

Deconvolution of Chemiluminescent Emission Curve Associated with Phagocytosis into Three Logarithmic Normal Distributions

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OKAZAKI, N., HIROSAWA, J., SAITO, N. and SATO, T. *Deconvolution of Chemiluminescent Emission Curve Associated with Phagocytosis into Three Logarithmic Normal Distributions.* Tohoku J. Exp. Med., 1998, **185** (4), 271-280 ——— The chemiluminescent emission reaction dependence on the activity of phagocytosis is well known. However, this method is not used to diagnostically in clinical assessment because the relationship between phagocytizing activity and chemiluminescent intensity has not been clearly established. Therefore, we attempted to analyze quantitatively the chemiluminescent emission curve by the phagocytosis of leukocytes. Mathematical assessment of the emission curve with respect to time was performed by fitting the curve to several regression models using the unweighed non-linear least squares method. A triple logarithmic normal distribution model provided a reasonable goodness of fit to the measured emission curve. The first component, about 5% of the calculated total counts, was assumed to arise from monocytes activity, the second component, about 20% from eosinocytes activity and the third component, up to 75%, from neutrophils activity. This method seems promising as a means for assaying whole blood without the need for pretreatment and for the providing a valid index that is independent of the technical differences between laboratories. ——— whole blood; phagocytosis; chemiluminescence; curve-fitting © 1998 Tohoku University Medical Press

The chemiluminescent technique for measuring the oxidative activity related to the phagocytizing ability of leukocytes has been known for about 25 years (Allen et al. 1972; Hodgson and Fridovich 1973; Anderson et al. 1978). However, the method is not widely used in clinical applications due to following reasons (Sakura et al. 1982). Generally, it is difficult to make repeated measures

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under the same conditions and to compare results obtained by different institutions because phagocyte activity varies widely. So far, a typical index reflecting the chemiluminescent emission curve has not yet been determined despite the fact that several values have been shown to be related to phagocytic activity such as total counts, peak counts, peak time, and so on. Also, for a long time, the only instrument available measuring chemiluminescent emission was the scintillation counter: and this was used mainly as a radiation counter. Moreover, the instrument was not capable of continuous measurement and was also expensive.

In recent years, simple and inexpensive instruments dedicated to measuring chemiluminescent emissions have been developed (Sakura et al. 1982; Suwa and Tanaka 1984), but these have not yet been applied to clinical assays. Then, the present paper attempts to contribute to the ongoing discussion concerning quantitative analysis and measurement of the zymosan-induced luminol-dependent chemiluminescence curve.

MATERIALS AND METHODS

Residual heparinized arterial blood samples taken in the extracorporeal circulation (ECC) for blood gas analysis before, during and after surgery (at 2–4 points on days 0, 1, 3 and 5 post-operation) were used (15 patients; total number of samples = 74). Informed consent was obtained from all patients. Blood samples containing 0.25 ml and 0.20 ml non-opsonized zymosan A (30 mg/ml, Sigma Chemical, St. Louis, MO, USA) were added to 1.4 ml HEPES buffer (135 mM NaCl, 3.0 mM KCl, 2.4 mM MgCl₂, 1.2 mM CaCl₂, 2.0 mM Na₂HPO₄ and 17 mM HEPES: 4-hydroxyethyl-1-piperazine ethansulfonic acid), together with 0.10 ml of 0.4 mM luminol and 0.01 ml of 1 mM glucose (all these chemicals were purchased from Wako Pure Chemicals, Osaka). This procedure was completed in less than 15 minutes after blood had been drawn. The light emission intensity was measured by integrating counts for 50 minutes (counts·min⁻¹ unit) using a Luminescence Reader BLR-201 (ALOKA, Tokyo), while the test tube was subjected to frequent and gentle mechanical agitation (1 cycle per second) at 37°C.

