

The Observation of Reactive Thrombocytosis in New Zealand White Rabbits in Response to Experimental *Pasteurella multocida* Infection

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ABSTRACT: Reactive thrombocytosis is an increase in the circulating thrombocyte count secondary to a physiologic process within the body, often an infection. Reactive thrombocytosis is different than primary or essential thrombocytosis which is usually related to myeloproliferative neoplasia. Essential thrombocytosis is most common in adults, whereas reactive thrombocytosis is most frequently observed in children. Reactive thrombocytosis has been occasionally reported in cats, dogs and horses but has not been previously reported in the rabbit. Rabbits were challenged with virulent *Pasteurella multocida*. Hematologic, clinical, and culture assessments were performed prior to challenge, enabling each animal to serve as its own control. The questions asked were whether reactive thrombocytosis was a consistent phenomena and whether its presence and/or intensity was related to disease severity. All challenged rabbits demonstrated some degree of thrombocytosis in response to the infection, but individual rabbits were varied in their pattern of thrombocytosis. Elevations varied from intense to mild to undulating with durations of 1 to 11 days above $500 \times 10^9/L$ and 0 to 5 days above $650 \times 10^9/L$. Correlation analysis was unable to demonstrate significant association between thrombocytosis, body temperature, leukocyte count, or the granulocyte lymphocyte ratio (all $r < 0.2$). No significant association between intensity of thrombocytosis and degree or type of pathologic lesions was observed. Thrombocytosis does not appear predictive of disease intensity or outcome. The data indicate that in the rabbit thrombocytosis is a consistent response to infection with *P. multocida*. Rabbits may serve as a model for the study of reactive thrombocytosis, in humans especially in children infected with *Haemophilus* sp., which are also a members of the bacterial family Pasteurellaceae. © 1999 Academic Press

Keywords: animal model; *Pasteurella multocida*; platelets; rabbits; reactive thrombocytosis

INTRODUCTION

Awareness of thrombocytosis has paralleled the development of automated cell counters (1). Primary thrombocytosis may be a manifestation of neoplasia or an idiopathic process referred to as essential thrombocytosis. Secondary or reactive thrombocytosis is an increase in the peripheral thrombocyte count due to metabolic processes within the body or a physiologic response to disease (2). The etiology and significance of reactive thrombocytosis are unknown and the condition is rarely associated with hemostatic abnormalities (3, 4), thus, reactive thrombocytosis is often ignored. Prior to the diagnosis of reactive thrombocytosis, essential thrombocytosis and myeloproliferative neoplasia must be excluded (5). Reactive thrombocytosis may be associated with infection, trauma, acute or chronic inflammation, renal disease and splenectomy (4). Reactive thrombocytosis is a variable phenomenon whose diagnostic and prognostic value is unclear but may be related to the secretory function of platelets during inflammation (6–9).

Reactive thrombocytosis is best described in humans (2), it is occasionally reported in cats, dogs and horses (10), and is previously unreported in rabbits. The mean and range for thrombocyte counts in the rabbit are more variable and wider than that reported for other species (10, 11). Depending on the researcher, thrombocyte counts in rabbits range from $250 - 1,100 \times 10^9/L$ (10–15). The factors governing this variability in rabbits are uncharacterized. Low end values may be related to sample handling (platelet clumping), and upper end values may be related to species specific qualities, health status, and variations in measurement technique or equipment (16).

In humans the physiologic associations of thrombocytosis vary with patient age. Prior to puberty thrombocytosis is often secondary to inflammation especially of infectious etiology. After puberty, thrombocytosis is most often due to neoplasia or essential thrombocytosis. Thrombocytosis may be observed with any condition generating an acute phase reaction. (3, 4, 17). The intensity of thrombocytosis among children varies with age. Greater intensity is found with younger age. As children approach puberty the response fades towards an

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adult-like response. Whether the intensity of the thrombocytosis reflects the intensity of physiologic derangement is undetermined.

In this study, reactive thrombocytosis was observed consistently in rabbits challenged with *Pasteurella multocida*. The thrombocytosis was analyzed to determine the relationship of reactive thrombocytosis intensity to disease processes, including other hematologic parameters and post-mortem culture and organ pathology.

