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COMPLEMENT ACTIVATION IN RATS BY LIPOSOMES AND LIPOSOME-ENCAPSULATED HEMOGLOBIN: EVIDENCE FOR ANTI-LIPID ANTIBODIES AND ALTERNATIVE PATHWAY ACTIVATION

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Summary: Intravenous injection of hemoglobin-containing liposomes (LEH) caused a significant reduction in plasma hemolytic complement activity in rats on a time scale of minutes. Liposomes without hemoglobin also caused complement consumption, but less than LEH, while free hemoglobin was without effect. Consistent with complement activation, the LEH-induced drop in plasma hemolytic complement activity was closely paralleled by an increase in plasma thromboxane B_2 level. Studies to determine the mechanism of complement activation demonstrated the presence of natural antibodies in rat serum against all lipid components of LEH, thus, the potential for classical pathway activation. Yet, *in vitro* incubation of LEH with rat serum showed that: 1) EGTA/Mg^{++}, which inhibits complement activation through the classical pathway, did not inhibit complement consumption by LEH, and 2) the use of serum preheated at 50°C, which inhibits C activation through the alternative pathway by selectively depleting factor B, effectively reversed the complement-consuming effect of LEH. Consequently, LEH-induced complement activation in rat serum seems to involve primarily the alternative pathway.

The complement (C) system provides the first line of defense against foreign cells or particles, ensuring their cytolytic and/or phagocytic removal (1). C activation represents therefore a primary concern with regard to the biocompatibility of foreign particulate materials exposed to the blood for therapeutic purposes (2). Liposome-encapsulated hemoglobin (LEH) has been proposed as an artificial red blood cell substitute (reviewed in: 3, 4); however, no study to date has analyzed the interaction of LEH with the C system. Certain types of liposomes are

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Abbreviations used: AP, alternative pathway; C, complement; CH_{50}, measure of hemolytic C activity; CP, classical pathway; Hb, hemoglobin; SRBC, sheep red blood cell; TXB_2, thromboxane B_2.

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recognized as foreign and removed from the bloodstream through C-mediated phagocytosis (5-7). Thus, activation of C may entail reduced functional lifetime for LEH. Furthermore, the anaphylatoxins (C3a, C5a) released following C activation cause a wide variety of physiologic changes, which, in species where the desarginine derivative of C5a retains substantial spasmogenicity (for example in swine (8)), can lead to lethal anaphylaxis (9). In man, apart from a mild pulmonary dysfunction, C activation is generally harmless (10, 11). However, anaphylatoxin stimulation of granulocytes has been shown to play an important role in the pathogenesis of complications of hemorrhagic and septic shock, particularly in the development of adult respiratory distress syndrome (ARDS) (11). As hemorrhagic shock is a major area where an application for LEH is projected, it seems useful to be aware of possible C activation that could potentially cause clinical deterioration of trauma patients.

The main goal of the present study was to test if LEH-induces C activation in rats. Having established such activation, we also wished to determine whether naturally occurring antibodies existed against LEH components that could account for it, and whether C activation proceeds through the classical (CP) or the alternative pathway (AP).

MATERIALS AND METHODS

Materials: Distearoyl phosphatidylcholine (DSPC), dimyristoyl phosphatidylglycerol (DMPG), cholesterol (Chol) and α-tocopherol were obtained from Avanti Polar Lipids (Alabaster, AL). Human hemolysate, prepared from outdated washed human erythrocytes as described elsewhere (12), was provided by the Blood Research Detachment of the Walter Reed Army Institute of Research. Anti-sheep erythrocyte antiserum was purchased from Difco Laboratories (Detroit, MI).

Preparation of LEH: Details of the preparation of LEH from DSPC/DMPG/Chol/α-tocopherol (50:4.5:45:0.5 mole%), using a microfluidizer, were described earlier (13-16). LEH was washed by repeated centrifugation to remove unencapsulated hemoglobin (Hb) and suspended in isotonic phosphate buffered saline (PBS) in a concentration of 167 mM LEH phospholipid. Other parameters of LEH were as follows; Hb concentration, 5.8 g/100 ml; metHb, 12.3 %; mean diameter, 388 ± 55 nm; endotoxin level, 5-10 EU/ml (0.5-1 ng/ml). Empty liposomes (L) were prepared similarly, except that Hb was replaced by PBS. The mean diameter of L was 275 ± 45 nm. The preparations were tested for sterility by inoculating aliquots in thioglycollate broth followed by plating on agar and blood agar. LEH and L were stored at 4°C until use.