The emission curve was assessed mathematically with respect to time using eight models consisting of 1 to 4 components having either normal or logarithmic normal distribution components. The models were applied for fitting using the unweighed non-linear least squares method with damping Gauss-Newton's algorithm and the goodness of fit for those models was judged by AIC (Akaike's Information Criterion or An Information Criterion: The best model is the one with the lowest values of AIC). Analysis revealed that the emission curve could be deconvoluted into three logarithmic normal distribution curves as represented by the following equation.

$$f(t) = \sum_{i=1}^{n=3} \frac{P_i}{\sqrt{2\pi}\sigma_i t} \exp\left\{-\frac{(\ln t - m_i)^2}{2\sigma_i^2}\right\}$$

Here t is time and \ln is the natural logarithm, and magnitude P represents the intensity parameter of each component. The “average” and “variance” parameters (m and σ , respectively) were used in determination of the authenticity of the fitness because each value showed little change within the same subject. The exponential m_i is equivalent to the peak time required to reach the apex of each distribution curve.

Three kinds of counts were used: “peak count” (counts \cdot min $^{-1}$ unit), which is the maximum number of measured counts; “cumulative count” (counts unit), which is the total number of counts accumulated over a 50-minute period; and “calculated count” (counts unit), expressed as a finite sum (i.e., the area under the curve) calculated in 1-minute intervals using the three logarithmic normal distribution models over a 150-minute period.

RESULTS

Judging from the results of deconvolution with a normal or logarithmic normal distribution model, the chemiluminescent emission curve was seen statistically to be composed of three distinct logarithmic normal distributions. The results of ten samples taken before and after the ECC of 5 patients chosen randomly were shown in Fig. 1.

An example of deconvolution was shown in Fig. 2. The emission counts per

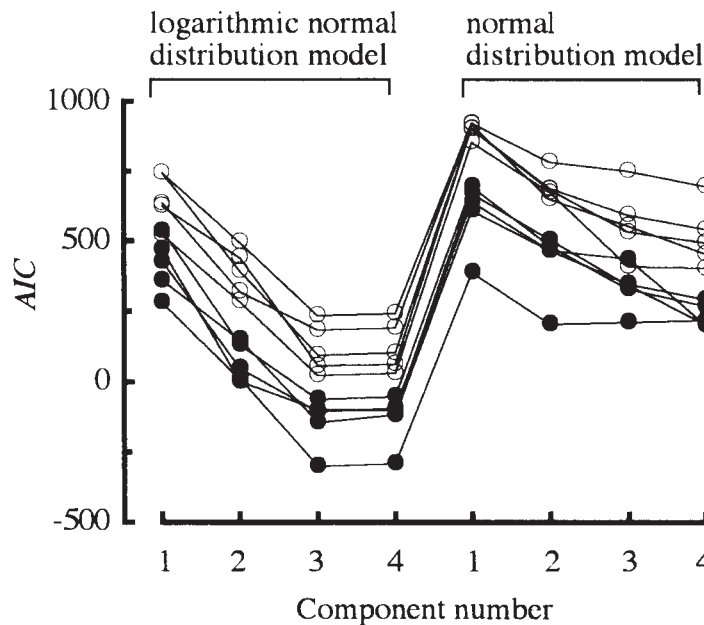


Fig. 1. Relation between AIC value and deconvolution models. The ten samples before and after extracorporeal circulation (ECC) of randomly five patients are shown individually. The abscissa is the components number either normal distribution or logarithmic normal distribution in complex model; the ordinate is the AIC value. Open circle, Samples before ECC; closed circle, Samples after ECC.