MATERIAL AND METHODS

Animals and Husbandry

Forty *P. multocida* specific pathogen free New Zealand White rabbits were purchased at 4 weeks of age from the Western Oregon Rabbit Company (Philomath, OR). Rabbits were housed in a portion of the animal care facility at Animal Resource Services, University of California at Davis isolated from conventional rabbits (those of unknown bacteriologic status). Barrier status was maintained by physical distance, a separate building, separate ventilation and designated animal care personnel having no contact with other rabbits. For the first ten days, rabbits were housed as pairs and fed free choice alfalfa hay. Afterwards, during the next two months prior to study commencement and during the study rabbits were individually caged and fed commercial rabbit pellets (Rabbit Chow, Purina Mills, St. Louis, MO). Water ad libitum was available at all times. The light/dark cycle through out the study was 12 hours and 12 hours. Physical parameters, such as appetite, weight, chest auscultation, dyspnea, body temperature and attitude were monitored prior to and after bacterial challenge. Rabbits were humanely euthanized with an intravenous injection of sodium phenobarbital (Euthasol, Delmarva Laboratories, Midothian, VA). The study protocol adhered to the NIH guidelines (NIH Publication No. 85-23) for the care of laboratory animals and was approved by the University of California at Davis IACUC.

Sample Collection

Blood sample collection began after a 10 day conditioning period. Initially the small size of the weanling rabbits prevented collection of volumes adequate for processing by electronic cell counter. Until 11 weeks of age peripheral blood specimens were collected and hematologic assessment consisted of a mechanical packed cell volume and a differential leukogram without a total cell count. Starting at 11 weeks of age blood was collected by jugular venipuncture.

Samples were placed in Vacutainer tubes with EDTA (Becton Dickinson, Rutherford, NJ) until performance of a complete blood count (CBC) by electronic cell counter (Serono-Baker Diagnostics, System 9000, Allentown, PA) in which gating was set individually for each sample to maximize discrimination between cell sizes. CBCs were carried out within 4 hours of collection to minimize platelet clumping. CBCs included a differential leukogram and scanning of slides for the presence of platelet clumping.

To establish base line parameters and allow each rabbit to serve as its own control, three CBCs and differential leukograms were carried out in the ten days prior to challenge. Blood was collected for an additional CBC just prior to inoculation of the rabbits with pathogenic *P. multocida*. In summary, blood was collected for CBCs on day -9, -6, -3, 0, 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36 and 39.

Four rabbits died during the study due to fulminant pasteurellosis, and the remaining 34 rabbits were euthanized on the fortieth day. Complete necropsies were performed on all rabbits including gross examination of tissues for abscesses and other evidence of infection. Tissue samples from the liver, heart, lung, kidney, testes or ovary, brain, and any abscesses were collected for histopathologic examination. Five micron sections of tissues were examined by light microscopy after hematoxylin and eosin staining. Postmortem cultures were carried out looking for *P. multocida*. Cultures included the frontal sinuses and both tympanic bullae as well as all of the tissues collected for histopathologic exam.

Challenge

To insure the *P. multocida* free state of the rabbits prior to challenge, the external nares of all rabbits were cultured by swabbing with a sterile cotton tipped applicator followed by streaking directly onto sheep blood agar plates (Remel, Lexana, KS) which were incubated at 37°C for 36 to 48 hours before reading. Nasal isolates were identified biochemically (18, 19). Two rabbits were designated as sentinels and sacrificed prior to challenge, to obtain cultures of internal organs and deep body cavities checking for *P. multocida*.

The *P. multocida* used for challenge was of rabbit origin. The strain was isolated from a rabbit that had died of disseminated abscesses within the abdominal and chest cavities. Consistent and reproducible pathogenicity for rabbits was established prior to initiating this study. A 24 hour culture of *P. multocida* grown in brain heart infusion broth (Difco Media, Detroit, MI) was used as inoculum for the study. *P. multocida* (0.1 ml of approximately 10⁸ colony forming units/ml) was topically instilled into each nostril and each lower bulbar conjunctival sac of all rabbits.

