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Review Article

Hypothesis: Hemolytic Transfusion Reactions Represent an Alternative Type of Anaphylaxis

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Abstract: Classical anaphylaxis is the most severe, and potentially fatal, type of allergic reaction, manifested by hypotension, bronchoconstriction, and vascular permeability. Similarly, a hemolytic transfusion reaction (HTR) is the most feared consequence of blood transfusion. Evidence for the existence of an alternative, IgG-mediated pathway of anaphylaxis may be relevant for explaining the pathophysiology of IgG-mediated-HTRs. The purpose of this review is to summarize the evidence for this alternative pathway of anaphylaxis and to present the hypothesis that an IgG-mediated HTR is one example of this type of anaphylaxis.
Key Words: hemolytic transfusion reaction, anaphylaxis, platelet activating factor

Introduction

The classical form of anaphylaxis is an immediate type allergic reaction that can be fatal in as many as 2% of cases [1]. Although anaphylaxis was described over a century ago, it is still under recognized, under reported, and under treated [2]. In addition, the mechanism by which a localized allergic response becomes rapidly generalized in only a select set of sensitized individuals is poorly understood [2]. Most human cases of anaphylaxis are caused by the IgE pathway [3] where a small amount of allergen cross-links IgE antibodies bound to their receptors (i.e. FcεRI) on mast cells and basophils [4]. This event activates these cells, thereby releasing multiple inflammatory mediators, most notably histamine, but also including serotonin, platelet-activating factor (PAF), and leukotrienes [4]. These mediators then cause the various manifestations of anaphylaxis such as hypotension, bronchoconstriction, urticaria, dyspnea, abdominal cramping, and diarrhea [3].

Despite the apparent simplicity of this explanation of anaphylaxis, there is anecdotal evidence describing anaphylactic reactions in humans without evidence of elevated plasma histamine or serum tryptase levels (the latter a marker of mast cell degranulation), and without IgE antibodies specific for the offending allergen [4, 5]. In addition, interestingly, histamine and tryptase levels do not correlate well with each other during acute allergic reactions [6]. Finally, a well-described animal model of IgG-mediated anaphylaxis is histamine- and mast cell-independent; instead, it depends on macrophages as the primary effector cell and PAF as the primary mediator responsible for the resulting clinical manifestations [7]. Although not yet clearly documented in humans, we hypothesize that this alternative pathway of anaphylaxis plays a central role in the transfusion medicine setting where some individuals mount severe and potentially fatal IgG-HTRs in response to the transfusion of incompatible blood.

Alternative Pathway of Anaphylaxis

Several murine models of the IgG-mediated alternative pathway of anaphylaxis have been described [7-10]. For example, this alternative pathway can be modeled by injecting mice with the 2.4G2 rat monoclonal antibody (Mab) which recognizes an epitope shared by two

mouse Fcγ receptors (FcγRs): FcγRII and FcγRIII. Binding of this antibody cross-links these receptors, thereby stimulating PAF release [8, 11]. Interestingly, these anaphylactic reactions still occur when the 2.4G2 Mab is injected into IgE-deficient, FcεRIα-deficient, or mast cell-deficient mice [7, 8]. The clinical manifestations seen with the alternative pathway are similar to those found with the classical IgE-dependent pathway, including significant mortality and various physiologic changes, such as tachycardia, hypotension, decreased body temperature, vascular leakage, and airway constriction [8-10, 12]. Thus, at least in mice, neither mast cells nor IgE antibodies are necessary for the expression of an active systemic anaphylactic reaction.

In another murine model of the alternative pathway, injection of goat anti-mouse IgD antibody initially induces interleukin (IL)-3 and IL-4 production, which promotes both mastocytosis and significant mouse IgE and IgG antibody responses specific for goat IgG [13, 14]. The subsequent injection of goat IgG leads to anaphylactic reactions in these sensitized mice, with signs and symptoms similar to classical anaphylaxis. However, these reactions also occur in IgE-deficient, FcεRIα-deficient, or mast cell-deficient mice [7]. A series of experiments in this model, using depletion of different inflammatory cell populations, various knockout mice, and several pharmacologic inhibitors, shows that the function of this alternative pathway requires IgG1 antibodies, the FcγRIII for IgG, macrophages, and PAF [7-9]. Using this approach, both classical IgE-mediated anaphylaxis and alternative IgG-mediated anaphylaxis are complement independent [7].

