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COMPLEMENT ACTIVATION BY LIPOSOME-ENCAPSULATED HEMOGLOBIN *IN VITRO*: THE ROLE OF ENDOTOXIN CONTAMINATION

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ABSTRACT

Incubation of liposome-encapsulated Hb (LEH) with rat serum at 37°C led to accelerated decay of serum complement (C) hemolytic activity (CH_{50}). Empty liposomes (L) caused less expressed decrease of CH_{50}, whereas free Hb had no effect on C hemolytic activity. The LEH- and L-induced increase in C consumption was unlikely due to endotoxin (LPS) contamination, as spiking of rat serum with LPS caused reduction in CH_{50} only at levels significantly higher than detectable in LEH or L. The LPS-induced C consumption was not potentiated by free hemoglobin.

INTRODUCTION

Liposome-encapsulated hemoglobin (LEH) represents an approach to provide an universal erythrocyte substitute that avoids many difficulties associated with the transfusion of human blood (1-3). *In vivo* experiments have shown the efficacy of LEH in terms of oxygen transport and survival benefit after exchange transfusion (4-7), while other studies demonstrated the feasibility of LEH for large-scale production and long-term storage (8-10). Concerning the toxicity aspects of LEH, studies by Rabinovici et al. revealed transient (30-120 min) hematological and hemodynamic changes following iv. injection in rats (11-14). Since some of these side effects are also hallmarks of complement (C) activation (15-17), we wished to test the hypothesis that LEH might activate C in rats.

Incubation with serum has been shown to be a quick and convenient assay to test for C activation by various biomaterials (18-20). We applied this method to assess the C activating capacity of LEH and of corresponding liposomes (L).

Having established C activation by LEH and L, we also wished to clarify whether it could be due to the omnipresent endotoxin (LPS) in these preparations. In view of the projected application of LEH as an emergency resuscitation fluid, a further aim of this study was to elaborate on the clinical implications of LEH-induced C activation.

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 as described in (21), was provided by the Blood Research Detachment of the Walter Reed Army Institute of Research. Anti-sheep erythrocyte antiserum was purchased from Difco Labs. (Detroit, MI). Phenol-extracted LPS (E. coli 0111:B4) was from Sigma Chem. Co., (St. Louis, MO).

Preparation of Liposomes and 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 (10, 13, 14, 22). LEH was washed by repeated centrifugation to remove unencapsulated Hb and was suspended in isotonic phosphate buffered saline (PBS). Empty liposomes (L) were prepared similarly, except that Hb was replaced by PBS. Typical parameters of the preparations used were as follows; Hb concentration, 7-8 g/100 ml; metHb, 10-12 %; osmolarity, 290 mOsm/l; phospholipid concentration, 160 mM; mean diameter, 0.3 ± 0.1 μm; polydispersity index, 0.4. The level of LPS contamination in the preparations was tested by the Limulus amebocyte lysate-based gel-clot, kinetic turbidimetric and kinetic chromogenic assays (Associates of Cape Cod Inc., Woods Hole, MA). A detailed evaluation of these methods is presented in a contribution by R. O. Cliff et al. in this volume. All assays showed the LPS level to be ≤10 EU (≤1 ng/ml) in the tested preparations. LEH and liposomes were stored at 4°C until use.

Measurement of complement consumption by LEH and liposomes *in vitro*. LEH or L was added to rat serum (20 volume %) and the suspension was incubated at 37°C in a shaking water bath. At the indicated times aliquots were taken, centrifuged at 3000 g for 2 min, and C hemolytic activity (CH_{50}) determination was performed in the supernatant (for LEH) or in the subnatant (for L) as described below. Control sera were diluted by 20% with PBS.

Assay of total C hemolytic activity CH_{50} was determined by the sheep red blood cell (SRBC) hemolysis assay (23). 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 (2-12 µl) was added to each milliliter 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 calculated from the regression lines of the log of the volume (µl) versus log[(y/1-y)] relationship, where µl stands for the amount of rat serum added to 1 ml SRBC suspension, and y denotes percent hemolysis (23). The regression lines were obtained from 2-3 values in the dynamic range of the assay (20-80% hemolysis).

