Published in final edited form as:

Cytokine. 2011 February ; 53(2): 158–162. doi:10.1016/j.cyto.2010.09.008.

Mathematical relationship between cytokine concentrations and pathogen levels during infection

Yue Zhang^{*} and James B. Bliska

Center for Infectious Diseases and Department of Molecular Genetics and Microbiology, Stony Brook University, Stony Brook NY 11794-5120, USA

Abstract

The relationship between concentrations of cytokines and microbial pathogen levels during infection is not clear. In a sub-lethal murine infection model using Gram-negative bacterial pathogen *Yersinia pseudotuberculosis*, the serum concentrations (C) of pro-inflammatory cytokines tumor necrosis factor α (TNF α), interferon γ (IFN γ), interleukine-1 β (IL-1 β) and interleukine-18 (IL-18) formed a mathematic relationship with the splenic pathogen levels (P) as measured by colony forming unit. Naming parameters “m” and “k” for magnitude and kinetics, respectively, the relationship is depicted as $C=mP^k$. When reanalyzing the TNF α and IFN γ concentrations and the bacterial levels that were determined by other groups during infection with another strain of *Y. pseudotuberculosis* or with *Y. pestis*, this relationship was maintained. Interestingly, the changes in the values of “m” and “k” were consistent with the progress of the host immune response during infection; while deviation from this relationship was observed in individuals that seemed to be unable to control infection. Furthermore, in a murine model of ricin intoxication the local concentrations of the cytokine monocyte chemotactic protein 1 (MCP-1) and the concentrations of injected castor bean toxin ricin also conform to this relationship. $C=mP^k$ could be a general relationship in host cytokine response to pathogens or pathogen-associated molecular patterns. If confirmed, this type of analysis will be very useful in identifying the steps in a host immune response with which a pathogen interferes. It will also help to determine the specific functions of a host factor in the immune response.

Keywords

cytokine; pathogen level; bacterial infection; bioinformatics analysis

1. Introduction

Cytokines orchestrate host innate and adaptive immune responses to pathogens. In response to the detection of pathogens, the host produces pro-inflammatory cytokines. The molecular patterns associated with various pathogens are recognized through conserved pattern recognizing molecules like the Toll-like Receptors and the intracellular Nod-like Receptors. Given the convergence of pathways downstream of these receptors, and the subsequent activation of NF- κ B and other transcription factors to initiate the production of cytokines

^{*}Corresponding Author. Telephone: 1-631-632-8783. Fax: 1-631-632-4294. yzhang@ms.cc.sunysb.edu.

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[1], we hypothesize that a quantitative relationship exists between the pathogen levels and the levels of cytokines.

Using a sub-lethal murine intragastric infection model with *Y. pseudotuberculosis* strain 32777, we investigated the relationship between the systemic bacterial load and the serum concentrations of cytokines. The pro-inflammatory cytokines IFN γ and TNF α were chosen initially for analysis as they are known to be important host protective elements during *Yersinia* infection [2,3].

2. Materials and Methods

2.1. Mouse infection

Y. pseudotuberculosis serogroup I strain 32777 (previously known as IP2777) was used here [4]. All animal procedures were approved by the Stony Brook University IACUC. For intragastric infection, 9 week-old female C57BL/6 mice (Taconic) were fasted for 16 h before orogastric inoculation with 200 μ l of 5×10^7 colony forming units (CFUs) of *Y. pseudotuberculosis* culture through a 20 gauge-feeding needle. To prepare bacteria, overnight cultures grown in Luria-Bertani at 26°C were washed once and re-suspended in Phosphate Buffered Saline to 25×10^7 CFUs/ml. Mice were provided with food and water ad libitum thereafter. At 4, 7 or 10 days post infection, groups of 4 or less surviving mice were euthanized by CO $_2$ asphyxiation. Blood was collected through cardiac puncture, and separated into serum after centrifugation in Z-Gel Micro tubes (SARSTEDT). Mouse spleens were dissected aseptically, weighed and homogenized in 5 ml of sterile PBS or Dulbecco's Modified Eagle Medium. One hundred μ l of homogenate was serially diluted and plated on LB agar to determine bacterial load.

2.2. Measurement of cytokine concentration with ELISA

Serum IFN γ concentrations were measured with a mouse IFN γ MAX set Deluxe kit from BioLegend. TNF α and IL-1 β concentrations were determined with either a Quantikine[®] Mouse TNF α Immunoassay kit or an IL-1 β Immunoassay kit, both from R&D Systems. The mouse IL-18 ELISA kit was from Medical & Biological Laboratories. Sera were routinely diluted 10-fold in an appropriate dilution buffer and further adjusted as necessary, following the manufacturers' instructions.

2.3. Statistical analysis

Logarithmic transformation and other calculations were performed in Microsoft Excel; linear regression was performed in Prism 4.0c.

