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Leukocyte Adherence in Arterioles following Extravascular Tissue Trauma

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To study the effect of tissue injury on leukocyte adherence in arterial microvessels, small regions of tissue adjacent to vessels in the wing of the bat were exposed to single doses of laser irradiation. The experimental design provided data on the effect of (1) tissue injury of variable extent but a fixed distance from the arterial vessel and (2) tissue injury of a constant extent but at varying distances from the arterial microvessels. Preirradiation controls showed that normally no leukocytes could be observed either adherent to, or rolling along, the vessel wall. Following injury and after a quite variable latency time there was a time-dependent increase in the number of observed leukocytes. Using the latency time as an index, an analysis was applied to test the hypothesis that the laser irradiation released some substance which subsequently diffused to the vessel wall. The results of this analysis have shown that all the experimental latency time data can be explained on the basis of a model in which a diffusable "leukotactic" substance is released from the laser site in an amount that is proportional to the surface area of induced injury. Though the precise nature of the released substance is speculative, analysis shows that the process is characterized by an effective diffusion coefficient of approximately 7.0×10^{-7} which is suggestive of high-molecular-weight substance.

INTRODUCTION

The adherence of leukocytes to the endothelium of microvessels is a commonly described hematological response by investigators who directly observe the microvasculature in the living animal. Described by Dutrochet (1824), the leukocyte sticking phenomena and in some instances the emigration to adjacent tissue is now considered an initial hallmark of an inflammatory process. Though the significant events which are part of this process are lucidly described in the now classic works of Cohnheim (1867), Metchnikoff (1893), and the Clarks (1935), the underlying mechanisms responsible for the initial leukocyte adherence is unknown. Recent methods employed to study this problem *in vivo* (Atherton and Born, 1972, 1973) have shed some light on the dynamics of this process but are complicated by the fact that in most preparations in which the microvessels are visible for microscopic study the associated surgery is itself sufficient to promote adherence. Thus the very initial events have been difficult to quantify. One preparation which may be an exception to this is the rabbit ear chamber used by Allison *et al.* (1955) to study and describe events associated with acute inflammation induced by local tissue heating. They report that the early sticking of leukocytes in response to the

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thermal stimulus had a latency period of from 10 to 15 min. Further they point out that such a long latency period would be inconsistent with a postulated mechanism which would attribute intravascular wall adherence to altered endothelial surface charge (Sawyer *et al.*, 1953). Since it is felt that the time element is crucial in assessing the viability of this, as well as alternative hypotheses, the present study was initiated to determine the latency time between laser-induced tissue injury and the onset of the leukocyte–vessel wall interaction process in arterioles in the wing of the small brown bat.

METHODS—EXPERIMENTAL

Animal Preparation

All experiments were performed on the wing of the little brown bat (*Myotis lucifugus*). The procedure for preparing the wing for microscopic observation is similar to that described previously (Wiedeman 1973). Briefly, the unanesthetized animal was placed in a tubular chamber with its wing extended over a large optical flat and secured in position using small spring-loaded clips. To enhance visibility a small amount of mineral oil was put on the under surface of the wing and a thin layer spread on the wing top surface. The animal was then placed on the stage of a trinocular microscope. To the vertical barrel of the microscope was attached a pulsed ruby laser from which a single 200 μsec pulse could be delivered to the preparation through the microscope optics. In addition a closed circuit TV (CCTV) was coupled to the laser so that the tissue and vessels could be seen on a CCTV monitor and also tape recorded.

Introduction of Leukocyte Adherence

A first-order branch of the main artery entering the wing was observed using a magnification of 900× for periods of up to 45 min to determine control levels of rolling or adhering leukocytes. Following this control period the microscope stage was moved perpendicular to the arterial vessel under observation and a single laser pulse was delivered to the tissue. Immediately after the laser irradiation, the stage was moved back to its original position and subsequent events were monitored directly and via the CCTV system. Only one irradiation was made for each animal used in the main study (14 animals).

