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Stochastic aspects of leukocyte transit in hamster cheek pouch arterioles

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Abstract. To clarify the dynamics of leukocyte delivery to the capillary network we measured leukocyte velocity (Vwbc), leukocyte flux and the intra-arrival times between successive leukocytes in terminal arterioles of the cheekpouch of eight hamsters. The sequences, generated electronically when fluorescent leukocytes passed a fixed point in the arteriole, were analyzed as stochastic point processes. Results showed that six sequences were stationary, and two with a linear trend in Vwbc, were not. Tests performed on the stationary sequences indicated conformity to a renewal process in all cases. Analysis of the distributional and sequential properties of the sequences indicated a good fit to a Poisson process. Overall these results show that leukocyte delivery to the capillary network can be characterized as a Poisson process if Vwbc remains relatively constant, if not, a non-homogeneous Poisson process is a more suitable model, with Vwbc being the explanatory variable. The demonstration of the applicability of these models may lead to a more complete understanding of both the physiological and patho-physiological dynamics of leukocytes within the microvasculature.

Introduction

Circulating leukocytes normally pass through capillaries without apparent functional impairment to the microcirculation. However, due to the size [31, 35] and stiffness [3, 10, 37, 42] properties of a subpopulation of the leukocytes there is a transient retardation of their transit [1, 2], and thereby the flow through the capillaries in some tissues. This retardation partially accounts for the to-and-from character of capillary flow as well as other features of capillary flow temporal and spatial variability. When the motion of ones own leukocytes in retinal capillaries are perceived via the blue field entoptic phenomenon [33, 40], one is impressed with the appearance of a random pattern with which the flowing leukocytes enter the various capillary pathways. Entry of the leukocytes into the capillary network is most directly dependent on the flux of these cells in the supplying terminal arteriole. Experimental observations reveal that leukocyte movement in the

arterioles [29, 36] and their entry into the capillaries is indeed a time dependent process influenced by a variety of factors [7, 22, 30, 39]. However, little is known concerning the underlying processes which give rise to these normally occurring dynamics. Such information would not only impact on our understanding of normal microcirculatory physiology but may also clarify features associated with abnormal conditions. Circulating leukocytes that undergo structural, mechanical or functional changes may become entrapped within the microvasculature and significantly effect the outcome of a variety of pathological conditions including hemorrhage [4–6, 43], tissue ischemia [17–19, 32] and lung injury [8, 21, 23].

In the present paper we attempt to penetrate the complex question of leukocyte movement within the microvasculature by first measuring the single file flow of leukocytes in the supplying arterioles of the hamster cheekpouch and then analyzing the leukocyte sequence as a stochastic point process. Our purpose is to characterize the statistical properties of leukocyte delivery into the capillary network and thereby gain basic information on the nature of this dynamic process.

Methods

Experimental preparation

The cheekpouch microvasculature of eight Syrian golden hamsters weighing 90–120 g (Charles Rivers Laboratory) were prepared for microscopic observation and studied as previously described [29]. Each animal was initially anesthetized with pentobarbital (0.6 ml/100 g i.p.) and supplemental doses given as necessary. The animal was secured to an observation board in a supine position and core body temperature was maintained by a heating mat throughout the surgical procedure as well as during the observation period to follow. A tracheal cannula (PE-200) was inserted to ensure patent airway during the experiment. The left femoral vein was cannulated (PE-20 tubing pulled to the desired diameter) and the patency of the femoral vein was maintained with heparinized Normosol (isotonic saline with pH 7.4). Following the surgical procedure the animal was transferred to the stage of a Laborlux 12 HL Leitz fluorescence microscope equipped with a 50-W ultrahigh-pressure mercury lamp used as an epiilumination system.

Most measurements were made using a 50 × water immersion objective with numerical aperture 1.00. Occasionally a 32 × long-working dry objective was used to get a larger view of the field. A 5 × or 10 × ocular was used in the trinocular tube of the microscope for the purpose of obtaining video

information. To this end an MTI-65 low-light level video camera was attached to the trinocular tube with an adapter which permitted rotating the camera and its projected image 360 degrees. The video image was recorded on a 3/4″ JVC video recorder and displayed on a 19 inch TV monitor.