Subsequent studies showed that a larger dose of antigen is needed to induce IgG-dependent anaphylaxis as compared to classical IgE-mediated anaphylaxis [15]. **Table 1** summarizes the characteristics of these two pathways of anaphylaxis. Most commonly, anaphylaxis is caused by a small dose of antigen (e.g. an insect sting), which would likely be too weak a stimulus for the alternative pathway; thus, the classical pathway is the most likely culprit in these situations and the alternative pathway is likely to have gone unnoticed. However, with the transfusion of a relatively large amount of incompatible blood, IgG-mediated HTRs may occur and this would be consistent with the large antigen dose necessary to initiate the alternative pathway of anaphylaxis.

Similar to the alternative model of anaphylaxis, the aggregates of immunoglobulins that may be found in intravenous immunoglobulin (IVIg) can also cause a similar phenomenon. Early trials with these immunoglobulin preparations caused severe anaphylactoid reactions in human patients [16]. Interestingly, polymeric and dimeric IgG in human IVIg induces dose-dependent neutrophil degranulation *in vitro* via FcγR-dependent signal transduction [17]. When infused into rats, these IgG aggregates cause profound hypotension requiring FcγRs, macrophages, and PAF [18]. Thus, it is likely that cross-linking activating receptors on macrophages causes the release of pro-inflammatory mediators such as PAF, which then lead to the symptoms of anaphylaxis.

PAF and the Alternative Pathway of Anaphylaxis

The primary mediator of anaphylaxis in the alternative pathway is PAF, a phospholipid with the chemical name of 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine [19]. PAF is a potent activator of platelet aggregation, leukocyte chemotaxis, inflammation, and classical anaphylaxis [20]. It is not stored in cells and is synthesized from lysophosphatidylcholine and

Table 1 Comparison of classical and alternative pathway of anaphylaxis in mice (adapted from [3])

Characteristics	Classical Pathway	Alternative Pathway
Antibody isotype	IgE	IgG
Antibody Fc receptor	FcεRI	FcγRIII
Main inflammatory mediator	Histamine	PAF
Effector cell type	Mast cell	Macrophage
Complement	Independent	Independent
Antibody concentration needed	Low	High
Antigen amount needed	Low	High

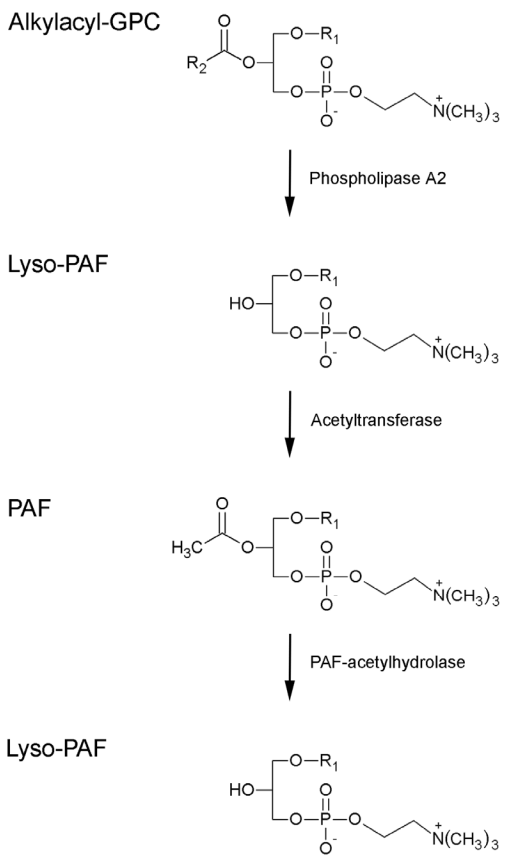

Figure 1 Important steps in the biosynthesis and degradation of PAF. R_1 and R_2 represent alkyl chains; GPC represents glycerophosphocholine.