Indices of Leukocyte Adherence

The principle index used to characterize changes in leukocyte adherence was obtained by the measurement of the number of leukocytes observed rolling along the wall of the vessel under observation. The number observed per minute at a fixed observation site is referred to as leukocyte flux. The change in leukocyte flux was monitored immediately after the laser irradiation for periods of time ranging from 30 to 80 min. From these measurements the change in leukocyte flux as a function of time and the time delay between the laser irradiation and initial appearance of rolling or adhering leukocytes (latency time) was determined. In some experiments the average residence time of the rolling leukocytes was determined by measuring their transit over a known axial distance and this time was used as an additional index of changes in leukocyte–vessel wall interaction. This residence time is in effect the reciprocal of the average leukocyte velocity.

Laser Energy Observed

In all experiments the input energy to the laser was maintained at 170 J. The total output energy from the microscope objective was measured in three animals using a substage thermopile and an energy density meter, and found to be approximately 10 mJ. By comparing the energy received at the substage thermopile with and without the batwing interposed, the energy absorbed by the wing was determined to be approximately 2 mJ.

Extent of Tissue Injury

The extent of tissue injury caused by the laser was estimated by measuring the area of frank destruction to the epithelium. The boundaries of this region were quite clear since the light transparency was much greater than in adjacent normal tissue. Fifteen laser irradiations of the wing epithelium on randomly selected sites in a single bat wing indicated reasonable uniformity and an average injury radius of 85.6 μm.

METHODS—ANALYTICAL

Calculation of Temperature Rise at Vessel

Although it is possible to calculate the effective damage radius when the absorbed energy is known, due to variability of wing pigmentation between animals the precise amount of energy absorbed is variable. Thus the injury radii and the surface area of damage were not always uniform even with the same irradiating dose. To calculate the maximum temperature rise of a vessel at a distance r from the center of the injury region of radius a, it was assumed that the initial temperature of the injury region (T_0) was uniform at 100°C. For such an initial temperature distribution it can be shown (Carslaw and Jaeger, 1959) that the temperature T at a distance r in a medium with thermal diffusivity σ is given by Eq. (1).

$$T = \frac{T_0}{2\sigma t}\, e^{-r^2/4\sigma t} \int_0^a e^{-r_1^2/4\sigma t}\, I_0\left(\frac{r\, r_1}{2\sigma t}\right) r_1\, dr_1. \qquad (1)$$

When utilized, Eq. (1) is evaluated numerically in accordance with Masters (1955).

Calculation of Diffusion Coefficient

To test the hypothesis that a leukotactic substance released from the irradiated tissue might be responsible for the observed leukocyte sticking, a calculation of the required diffusion coefficient necessary to account for the measured latency time was made. Using latency time data obtained at equal vessel-injury site distances, the diffusion coefficient was calculated with the assumption that (1) the amount of substance released at the irradiated tissue site was proportional to the surface area of injury and (2) the threshold concentration at the vessel wall for leukocyte adherence was constant. Using the following definitions, the diffusion coefficient (D) was calculated as that value of D which satisfied Eq. (2) which represents a simultaneous application of the standard one-dimensional diffusion equation (Carslaw and Jaeger, 1959).

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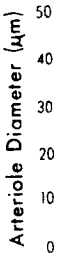

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A_1, A_2 = two different areas of injury at the same distance (r) from the vessel wall
δ_1, δ_2 = corresponding measured latency times
C_0 = threshold of leukotactic substance
K = constant of proportionality linking amount of substance released to surface areas of injury.

$$\frac{C_0}{K} = A_1 \operatorname{erfc}\left(\frac{r}{2\sqrt{D\delta_1}}\right) = A_2 \operatorname{erfc}\left(\frac{r}{2\sqrt{D\delta_2}}\right). \tag{2}$$