In vivo administration of liposomes and LEH in rats: Female Sprague-Dawley rats (280-340 g) were anesthetized with Metofane (Pitman-Moore, Mundelein, IL) inhalation, and 1-1.5 ml blood samples were taken from the tail artery for baseline parameters. LEH (n=5), L (n=5), Hb (n=4) and PBS (n=3) were injected i.v. into the femoral vein (5.6 ml/kg, which is approximately 10% of the blood volume of rat (15)) at a rate of 1 ml/min. Further 1-1.5 ml blood samples were taken from the tail artery at 10, 60, and 120 min into precooled EDTA/indomethacin-containing tubes (0.2 mM indomethacin to prevent prostaglandin metabolism), and, after separation of blood cells by centrifugation, plasma samples were stored at -80°C until further analysis. For *in vitro* studies serum was collected from untreated rats by allowing the blood to clot on ice before centrifugation at 4°C. Serum was also stored at -80°C.

Assay of total C hemolytic activity: The 50% hemolytic C activity (CH_{50}) was determined by the sheep red blood cell (SRBC) hemolysis assay (17). In brief, washed SRBCs were suspended

in veronal buffer containing 0.2% gelatin at a cell density of 10^9/ml. Hemolysin was added at a dilution of 1/1000, and the suspension was allowed to stand for 30 min at room temperature. Rat serum was added (2-12 µl to 1 ml of SRBC suspension), and the mixture was incubated in a shaking water bath for 60 min at 37°C. Samples were chilled on ice, centrifuged, and the degree of hemolysis was determined spectrophotometrically. CH_{50} was obtained from the intercepts of the regression lines of the log x versus log (y/100-y) plots, where x stands for the volume of rat serum (µl) added to 1 ml SRBC, and y denotes percent hemolysis (17). The regression lines were obtained from 2-3 values in the dynamic range of the assay (20-80% hemolysis). Their R^2 were in the 0.97-1.00 range.

Plasma TXB_2 assay: Circulating levels of plasma TXB_2 were determined by an ELISA kit obtained from Amersham Life Sci (UK).

ELISA for detection of natural antibodies against LEH and its lipid constituents in rat serum: Details of the solid-phase ELISA developed to detect lipid antigens were described earlier (18-20). In brief, wells of "U" bottom 96-well plates (Dynatech Labs., Alexandria, VA) were coated with antigen by incubation overnight at room temperature with LEH, L, DSPC, DMPG, and Chol. LEH and L were suspended in PBS (5 nmole phospholipid/50 µl); the lipids were dissolved in ethanol (5 nmole/50 µl). Plates were blocked with 100 µl of 0.3% gelatin in PBS (3-5 h at room temperature), washed with PBS, and incubated with 50 µl serum samples at various dilutions overnight at 4°C. Plates were then washed three times with PBS, and 50 µl of 500-fold diluted, affinity-purified, horseradish peroxidase (HPR)-conjugated goat anti-mouse IgG (H+L) (Bio Rad Lab, Hercules, CA), or HPR-conjugated goat anti-rat IgM (anti-µ chain) (Cappel, Organon Teknika, Corp. West Chester, PA) were added. After incubation for 4 h at room temperature or overnight at 4°C, plates were washed 3 three times with PBS and 100 µl of the substrate solution was added. After 15-60 min incubation in the dark, absorbance at 405 nm was read in a microtiter plate reader. The values were corrected for serum background, i.e., for positivity obtained in wells without antigen.

Adsorption of anti-LEH antibodies: LEH was incubated with rat serum (2:10 volume ratio) at 4°C for 2 min, followed by centrifugal separation at 2000 g, 4°C. This procedure was repeated two more times with the supernatants.

In vitro studies on complement activation by LEH: LEH was added to rat serum at a volume ratio of 2:10 LEH, and the suspension was incubated at 37°C in a shaking water bath (80 rpm/min) for 1 h. Samples were centrifuged and CH_{50} was determined in the supernatant as described above.