acetyl CoA by an acetyltransferase; the latter is the key regulator of PAF synthesis in macrophages [21]. PAF is degraded and inactivated by PAF acetylhydrolase (PAF-AH), a Ca^{2+}-independent phospholipase A_2 (PLA_2) [22] that hydrolyzes the acetate moiety in the sn-2 position of PAF [23]. **Figure 1** depicts PAF structure and the pathways of its biosynthesis and inactivation. Due to the presence of PAF-AH in plasma, the circulatory half-life of PAF is only a few minutes [24]; thus, PAF appears in measurable quantities in blood for only a very brief time. For example, in response to IgE-mediated anaphylaxis in rabbits, the serum level of PAF begins to rise approximately 30 seconds after antigen challenge, peaks at approximately 120 seconds, and returns to baseline by 300 seconds after antigen challenge [25].

PAF mediates its biological effects through binding to the PAF-receptor (PAF-R), a G protein-coupled receptor linked to several signal transduction pathways [26]. Mice lacking this receptor have impaired anaphylactic responses [26]. Aerosolized PAF induces bronchoconstriction in humans [27]. Infusion of PAF into animals produces the physiologic events associated with anaphylaxis, such as bronchoconstriction [28], increased vascular permeability [29], hypotension, and death [30]. In addition, PAF is the downstream mediator of the effects of tumor necrosis factor-alpha (TNF-α) and lipopolysaccharide (LPS), activates the complement system [31], and synergizes with components of the complement system (e.g. the anaphylatoxin C5a) to produce shock, tissue injury, and death [30]. Finally, PAF enhances phagocytosis of human red blood cells (RBCs) by monocytes in a model of complement-dependent clearance of oxidant-damaged RBCs [31].

PAF is produced by multiple cell types, including macrophages, neutrophils, basophils, platelets and endothelial cells [32-35]. However, the trigger for its release is specific for the individual cell type [32]. For example, neutrophils release PAF in response to stimuli to which monocytes are insensitive, such as C5a; however, both cell types release PAF in response to a phagocytic stimulus, with monocytes secreting the most PAF on a cell-for-cell basis (i.e. >100 times more per cell than neutrophils) [32].

The PAF inactivating enzyme, PAF-AH, was cloned by Tjoelker *et al* [36], and circulating enzyme originates from cells in the hematopoietic lineage, such as macrophages, mast cells, and activated platelets [22, 37]. Plasma PAF is primarily inactivated by the activity of PAF-AH [38]. Circulating PAF-AH levels are affected by both total cholesterol concentration [37] and a relatively common missense mutation in the PAF-AH gene (valine to phenylalanine at position 279); the latter is present in heterozygous form in up to 30% of the Japanese population (up to 5% of the population is homozygous) [39]. Decreased levels of PAF-AH activity, with resulting higher levels of circulating PAF, are associated with asthma [40], sepsis [24], and fatal anaphylaxis [41]. A recombinant form of PAF-AH has been tested in numerous animal disease models and has therapeutic benefit in animal models of inflammation, asthma, and sepsis [22, 38]. Unfortunately, as of yet,

recombinant PAF-AH has not been effective in human trials of sepsis or asthma [22] suggesting that PAF may not be the only relevant mediator in these conditions.

In addition to varying levels of PAF-AH, which may modify the severity of allergic reactions, the levels of certain cytokines may also modulate these reactions. For example, IL4 and IL13 potently enhance anaphylaxis induced through either the classical or alternative pathway; whereas IL12, IL18, and interferon-gamma (IFN-γ) inhibit allergic inflammation [42]. Thus, mice infected with the parasite *Trichinella spiralis*, which is associated with elevated IL4 and IL13 levels, have more severe and frequently fatal IgG-mediated anaphylactic reactions [42]. One mechanism for this enhancement is by increasing the sensitivity of the vascular endothelium to vasoactive mediators, thereby exacerbating vascular leak [42]. Thus, circulating cytokine profiles resulting from a concomitant illness may determine whether a patient experiences either a mild or severe anaphylactic reaction upon challenge with antigen.