Since C_0 and K are assumed constant, the unknown quantities (A, δ) in the right-hand members of Eq. (2) may be evaluated separately in two independent experiments. In one of these values A_1 and δ_1 are determined for a given value r. A second experiment will yield corresponding values for A_2 and δ_2. A simultaneous solution of these two equations will yield the value of D the diffusion coefficient. When this procedure is applied to all combinations of paired surface areas of injury one may then obtain an average diffusion coefficient. Armed with this information the latency time (δ_i) for any combination of measured surface area of injury (A_i) and vessel-injury site distances (r_i) is then calculable from Eq. (3) below in which the quantity erfc^{-1} denotes the argument of the complementary error function:

$$\delta_i = \left[\frac{\frac{r_i}{2D^{1/2}}}{\operatorname{erfc}^{-1}\left(\frac{C_0/K}{A_i}\right)}\right]^2. \tag{3}$$

RESULTS

1. General Description of Vascular Events following Tissue Radiation Vessel Diameter

One common event seen following the laser irradiation was an almost immediate change in the arteriole diameter. The most frequent effect was vasoconstriction as shown in the top part of Fig. 1, but occasionally vasodilation was seen as shown in the top part of Fig. 2. Following these initial diameter changes the

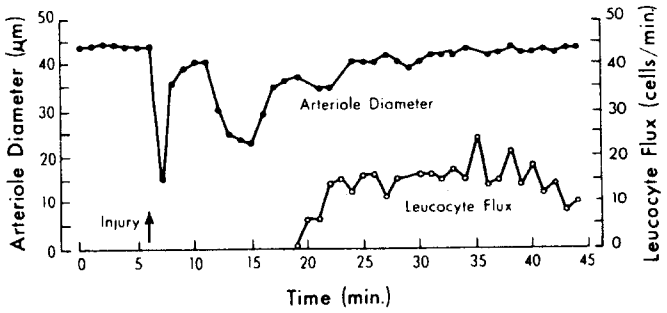

FIG. 1. Arteriole diameter and leukocyte flux following laser injury. The arteriole initially constricted then after several damped oscillatory cycles its diameter returned to control. Leukocytes were detected 13 min after injury and the luekocyte flux rose rapidly and remained elevated.

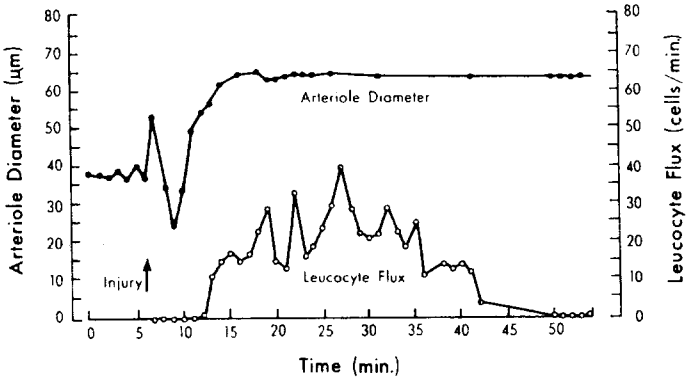

FIG. 2. Parameters shown are the same as in Fig. 1. In this example the arteriole initially dilated in response to the laser injury then constricted and finally dilated. Leukocytes were detected 6 min after injury and the leukocyte flux rose rapidly, peaked, and returned to the zero control value 38 min after the initial rise.

subsequent time course was nonuniform. Vessel diameter sometimes returned to its control value as shown in Fig. 1, or as shown in Fig. 2, after a transient contraction, the vessel relaxed to reach a steady state diameter as much as 75% larger than control.

Leukocyte adherence. Quite independent of the initial diameter response, leukocytes were observed to become transiently adherent to the vessel wall following the laser injury to the adjacent tissue. The latency time which represents the time delay between the laser irradiation and the first appearance of adherent leukocytes was variable and ranged from 15 sec to approximately 15 min. Once initiated the number of observed rolling leukocytes increased with time, peaked, and as shown in Fig. 2, returned to the zero control value or in some cases remained elevated as shown in Fig. 1.

