<td>6.11±0.23</td>
<td>3.95±0.09†</td>
<td>3.45±0.04† ‡</td>
</tr>
<tr>
<td>TG, mmol/L</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>16 wk</td>
<td>0.74±0.11</td>
<td>0.66±0.05</td>
<td>0.52±0.05</td>
</tr>
<tr>
<td>TC, mmol/L</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>16 wk</td>
<td>3.21±0.10</td>
<td>3.05±0.06</td>
<td>2.61±0.05† ‡</td>
</tr>
<tr>
<td>NEFA, g/L</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>16 wk</td>
<td>0.0126±0.0007</td>
<td>0.0100±0.0007†</td>
<td>0.0077±0.0009†</td>
</tr>
</tbody>
</table>

Blood samples were taken 17 hours after fasting. Data are expressed as mean±SEM. †P<0.05 vs AL, ‡P<0.05 vs VC. TG indicates triglycerides.
tions, we investigated the expression levels of several factors using RNA isolated from the aorta of each group. Although it is widely recognized that intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 play major roles in monocyte adhesion to endothelial cells, we could not find changes of their mRNA expression levels among the groups (Figure 3). Next, we investigated their protein expression level. However, we could not observe the obvious differences of ICAM-1 and VCAM-1 expression in each group (supplemental Figure VII).

Among the factors investigated in the present study, we found the increased expression of connecting segment (CS)-1 fibronectin that can act as a monocyte ligand in the VC group. Furthermore, we also found the increased expression of very-late-acting antigen (VLA)-4, a counter-receptor for VCAM-1 and CS-1 fibronectin (Figure 3). Although the differences were not statistically significant, these data suggest that monocyte adhesion to the vascular endothelium stimulated by fluctuations in glucose concentrations might be associated with increased arterial expression of CS-1 fibronectin.

Fluctuations of Blood Glucose Promote Intimal Thickening of Rat Thoracic Aorta

As shown in supplemental Figure VIII A and VIII B, we frequently observed intimal thickening, including cells as a component in aorta of GK rats at the age of 22 weeks. Most of the cells located in this region were $\alpha$-SMA positive (supplemental Figure VIII C). We occasionally observed CD68-positive cells in the lesion (data not shown). Oil Red O staining showed that there were no obvious lipid deposition in such lesions (data not shown). However, increased oxidative stress was observed in cells which construct the lesion, by immunostaining with 8-OHdG (supplemental Figure VIII E through VIII J). Measurement of the intimal thickness in each group identified significantly larger intimal lesions (thickening) in the VC group than AL and PZ groups (Figure 4).

**Discussion**

Our aim in this study was to investigate the effect of glucose fluctuation per se on monocyte adhesion to vascular endothelial cells by comparing with that of consistent hyperglycemia with small repetitive fluctuations in blood glucose concentrations. Our data demonstrated that consistent hyperglycemia with hyperinsulinemia increases monocyte adhesion to endothelial cells, because the number of adherent monocytes in the AL group was higher than the PZ group. More importantly, our data demonstrated that the VC group showed circadian blood glucose fluctuation but, with lower total glycemic exposure, demonstrated markedly increased monocyte adhesion to endothelium compared with the AL group. Prevention of fluctuations in blood glucose concentrations by phloridzin...
efficiently reduced monocyte adhesion to endothelial cells to a level lower than that in the model of persistent hyperglycemia. Although our results suggest that exposure to consistent hyperglycemia modestly enhances monocyte adhesion to endothelial cells, fluctuations in blood glucose concentrations seem to have more deleterious effects on this process.

The exact mechanism of the deleterious effect of glucose fluctuations on atherosclerosis is unknown. However, recent studies demonstrated that intermittent hyperglycemia induces increased rate of apoptosis, oxidative stress, protein kinase C activation, and NAD(P)H-oxidase activation in cultured endothelial cells, which are more pronounced than those of persistent hyperglycemia.16,17 Although we could not find any increase in extracellular oxidative stress in the VC group as assessed by urinary 8-OHdG concentrations (supplemental Figure V), this does not completely rule out increased oxidative stress at arterial wall level. Indeed, we found increased intimal thickening in the VC group (Figure 4), and this abnormality was associated with enhanced local oxidative stress (supplemental Figure VIII).

