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Increased In Vitro and In Vivo Generation of Procoagulant Activity (Tissue Factor) by Mononuclear Phagocytes After Intralipid Infusion in Rabbits

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Intralipid, a fat emulsion widely used in parenteral nutrition, can produce marked functional changes of the mononuclear phagocyte system. We investigated the effect of Intralipid administration on the generation of procoagulant activity by rabbit mononuclear phagocytes. Two groups of ten rabbits given either a single infusion of Intralipid 10% or a similar volume of sterile saline were studied before and after infusion. Procoagulant activity was measured on isolated blood mononuclear cells after incubation with and without endotoxin, using a one-stage clotting assay. Cells from animals infused with Intralipid produced significantly more procoagulant activity than controls ($P < .01$). Results were similar when freshly collected whole blood was incubated with and without endotoxin, and procoagulant

activity was measured on subsequently isolated mononuclear cells ($P < .01$). In addition, when rabbits were given a single injection of endotoxin, blood and spleen mononuclear cells harvested 50 to 60 minutes after the injection from animals pretreated with Intralipid expressed five to seven times more procoagulant activity than did cells from animals pretreated with saline. In all instances, procoagulant activity was identified as tissue factor. These findings suggest that Intralipid may cause functional changes in mononuclear phagocytes, resulting in increased production of tissue factor on incubation in short-term culture in vitro and in response to endotoxin in vivo.

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INTRALIPID, an emulsion of soya bean oil, egg lecithin, and glycerol, is widely used as a standard component of most parenteral nutrition regimens. Some recent studies have shown that it can produce profound changes in the functional state of the mononuclear phagocyte system, including depressed monocyte chemotaxis and phagocytosis,¹ impaired bacterial clearance in mice,² reduced synthesis of complement components,³ and inhibition of macrophage cytotoxicity for virus-infected cells.⁴ Other investigators reported normal or even increased function of mononuclear phagocytes as judged by their oxidative metabolism, chemotactic response, and phagocytic capacity.⁵

Recently, evidence has accumulated that on exposure to different stimuli (bacterial endotoxin, immune complexes, complement proteolytic products, antigens, and others), peripheral blood monocytes and macrophages from diverse anatomical sites produce procoagulant activity in vitro.⁶⁻¹⁰ This activity has usually been identified as tissue factor,⁶⁻¹⁰ although prothrombinases and factor X activators have been described.¹¹⁻¹⁴ In view of this ability to produce procoagulants, mononuclear phagocytes have been implicated in the activation of intravascular or extravascular coagulation occurring in malignancy, endotoxemia, immunologic diseases, and after major surgery.¹⁵⁻²⁰

In the experiments described here, we used a rabbit model to assess the effect of Intralipid on the capacity of mononuclear phagocytes to produce procoagulant activity. The in vitro generation of procoagulant activity by rabbit peripheral blood monocytes was found significantly increased after Intralipid administration. In addition, Intralipid significantly enhanced the pro-

coagulant response of blood and spleen mononuclear phagocytes to endotoxin in vivo.

MATERIALS AND METHODS

Animals

Male New Zealand white rabbits (Charles River, Calco, Italy), weighing 2.5 to 3 kg were used, fed pelleted rabbit food with water ad libitum. The animals were anesthetized with intravenous sodium pentobarbital (20 mg/kg body weight). A polyvinyl catheter was inserted into the right jugular vein for infusing agents and a second catheter into the central artery of the left ear for blood sampling. The arterial catheter was continuously perfused with saline (1.2 mL/h) to prevent occlusion. All the animals were treated in the same manner with regard to operation, timing, and blood sampling.

The rabbits were divided into two groups. In group 1, ten animals were continuously infused with Intralipid 10% (Kabi Vitrum Ltd, Stockholm), 7 mL/kg over two hours. In group 2, ten animals were infused with the same volume of sterile isotonic saline. Blood samples (10 mL) were collected immediately before and at the end of the infusions using trisodium citrate as anticoagulant (0.015 mol/L final concentration).

Ten to 15 minutes after infusion and blood collection, five animals in each group were given a single endotoxin injection (50 μ g/kg of *Escherichia coli* 0111:B4 LPS, W, obtained from Difco Laboratories, Detroit) into the lateral ear vein. The remaining animals received saline. Blood samples were again collected 50 to 60 minutes

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after the endotoxin or saline. Thereafter, the animals were killed with an overdose of anesthetic, and the spleen was removed and placed in Petri dishes containing saline at 4 °C.