In addition to the increase in the number of leukocytes becoming adherent (leukocyte flux) a time-dependent change in the average residence time of these rolling leukocytes was often found. As shown in Fig. 3, the change in residence time closely parallels the change in leukocyte flux. In this example both leukocyte flux and residence time are seen to first increase and then decrease in the presence of a continuously decreasing arteriole diameter. The close relationship between

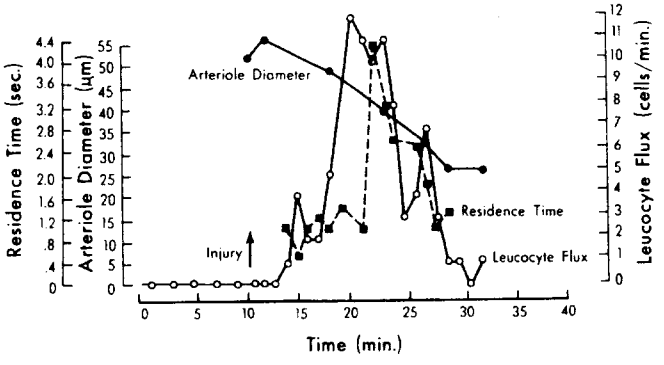

FIG. 3. Temporal relationship between arteriole diameter, leukocyte flux, and average leukocyte residence time in response to laser injury. Residence time is defined as time required for the leukocyte to transit a fixed axial distance (70 μm). Note that in this example leukocyte flux and resistance time have a similar temporal pattern.

the leukocyte flux and residence time is not, however, always obtained. Thus in experiments in which a laser stimulus was used in the presence of preexisting tissue trauma (denuding of the epithelium) the now nonzero control leukocyte flux was not changed by the laser but the mean residence time of these rolling leukocytes was significantly increased as illustrated in Fig. 4.

2. Latency of Leukocyte Response

To focus on the initial events of leukocyte adherence two aspects of latency time were studied: (1) its dependence on the distance between the site of tissue laser irradiation and the arteriole under observation and (2) its dependence on the extent of tissue injury.

Dependence on the extent of tissue injury. In seven animals the laser injury was produced at a distance of 110 ± 10 μm from the adjacent arteriole. Due to variability in wing pigmentation from animal to animal the same incident laser energy resulted in varying degrees of frank tissue injury and ranged from 4,000 to 24,000 μm^2 of wing surface. Because of this it was possible to determine the relationship between the extent of injury surface area and the latency time of leukocyte adherence in response to this injury. This relationship is displayed in Fig. 5. The data points labeled experimental are those obtained by direct measurement of the latency time and each data point represents a determination from a different animal. These data show that the latency time is inversely related to surface area of injury and for the same vessel-injury site distance (in this case 110 ± 10 μm) ranged from 15 to 760 sec. In addition to the experimental data, Fig. 5 displays a plot of Eq. (3). Since Eq. (3) is based upon an assumed model to describe the leukotactic process, the significance of these calculations will be deferred until the model and its formulation is discussed below.

Dependence on vessel-injury site distance. Using an additional seven animals latency time data were obtained for a uniform surface area of injuries (19000 ± 200 μm^2) at different vessel-injury site distances ranging from 80 to 360 μm. The results obtained are plotted in Fig. 6 in which each solid point represents a determination in one animal. As may be seen the latency time is a nonlinear

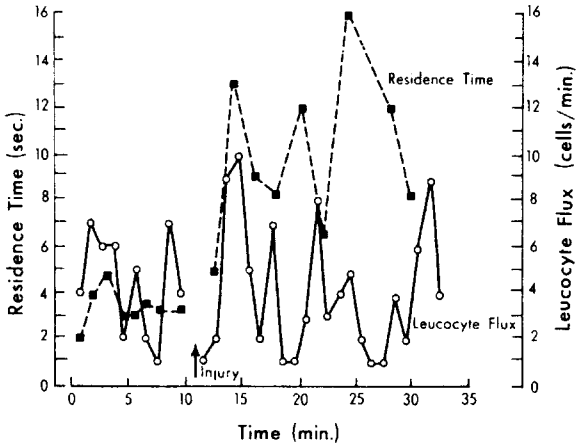

FIG. 4. Effect of laser injury on leukocyte flux, arteriole diameter, and residence time when control values were made different from zero. Laser injury did not significantly alter the average flux but did increase the residence time.