(AIC = $N \times \log(ss) + 2 \times M$, N , number of data points; ss , residual sum of squares; M , number of parameters in the model)

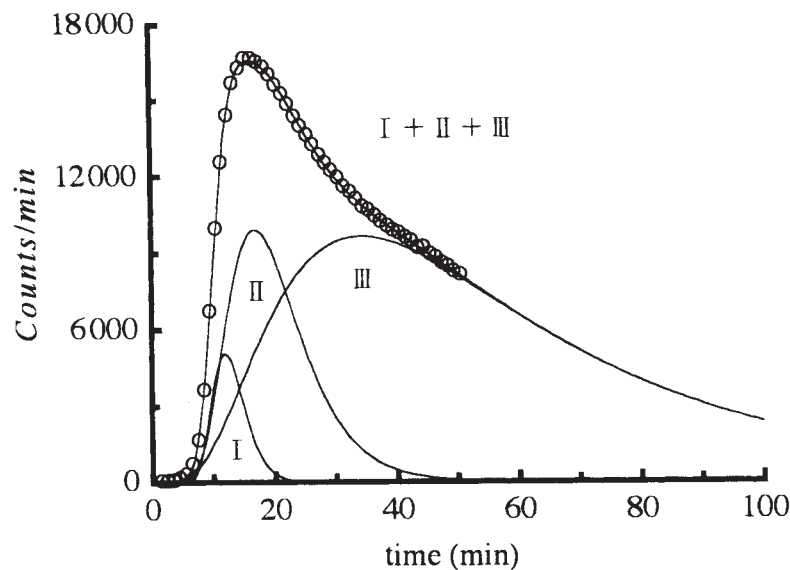


Fig. 2. A typical chemiluminescent intensity curve. Open circles indicate the “measured counts” represented in 1-minute intervals. I, II, III are deconvolution curves analyzed using the three-logarithmic normal distribution model. I + II + III is a synthetic curve of these three components.

minute at 50 minutes still exceeded about half of the peak counts or about 1.5% of the total counts.

“Cumulated” counts measured over 50 minutes, “calculated” counts finite summed over 150 minutes by the three-component model, percentage of each component accounted for by the “calculated” total counts, and the ratio of the residual sum of squares by the three-component model to the “cumulated” counts were shown in Table 1. The composition ratio of each component was about 5%, 20% and 75%, and the peak time value of each component was 14.3 ± 4.5 , 20.5 ± 5.0 and 43.7 ± 8.6 minutes, respectively. Each peak time of before ECC was shorter than that of after ECC, but after operation, it returned gradually to each original value of before ECC in all cases.

The correlation between “calculated” counts over 150 minutes and “cumulated” counts for 25 or 50 minutes were shown in Fig. 3, and peak counts were shown in Fig. 4. In Fig. 3, the relationship between the “cumulated” counts and “calculated” counts would correlate nicely in proportion to measuring time. These results indicate that the scattering values in the measurement over 25 minutes were caused by the different position of peak time in each individual, that is, the emission curve were not uniformly.

In Fig. 4, the coefficient of determination (r^2) between “peak” counts and “calculated” counts was 0.845. Also, the measured peak time, ranging from 12.5 minutes to 33.0 minutes, was 19.3 ± 4.8 minutes. Therefore, it needs about 40 minutes to measure the peak value in the non-opsonized zymosan-induced chemiluminescence. On the other hand, in the opsonized method, it is considered that it would not be able to use the peak value as one of parameters expressing the

TABLE 1. Cumulative counts and calculated counts by deconvolution method

ECC	Cumulated counts	Components (Peak time)	Calculated counts (%)	SS (SS ratio)
Before <i>n</i> = 15	366.8 ± 172.5	1st	31.8 ± 22.0 (16.4 ± 5.0)	0.55 ± 0.66 (0.0015 ± 0.0018)
		2nd	112.4 ± 56.1 (22.2 ± 5.0)	
		3rd	480.0 ± 223.4 (49.1 ± 10.7)	
During or after <i>n</i> = 59	2048.1 ± 731.3	1st	128.6 ± 99.6 (13.7 ± 4.3)	5.74 ± 3.89 (0.0028 ± 0.0019)
		2nd	471.1 ± 257.4 (20.1 ± 5.0)	
		3rd	2461.5 ± 1016.1 (42.3 ± 7.5)	

The “cumulated” counts over 50 minutes, the “calculated” counts over 150 minutes and the residual sum of squares to a regression curve are shown. The ratio of the residual sum of squares to the “cumulated” counts for 50 minutes.