Selection criteria for the arteriolar vessels was determined by three factors; (1) the diameter of the vessel was within a range of 8–12 microns; (2) each of the vessels chosen for observation showed single-file leukocyte flow, and (3) the vessel had no branches within the segment of interest. The choice of the 8–12 μm diameter range was based on previous *in vivo* measurements of the rodent leukocyte size distribution [31].

Acridine orange (50 μg/ml filtered and pH balanced to 7.4, Aldrich Co.) was infused via the cannulated femoral vein at a constant rate of 0.00866 ml/min (Harvard apparatus pump, Model 902). Leukocytes stained with the acridine orange and brilliantly fluorescing, were observed travelling along the arteriole. Data was recorded through the video system for periods not less than 10 min.

Data acquisition

The parameters measured were; (1) arteriole diameter D_a, (2) leukocyte velocity V_{wbc}, (3) time intervals between successive leukocytes x_i (intra-arrival times) and (4) leukocyte flux (Φ) (number of leukocytes in contiguous 10 second intervals). Diameter measurements were taken at several sites along the vessel through a reticule in a 16× ocular previously calibrated with a stage micrometer. V_{wbc} was obtained by superimposing two video cursors, sufficient in width to cover the vessel diameter, on the video image axially separated by a known distance. As the leukocytes passed each cursor, a change in the optical density was recorded and displayed onto a dual-channel chart recorder. Each cell's passage by the cursors generated clearly discernible signal peaks on each channel of the recorder, one representing the upstream passage and the other its downstream passage. The distance between the cursors divided by the transmit time yielded V_{wbc}. The time interval between the arrival of successive leukocytes at a given cursor site (x_i) was determined from the chart recording. The number of cells in each 10 second consecutive interval was counted and defined as leukocyte flux.

Analysis procedures

Stationarity

We treat the time intervals x_i between successively arriving leukocytes as random variables which we hypothesize to define a stochastic point process.

A stochastic process may be thought of as a family of random variables that describes the evolution through time of some process [34]. A stochastic point process is one such process consisting of a series of events which are distinguishable one from the other, only by their placement in time. The thrust of this work was to identify the specific process which characterizes the sequential leukocyte flow through the terminal arterioles.

Since we are characterizing a univariate series of events, the x_i completely characterize the transit of leukocytes in the observed arterioles. These x_i are considered to be drawn from some underlying probability distribution and if it is found that the distributions, along with its parameters, do not depend on the time when the sequence was observed, the stochastic point process may be viewed as stationary. Tests herein employed determine whether the process of leukocyte passage is stationary in the wide-sense by testing for trends in the series of x_i. To reach a conclusion of wide-sense stationarity requires only the demonstration that the first and second-order properties of the process are invariant under a time shift. In the present case these time shifts corresponds to the intervals between adjacent and successive pairs of x_i. The test procedures used are the Wald-Wolfowitz runs test, regression analysis and the Cox-Lewis U-statistic [25].

The test statistic for the runs test is computed by counting the number of "runs" above and below the median value of the x_i. For large sample sizes, the test statistic approximates a standard normal variate and is tested against the null hypothesis of no trend in the process. Least squares regression analysis was also performed on the data using a log transformation of the x_i [15], and testing for a trend in the rate of occurrence of x_i over time. For convenience, the logarithmic transformation was performed on sums of eight or 20 contiguous x_i (depending on the data) and used as the dependent variable in the regression analysis. Results are reported as Pearson's r.

A more efficient test for stationarity in the series of x_i can be accomplished by using Cox and Lewis' U-statistic. This procedure assumes a Poisson model and tests for a trend in the rate of occurrence represented by a smooth change in time. Since the primary objective of this study is to characterize leukocyte transit as a point process, testing against the assumption of the Poisson model yields a more efficient test. In this method, instead of the rate parameter, λ (mean x_i), in a Poisson process being assumed constant in time, it is assumed to have the functional form

$$\lambda(t) = e^{\alpha+\beta t}$$

where α is a scale factor and $\beta = 0$ is the null hypothesis tested against $\beta \neq 0$ [25]. The U-statistic can only detect a linear form of time-dependency

based on independent and exponentially distributed x_i, a basic assumption of the Poisson process. If the null hypothesis is rejected, we are not just rejecting stationarity but more specifically we are rejecting a stationary Poisson process. These tests for stationarity are essential in further analysis of the process. If stationarity cannot be established, then a non-homogeneous (time-dependent) stochastic point process is inferred. The simplest generalization of the stationary Poisson process is the non-homogeneous Poisson process in which the rate is a function of time. This is sometimes due to the rate being a function of some observed explanatory variable v(t) which is a function of time [16].