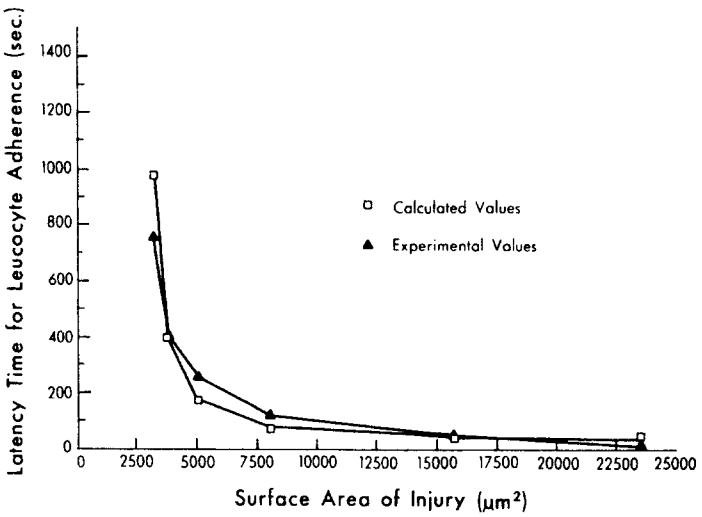

FIG. 5. Latency time for leukocyte response as a function of the surface area of laser-induced tissue injury. Calculated values are those obtained using the analytical model shown in Fig. 7 and calculated from Eq. (3). Vessel-injury site distance is 110 ± 10 μm.

function of the vessel-injury site distance with a range of 13–780 sec corresponding to the minimum and maximum distances. The solid line is a plot of Eq. (3) and is discussed in the next section concerned with the model of the leukotactic process.

3. *Analytical Model*

The essential details of the model used to analyze and interpret the experimental latency time data are summarized in Fig. 7. The model is formulated to test the hypothesis that the observed leukocyte–vessel wall interaction is mediated by the diffusion of some undefined leukotactic agent released at the injury site within the tissue. Two important assumptions are made: (1) that the amount of substance released at the injury site is proportional to the surface area of injury and (2) that once this substance diffuses to the vessel wall there is a fixed threshold concentration (C_0) of diffusing substance which is necessary to cause adherence. This threshold level is assumed constant. For the thin tissue of the wing surface the concentration of such a substance both as a function of position (r) and time (t) can then be expressed according to diffusional mathematics as being proportional

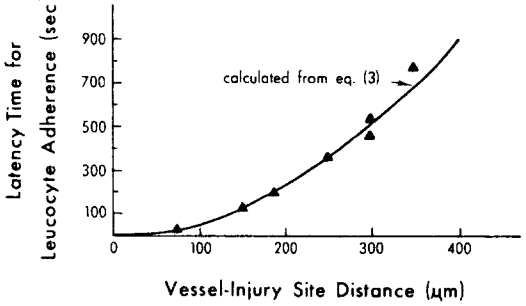

FIG. 6. Latency time for leukocyte response as a function of the distance between the vessel wall and the closest point on the surface of injury. Solid triangles correspond to experimental values. The solid line is determined from the analytical model summarized in Fig. 7 and calculated from Eq. (3). Data corresponds to a tissue injury radius of 78 ± 7 μm.