RESULTS

Activation of C by injection of liposomes and LEH in rats. Fig. 1 shows that i.v. injection of empty liposomes (L) and LEH in rats led to a significant drop of hemolytic C activity in the plasma within 10 min, while free Hb or PBS caused no changes in CH_{50}. The C-consuming effect of LEH tended to be more expressed than that of L, the difference reaching statistical significance at 60 min. Importantly, the LEH-induced rapid decline of CH_{50}/ml was closely paralleled by a pronounced rise of plasma TXB_2, a marker of TXA_2 production (8, 21). Since the anaphylatoxin byproducts of C activation (C3a and C5a) are known to cause TXA_2 release from blood cells (8, 21), the demonstration of increased plasma level of this metabolite provides supporting evidence for LEH-induced C activation.

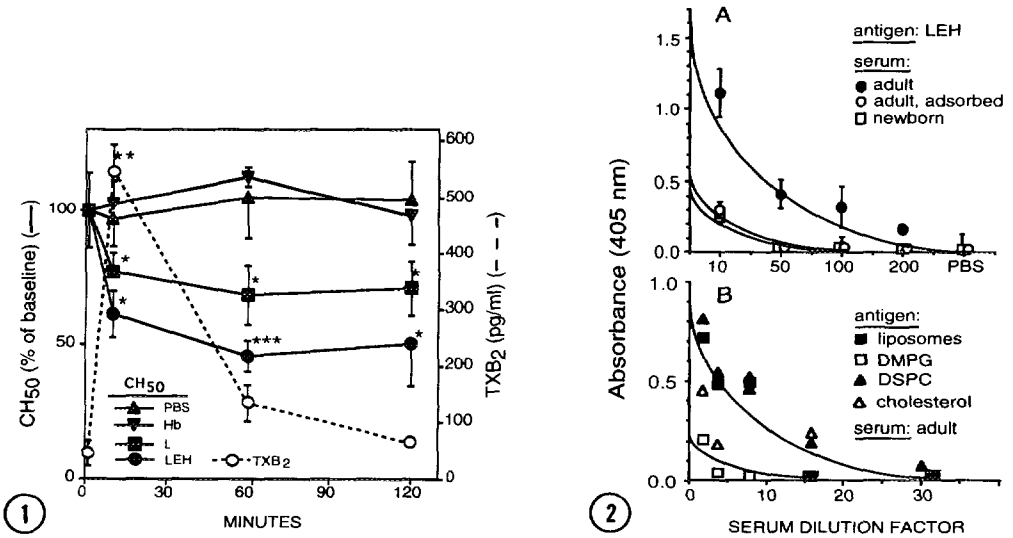

Figure 1. Complement consumption in rat plasma following injection with LEH, L, free hemoglobin (Hb), and PBS, and plasma thromboxane B_2 levels in rats injected with LEH. Rats were injected i.v. with volumes corresponding to 10% of their calculated circulating blood volume (10% top-load). Blood samples (1.0-1.5 ml) were taken at the indicated times; the plasma was separated and analyzed for CH_{50} and TXB_2 levels. Mean ± SE are shown for 5, 5, 4 and 3 animals in the LEH, L, Hb and PBS groups, respectively, except in the LEH group at 120 min, where n=3. The baseline error bars represent the variation coefficient for pretreatment CH_{50} values, which averaged 278/ml and 286/ml in the L and LEH groups, respectively. *, significant ($p \leq 0.05$) decrease from baseline, as determined by two-sample paired t-test (one-tailed). **, significant increase relative to baseline at $p<0.01$; ***, significant ($p \leq 0.05$) decrease from baseline, as determined by paired t-test, and also, significant ($p \leq 0.05$, two-tailed) decrease relative to L, as determined by two-sample t test.

Figure 2. Natural antibodies in rat serum having reactivity with LEH and liposomal lipids. A: adult serum represents pooled serum from four 4-month-old rats; adult adsorbed: serum represents adult pooled rat serum adsorbed with LEH at 4°C; newborn serum represents pooled serum from three 8-day-old rats. Values represent mean ± SD for triplicate wells; B: the key shows the lipid antigens used.