Renewal process

A stationary series of events in which the times between events (x_i) are statistically independent is a renewal process for which there is some cumulative distribution, F(x). Tests for a renewal process are essentially those which establish the independence of the x_i. Primarily we are concerned with the serial correlation coefficients (ρ_i) which characterize the second order, joint probabilities of the intervals between leukocyte arrivals, and tests based on estimates of the power spectral density (periodogram). The serial correlation coefficients are the Fourier coefficients of the spectral density function which provides a means of testing for the presence of serial correlation provided the marginal distributions of the x_i are not too highly skewed [25]. The expected value of the serial correlation should be approximately zero if the x_i come from a renewal process. Tests on individual correlation coefficients are based on the result that the estimates can be considered to follow a normal probability distribution. This test is based on the fact that the periodogram is constant for renewal processes. However, we can test the null hypothesis of a constant spectral density function by the (K-S) test [26]. Here we use the real and imaginary coefficients of the periodogram that have been determined by the finite Fourier transform of the x_i and test a variate (C_j) [24], which represents the order statistics from a uniform distribution, for uniformity. This test is, in general, more exact and probably more powerful than the test based on the estimated serial correlation coefficients [26].

Poisson process

A Poisson process is a special case of the renewal process in which the cumulative distribution function is given by $F(x) = \text{prob}(X \leqslant x) = 1 - e^{-\lambda x}$. The Poisson process has, in addition to the requirements of a

Table 1. Descriptive statistics

EXP	D_a (μm)	T_{obs} (s)	WBC (#)	x_i (s)	V_{wbc} ($\mu m \cdot s^{-1}$)
1	11.2	911	360	2.5 ± 2.8	594 ± 83
2	11.2	622	1224	0.5 ± 0.5	1009 ± 136
3	12.0	854	556	1.5 ± 1.5	168 ± 26
4	8.0	662	380	1.7 ± 1.6	623 ± 109
5	8.0	720	385	1.8 ± 2.1	905 ± 239
6	10.7	900	743	1.2 ± 1.3	497 ± 57
7	10.0	450	460	1.0 ± 1.0	620 ± 137
8	10.4	726	1048	0.7 ± 0.7	1574 ± 215

EXP = experiment, D_a = arteriole diameter, T_{obs} = duration of arteriole observation, WBC = total number of white blood cells consecutively counted during T_{obs}, x_i = Mean intra-arrival times ± standard deviation, V_{wbc} = Mean velocity of the white blood cells ± standard deviation during T_{obs}.

renewal process, the requirement that the number of events in any set of non-overlapping intervals be independent random variables with a Poisson distribution and the intervals between events (x_i) be independent random variables with an exponential distribution. In addition, a Poisson process cannot have two or more events occurring simultaneously nor can the probability of occurrence of an event be affected by past events. The non-homogeneous Poisson process characteristics differ from the homogeneous process in that: (1) non-stationarity of the series of x_i may be present, (2) the x_i are not necessarily independent although the number of cells in any non-overlapping intervals are independent with a Poisson distribution, and (3) the x_i no longer have the simple exponential distribution.

We tested the Poisson hypothesis, based on the uniform distribution of times to events (U_i) using two distribution free goodness-of-fit tests (Kolmogorov-Smirnov (K-S) and Anderson-Darling (A-D)). Another index of the x_i conformity to a Poisson process that we used was the coefficient of variation (CV). For a Poisson process the CV should be 1; however, it has been stated [15] that a range of $0.8 \leq CV \leq 1.2$ may be expected from experimental data for a completely random process [24]. A majority of the tests described above in the analysis of the leukocytes sequence were performed by the SASE IV program (Statistical Analysis of Series of Events) [26].

Results

Table 1 summarizes the descriptive statistics of all experiments. The group mean diameter herein studied was 10.2 ± 1.5 with a range of 8–12 microns.