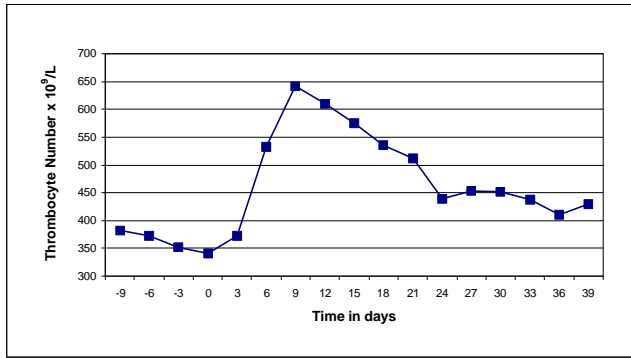


Figure 1. Mean thrombocyte count in rabbits challenged with *P. multocida*. The prechallenge baseline mean calculated from days -9, through 0 was $371 \times 10^9/L$. The mean thrombocyte count based on days 24 through 39, after the period of maximal thrombocytosis, was $507 \times 10^9/L$ which was significantly different from the prechallenge baseline count ($p < 0.0001$).

Hematology

The predominant granulocyte of the rabbit is the heterophil. Due to its polychromatic staining the heterophil has earned itself many names including pseudoeosinophil (13), amphophil (12), fine granular oxyphil, micro-oxycte, oxyphil, micro-oxyphil, amphi-oxyphil [authors of the latter names cited in Scarborough (12)] and heterophil (10). The polychromatic staining properties make it difficult to differentiate heterophils from eosinophils (10, 13). As other researchers have done (15, 20), all polymorphonuclear leukocytes are reported collectively as granulocytes.

Definition of Reactive Thrombocytosis

The definition of reactive thrombocytosis in humans differs with the critical point varying between $500 \times 10^9/L$ and $1,000 \times 10^9/L$ depending on the researcher (2,17,21–24). Reactive thrombocytosis is poorly defined and arbitrary in animal species. The defining point for reactive thrombocytosis should reflect the mean and the ranges in thrombocyte count for a species under the conditions of good health. The thrombocyte range in a state of good health for most laboratory animal species (11) and domestic animal species (10) is between $250 \times 10^9/L$ and $400 \times 10^9/L$, similar to that seen in humans, thus, based on extrapolation from these species and the pre-challenge mean thrombocyte count of $371 \times 10^9/L$ for rabbits in the study, a count greater of $650 \times 10^9/L$ was chosen to define reactive thrombocytosis. Less dramatic increases in platelet numbers are also included in the graphic descriptions of study observations to provide perspective with regards to the variability of response and its graded nature.

Data Analysis

A granulocyte/lymphocyte ratio was calculated, reported and compared to the thrombocyte count. Thrombocyte counts, leukocyte counts and body temperature pre- and post- infection were compared using Student's "t" test. Correlation analysis was used to look for associations between thrombocyte numbers, leukocyte counts, granulocyte lymphocyte ratio, and body temperature.

To examine the predictive potential of thrombocytosis on disease outcome, reactive thrombocytosis was dichotomously categorized as intense when an elevation of $650 \times 10^9/L$ or greater was present on at least three consecutive measurements. If rabbits exhibited a thrombocytosis of $650 \times 10^9/L$ or greater on only a single occasion and/or on non-consecutive measurements, then rabbits were considered to have exhibited milder reactive thrombocytosis. Rates of positive cultures and frequencies of histopathologic lesions were then compared in rabbits with intense and mild thrombocytosis.

RESULTS

Prechallenge Activities

Nasal cultures and internal cultures on the 2 sentinel rabbits were all negative for *P. multocida*. Prechallenge nasal cultures on the other 38 rabbits were uniformly negative for the presence of *P. multocida*. No rabbits demonstrated thrombocytosis prior to challenge with *P. multocida* (Figure 1).