RESULTS

C-consumption in rat serum by liposomes and LEH *in vitro*

Fig. 1 shows that the C hemolytic activity in PBS-diluted rat serum gradually decreased over time upon incubation at 37°C. Addition of LEH accelerated the decay of CH_{50}, with the difference reaching statistical significance at 60 min. Empty liposomes also tended to decrease CH_{50}, although less than LEH. As shown in Fig 2 (in the context of LPS), free Hb caused no change in serum C hemolytic activity. Thus, encapsulation of Hb seemed to accentuate an intrinsic tendency of liposomes to cause C consumption.

The effect of endotoxin

LPS is one of the best known C activators (24), and a low level of LPS contamination was invariably (and unavoidably) present in the LEH and L preparations used in this study (≤10 EU/ml, ≤~1 ng/ml). To test if the level of LPS contamination in LEH could explain C activation, we have measured C consumption in rat serum spiked with increasing amounts of exogenous LPS. In light of the information on a potentiating effect of Hb on various biological activities of LPS (25, 26), the C-consuming effect of LPS was examined both in the presence and absence of human Hb. As shown in Fig. 2, E. coli LPS led to decreased CH_{50} values in the 100-500 µg/ml range, i.e., at concentrations several orders of magnitude higher than the LPS contamination of LEH or L. Hemoglobin did not augment the C-activating effect of LPS, but appeared to attenuate it.

DISCUSSION

In vitro evaluation of the C-activating capacity of particulate materials exposed to the blood for therapeutic purposes is an important part of assessing their biocompatibility (18-20). Liposome-encapsulated Hb is a prototype particulate material that is being developed as an oxygen-carrier red blood cell substitute (1-10). Hence, it is essential to explore its effect on C, as it has been done in the case of other oxygen carriers, including free tetrameric and crosslinked Hb (27-30). Yet, LEH has not been evaluated from this aspect, although liposomal activation of C has been demonstrated previously in many laboratories (31-40).

The data presented here show that LEH can activate C in a very similar time course that was reported for the LEH-induced hematological and hemodynamic changes *in vivo*, including hyperventillation, tachycardia, decreased cardiac output, systemic hypotension with rebound hypertension, hemoconcentration, leukocytosis, thrombocytopenia and increased plasma level of TXB_2 (11-14). The observation in our experiments that L tended to cause less C consumption than LEH is consistent with the relatively smaller thrombocytopenic, tachycardic (11) and TXB_2-increasing effects (13) of L relative to those of LEH. The finding that free Hb caused no C activation is also in keeping with a lack of Hb-induced hematological and hemodynamic changes *in vivo* (13). These correlations with the *in vivo* studies, taken together with the fact that the above mentioned pathophysiological changes are also known to occur following C activation (15-17), strongly suggest a causal relationship between these processes.

The mechanism by which LEH and L causes C activation is currently under study. According to the present results the low level of LPS contamination in LEH and L is not responsible for C activation. Some biological activities of LPS have been reported to be potentiated in the presence of Hb (25, 26), however, in our experiments free Hb did not increase LPS-induced C consumption. Nevertheless we can not completely exclude the possibility that liposome-bound, or liposome-associated Hb-bound LPS could play a role in C activation.

The practical implications of C activation by LEH are still unknown. One consequence, increased phagocytic uptake by RES cells is certainly unwanted, as it decreases the dwell time of LEH in the blood. As mentioned, C activation-related liberation of anaphylatoxins leads to a wide variety of pathophysiological changes, which, in species where the spasmogenic action of C5a-desArg is most expressed (e.g., swine, guinea-pig) (16)) may lead to lethal anaphylaxis (41). Human C5a-desArg, fortunately, does not belong to this category (16). C activation in healthy man is in most cases asymptomatic. Rarely it causes mild pulmonary dysfunction-related transitory symptoms, like chest-tightness and/or flushing (42-44). Nevertheless, in a situation which is of particular relevance to LEH, i.e., hemorrhagic shock, special attention might be warranted to the consequences of LEH-in-

duced C activation, as anaphylatoxin-mediated neutrophil activation has been shown to play an important role in shock-related tissue damages (17, 42, 43, 45, 46).