ECC; extracorporeal circulation. The counts unit is kilo-counts, the peak time unit is minutes, and data are expressed as the mean ± s.d. SS; residual sum of squares by curve fitting. SS ratio; SS/cumulated total counts.

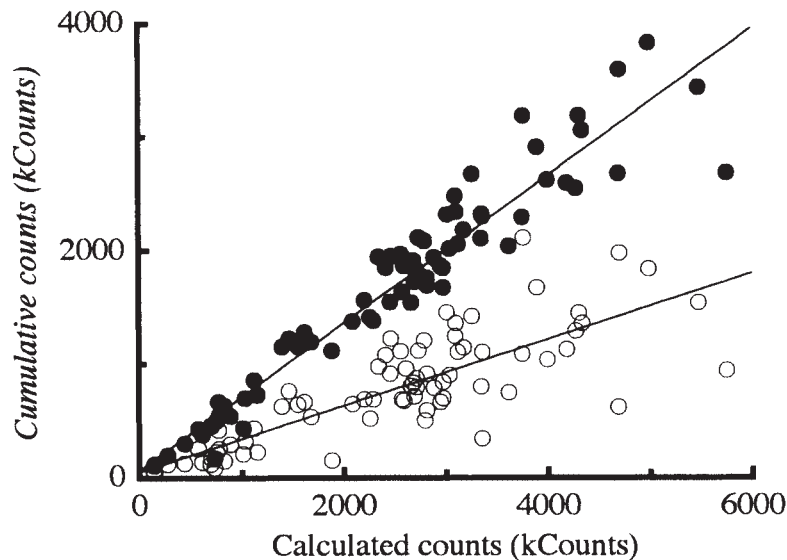


Fig. 3. Relationships between “calculated” counts over 150 minutes using a three element complex model and “cumulated” counts for 25 or 50 minutes. The open circle shows the “cumulated” counts for 25 minutes (*r* = 0.81) and the closed circle shows the “cumulated” counts over 50 minutes (*r* = 0.96). *n* = 74.

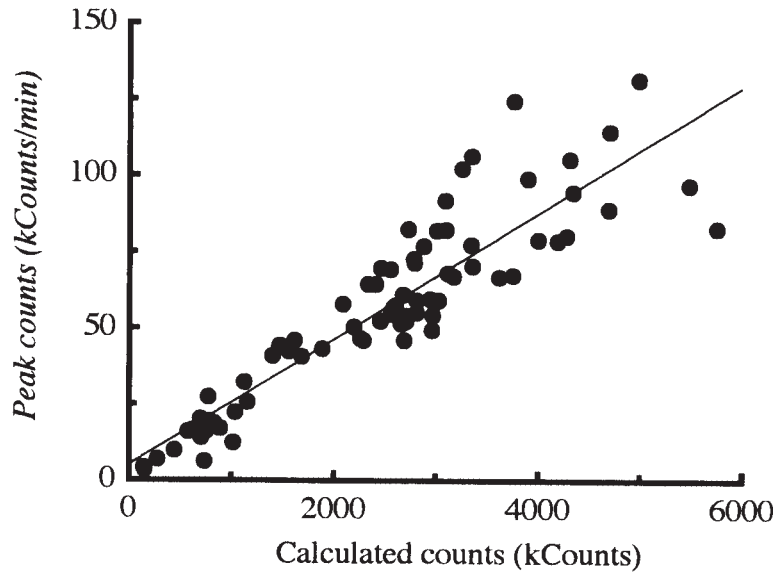


Fig. 4. Correlation between “calculated” counts (abscissa) which was a finite sum using the three-logarithmic normal distribution model over a 150-minute period and measured peak value (ordinate). $n=74$, $r=0.91$.