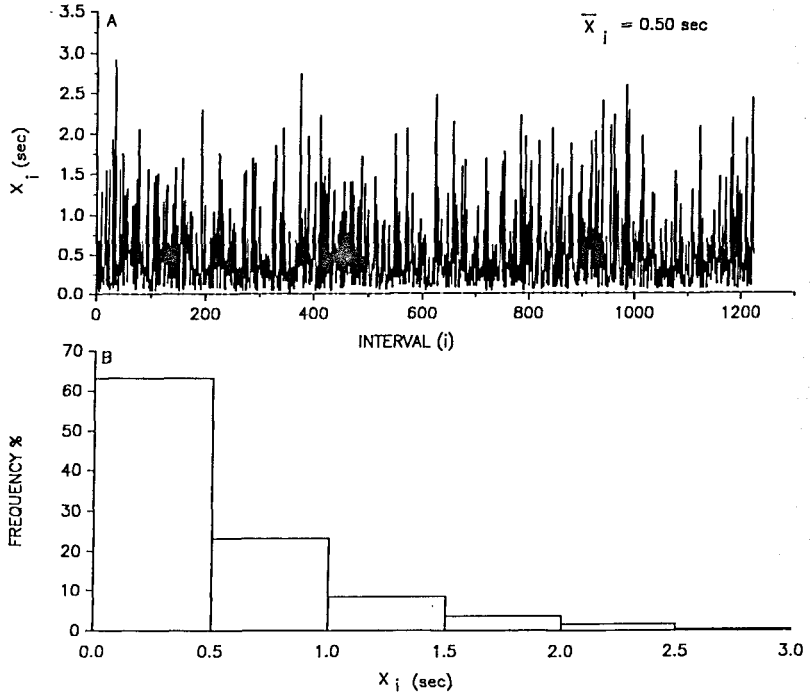

Fig. 1. (A) Leukocyte intra-arrival sequence (x_i) with the smallest mean x_i of all sequences measured. (B) Corresponding frequency distribution of x_i.

The mean observation time was 731 s with a range of 450–911 s. The averaged measured velocity was 749 μm/s with a range of 168–1574 μm/s. The CV is the ratio of the standard deviation of x_i divided by x_i and has a mean of 1.04 with a range of 0.90–1.16. This range is well within the acceptable limits for a Poisson process.

Examples of recorded intra-arrival sequences are illustrated in Figs. 1, 2 and 3. The sequences shown are for the smallest mean x_i (Fig. 1a) and the largest mean x_i (Fig. 2a) observed in the study. Figure 3 is an example of one of two non-stationary sequences which were detected as will subsequently be discussed.

Stationarity

Results of the tests for stationarity are compiled in Table 2. Based on the tests on the intra-arrival times (x_i), a significant deviation from stationarity was detected in only two of the eight sequences (# 3 and 5). In all cases the results obtained with the U-statistic and the Pearson r (obtained through the regression analysis of the log transformation of the x_i) yielded the same conclusion. In the two sequences which we reject as being stationary based

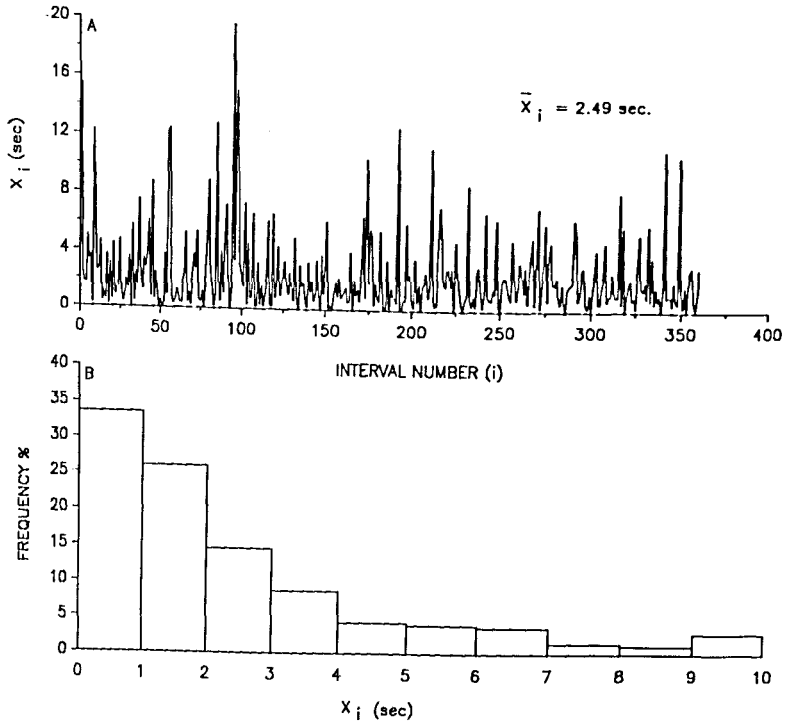

Fig. 2. (A) Leukocyte intra-arrival sequence (x_i) with the largest mean x_i of all sequences measured. (B) Corresponding frequency distribution of x_i.