There are many possible approaches to prevent or alleviate the adverse consequences of C activation. Strategies in this regard include pharmacologic inhibition of secondary mediators of anaphylatoxin action, e.g. by indomethacin, antihistamines, etc., (16, 41); inhibition of C-receptor-mediated phenomena, and the design of LEH invisible for C, for example by using stealth lipids (47, 48) or by adopting the C evasion strategies of mammalian cells and microorganism (49) (e.g., surface modification with sialic acid, complement regulatory proteins). Clearly, there is ample space for creativity in this area.

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Legend to the Figures

FIGURE 1. Complement consumption in rat serum following incubation with LEH, empty liposomes (L) or an equal volume of PBS. Results (mean ± SE for sera from 4 rats) are expressed as % of baseline, i.e., the CH_{50} level in PBS-diluted sera before incubation, which averaged 174.4 ± 6.5 CH_{50}/ml. *, significant ($p \leq 0.05$) decrease relative to PBS control, as determined by two-sample t test.

FIGURE 2. The effect of endotoxin on rat serum C hemolytic activity in the presence and absence of human hemoglobin. LPS, at concentrations shown in the key, was added to rat serum which contained 2.4 g/100 ml human Hb (Hb group), or was diluted with a corresponding volume of PBS (PBS group). After incubation for 60 min at 37°C, samples were analyzed for C hemolytic activity in the SRBC assay. The bars represent C consumption, related to control (i.e., without LPS). The LPS content of Hb was 1.3 ng/ml. R^2 values for the regression lines from which the CH_{50} values were derived were in the 0.9-1.00 range.

Fig. 1.

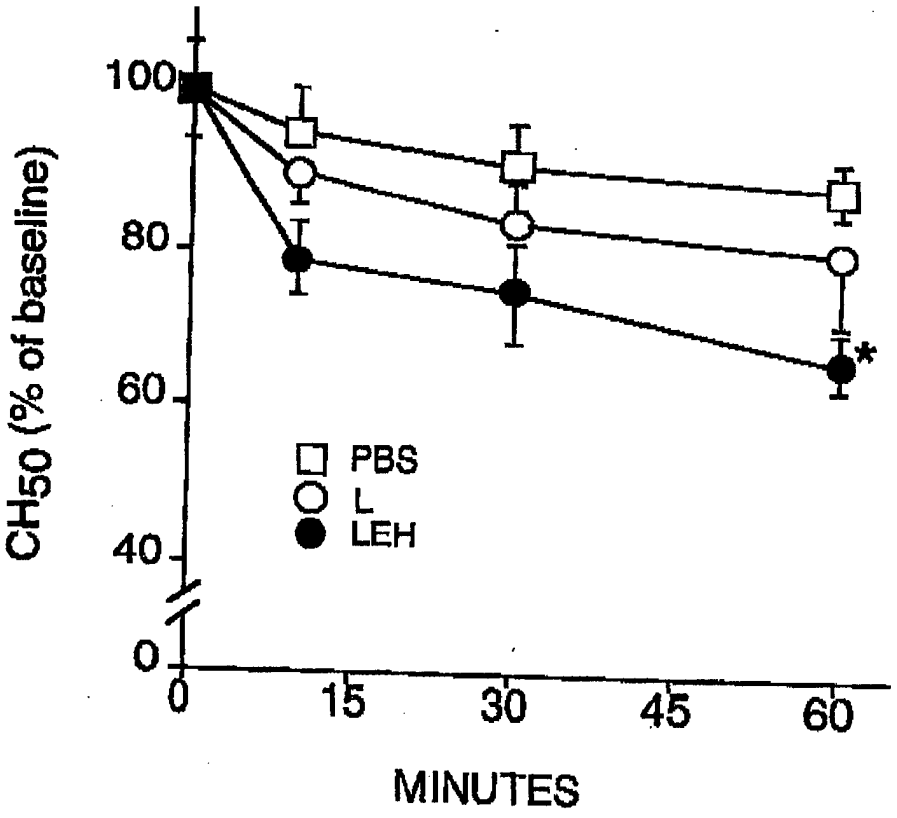

Fig. 2.