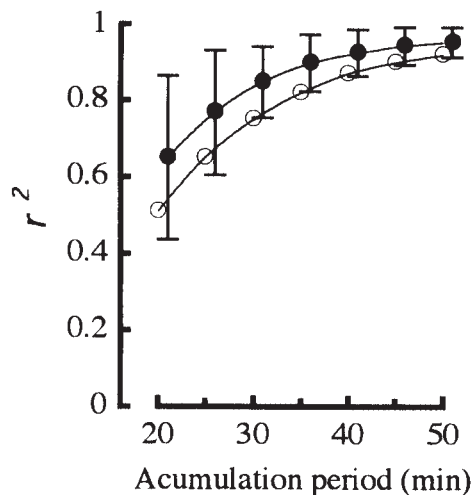


Fig. 5. Changes in coefficient of determination (r^2) between “cumulated” and “calculated” counts. The “cumulated” counts and the “calculated” counts were presented by cumulating measured counts for 25 to 50 minutes in 5-minute steps and by calculating counts in 1-minute intervals over 150 minutes using three element model respectively. The open circle shows the estimated r^2 in a lump (aggregated data: $n=74$) and the closed circle shows the data divided into each patient (individual data: $n=15$, mean \pm s.d.).

emission curve any longer, since the correlation coefficient in Fig. 4 would vary by the degree of opsonization.

Fig. 5, shows the change in the coefficient of determination (r^2) between the “calculated” and “cumulated” counts in 5-minute steps from 20 to 50 minutes aggregated for all 74 samples (open circle in Fig. 5). Also, the change in the coefficient of determination (r^2) between the “calculated” counts and “cumulated” counts is shown in the same manner for the same data classified according to each

patient (15 patients. approx. 4-6 samples per patient: Closed circle, mean \pm s.d.).

DISCUSSION

Although the luminol-dependent chemiluminescent response reflects the generation of oxygen radicals induced by phagocytosis, the magnitude of the response is not equivalent to the phagocyte activity in a strict sense. The reason is that the duration of the luminol-dependent emission is longer in comparison with the duration of O_2^- production detected directly by electron paramagnetic resonance (EPR) spectrometry (Black et al. 1991). Furthermore, it is conceivable that this difference is caused by the luminol-based measurement system itself, that is, by the luminol-dependent peculiarly chain reaction derived by O_2^- (Hodgson and Fridovich 1973; Misra and Squatrito 1982). Therefore, we can considered that chemiluminescent intensity represents the sum between the real intensity due to phagocyte-dependent activity and the amplified intensity due to the chain reaction, and that the intensity of the latter is proportional to the former. This relation is further complicated by the fact that phagocytosis activity differs according to the species of white blood cell.

In addition to the problem of the relation between phagocytosis and chemiluminescence of mentioned above, there is no typical parameter for accurately expressing the emission curve, and it is virtually axiomatic that several parameters must be used such as total count, peak count, instantaneous maximal intensity (Anderson et al. 1978; Sakura et al. 1982; Suwa and Tanaka 1984; Akao et al. 1992). Furthermore, several unnegligible problems remain to be solved. For example, it has been pointed out that (1) most investigations have been carried out with isolated white blood cells in vitro, and it is uncertain how such isolation influences cell activity, (2) it is unknown to what extent the storage temperature and time lag until measurement affect the chemiluminescent intensity, (3) the concentration of phagocytized stimulant is known to influence to the peak count and instantaneous maximal intensity, and (4) the limited measuring time, such as 20 minutes generally used, amplifies errors for true emission counts. Therefore, it is conceivable that none of these parameters actually expresses a representative value of the emission intensity. Because of this, the authors chose to investigate the chemiluminescence curve to analyze mathematically.