on the tests on x_i, there was also a significant trend in V_{wbc} as evidenced by the Pearson r values. Based on this, it appears that the absence of stationarity in the process in sequences 3 and 5 is due to a linear monotonic trend in V_{wbc} over the observation interval. Though a monotonic trend was also detected in sequence 6, its impact on the stationarity of the x_i was apparently insufficient to result in rejection of the null hypothesis.

We additionally used the runs test which apply to a wide variety of trends including cyclic variability. These results are tabulated for Φ in Table 2. Primarily, the runs test is testing for randomness of the sequence. All cases strongly exhibited randomness. Based on the composite results of all tests for stationarity it appears that all sequences, except 3 and 5, may be reasonably accepted as stationary.

In these two cases, knowledge of the observed explanatory variable v(t) (velocity) causing the trend in the series of x_i, allows the non-stationarity sequence of x_i to be analyzed after a transformation based on uniform velocity. After the transformation, the previous non-stationary sequences were tested for stationarity by the same means as before (U-statistic and Pearson r). The results clearly indicate the elimination of any trend (p-values

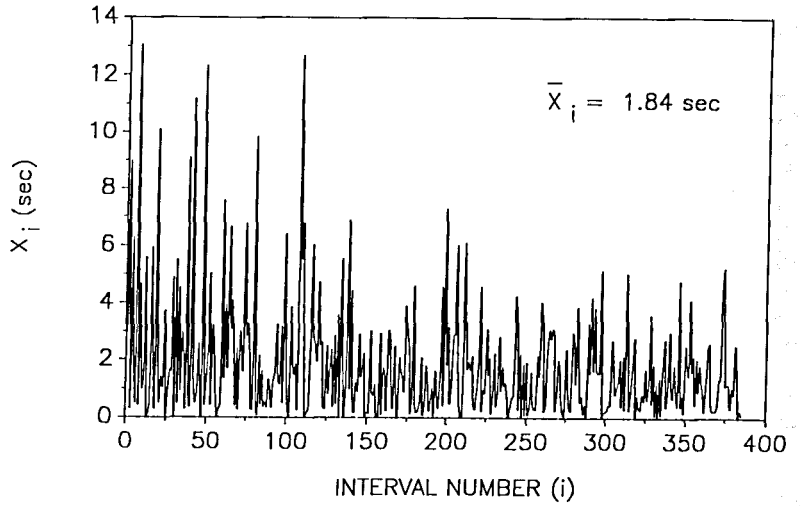

Fig. 3. Non-stationary sequence of intra-arrival times (x_i) between consecutive leukocytes. The graph indicates a decrease in x_i over time. This decrease in x_i corresponded with an increase in cellular velocity.

for U-statistic become 0.56 and 0.53 respectively and 0.80 and 0.64 for the Pearson r) and the initial non-stationarity is no longer evident.

Renewal process

In Table 3 the p-values of the tests for the first three serial correlation coefficients are listed. The non-stationarity of experiments 3 and 5 precluded

Table 2. Results of tests for stationarity

	Tests on x_i		Test on Φ	Test on V_{wbc}
	U-statistic	Pearson r	Runs test	Pearson r
1	0.05	0.05	0.11	0.30
2	0.65	0.76	0.27	0.21
3	0.03*	0.01*	0.93	0.001*
4	0.05	0.05	0.85	0.06
5	0.01*	0.01*	0.10	0.001*
6	0.05	0.25	0.14	0.003*
7	0.09	0.14	0.20	0.705
8	0.41	0.55	0.34	0.37

The tests on x_i test for stationarity of the leukocyte intra-arrival sequence, the test on Φ tests for stationarity of the number of leukocytes in 10 second contiguous intervals and the test on V_{wbc} is based on regression analysis and tests for a trend in velocity vs. time. In all tests the null hypothesis is stationarity. Entries in the table are reported as p-values. Entries < 0.05 indicate rejection of the hypothesis of stationarity and are denoted by *.