Hemolytic Transfusion Reactions (HTRs)

HTRs are immunological reactions caused by the presence of antibodies in the transfusion recipient that recognize foreign alloantigens on the surface of donor RBCs. These reactions can be either acute (AHTR) or delayed (DHTR). AHTRs are medical emergencies and are usually caused by a clerical or managerial error leading to the transfusion of ABO-incompatible RBCs [43]. These reactions are caused by preformed, "naturally-occurring" IgM antibodies that fix complement and cause rapid intravascular destruction of transfused RBCs leading to the classical triad of fever, flank pain, and hemoglobinuria. These reactions can also lead to disseminated intravascular coagulation (DIC), shock, acute renal failure (ARF) due to acute tubular necrosis, and death [44, 45].

DHTRs usually occur 1-14 days after transfusion of alloantigen mismatched blood, and are caused by the development of significant titers of IgG antibodies due either to an anamnestic response in a previously sensitized individual or to a rapid primary antibody response [46]. These antibodies opsonize alloantigen-positive RBCs leading to extravascular hemolysis through phagocytosis by macrophages of the reticuloendothelial system [47]. The symptoms can be clinically inapparent other than the detection of a new alloantibody and a positive direct antiglobulin test (DAT), in which case the reaction is termed a delayed serological transfusion reaction (DSTR) [48]. The DAT in DSTRs typically demonstrates the presence of an IgG antibody coating the transfused RBCs, although complement deposition may also occur [49]. DHTRs are similar to DSTRs except that they are associated with clinically significant hemolysis, which can be manifested by a drop in hemoglobin, fever, rigors, dyspnea, nausea, vomiting, malaise, pain, jaundice, shock, and renal insufficiency [47, 49]. DSTRs are not uncommon, with an incidence of 1 in 1612 RBC transfusions, whereas DHTRs have a lower incidence of 1 in 6715 transfusions [50]. Despite the relatively infrequent occurrence of DHTRs, these reactions can occasionally be severe and even fatal [51, 52]. Rarely, DHTRs demonstrate severe and persistent hemolysis, not only of the transfused incompatible RBCs, but also of autologous RBCs; this severe and often fatal complication is termed the "hyperhemolysis syndrome" [53].

In vitro models of DHTRs suggest that cytokines may be responsible for many of the clinical aspects of these transfusion reactions [47]. In these models, macrophage phagocytosis of IgG-opsonized RBCs leads to the elaboration of pro-inflammatory cytokines such as IL-1, IL-6, IL-8, monocyte chemoattractant protein (MCP)-1, and TNF-α [46]. When injected into animals, these cytokines can cause fever, the acute phase response, shock, thrombosis, tissue damage, and death [54-58]. The severity of this reaction may be modulated by the competitive IL-1 inhibitor, IL-1ra, which is also released from monocytes engaged in erythrophagocytosis [59, 60]. Thus, the clinical variability seen with DHTRs may result from the relative balance of pro- and anti-inflammatory cytokines [61]. Indeed, anecdotal evidence in humans receiving anti-Rh(D) (i.e. RhIg) treatment for chronic autoimmune thrombocytopenic purpura (AITP) [62, 63], or experiencing a DHTR [64], indicates that these cytokines are relevant and that the induced "cytokine storm" may mediate the adverse clinical symptoms.

The macrophage cytokine response elicited by phagocytosis of IgG-coated RBCs appears to be mediated largely by the low affinity FcγRI, although binding of IgG-coated RBCs to splenic macrophages can also be blocked by inhibition of FcγRIII [47]. In addition, the complement cascade may be involved in IgG-mediated HTRs [49]. With extravascular hemolysis, IgG-mediated activation of the complement cascade arrests after cleavage of C3, thereby coating the RBCs with C3b. The C3bi-coated RBCs interact predominantly with complement receptor CR3 on macrophages leading to phagocytosis, thereby resulting in their removal from the circulation [65, 66]. IgG antibodies to certain RBC antigens, most notoriously to those in the Kidd blood group system, are associated with severe HTRs, which may be immediate or delayed, and are thought to be due to complement-mediated intravascular hemolysis [67, 68]. It is still unknown why some IgG antibodies cause such severe life-threatening reactions while others are clinically silent.