Isolation of Mononuclear Cells

Citrated blood was centrifuged for 15 minutes at 200 g and platelet-rich plasma was removed. Mononuclear cells were isolated from the remaining blood diluted with citrated phosphate-buffered saline (PBS) (9 vol PBS plus 1 vol 0.15 mol/L trisodium citrate) by the Ficoll-Hypaque (Lymphoprep, Nyegaard, Oslo) gradient technique.²¹ Cell preparations were washed with citrated PBS and suspended in Hanks' balanced salt solution (HBSS; Difco). Spleen cells were obtained by gentle teasing, washed, and suspended in PBS. Mononuclear cells were isolated by the Ficoll-Hypaque gradient technique as above.

Final cell suspensions contained more than 97% mononuclear cells. The ratio of platelets to white cells in each preparation was always less than 0.5:1 as determined by light microscopy. Mononuclear phagocytes were identified by cytochemical reactivity for alphanaphthyl acetate esterase. In cell preparations from peripheral blood, monocytes were 14% to 24% (mean, 18%). There were no changes after intralipid or saline infusion. Spleen mononuclear cells contained 28% to 37% macrophages (mean, 32%). Blood and spleen cell populations harvested after challenge with endotoxin contained, respectively, 8% to 13% (mean, 11%) and 24% to 35% (mean, 27%) mononuclear phagocytes.

Cell viability, assessed by the trypan blue test, was always more than 95%. All reagents used were free of endotoxin as determined by the limulus amoebocyte lysate assay (Microbiological Associates, Waltersville, Md). The sensitivity of the assay in our hands ranged from 0.1 to 0.2 ng/mL of LPS (*E coli* 0111:B4; Difco).

Incubation of Mononuclear Cells

The capacity of mononuclear cells to produce procoagulant activity (PCA) in vitro was studied using two experimental systems.

Isolated mononuclear cell suspensions. Aliquots of mononuclear cell preparations isolated from freshly collected blood and suspended in HBSS were mixed with either endotoxin (1 µg/mL of *E coli* LPS) or a similar volume of sterile isotonic saline and incubated at 37 °C in plastic tubes. PCA generated in the incubation mixture was measured after four hours of incubation.

Whole blood. Citrated whole blood was mixed with either endotoxin (10 µg/mL of *E coli* LPS) or sterile isotonic saline and incubated at 37 °C in plastic tubes. After four hours, mononuclear cells were isolated from these samples, washed, suspended in HBSS, and tested immediately for PCA. This experimental system provides a simple method for studying the procoagulant response of native monocytes suspended in their physiologic environment.^{22,23}

Assay for PCA

All assays were performed with cells disrupted by repeated freezing and thawing. PCA was evaluated by a one-stage plasma recalcification time. Clotting time was determined in duplicate in prewarmed plastic tubes using the following test system: 0.1 mL cell material, 0.1 mL rabbit plasma, and 0.1 mL 0.025 mol/L calcium chloride. Duplicate times with cells differed by less than 5%. Buffer blanks were >400 seconds. To characterize PCA, human plasma from healthy donors or from patients with congenital deficiency of factor VII, IX, or X was used as substrate. Results were expressed in arbitrary units by comparison of the clotting times of disrupted cells with a standard curve of clotting times produced by dilutions of a rabbit brain thromboplastin suspension. One thousand units of thromboplastin causes normal rabbit plasma to clot in 23 seconds. Because PCA is generated exclusively by mononuclear phagocytes in this system, data are expressed as units per 10⁵ monocytes-macrophages.

Statistical Methods

Results are always expressed as the mean ± SEM. Statistical analysis was performed by the analysis of variance (ANOVA). The split-plot design was used for comparisons of results before and after the infusion of Intralipid or saline and the two-ways analysis of variance for comparisons of data after endotoxin or saline injection.²⁴

RESULTS

PCA was first measured in peripheral blood mononuclear cells immediately after isolation (Table 1, line 1). Cell preparations from the control and treatment groups before infusion and from the control group after infusion of sterile saline expressed low PCA. This was slightly but not significantly increased after Intralipid infusion.

Table 1, lines 2 and 3, shows the PCA generated in vitro by mononuclear cell preparations after four hours of incubation with and without endotoxin. Cells isolated from ten animals infused with Intralipid produced significantly higher PCA than cells from control animals infused with saline and cells before infusion ($P < .01$).