Table 3. Results of tests for independence of intra-arrival times

EXP	ρ_1	ρ_2	ρ_3	(K-S)
1	0.51	0.69	0.67	0.68
2	0.32	0.34	0.99	0.49
3′	0.70	0.88	0.90	0.94
4	0.75	0.76	0.10	0.62
5′	0.81	0.46	0.61	0.57
6	0.90	0.94	0.53	0.99
7	0.95	0.13	0.36	0.92
8	0.06	0.83	0.36	0.07

The first three serial correlation coefficients (ρ_1, ρ_2, ρ_3) and the K-S test are tested under the null hypothesis of independence of x_i. Entries in the table are the associated p-values. A p-value > 0.05 indicates that independence cannot be rejected at the 0.05 level. ′ denotes a detrended sequence.

analysis of the original data for independence. However, the detrended data (as previously described) were examined and the results denoted as 3′ and 5′ in the table. Since the null hypothesis for all tests is the statistical independence of the x_i, a large p-value (i.e. $p > 0.05$) implies independence of the intervals between passing leukocytes. All values exceed this level and most are significantly greater thus strongly suggesting the x_i are independent. The p-values for the (K-S) test indicate that the hypothesis of a constant spectral density function cannot be rejected. Since this is true only when the serial correlation coefficients are zero, these tests support the independence of the x_i.

A renewal process requires that both the properties independence and stationarity be established. The property of stationarity was previously confirmed when there was no significant trend in V_{wbc}. Further analysis which depend on these properties may now proceed.

Poisson process

Results of testing the leukocyte intra-arrival sequence as a Poisson process are listed in Table 4. The distributional properties of the series of x_i and Φ are reported in Table 4 based on consecutive but non-overlapping 10 second intervals. If the x_i conform to a Poisson process then the x_i would have an exponential distribution whereas the fluxes would have a Poisson distribution. The distribution of the stationary sequences with the smallest and largest mean are shown in Figs. 1b and 2b respectively. The Kolmogorov-Smirnov (K-S) goodness-of-fit test was employed to compare the observed distribution to the theoretical distribution of sequences. The null hypothesis for the test is conformity to the associated Poisson distribution,

Table 4. Results of tests for a Poisson process

Exp	x_i	Φ	U_i	
	(K-S)	(K-S)	(K-S)	(A-D)
1	0.59	0.99	0.01*	$p < 0.05$*
2	0.99	0.61	0.81	$p > 0.05$
3	0.47	0.61	–	–
3′	0.61	0.96	0.35	$p > 0.05$
4	0.81	0.99	0.05	$p > 0.05$
5	0.10	0.86	–	–
5′	0.85	0.99	0.93	$p > 0.05$
6	0.99	0.33	0.01*	$p < 0.05$*
7	0.91	0.63	0.07	$p < 0.05$*
8	0.93	0.39	0.35	$p > 0.05$

The null hypothesis for x_i is conformity to an exponential distribution and for Φ to a Poisson distribution. The null hypothesis for U_i is a uniform distribution. Entries in the table are p-values obtained from the two-sided Kolmogorov-Smirnov (K-S) test and the Anderson-Darling (A-D) test. Since complete probability tables for the (A-D) test are unavailable, appropriate ranges were tabulated. Experiments 3 and 5 had an initial trend and therefore the (K-S) and (A-D) tests were not run. The * denotes rejection of the Poisson hypothesis at the 0.05 level.

thus large values of the test statistic which are associated with low significance levels, result in rejecting the null hypothesis. From Table 4 it may be seen that all stationary sequences strongly indicate both an exponential distribution of x_i and a poisson distribution of fluxes. In those sequences which are not stationary (#3 and 5), the fluxes do follow a Poisson distribution but the x_i do not follow the simple exponential distribution in sequence # 5. However, as previously stated the non-homogeneous Poisson process will have a Poisson distribution of fluxes but not necessarily the simple exponential distribution of x_i.

Table 4 also lists results of the tests based on sequential x_i. The remaining tests are based on the order statistics of the times-to-events (U_i) and are, for a Poisson process, independent observations from a uniformly distributed population [25]. A test for the Poisson hypothesis based on testing the uniform distribution of the U_i is called a uniform conditional test. Both the K-S and A-D (Anderson-Darling) distribution free procedures are performed on the U_i with the p-value being reported in Table 4. There is no evidence indicating superiority of either the K-S or A-D tests. Based on these tests only experiments 1 and 6 reject the hypothesis of a uniform distribution, but we can accept an exponential distribution of the x_i and a Poisson distribution of the fluxes.