Despite the results from predominantly *in vitro* studies, we clearly still do not understand very much about the pathophysiology of HTRs. This lack of understanding has prevented the development of new, specific, and effective therapies to treat individuals experiencing DHTRs. For example, the role of PAF in these reactions has not been studied. We propose that our understanding of HTRs should be reconsidered in light of the new concept of an alternative pathway of anaphylaxis.

Hypothesis

Our hypothesis is that the mechanisms underlying the signs, symptoms, and consequences of IgG-HTRs, such as DHTRs, result from this phenomenon exemplifying an instance of the alternative pathway of anaphylaxis. Thus, crosslinking of FcγRs on macrophages mediates both IgG-HTRs and IgG-mediated anaphylactic reactions and the resulting clinical symptoms are believed to be due to the elaboration of pro-inflammatory mediators such as cytokines and PAF. Complement does not seem to play a major role in either reaction and the antigen dose required to initiate anaphylaxis by the alternative pathway in mouse models is consistent with the large antigen dose of transfused RBCs necessary to cause IgG-HTRs. Table 2 summarizes the key similarities and differences between these two situations.

Clinically, both IgG-mediated anaphylaxis and IgG-HTRs can cause death, shock, thrombosis, and gastrointestinal symptoms. Pulmonary symptoms seem to be more apparent in anaphylaxis; however, in certain settings, it appears as if murine airways are relatively unresponsive to circulating PAF, and only PAF produced, or introduced, in the lung acts in a paracrine fashion to induce airway obstruction [26]. This may explain why pulmonary symptoms are not as apparent in IgG-HTRs, in which we hypothesize that PAF is released directly into the circulation.

The main question remaining to be answered is whether PAF is involved in IgG-HTRs. Given the importance of macrophages in the clearance of IgG-coated RBCs, and the finding that rat macrophages release PAF in response to phagocytosis of antibody-coated RBCs [69], it is reasonable to assume that PAF is secreted in large amounts in IgG-HTRs; nonetheless, this has yet to be shown. Although PAF is probably not the sole mediator of IgG-HTRs or IgG-mediated anaphylaxis, nonetheless, PAF is most likely an early mediator and may play a central role in these reactions.

Table 2 Comparison of IgG-HTRs and the alternative pathway of anaphylaxis

Characteristics	Alternative Pathway	IgG-HTRs
Antibody isotype	IgG	IgG
Antibody Fc Receptor	FcγRIII	FcγRI, FcγRIII
Main inflammatory mediator	PAF	Cytokines; PAF?
Effector cell type	Macrophage	Macrophage
Complement	Independent	Independent
Antigen amount needed	High	High
Symptoms	Death, shock, gastrointestinal symptoms, thrombosis, airway constriction and vascular leakage	Death, shock, gastrointestinal symptoms, disseminated intravascular coagulopathy, dyspnea and acute renal failure