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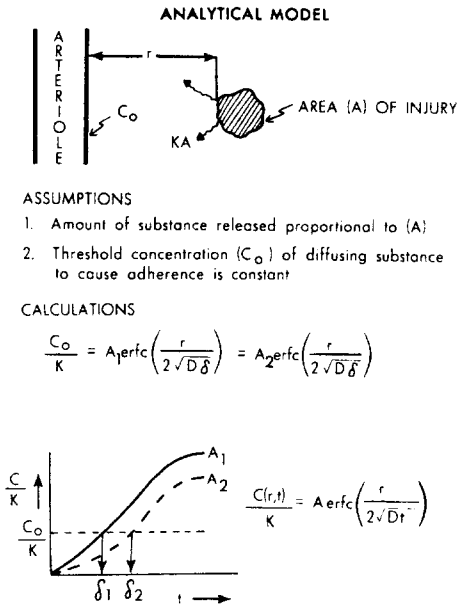

FIG. 7. Analytical model to test the hypothesis that latency time data can be explained on the basis of diffusion from the tissue injury site to the arteriole wall. Calculated latency times are based on this model and calculated from Eq. (3).

to the error function of the argument $r/(Dt)^{1/2}$. The intrinsic consequence of this model as illustrated by the two curves labeled A_1 and A_2 in Fig. 7 is as follows. At any fixed vessel-injury site distance (r) a larger surface area of injury (A_1) will reach the threshold concentration sooner than will a smaller surface area of injury characterized by the dash curve labeled (A_2). As a consequence the larger surface area of injury A_1 will result in a smaller latency time (δ_1) as compared to a larger latency time characterized by (δ_2) for A_2.

As may be noted from Eq. (2) (also shown in Fig. 7) if a fixed distance r is maintained for two different surface areas of injury (A_1 and A_2) and two corresponding latency times (δ_1, δ_2) are measured, then it is possible to calculate the effective diffusion coefficient (D) without knowledge of either the constant of proportionality (K) or the threshold concentration (C_0). The calculated average diffusion coefficient using Eq. (2) was 7.0×10^{-7} CGS units. Using this value for the diffusion coefficient, Eq. (2) may be rearranged in the form of Eq. (3) to calculate the latency time associated with any vessel-injury site distance r_i and surface area of injury A_i. The results of these calculations for a vessel-injury site of 110 ± 10 μm is shown in Fig. 5 together with the experimental data points.

If instead of using a constant r in Eq. (3), r is treated as a variable and the surface area of injury is held constant (and equal to measured values) then the calculated latency times can be compared with those measured at different vessel-injury site distances. The result of this calculation produces an empirical formula which relates latency time to vessel-injury site distance. This relationship is graphed in Fig. 6. The latency times calculated for variable surface areas of injury at fixed vessel-injury site distances and those calculated for fixed surface area of injuries and variable vessel-injury site distances are consistent with the premise that an assumed leukotactic substance diffuses from the laser injury site in a manner characterized by Eqs. (2) and (3).

DISCUSSION

From their studies on amphibians and in the rabbit ear chamber, the Clarks (1935) concluded that leukocyte sticking to the wall of a blood vessel was not in itself a pathological process. It appears that this conclusion was based in part on the frequency with which this phenomena was observed and on the relatively slight stimuli required to produce it. Grant (1973) has suggested that perhaps all preparations employed in the study of leukocyte sticking had a built in artifact which renders leukocyte sticking omnipresent in "normal" circumstances. In the present study the use of the bat wing preparation which requires neither anesthesia, surgical intervention, nor vessel exposure shows that leukocyte sticking is in fact an extremely rare occurrence in arterial vessels in the absence of trauma. However, as shown by the present work, following extravascular trauma in the form of laser irradiation, leukocytes do become adherent to vascular endothelium with a latency time that depends on the distance from the injury site to the vessel and on the extent of the injury. One of the implications of this finding is that intravascular adherence can and is induced by extravascular stimuli. Prior to the onset of the leukocyte reaction there was invariably observed a change in arteriolar diameter following the laser irradiation. The mechanism responsible for this change in diameter is speculative and may have occurred due to transient temperature changes at the vessel, or possibly to the pressure impulse induced by the laser irradiation being transmitted to the vasoactive segment of the vessel. However, since initial leukocyte adherence was observed following laser-induced vasoconstriction and vasodilation the present findings support the view originally advanced by Allison *et al.* (1955) that vasodilation is not a required forerunner of leukocyte adherence. However, it is likely that changes in vessel diameter will affect the adherence process due to alterations in local hemodynamics (Mayrovitz *et al.*, 1977a,b, 1979).