An important component of monocyte adhesion to endothelial cells is the activation and upregulation of adhesion molecules on the endothelial surface. Whereas several studies reported the importance of ICAM-1 and VCAM-1 as adhesion molecules, the significant change of mRNA and protein expression of ICAM-1 and VCAM-1 were not observed in this study. A previous study demonstrated that chronic exposure of cultured endothelial cells to glucose did not induce endothelial cell surface expression of ICAM-1 and VCAM-1. Instead, they found an increase in the cell surface deposition of CS-1 fibronectin, which is a counter-receptor for VLA-4.18 Another previous work showed that the levels of VLA-4 are increased in the endothelium of human atherosclerotic lesions.19 Furthermore, blockade of VLA-4 with anti-α4 integrin antibodies completely inhibited monocyte adhesion induced by high glucose concentrations.20 Thus, the interaction of VLA-4 on monocytes with CS-1 seems to play a major role in monocyte adhesion to endothelial cells induced by hyperglycemia in vitro. On the other hand, our study design is completely different from the abovementioned studies. Our quantitative RT-PCR data showed increased CS-1 and VLA-4 mRNA expression levels in the thoracic aorta of rats exposed to repetitive fluctuation of glucose concentrations. Although the number of adherent monocytes in the AL group was higher than that in the PZ group, we could not find the difference of CS-1 and VLA-4 mRNA expression level. Thus, although our data only showed the association of their expression levels in the aorta and monocyte adhesion to endothelial cells, the interaction of VLA-4 on monocytes with CS-1 might be involved in monocyte–endothelial interaction promoted by glucose fluctuation in vivo not by sustained hyperglycemia in vivo. In addition, it is possible that the changes of the expression of several proteins in monocytes or structural changes or changes of cell surface expression of adhesion molecules without the changes of mRNA expression might be involved in this process. Thus, further assessment will be needed to complete our understanding of this process.

When estimating the nocturnal insulin level, we found that total serum insulin secretion in the AL group was higher than the VC group. However, we found the fluctuation of serum insulin level in the VC group. As same as the fluctuation of glucose level, the fluctuation of insulin level could promote the monocyte adhesion to endothelial cell. However, the fluctuation of insulin level is physiologically observed at least in human. In addition, we recently found that temporal hyperglycemia not temporal hyperinsulinemia induced the increased monocyte adhesion to endothelial cells.9 Furthermore, we observed that the treatment of the rats with restricted diet with exogenous insulin injection just before each feeding prevented the monocyte adhesion to endothelial cells (K.A. and H.W., unpublished observation, 2006). Thus, it is likely that the fluctuation of blood glucose not insulin induced monocyte adhesion to endothelial cells.

To investigate the role of glucose fluctuation on the progression of atherosclerosis, the model rats showing exaggerated glucose fluctuation were used in this study. In addition, this study was designed to set other risk factors comparable among each group. In fact, although we still find the significant differences of TC and body weight between the AL and VC groups, the absolute values of the difference were relatively small. Thus, whereas our data suggested that the AL group, with more weight gain and more atherogenic lipid profile, was less prone to the development of atherosclerosis, this does not mean that glucose fluctuation play more important role than other major risk factors on early stage of atherosclerosis.

In conclusion, we have demonstrated in the present study that fluctuations in blood glucose concentrations in rats enhanced both monocyte adhesion to the endothelium and intimal thickening in vivo. Furthermore, reduction of postprandial hyperglycemia effectively improved these changes. Although extrapolation of these animal results to diabetic patients is premature, the data suggest that inhibition of fluctuations in circadian glucose concentrations is a potentially important goal, along with reduction of HbA1c, in the prevention of cardiovascular disease in diabetic patients.

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Disclosures
None.

References
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Fig. I

A

Age (Weeks)
6  8  10  16  22

NEMOes

Quantitative RTPCR
Western Blotting

Ad lib Fed (AL)
Restricted Fed (RD)
Restricted Fed + Vehicle (VC)
Restricted Fed + Phloridzin (PZ)

Daily Feeding time of AL group
light  dark  light
7:00 9:00 10:00 15:00 16:00 19:00
Feeding

Daily feeding Protocol of VC and PZ groups
light  dark  light
7:00 9:00 10:00 15:00 16:00 19:00
VC: Vehicle
PZ: 100mg/kg PZ

VC: Vehicle
PZ: 100mg/kg PZ
Fig. II

B

Blood Glucose (mg/dl)

Blood Insulin (ng/ml)

min

Wister
AL
RD
Fig. IV

A

Diet Intake (g/day)

Weeks of age

B

Body Weight (g)

Weeks of age

C

Body Weight gain (g)

10 to 16 weeks
16 to 22 weeks

AL
VC
PZ

AL
VC
PZ
Fig. V

8OHdG (ng/mg Creatinin)
Fig. VI

![Bar chart showing cell counts per mm² for AL, VC, and PZ at 10w, 16w, and 22w.](image)
Fig. VII

A

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B

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<td>VCAM-1</td>
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Fig. VIII

A

B

C

D

E

F

G

H

I

J