Discussion

Results of the present study show that leukocyte transit through arterioles, en route to the capillary network, can be suitably modeled as a stochastic point process. When the cellular velocity is relatively constant over the observation time of interest, the sequence of arrival times between consecutive leukocytes conforms to that of a renewal process and more specifically to that of a Poisson process. If cellular velocity has a significant trend over the observation time of interest, non-stationarities in the series of leukocyte arrivals are to be expected, and the sequences are best characterized by a non-homogeneous Poisson process.

Previous applications of stochastic point process models to study the properties of other physiological processes have yielded varying results [9, 12–14, 24]. In our analysis we successfully showed that the intervals between leukocyte transit are independently and exponentially distributed if the sequence is stationary and we eliminated the potential for simultaneous occurrences by studying arterioles $< 12\,\mu$m in diameter thus eliminating simultaneously passing leukocytes.

Studies of erythrocyte passage in capillaries [9] led the authors to conclude that neither a renewal nor a Poisson process described these events because of non-stationarities in transit times probably due to microvascular vasomotion. We have also found some non-stationarities (in two of eight experiments). The source of the non-stationarity in these two cases was not likely caused by vasomotion as no diameter modulations were observed. However, a measured linear trend in the blood velocity was found to correlate with the observed non-stationarity in the intra-arrival sequence. Though the cause of the velocity change is not known, the incorporation of its perturbing effect into the model to produce a detrended sequence allowed us to estimate the properties of the detrended sequence. In the absence of the confounding effect of velocity changes the analyses revealed that the series conformed to a renewal and Poisson process.

Our demonstration of the applicability of a Poisson model to describe leukocyte delivery into the capillary network represents an important first step in the characterization of the dynamics of this complex physiological process. Understanding of leukocyte delivery patterns has broad implications. It may help clarify the interpretation of the movement of these cells through retinal microvasculature as with the blue field entoptic procedure [40] and it may lead to a better understanding of certain features related to leukocyte impact in capillaries. Tissue damage due to ischemia has been linked to entrapment of leukocytes in the microvasculature. When this occurs within the capillaries, then the capillary, the associated microvessels and the surround-

ing tissue will be exposed to a number of leukocyte related pathological processes. Knowledge of the nature of the process distributing leukocytes into the capillary network allow a proper estimate of the probabilities associated with leukocyte occupancy within the capillaries under various conditions.

In spite of our successful use of stochastic theory to characterize the leukocyte dynamics our findings do not necessarily imply that leukocyte delivery to the capillary mesh is governed purely by random principles. Clearly the number of leukocytes delivered to any given terminal arteriole depends on all the physical, topographical and hemodynamic factors which influence the cellular distribution within the microvasculature [7, 22, 29, 36], the physiological state of the microvasculature itself [27, 28] and that of the leukocytes [11, 20, 41]. For any terminal arteriole, the source of leukocytes is the immediately proximal parent arteriole in which there is present a concentration of leukocytes (leukocrit). This leukocrit is itself time dependent and some portion of these cells are distributed to the arteriolar branch under observation. The distribution depends, in part, on the blood flow in the observed vessel, relative to the blood flow in the segment of the parent vessel which is immediately distal to the site of branching. For a given set of hemodynamic conditions the leukocyte distribution to the terminal arteriole is also importantly influenced by the details of the branch junction and the radial position of the leukocyte as it approaches the junction [38]. The size of the leukocyte, either its absolute value or relative to the branch diameters, also plays some role in determining distribution selectivity. The presence of a broad statistical distribution of circulating leukocyte sizes could thus effect the temporal sequence of leukocyte entry into the observed branch. Similarly, a statistical distribution in the physical and mechanical properties of the circulating leukocytes would likely effect the observed sequence since these features influence distribution at branch points.

The above statements concerning events within the observed terminal arteriole and its parent vessel apply to varying degrees to each branch site along the vascular path which joins this branch to the main entering arteriole, thus influencing the dynamic leukocrit at all distal sites. The consequence of this vast number of interacting factors (many of which may be deterministic at a local level) is to give rise to an observed sequence which exhibits the stochastic features herein described. The stochastic characterization thus represents a concise formulation which allows us to deal with leukocyte dynamics within the terminal microvasculature.

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