Because the preirradiation levels of observable leukocytes were sensibly zero it was possible to characterize the latency time of the leukocyte response quite accurately. Latency times spanned a wide range but were frequently below the 10 min figure reported by Allison *et al.* (1955) for thermal injury in the rabbit ear chamber. In view of this finding it appears that whatever the mechanisms responsible for altering the mutual interaction between leukocytes and vessel wall are, they can act rapidly. Therefore, although the present experiments offer no specific evidence supporting any particular hypothesis concerning the basic mechanisms, it would appear unwarranted to exclude rapidly acting mechanisms such as altered endothelial surface charge to account for this initial leukocyte adherence.

Since the laser irradiation causes local thermal effects it is necessary to consider briefly the possible role of thermal factors in mediating the observed leukocyte response. The dependence of leukocyte adherence on thermal factors has long been recognized. In 1935 the Clarks increased the temperature of the rabbit ear chamber for a period of 1.5 hr in the range of 100–114°F and found an increase in leukocyte adherence following removal of the thermal source over a period of about 2 hr. However, in these experiments it is unclear whether the leukocyte effect was produced directly by the thermal source on the vessels or possibly indirectly via an acceleration of a biochemical process within the tissue itself. Allison *et al.* (1955) using a more intense and more localized thermal stimulus for a

shorter period of time in the rabbit ear chamber found an increase in leukocyte adherence but also produced platelet sticking and aggregation indicative of vessel injury. Further, the magnitude of the stimulus they used was sufficient to produce total occlusion of flow in the entire chamber for a period of some minutes following application of the thermal source. Contrastingly in the present study where the thermal energy was supplied over a period of a few hundred microseconds (as compared with 15 sec for Allison and 1.5 hr for the Clarks) and over a small segment of tissue (50 μm as compared to Allison's 500 μm) there was no platelet aggregation or observable platelets in the vicinity of the vessel wall. Further, calculations of the maximum temperature rise (Eq. (1)) substantiated by measurements on the wing surface using fast reacting thermal sensors show that for the smallest injury site-vessel distance the maximum temperature rise at the vessel was less than 3°C. Thus it is felt that thermal factors are not the primary mediator of the leukocyte dynamics observed in the present experiments.

A more tenable hypothesis to explain the intravascular leukocyte adherence induced by focal tissue injury states that the vascular process is mediated by the diffusion of a leukotactic substance released at the injury site. From a qualitative as well as quantitative point of view this hypothesis is supported very well by the calculated latency times both for variable distance between injury site and vessel as well as variable surface areas of injury at fixed distances from the vessel under observation. The assumptions made in order to carry out the calculation, namely, that the amount of substance released at the injury site is proportional to the surface area of injury and the presence of a threshold concentration at the vessel necessary to cause adherence, are open to criticism but appear to be quite reasonable. One possible difficulty in the calculation of the effective diffusion coefficient for the leukotactic substance is that the effects of restricted diffusion, if present, have not been fully taken into account. This would include diffusion from the site of injury through the vessel wall to the site of principal activity. Thus if these regions are associated with significant nonuniformity in diffusional properties for the leukotactic substance, the calculated diffusion coefficient must be viewed as an effective diffusion coefficient. Specifically, if diffusion through the vessel wall is necessary for leukocyte adherence, the calculated diffusion coefficient will differ from that obtained in free diffusion by an amount which depends on the extent to which the vessel wall represents a threshold barrier. If the time required for the diffusing substance to cross the wall and reach threshold concentrations is small compared to the time to diffuse from the injury site to the wall, then the wall effect is negligible. Since the minimum latency time found in the present experiments was approximately 15 sec the characteristic diffusion time for transport across the vessel wall must be less than this value and would appear not to alter significantly the diffusion coefficient calculation. However, since neither the intravascular threshold concentration nor extravascular concentration of leukotactic substance is known as yet, no definitive statement can yet be made on this point.

ACKNOWLEDGMENTS

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