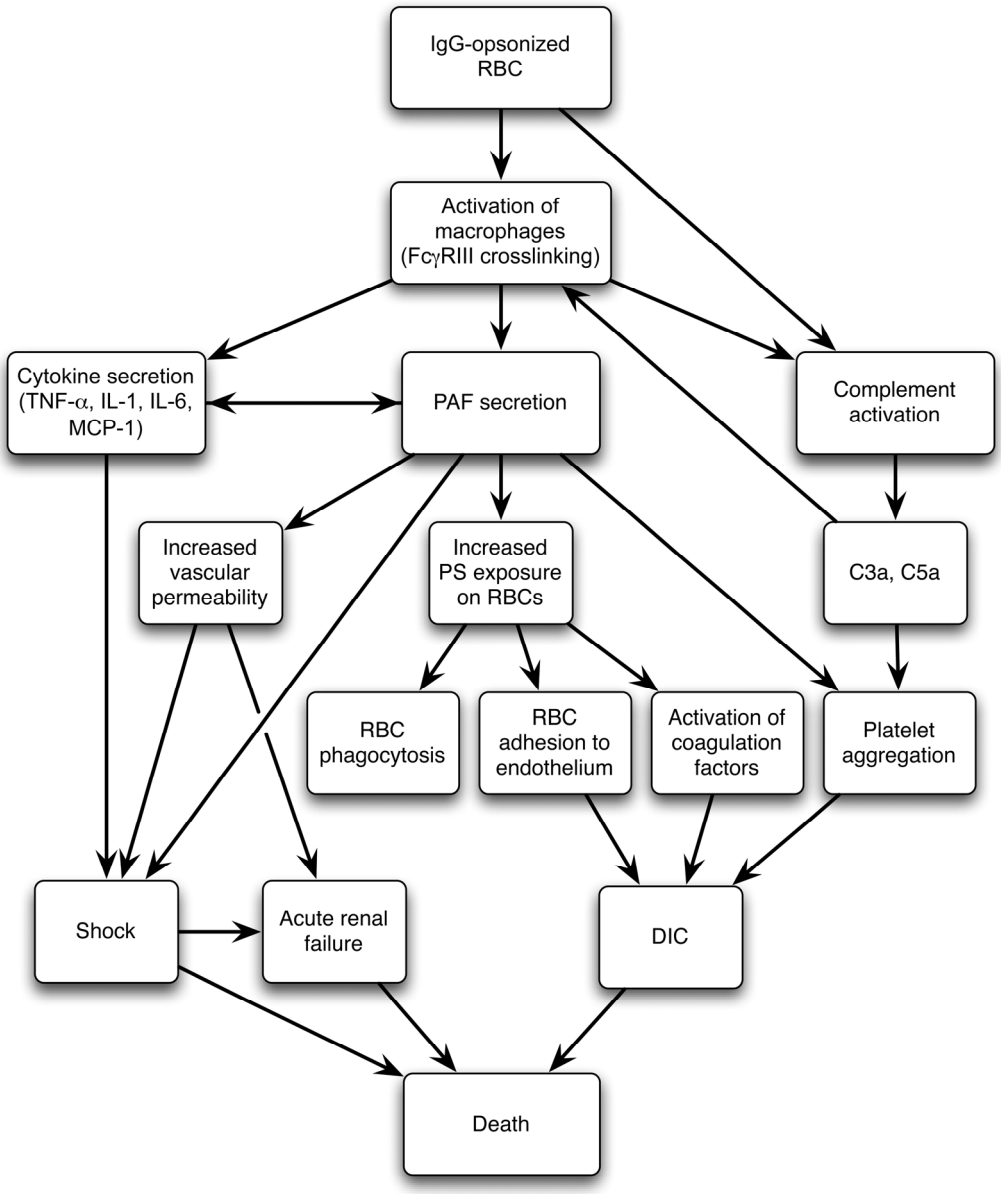

Figure 2 Proposed mechanistic pathway for IgG-mediated HTRs

Thus, the mechanistic pathway explaining the pathophysiology of IgG-HTRs is illustrated in **Figure 2**. IgG-coated RBCs are first phagocytosed by macrophages (presumably in the spleen and liver) through crosslinking of FcγRs. Massive activation of the FcγR signal transduction pathway then leads to PAF secretion by macrophages, along with secretion of other pro- and anti-inflammatory factors (e.g. cytokines such as TNF-α). Finally, PAF and cytokines bind to receptors on various target tissues causing the associated clinical signs and symptoms.

PAF-induced signal transduction leads to increased intracellular calcium levels in macrophages and RBCs [70, 71]. In addition to inducing of cytokine production in macrophages [72, 73], this leads to a loss of phosphatidylserine (PS) asymmetry in the RBC membrane [74]. Cell surface exposure of PS, by translocation from the inner leaflet to the outer leaflet of the RBC membrane, can also occur by the activation of a sphingomylinase, thereby increasing the cellular concentration of ceramide and the subsequent activation of a scramblase [75]. PS exposed on the RBC

surface enhances macrophage phagocytosis [76], increases adhesion to endothelial cells [77], and activates the coagulation cascade [78]. If IgG-HTRs produce increased circulating levels of PAF, then autologous RBCs will also be exposed to PAF; thus, this proposed mechanism also has the potential to explain innocent bystander hemolysis, where, during an IgG-HTR, autologous, compatible, recipient RBCs are cleared along with the incompatible transfused RBCs [49]. **Figure 3** depicts this hypothetical mechanistic pathway for innocent bystander hemolysis.