3. Results

3.1. The relationship between serum cytokine concentration and bacterial colonization level in *Y. pseudotuberculosis* infection

Intragastric infection with *Y. pseudotuberculosis* resulted in persistent bacterial colonization in the spleens and progressive spleen weight increase for up to 10 days. However, of the 80% of mice that survived such infection, the majority (~71%) cleared the bacteria from their spleens after 14 days [5]. Serum cytokine concentrations (C) of TNF α or IFN γ and splenic pathogen loads (P) were determined 4, 7 and 10 days post infection. The levels of these cytokines 4 and 7 days post infection have been published before [5]. A simple linear relationship was observed between the values of logC_{IFN γ} or logC_{TNF α} and that of logP_{CFU} (Fig 1AB. and Table). This observation confirms the general notion that the immune system responds to higher levels of infection by producing higher concentrations of pro-inflammatory cytokines.

The slope and the intercept determine a linear relationship. A larger slope indicates that with a similar increase in the value of $\log P$, a faster increase in the value of $\log C$ will result. Therefore, the slope reflects the kinetics of $\log C$ accumulation, and is named as parameter of kinetics or “k”. During the course of infection in our sub-lethal model, the average bacterial loads remained steady during the first 10 days before a measurable decline on day 14 [5]. Accordingly, it was expected that the host immune response would initiate, peak and subside during these two weeks. Consistently, the “k” values for both $\text{IFN}\gamma$ and $\text{TNF}\alpha$ increased first and then decreased (Table).

With the same slope, two linear relationships could differ in the intercept. Here, the value of the intercept is the $\log C$ value when $\log P$ is 0. When $\log P=0$, $P=1$, so the intercept reflects the amount of the cytokine that already exists when one “new” bacterium is detected at the systemic site at a given time. Therefore, the intercept is named “b” for baseline. In mice left uninfected, the serum levels of cytokines were below the detection limit. For $\text{TNF}\alpha$ from 4 days post infection to 10 days post infection, the b value followed the same trend as that of k: it increased first, then decreased. For $\text{IFN}\gamma$, however, it increased for the first 10 days post infection. This difference could reflect a difference in the production of these two cytokines-- $\text{TNF}\alpha$ is mainly produced from the cells of the innate immune system while $\text{IFN}\gamma$ is produced from the cells of both the innate and the adaptive system.

In a linear relationship, the value of one variable can be calculated from the other if the slope and the intercept are known, so that:

$$\log C = k \log P + b$$

After transformation,

$$\begin{aligned} \log C &= \log(10^b P^k) \\ \text{or } C &= 10^b P^k \end{aligned}$$

If naming magnitude or $m=10^b$, then:

$$C = m P^k.$$

Cytokine and bacterial colonization levels have long been associated with the progress of infection, and as demonstrated here, these two properties can be expressed in the simple mathematical relationship of $C = m P^k$ during infection.

3.2. The relationship of serum $\text{TNF}\alpha$ and $\text{IFN}\gamma$ concentrations and bacterial levels in other *Yersinia* infection models

Next, we investigated whether this relationship is maintained in mouse infections with other *Y. pseudotuberculosis* strains. Brodsky and Medzhitov recently reported serum cytokine and splenic CFU levels in mice after intragastric infection with a strain, $Yp\Delta yopJ/pYopP$, which is derived from *Y. pseudotuberculosis* IP2666 [6]. In that report, 5 days post infection 90% of the mice infected with this strain were alive, while overall 40% survived until 14 days post infection. The serum $\text{IFN}\gamma$ and $\text{TNF}\alpha$ concentrations, and bacterial levels as measured on 3, 5 and 7 days-post infection were in good linearity after logarithmic transformation (Fig. 2A; for simplicity, only the day 5 data were shown). Specifically, the R^2 values (which indicate the goodness of correlation) of $\text{TNF}\alpha$ are 0.60, 0.88 and 0.93, and those of $\text{IFN}\gamma$ are 0.44, 0.65 and 0.90, respectively for days 3, 5 and 7-post infection. This result indicated that

this relationship between cytokine and bacterial levels exists in other *Y. pseudotuberculosis* infection models.

In a rat model of bubonic plague established using *Y. pestis* strain 195/P, Sebbane et al. also reported the levels of $\text{IFN}\gamma$ and $\text{TNF}\alpha$ in serum and the bacterial load in blood 48 to 72 hours post infection [7]. In this model, all infected rats developed signs of terminal plague between 2–4 days. Interestingly, while the linearity between $\log C_{\text{TNF}\alpha}$ and $\log P_{\text{CFU}}$ is medium ($R^2=0.56$), the value of the slope is negative (Fig 2B, squared points). So with the increase of blood colonization by *Y. pestis*, the serum concentration of $\text{TNF}\alpha$ decreased, suggesting an inhibitory effect of the bacteria on $\text{TNF}\alpha$. This phenomenon is consistent with the non-stimulatory lipopolysaccharide that *Y. pestis* synthesizes at 37°C [8] and that $\text{TNF}\alpha$ is protective of *Y. pestis* infection [3]. This result not only supports the existence of $C=mP^k$ during infection with *Y. pestis*, but also suggests that a distorted relationship could be used to deduce the specific host response with which a pathogen interferes.