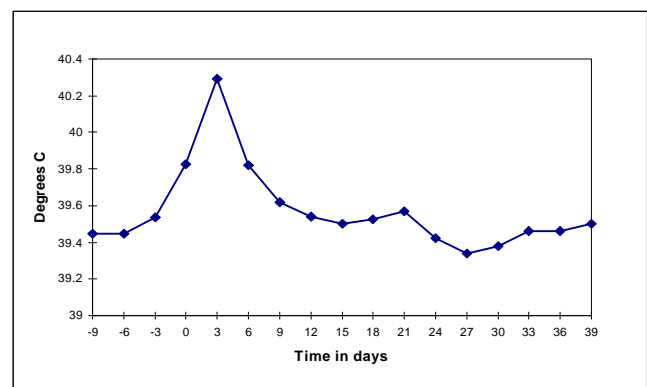


Figure 2. Mean body temperatures in rabbits challenged with *P. multocida*. The average body temperature for 2 months prior to study initiation was $39.5^\circ C$ (this information is not included on the graph). After day 12 the mean body temperature had returned to baseline levels where it remained throughout the remainder of the study.

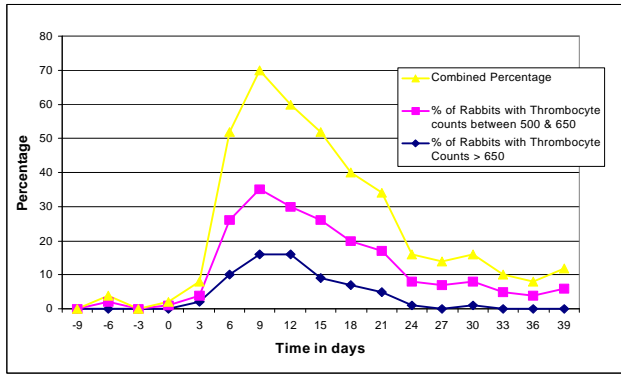


Figure 3. Temporal display of the percentage of *P. multocida* challenged rabbits in two different thrombocytosis ranges on each day of the study. The ranges are greater than $650 \times 10^9/l$ and between $500 \times 10^9/l$ and $650 \times 10^9/l$. Also presented is a combined total of all rabbits greater than $500 \times 10^9/l$. On day 9 after challenge there was greatest prevalence of thrombocytosis.

Body Temperature

Pyrexia was observed within 24 hours of *P. multocida* challenge. The pyrexia in most rabbits lasted approximately 72 hours (Figure 2).

Thrombocytosis

Around the third day after challenge all rabbits to varying degree exhibited an increase in their thrombocyte thrombocytosis which would have been missed without baseline data for comparison (Figure 4). Maximum thrombocytosis was observed on the sixth day, with numbers gradually declining over the next 2 weeks (Fig 1 and 3). with numbers gradually declining over the next 2 weeks (Fig 1 and 3). At study end, although, thrombocyte counts approached baseline levels, they were still significantly greater than initial values ($p < 0.0001$). Prior to *P. multocida* challenge (days -9, -6, -3 and 0) the mean thrombocyte count was $371 \times 10^9/l$. The mean thrombocyte count during the period of maximal thrombocytosis, i.e., day 6 through 21, was $592 \times 10^9/l$. Maximal thrombocytosis subsided on about day 21, the mean thrombocyte count after this period, i.e., day 23 until day 39 the study

endpoint, was $507 \times 10^9/l$. This mean was significantly higher than the pre-infection average ($p < 0.0001$) and in greater agreement with average thrombocyte counts commonly reported for rabbits (10, 11).

Hematology

Leukocytosis was observed starting around 6 days after challenge (Figure 5). The average leukocyte count (mean = $10.5 \times 10^9/l$) was significantly increased above baseline levels (mean = $7.3 \times 10^9/l$) ($p = 0.0344$). Similar to the thrombocyte counts, the leukocyte counts for individual rabbits at study completion were higher than the initial leukocyte counts ($p = 0.0386$).

Changes in the granulocyte/lymphocyte ratio were observed (Figure 6). Baseline differential leukograms showed granulocytes and lymphocytes in the 30% and 60% range, respectively, resulting in a granulocyte lymphocyte ratio less than 1, i.e., an average ratio of 0.8386. After challenge the granulocyte and lymphocyte percentages became inverted and the granulocyte lymphocyte ratio was greater than 1. The inversion was biphasic with an initial change being observed soon after challenge and lasting approximately 6 days. The granulocyte lymphocyte ratio was then normal (< 1) for the next 3 to 5 days after which the ratio proceeded to undergo a more sustained inversion (i.e., day 18 until study end at day 39 the mean ratio was 1.4967).