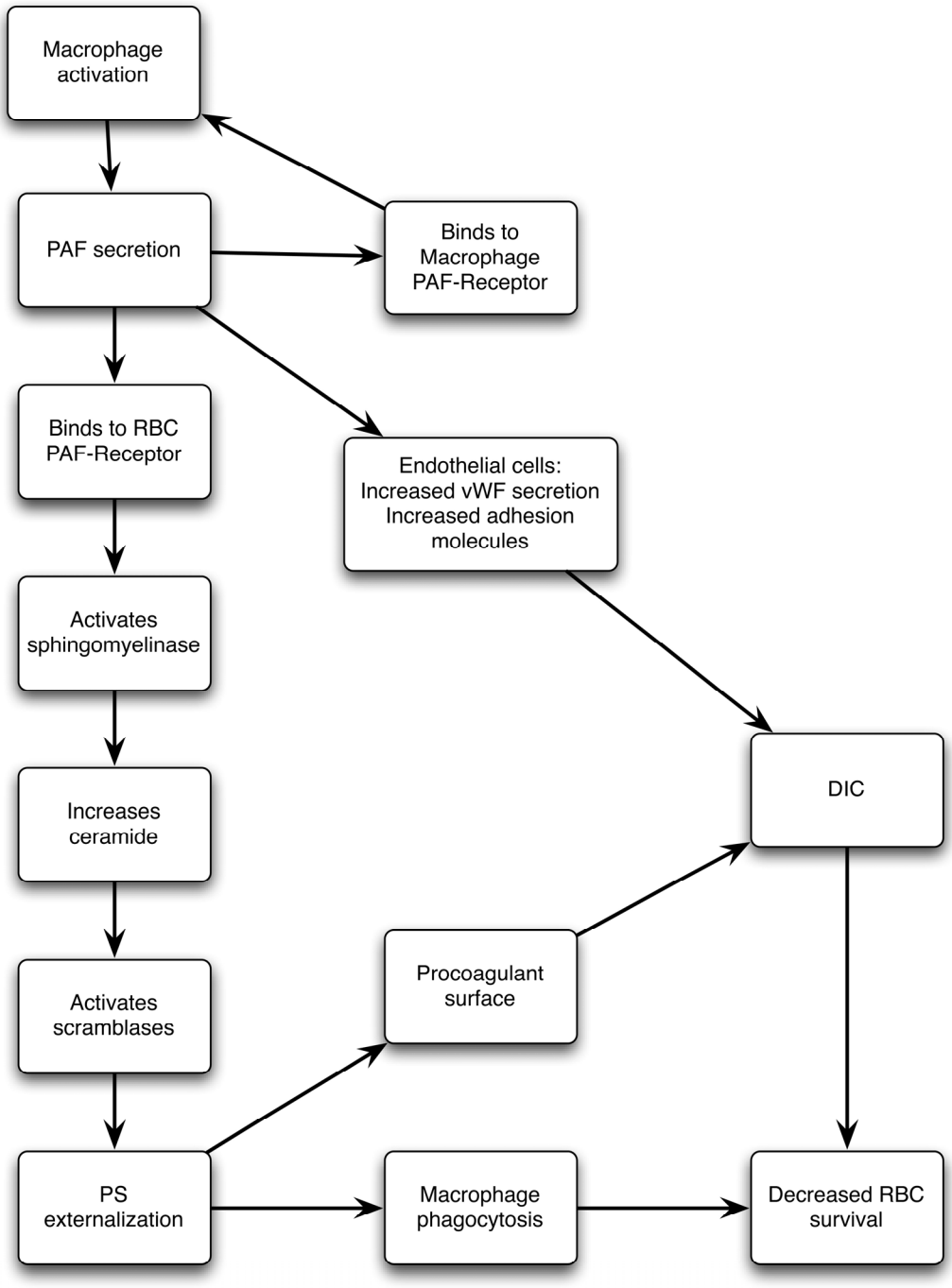

Figure 3 Proposed mechanism for innocent bystander hemolysis.