In contrast to $\text{TNF}\alpha$, the linearity between $\log C_{\text{IFN}\gamma}$ and $\log P_{\text{CFU}}$ of this rat infection model is poor ($R^2=0.05$). However, close analysis revealed that one rat carried a high level of bacteria in its blood; the bacterial count reached 10^9 CFU/ml, a level seen in saturated *Y. pestis* culture in optimum growth medium. The $\text{IFN}\gamma$ concentration in this mouse did not follow the trend established by the rest (Fig. 2B, data point in circle). Disregarding this data point, the R^2 value increased from 0.05 (dashed line in Fig. 2B) to 0.63 (solid line in Fig. 2B). This phenomenon indicated that when an animal is closer to terminal disease, some cytokines may deviate from normal regulation. This phenomenon is probably due to the depletion of immune cells responsible for cytokine regulation [9,10].

3.3. Relationship between other cytokines and bacterial colonization levels during *Yersinia* infection

We wondered whether other cytokines also follow this trend during infection. Using the samples obtained from our initial study, we determined the serum concentrations of IL-1 β , a pyrogen and a pro-inflammatory cytokine typically highly expressed after infection. Of the 15 samples collected 4 days post infection, only two yielded IL-1 β concentrations higher than the detection limit (50 pg/ml). However, in the majority of the samples collected 7 days post infection, the IL-1 β concentrations were higher than the detection limit. More importantly, $\log C_{\text{IL-1}\beta}$ and $\log P_{\text{CFU}}$ values also followed the trend (Fig. 1C, $R^2=0.54$, $k=0.36$).

Like IL-1 β , the cytokine IL-18 also belongs to the IL-1 superfamily [11]. We recently observed high serum concentrations of IL-18 during infection of mice with *Y. pseudotuberculosis* [5]. From 4 days to 7 days post infection, the linearity between $\log C_{\text{IL-18}}$ and $\log P_{\text{CFU}}$ increased from low to medium (Fig. 1D; $R^2=0.33$ and 0.48 respectively), while the k value increased from 0.18 to 0.34. Collectively, the concentrations of IL-1 β or IL-18 also follow the relationship with the bacterial load.

3.4. Relationship between cytokine and other pathological reagents

Given the conserved pathways leading to the production of cytokines, it is reasonable that $C=mP^k$ is valid under other pathological conditions. In an attempt to characterize the castor bean toxin ricin, Yoder et al analyzed the cytokine monocyte chemotactic protein 1 (MCP-1) in the mouse duodena homogenate after ricin challenge [12]. When their results were re-analyzed here, the concentrations of MCP-1 and injected ricin also formed a straight line after logarithmic transformation (Fig. 4, $R^2=0.8399$). This result suggests that the relationship between cytokines and the levels of pathogenic agents described here could be universal.

4. Discussion

Using data from mouse infections with the Gram-negative bacterial pathogen *Y. pseudotuberculosis* and published results from other groups, we showed here that the serum concentrations of several cytokines and the concomitant loads of pathogenic reagents can be expressed in a simple relationship of $C = mP^k$, where C represents the concentration of a cytokine and P the pathogen load. Because a limited number of receptors, adaptor proteins and kinases regulate the innate immune response [1], it is expected that such a relationship is universal.

The linear relationship between $\log C$ and $\log P$ suggests that the regulation of the cytokine network can be assessed with changes in the values of k and m for each individual cytokine. In the early phase of inflammation, by increasing the slope, which is represented by the value of “k”, the host increases the speed of the response and by increasing the intercept, which is represented by “b” or “m”, the magnitude of the response is increased. On the other hand, at the resolution of infection, the immunosuppressive cytokines can reverse the process by decreasing the slope, the intercept, or both simultaneously, of a pro-inflammatory cytokine. Modulation of either “k” or “b” will result in exponential changes in cytokine concentrations, so the alteration is very rapid and dramatic. This could be the mathematical basis by which the cytokine network is regulated.

General concepts established in Immunology could explain the changes in the values of either “k” or “b”. For example, both natural killer (NK) cells and T cells secrete $IFN\gamma$. In our study, when the dominant host immune response is transitioning from innate to adaptive from 4 to 7 days post infection, an increased “k” value is observed (Table). The difference in the value of k could reflect the difference in kinetics with which these two types of immune cells are activated. In contrast, in any given animal, the concentration of $IFN\gamma$ on day 7 was not necessarily higher than it was on day 4. Clearly, the state of the immune response is better depicted with the parameters of “k” or “m” than the absolute concentrations of the cytokines.