The three-component logarithmic normal distribution model provided an excellent fit compared with four-component logarithmic normal distribution model with a difference of about 6 in AIC value, corresponding to a difference of the parameter number (equal to 3) without decreasing the residual sum of squares. It was therefore concluded that further dividing the model into more components would have no significant effect in practical applications.

The ratio of the residual sum of squares after fitting the three-logarithmic normal distribution model to the "cumulative" total counts for 50 minutes was 0.0025 on average (s.d.=0.0019, $n=74$). This small value indicates that the

model is sufficiently effective as an approximate formula.

Although several stimulants such as opsonized zymosan or bacterial substances are used (Akao et al. 1988) and lucigenin or luminol is employed as an oxidizing indicator (Ewetz et al. 1981), different reports show that the correlation of emission intensity between both phagocytal stimulants occurred, or was observed only in healthy subjects (Akao et al. 1988). Also, the superiority in these indicators has been discussed in relation to the different mechanism whereby lucigenin acts outside the cell, and luminol acts both inside and outside the cell (Dahlgren et al. 1985). It should be understood that a conventional method which is examined over a short period promotes many inevitable differences in measurement conditions. Actually, in this study as shown in Fig. 2, the chemiluminescent emission for all practical purposes was not finished by 50 minutes. Therefore, it is understood that an appropriate value for the emission curve results in improved clinical utility.

Although the luminol chemiluminescent reaction depends on the radical species produced by phagocytosis (Misra and Squatrito 1982), the relation between the components discriminated by this method and the oxygen radical is unclear. However, a logarithmic normal distribution expressed on a logarithmic time scale is frequently observed in vital reactions. It would therefore seem appropriate that the chemiluminescence dependence on the luminol oxidative reaction was conjectured to be the one phenomenon that might represent the activity life span of three kinds of phagocytes. Thus, it is conceivable that the deconvolution to logarithmic components is adequate.

In the pre-operation blood samples, the peak time required by each cell type to reach the apex of the distribution curve was 16.4 ± 5.0 minutes, 22.2 ± 5.0 minutes, 49.1 ± 10.7 minutes, and the contribution ratio of each component to the total emission quantity was about 5%, 20% and 75%, respectively. The peak time for luminol-dependent non-opsonized zymosan-induced chemiluminescence in isolated blood cells has been reported to be 18.5 minutes for monocytes, 41.9 minutes for eosinophils and 48.3 minutes for neutrophils (Lindena et al. 1987). Furthermore, the activity ratio is about 0.3–0.5: 1: 1 (Shult et al. 1985; Lindena et al. 1987; McNally and Bell 1996). Taking into account the ratios of the 3 components in white blood cells, it is thought that the second component showed eosinocyte activity. The third component is about 75% and is therefore thought to be similarly representative to neutrophil activity. It was also suggested that the component of about 5% at the early phase may indicate phagocytizing activity caused by monocytes.

As mentioned above, the emission intensity does not directly indicate phagocyte activity because this "total" activity differs according to the white blood cell species. However, in this deconvolution method, the count value are thought to be adequate as indexes of activity because it was possible to determine the significance of emission intensity by assigning a specific emission intensity to

each type of phagocytic activity.

Since the coefficient used for determination remained almost unchanged after 40–50 minutes in the same patient, it was conceivable that “peak” counts or “cumulative” counts made for 40–50 minutes were sufficient as phagocytic parameters in consideration of time clinical use in requiring a relatively quick and accurate test so as to prevent misdiagnosis and subsequent inappropriate therapy.

In summary, the present results confirm that the time sequential curve of phagocytic activity as determined by the oxidative reaction could be adequately represented by a complex model consisting of three-logarithmic normal distributions. The present method is thought to be useful for numerical assessment. Furthermore, this method offers promise as an extremely effective analytical method suitable for use with whole blood without pre-treatment and that is independent of the inevitably differences in opsonization and conditions of measurement.

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