The relationships between the thrombocyte count and other continuous variables (leukocyte count, body temperature and granulocyte lymphocyte ratio) were examined by correlation analysis. Additionally, the relationship between body temperature and leukocyte count was also examined by correlation analysis. All correlation coefficients were low (all $r < 0.1$).

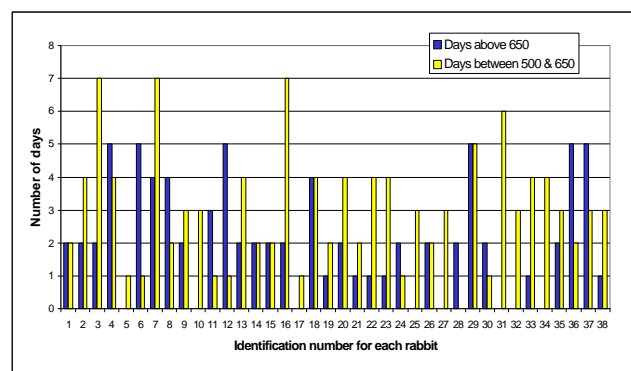


Figure 4. The number of days of elevated thrombocytes in each individual rabbit challenged with *P. multocida* categorized into two levels, above $650 \times 10^9/l$ and between 500 to $650 \times 10^9/l$. All rabbits exhibited some degree of increased thrombocytes, although the intensity and duration of that increase varied.

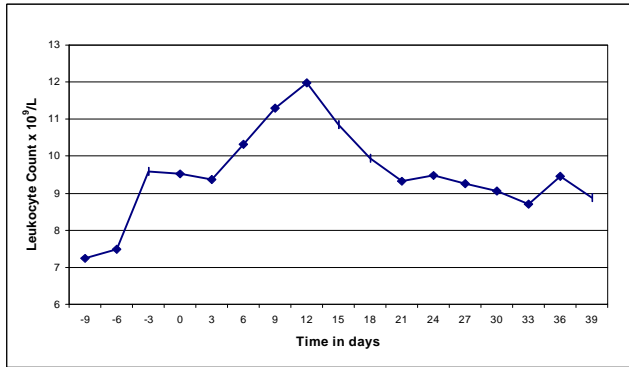


Figure 5. Mean leukocyte counts in rabbits challenged with *P. multocida*. A pattern of leukocyte elevation similar to that seen with thrombocytes was observed. It should be noted that like the thrombocytes the leukocyte count did not return to base line levels by the time of study end but was within the range of what is considered normal for total leukocyte count in rabbits. The prechallenge mean was $7.3 \times 10^9/L$ and the postchallenge mean was $10.3 \times 10^9/L$. The difference is statistically significant ($p=0.0386$).

Postmortem Cultures

The majority (84%) of rabbits were culture positive for *P. multocida* at postmortem at one or more sites. There was no statistical difference in the rate of culture positivity when rabbits with intense and mild reactive thrombocytosis were compared regardless of site (all $p>0.05$). The two most common sites of *P. multocida* recovery were the frontal sinuses (79% prevalence) and the tympanic bullae (middle ear) (34% prevalence). There were few clinical symptoms associated with any lesions and most rabbits continued to grow and gain weight throughout the study.

Histopathology

Histopathologic lesions were minimal and changes showed no statistically difference in prevalence between rabbits with intense and mild reactive thrombocytosis (all $p's > 0.05$). Nonpathogenic splenic hyperplasia, compatible with immunologic response to infection was found in 70% of the rabbits. The most common pathologic change was the presence of protein casts within the kidney tubules. Casts were found in 71% of the rabbits without difference in prevalence regardless of thrombocytosis intensity.