Natural antibodies against the lipid constituents of LEH. In an attempt to establish the mechanism of LEH-induced C consumption, we tested whether antibody-mediated C activation via the CP would occur in rat serum. As the ELISA results in Fig. 2A show, anti-LEH antibody was detectable in adult rat serum at dilutions ≤ 200, while the level of such antibodies in newborn rat serum was 4-5-fold less (seen only at 10-fold serum dilution). Furthermore, preadsorption of the serum with LEH at 4°C removed approximately 80% of the antibodies, suggesting that these antibodies were specific for LEH. Fig. 2B demonstrates antibodies in adult rat serum with reactivities against L as well as against each lipid constituent of LEH. These antibodies were detectable at lower (≤ 16) dilutions than the anti-LEH antibodies (Fig. 2A), which is consistent with the greater C activation by LEH than by L. Antibody binding to liposomal lipids was also demonstrated when HPR-conjugated goat anti-rat IgM (anti-μ chain) was used as second antibody, which detects only IgM (data not shown).

Effects of selective inhibition of the classical and alternative pathways on LEH-induced C activation. With the demonstrated presence of C-binding IgG and IgM antibodies against LEH, we anticipated that C activation by LEH would proceed through the CP. To verify this assumption, experiments were carried out in which LEH was incubated with rat serum *in vitro* in the presence and absence of 10 mM EGTA/2.5 mM Mg^{++}. The latter treatment is known to selectively inhibit C activation through the CP (2, 22-25). As shown in Fig 3A, *in vitro* reduction of serum CH_{50}/ml by LEH (ca. 40%) was comparable to its *in vivo* effect. Addition of 10 mM EGTA/2.5 mM Mg^{++} did not reverse the significant C consuming effect of LEH relative to serum controls (i.e., PBS-diluted serum incubated in the presence of 10 mM EGTA/2.5 mM Mg^{++}). In contrast, 10 mM EDTA, which inhibits both pathways of C activation, completely reversed the effect of LEH. The partial reduction of CH_{50}/ml in sera containing EGTA/Mg^{++} or EDTA was due to transfer of 20-100 μM chelator to the SRBC assay medium. Since 10 mM EGTA/2.5 mM Mg^{++} significantly inhibited the antibody-dependent hemolytic activity of rat C when tested with antibody-coated SRBC (data not shown), these observations argued for AP, rather than CP activation of C by LEH.

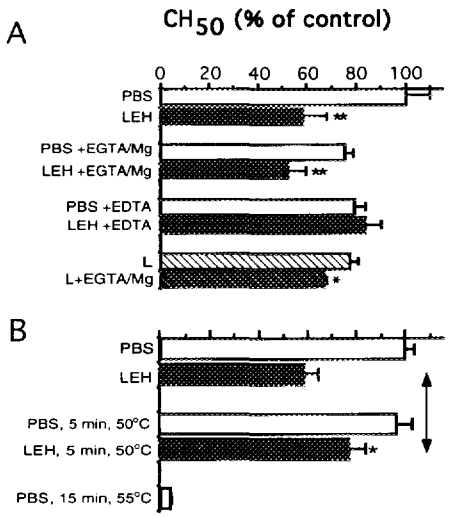

Figure 3. C consumption by LEH in rat serum *in vitro* following inhibition of the classical (A) and alternative pathway (B). A: Bars represent the CH_{50}/ml values (mean ± SE for 4-6 animals) incubated with LEH ± chelators and PBS + chelators, related to control serum which was diluted with PBS without chelators. **, significant decrease relative to control at $p<0.01$, and *, significant decrease relative to L at $p<0.05$, as determined by two-sample t test. The latter difference suggests acceleration of C consumption by Mg^{++} (23). The p values for differences between LEH and LEH+EGTA/Mg or between PBS+EDTA and LEH+EDTA were >0.05. B: Bars represent the CH_{50}/ml values (mean ± SE for 4 animals) relative to control (PBS-diluted, non-preheated serum) obtained in serum preincubated at 50°C for 5 min and then incubated for 1 h at 37°C with LEH or PBS. *, significantly less decrease of CH_{50}/ml relative to that in PBS control at $p<0.05$ (arrow), as determined by two-sample t test. Incubation of rat serum for 15 min at 50°C (23) led to a decrease in CH_{50}/ml even in the absence of LEH, most likely a consequence of partial depletion of CP factors in addition to factor B.

To test the involvement of the AP in LEH-induced C consumption, we incubated rat serum at 50°C and used these preheated sera for measuring C activation by LEH. This treatment has been used to selectively deplete factor B, a key component of the AP (23, 26). Fig. 3B shows that incubation of serum for 5 min at 50°C had no effect on hemolytic C activity, whereas LEH-induced C consumption in preheated serum was significantly less than that seen in nonpreheated serum. These observations are consistent with a critical role of factor B, and, hence, with the involvement of the AP in LEH-induced C activation.