Future Studies Needed to Support the Hypothesis

In order to address this hypothesis, the role of PAF in HTRs must be evaluated. Human studies of HTRs are difficult to perform because their occurrence is sporadic and unpredictable. In addition, due to the short half-life of PAF in plasma, special collection procedures are required to rapidly inactivate PAF-AH activity (i.e. immediate acidification of the sample) making the logistics of collecting samples from human patients especially difficult. It may also be ethically inappropriate to try to obtain consent and collect samples from patients in the midst of a severe HTR. However, in parallel to a recent study showing that low PAF-AH activity is associated with more severe anaphylactic reactions [41], testing whether low PAF-AH activity is associated with patients manifesting severe HTRs would provide evidence for the importance of PAF in the pathophysiology of HTRs. Therefore, measuring PAF-AH levels may be more feasible in sporadic cases of HTRs because this analyte is more stable and easier to measure.

Interestingly, clinical situations exist where HTRs are an expected consequence, and these settings may be useful for determining the role of PAF in human IgG-HTRs. For example, one treatment for AITP in Rh(D)-positive patients is administration of antibodies against Rh(D) (i.e. RhIg). Thus, patients receiving RhIg for AITP may be good candidates for such studies because treatment induces IgG-mediated clearance of Rh(D)-positive RBCs and elicits a transient cytokine storm [63] along with rare severe HTRs [79, 80]. Another potential patient population that may be useful to study is transplant recipients (i.e. those with solid organ or hematopoietic stem cell transplants). Due to the presence of passenger lymphocytes in the transplant, it is possible to develop IgG-HTRs due to subsequent alloantibody production by donor lymphocytes against "foreign" antigens on the recipient's RBCs [81-83]. Nonetheless, because of difficulties inherent in human research, animal studies are clearly necessary.

Animal models of HTRs have been described and may prove useful in studying the role of the alternative pathway of anaphylaxis in the pathophysiology of IgG-HTRs. For example, we developed a model of HTRs in which mice transgenic for the human glycophorin A gene (the carrier of the human M/N blood group system) are used as blood donors to transfuse wild-type mice. Subsequent passive immunization with anti-glycophorin A monoclonal antibody leads to HTRs [84], including cytokine storm [85]. A mouse model of HTRs using mice transgenic for the human RBC Duffy(b) antigen has also been developed [86] and there is a rabbit model of HTRs [49]. In addition to measuring PAF levels in these animal models, experiments using pharmacologic inhibitors of PAF, recombinant PAF-AH, and mice deficient in the PAF-receptor will help determine the importance of PAF in the pathophysiology of IgG-HTRs.

The importance of macrophages in these IgG-HTR models can be studied by depletion of the macrophage population with agents such as liposomal chlodronate [87] or gadolinium [88]. In addition, a transgenic mouse expressing the diphtheria toxin receptor gene driven by the monocyte/macrophage-specific CD11b promoter sequence has been developed in which administration of diphtheria toxin leads to macrophage and monocyte depletion [89]. Finally, the roles of activating FcγRs and complement in the pathophysiology of IgG-HTRs can be determined using the relevant knockout mice.

Conclusion

We currently do not have good treatments for HTRs because they occur sporadically and are, thus, difficult to study. The similarity between HTRs and the alternative pathway of anaphylaxis makes it possible that they share the same pathophysiology. In addition to the human studies that will eventually be required to address this hypothesis, relevant mouse models exist and should be utilized. If the alternative pathway of anaphylaxis and PAF prove to play a significant role in human HTRs, the implications for novel therapies is vast as there is a myriad of available pharmacologic inhibitors of PAF; in addition, recombinant human PAF-AH has already been used in clinical trials. Regardless of whether this hypothesis proves correct, there is much to be gained from performing the proposed experiments, as studies into the pathophysiology of IgG-HTRs will shed light on important aspects of the human immune system.

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