DISCUSSION

This study demonstrates reactive thrombocytosis in association with experimental *P. multocida* infection of

specific pathogen free rabbits by comparing pre-infection baseline thrombocyte counts with post-infection counts (Figure 1). Most literature to date reports thrombocyte counts in rabbits with wide ranges and rarely mentions the *P. multocida* status of the rabbits (10–15). Pasteurellosis can be difficult to diagnose antemortem with its prevalence often approaching 100% in conventional rabbits (25, 26, 27). Observation of reactive thrombocytosis in specific pathogen free rabbits after infection with *P. multocida*, leads to the question of the impact of pasteurellosis in conventional rabbits on thrombocyte counts in rabbits sampled to determine normal values. Supporting the hypothesis that pasteurellosis impacts the platelet count is the report of Kabata et al. (16), in which rabbits culture negative for gram-negative pathogens, including *P. multocida*, possessed thrombocyte counts ranging from $160\text{--}400 \times 10^9/L$.

Prior to challenge with *P. multocida* the mean thrombocyte count was $371 \times 10^9/L$, an average similar to that observed in other mammalian species. All rabbits experienced some degree of thrombocytosis (Figure 4) after challenge. The average thrombocyte count after the period of maximal reactive thrombocytosis until study end (i.e., days 22 to 39), was $507 \times 10^9/L$ which was significantly higher than the preinfection average ($p<0.0001$). Necropsy cultures at study end revealed that 84% of the rabbits were positive for *P. multocida* indicating that most rabbits were still infected, suggesting a possible etiology for the continued thrombocyte elevations above baseline levels. The thrombocytemean at study end ($507 \times 10^9/L$) was in

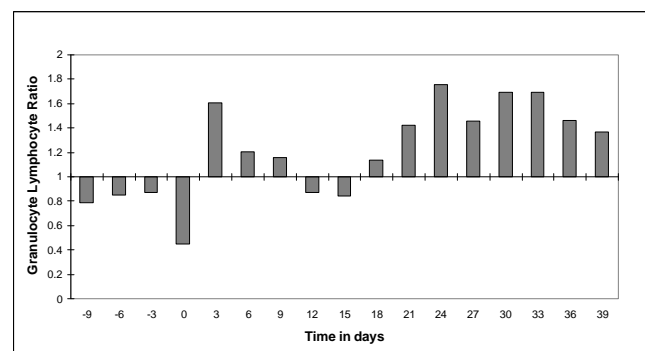


Figure 6. The granulocyte/lymphocyte ratio (GLR) in rabbits challenged with *P. multocida*. The mean GLR in all rabbits for the three months prior to challenge was 0.8386. A biphasic response occurred after challenge. Initially the GLR increased immediately after inoculation, returned to baseline after 10 days, and then exhibited a prolonged elevation throughout the remainder of the study. The mean GLR from day 18 through day 39 was 1.4967 this was significantly different than the mean baseline GLR ($p < 0.0001$).

keeping with many of the previously reported thrombocyte averages for rabbits (10, 11, 28). It should not be construed from the observation of thrombocytosis in association with pasteurellosis that an increased platelet count can be used as a diagnostic tool. The previously reported wide ranges in thrombocyte counts, the current observation of day to day vacillations in thrombocyte numbers in some individuals, and spontaneous resolution of reactive thrombocytosis precludes use of thrombocytosis predictively or diagnostically.

When researchers working with mice (29, 30) observed low platelet numbers their technique and methodology was questioned (31). As performed by Longmore, et al. (30), we used a Sero-Baker 9000 with the ability to detect thrombocytes of small size from many different animal species. Slides were scanned for platelet clumping to ensure accuracy of the automated count. Additionally, rabbits served as their own controls; pre-infection rabbits were compared to post-infection rabbits, thus, the influence of individual animal, technical, and mechanical variations on comparisons was minimized. Samples were collected by jugular venipuncture, reducing the likelihood of platelet clumping as compared to small peripheral vessel collection techniques such as “tail vein milking” in rodents (29) or the typical ear vessel collection used in rabbits.