The establishment of this relationship, or the deviation from it, could also be helpful in other areas of research. For pathogenesis studies, by comparing the “k” and “m” values of key cytokines, it is possible to identify or confirm the steps in host response that the pathogen interferes with, as exemplified by the negative “k” value of $TNF\alpha$ during *Y. pestis* infection in Fig. 2B. In addition, with the accumulation of data, by observing deviation from established trends, it may be possible to foretell if an individual, either a patient or an experimental animal, might no longer withstand an infection. On the side of the host, analyzing the changes in the values of “k” and “m” could be useful in deciphering the functions of specific genes when knockout animals were studied.

There are potentially other important implications. In our study, the P value in the equation was calculated as the quantity of culturable microbes, but in fact it represents the combination of pathogen associated molecular patterns and other danger signals that the immune system detects. In this sense, the formula can be used to correlate or even identify the cause of inflammation in sterile inflammatory diseases. This may lead to better therapeutic strategies.

Overall, the relationship of $C = mP^k$ represents not only the potential of a universal law regulating the concentrations of cytokines, but a powerful method to address some key questions in both immunology and infectious diseases. However, pathogens have diverse strategies to perturb the host cytokine network [13,14] and chronic or localized infections may result in different host responses from that of acute, systemic infections. Because there

are important medical implications of these findings, it is crucial to test in other fields whether $C=mP^k$ is a universal law for host cytokine response.

Acknowledgments

The authors are grateful for Dr. Wei-Yann Tsai for mathematical analysis, Dr. Igor Brodsky for sharing raw data and editorial suggestions, Drs. Jorge L. Benach, Martha B. Furie, and Adrianus W. M. van der Velden for critical reading of the manuscript, and for editing. This work was supported by NIH award AI043389 to JBB.

Abbreviations

CFU	colony forming unit
TNFα	tumor necrosis factor
IFNγ	interferon γ
MCP-1	monocyte chemotactic protein 1
IL-1β	interleukin-1 β
IL-18	interleukin-18

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Fig. 1.

The relationship between systemic cytokine concentrations and *Y. pseudotuberculosis* colonization levels. Female C57B/6 mice of 9 weeks old were intragastrically infected with 5×10^7 colony forming units (CFU) of *Y. pseudotuberculosis* strain 32777 for 4, 7 or 10 days. The serum TNF α (A), IFN γ (B), IL-1 β (C) and IL-18 (D) concentrations were determined using ELISA. Colonization levels in the spleens were determined after homogenization, serial dilution and plating. Each symbol represents the value from one mouse. The results shown are the summary of three to five independent experiments at each time point. Results shown for A, B and D are derived from published data with the exception of cytokine levels 10 days post infection [5].

Fig. 2.

The relationship between cytokines and pathogen load during infection with another *Y. pseudotuberculosis* strain (A) or *Y. pestis* (B). A. Distribution of serum logC_{IFN γ} or logC_{TNF α} and splenic logP_{CFU} values 5 days-post infection with *Y. pseudotuberculosis* strain Yp Δ yopJ/pYopP. Intra-gastric infection of mice, and the determination of serum cytokine and splenic CFU levels have been described by Brodsky and Medzhitov [6]. B. Distribution of serum logC_{IFN γ} or logC_{TNF α} and logP_{CFU} values during infection with *Y. pestis*. Values were calculated from published work of Sebbane et al [7]. Data points were omitted if the cytokine concentration was listed as below detection limit (<40 pg/ml). In either A or B, each symbol represents the value from one mouse.

Fig. 3.

The relationship between MCP-1 and the concentration of ricin in a mouse model of intestinal ricin intoxication. The MCP-1 concentrations were estimated from published work of Yoder et al [12], the concentration of ricin shown corresponds to the amount used to challenge mice intragastrically. MCP-1 concentration from duodena homogenate was determined 24 h post challenge.

Table

Values of some basic parameters of the lines depicted in Fig. 1.

TNFα	Day 4	Day 7	Day 10
R ²	0.66	0.46	0.37
Slope (k)	0.31 $\pm$ 0.060	0.40 $\pm$ 0.14	0.27 $\pm$ 0.20
Intercept (b)	-0.44 $\pm$ 0.29	0.11 $\pm$ 0.73	-0.17 $\pm$ 1.0
IFN γ			
R ²	0.82	0.62	0.70
Slope (k)	0.26 $\pm$ 0.030	0.36 $\pm$ 0.090	0.14 $\pm$ 0.053
Intercept (b)	1.57 $\pm$ 0.14	1.76 $\pm$ 0.47	2.08 $\pm$ 0.28