DISCUSSION

Liposome-encapsulated hemoglobin (LEH) has been studied for the past decade as a red cell substitute that may avoid many difficulties associated with human blood transfusions (3, 4). Numerous studies have proved the efficacy of LEH in terms of oxygen transport and survival benefit in various animal models (27-30), or demonstrated its other advantages as a blood substitute (16, 31, 32). Until recently, however, little attention has been paid to its possible adverse effects *in vivo*. Studies in this context demonstrated transient (30-120 min) hematological and hemodynamic changes following injection of LEH in normovolemic rats (14, 15, 33, 34), which were linked, indirectly, to TXA_2 and platelet activating factor (PAF) production (14). However, many of the reported changes, including hyperventillation, tachycardia, decreased cardiac output, systemic hypotension with rebound hypertension, hemoconcentration, leukocytosis, thrombocytopenia and increased plasma level of TXB_2, are also known to occur upon C activation (8, 21), and PAF can play a mediator role in anaphylatoxin-caused pathophysiological changes (35, 36). These facts, taken together with our previous observation of C activation in pigs injected with liposomes composed of dimyristoyl phosphatidylcholine, dimyristoyl phosphatidylglycerol and cholesterol (9) prompted us to explore the effect of LEH on rat C.

We have administered LEH in a similar model in which the above side effects were reported (14, 15, 33, 34), and found a significant decline in plasma CH_{50}/ml with a parallel rise of TXB_2. The time course of these changes closely coincided with the hematological and hemodynamic changes, as well as with the rise of plasma TXB_2 in previous studies (maximal rate within 10 min) (9, 14, 15, 33, 34). Empty liposomes also caused C activation, which tended to be less expressed than with LEH. Importantly, a similar difference was observed between the effects of LEH and L on postinjection platelet count, tachycardia (33) and plasma TXB_2 levels (14). The finding that free Hb caused no C activation in our model is also in keeping with a lack of Hb-induced hematological and hemodynamic changes *in vivo* (14). Thus, taken together with previous information, the observations presented in this study are strongly suggestive of a causal relationship between C activation and LEH-induced pathophysiological changes.

The interactions of liposomes with the C system have been extensively studied in the past, leading to the use of antibody-coated liposomes as a means to study the mechanism of C-induced membrane damage (37). *In vivo*, liposomal activation of the CP occurs when antibodies to liposomal phospholipids, cholesterol or glycolipids bind to the vesicles (37). Natural antibodies to phospholipids and cholesterol are widespread in all animal species, although the

specificities and titers show substantial interspecies and intersubject variation (6, 18, 20, 38). Liposomes can also activate C through non antibody-mediated mechanisms via the CP (39-42), as well as through the AP (22, 23, 26, 42-45).

In this study we have demonstrated the presence of natural antibodies in rat serum against the lipid components of LEH, thus raising the potential for CP activation. Nevertheless, our *in vitro* studies, which essentially reproduced the *in vivo* C-consuming effect of LEH, presented two lines of evidence that suggest AP activation; 1) EGTA/Mg^{++}, which inhibits CP activation of C (2, 22-25), did not reverse the C consuming effect of LEH, and 2) the use of serum preheated at 50°C, which inhibits AP activation by selectively depleting factor B (23, 26), did inhibit LEH-induced C activation.

The mechanism by which LEH activates C through the AP in the presence of natural antibodies remains to be understood. There are numerous deviations from the paradigm of antibody-induced CP versus antibody-independent AP activation of C, which include cases when antibodies enhance the rate and/or initiate AP activation in an Fc-fragment-independent manner (46-50). In fact, among the earliest analyses of the AP of C activation the initiator was identified as the $F(ab')_2$ portion of IgG_1 and IgG_2 (51, 52). Thus, one possible mechanism for our observations is that antibody-mediated or antibody-augmented AP activation prevails for some reason over CP activation in our experimental system. Of particular relevance in this context, a recent study described a plasma factor that enabled cetylmannoside-containing liposomes to activate C via the AP (53). The effect was abolished by preadsorption of the plasma by the same, but not by other types of liposomes (53), attesting to the involvement of specific antibodies.

In summary, results of the present study suggest that activation of C by LEH could explain some previously observed side effects of LEH administration in rats. Despite the presence of natural antibodies against liposomal lipids, C activation by LEH seems to involve primarily the AP.

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