With the observations of high thrombocyte counts in association with pasteurellosis, the impact of *P. multocida* infection in other studies on thrombocytes parameters needs to be reviewed (32–34). These studies utilized conventional rabbits from a source with endemic pasteurellosis. Whether this confounded the observations on variations in platelet parameters from dietary hyperlipidemia is unknown. It is known that the secretory products of activated platelets modulate the metabolism of low density lipids, lipoproteins and cholesterol (6–9). The reported thrombocyte means in these studies (34) was close to the post-infection mean of the current study suggesting some degree of platelet activation. The study of Scherer, et al., (35) reporting on the functional changes in platelet activity after endotoxin exposure also fails to describe the *P. multocida* status of the rabbits. Future studies of the impact of endotoxin on platelet function must address the *P. multocida* status of blood donor rabbits because of the possibility of physiologic priming or cross reaction of exogenous endotoxin with endotoxin from the bacterial wall of *P. multocida*. None of these studies (32–35) describe the bacterial status of the rabbits used. On consultation with the suppliers listed in their material and methods sections (personal communication) the presence of endemic pasteurellosis was acknowledged. What was the impact of the pasteurellosis status of the rabbits on the thrombocyte changes observed in these experiments?

The significant leukocytosis ($p=0.0386$) observed in this study (Figure 5) was in contradiction to other studies of

rabbits with bacterial infections (15). The difference may reflect a difference in bacterial etiology, or the usage of specific pathogen free rabbits rather than conventional rabbits and comparison of pre-infection baseline values with post-infection measurements. The leukocytosis observed was mild compared with the response of other mammalian species such as cats and dogs (10), and would have been missed without baseline information. Like the lingering thrombocytosis, leukocyte counts did not return to preinfection levels by the time of study termination.

The typical granulocyte/lymphocyte ratio of circulating peripheral blood in healthy rabbits is less than 1. In normal rabbits the predominant circulating leukocyte is the lymphocyte comprising 50 to 70% of the total, with granulocytes comprising 30 to 40% of the total. The leukocyte differential of rabbits may vary in response to physiologic conditions (14,36), infection, and inflammation (15). Changes in the differential leukogram consisting of a relative increase in the percentage of granulocytes and relative decrease in the percentage of lymphocytes is typically observed in rabbits responding to infection (15). This results in an inversion of the granulocyte lymphocyte ratio, which then becomes greater than 1, as was evident in the rabbits in this study (Figure 6).

Reactive thrombocytosis in children is most commonly associated with respiratory *Haemophilus influenzae* infection (3, 4, 20), which like *P. multocida* is a member of the bacterial family Pasteurellaceae. Rabbits in this study were young and may have been showing similar age related response to infection of Pasteurellaceae etiology as that observed in children.

Some researchers attribute the transitory nature of thrombocytosis to antibiotic intervention (20,37). Rabbits, in spite of demonstrable pasteurellosis at necropsy, showed spontaneous partial decline in thrombocyte counts in 10 to 20 days without therapeutic intervention. Thus, decreasing thrombocyte numbers cannot be attributed to resolution of infection.

Factors known to stimulate thrombocytosis are inflammation, infection, endotoxemia, trauma, neoplasia and surgery (1,4,17,21). These factors are identical with those initiating an acute phase response (38,39). The relationship between the acute phase response and thrombocytosis is unclear, as is the pathophysiology of reactive thrombocytosis. The acute phase response is mediated by cytokines such as TNF, IL-1 and IL-6. Thrombocytosis likely originates in response to the inflammatory cytokine milieu and the platelets released secrete additional inflammatory products (6-9). Investigation and clarification of the role of cytokines in reactive thrombocytosis of the rabbit must await development of cytokine assays specific for the rabbit.

The current study suggests many potential future explorations. Thrombocyte numbers in specific pathogen free rabbits and conventional rabbits without experimental

infection could be compared. If thrombocyte counts are significantly different this would lend credence to the notion that chronic pasteurellosis leads to elevations and fluctuations in thrombocyte ranges in the rabbit. To further clarify the nature of thrombocytosis, additional experimental infections with *P. multocida* and other pathogens in rabbits at younger and older ages are needed. These studies could also include histopathologic examination of bone marrow to assess megakaryocyte changes. Depending on these results and development of rabbit cytokine assays, it is possible that the rabbit may serve as a model to study the physiology of reactive thrombocytosis in young children and answer questions regarding the relationship between reactive thrombocytosis and the acute phase response.

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