In a parallel series of experiments, whole blood freshly collected from control and treatment groups before and after infusions was incubated at 37 °C with and without endotoxin. After four hours, mononuclear

Table 1. PCA in Peripheral Blood Mononuclear Cells

Mononuclear Cell Preparations	PCA (U/10 ⁵ Monocytes)			
	Saline		Intralipid	
	Before Infusion	After Infusion	Before Infusion	After Infusion
Freshly isolated	0.77 ± 0.16	0.86 ± 0.13	0.85 ± 0.16	1.51 ± 0.41
Incubated in medium without stimulus	19.0 ± 4.3	17.3 ± 4.4	19.9 ± 4.4	48.3 ± 11.2
Incubated in medium with LPS	67.6 ± 10.0	66.8 ± 11.9	68.7 ± 11.8	239.6 ± 29.2
From whole blood incubated without stimulus	2.5 ± 0.49	2.8 ± 0.6	2.6 ± 0.45	11.2 ± 4.3
From whole blood incubated with LPS	29.7 ± 4.5	27.8 ± 5.5	32.5 ± 4.4	124.4 ± 29.5

PCA was measured in animals infused with saline or intralipid before and after incubation (four hours at 37 °C) without stimulus or with endotoxin. Results are the means ± SEM of ten separate experiments.

Table 2. In Vivo Procoagulant Response of Blood and Spleen Mononuclear Cells to Endotoxin

Group	PCA (U/10 ⁵ Mononuclear Phagocytes)			
	Blood Cells		Spleen Cells	
	Saline	Intralipid	Saline	Intralipid
Control	0.94 ± 0.2	1.5 ± 0.5	3.7 ± 0.6	4.8 ± 0.6
Endotoxin	12.8 ± 2.9	92.3 ± 22.9	39.2 ± 5.3	188.8 ± 21.0

Endotoxin or saline (control) was given to animals at the end of intralipid or saline infusion. Mononuclear cells were isolated 50 to 60 minutes after injection and tested immediately for PCA. Results are the means ± SEM of five separate experiments.

cells were isolated and tested immediately for PCA. As shown in Table 1, lines 4 and 5, PCA was higher in cells from animals infused with Intralipid ($P < .01$).

To assess whether Intralipid modifies the response of mononuclear cells to endotoxin in vivo, animals infused with sterile saline or Intralipid were given a single injection of endotoxin or saline (control). Freshly isolated peripheral blood and spleen mononuclear cells harvested 50 to 60 minutes after endotoxin challenge consistently expressed marked PCA compared with controls (Table 2). However, in animals preinfused with Intralipid, the procoagulant response of blood and spleen mononuclear cells to endotoxin was significantly greater than in the group preinfused with saline ($P < .01$).

In all instances, the PCA produced by mononuclear cells after challenge with endotoxin in vivo was identified as tissue factor, since it required factor VII for its expression (Table 3). Similar results were obtained with all cell preparations shown in Table 1 (data not shown).

DISCUSSION

This study shows that Intralipid modifies the ability of rabbit mononuclear phagocytes to generate PCA. Intralipid per se, at least when given to rabbits as a single infusion, does not appear to be a powerful inducer of monocyte PCA, since peripheral blood mononuclear cells harvested after the infusion and assayed without preliminary culture or stimulation steps, expressed low PCA, comparable with that of

control cells. However, when incubated in nutrient medium or in whole blood with and without endotoxin, mononuclear cells from animals infused with Intralipid generated significantly higher levels of PCA than did cells from control animals. This suggests that Intralipid causes functional changes in mononuclear cell populations, resulting in increased production of PCA in vitro.

Previous work has shown that rabbit peripheral blood mononuclear cells^{18,19} and macrophages from diverse anatomical sites,^{25,26} harvested after endotoxin administration, express strong PCA immediately after isolation, suggesting direct cellular stimulation by endotoxin in vivo. Therefore, we used a single endotoxin injection as a standard stimulus to assess whether Intralipid modifies the procoagulant response of mononuclear phagocytes in vivo. In agreement with previous results in rabbits,^{18,19,25,26} endotoxin administration consistently resulted in the expression of strong PCA by freshly isolated blood and spleen mononuclear phagocytes. However, cells from animals pretreated with Intralipid expressed about five to seven times more PCA than did control cells. Thus, Intralipid may increase the responsiveness of mononuclear phagocytes to endotoxin and, possibly, to other stimuli in vivo.

In all instances the PCA produced by mononuclear phagocytes on incubation in short-term cultures in vitro or in response to endotoxin in vivo was identified as tissue factor.

The mechanisms through which Intralipid influenced monocyte-macrophage PCA in our experimental conditions are not clearly understood. Studies in animals and humans have shown the presence of intracellular lipid particles in mononuclear phagocytes after infusion of Intralipid or exposure of these cells to Intralipid in vitro.^{1,27} It was suggested that phagocytosis of Intralipid might affect the function of these cells. Van Ginkel et al²⁸ have shown that bacterial phagocytosis by human monocytes enhances the production of tissue factor by these cells. Phagocytosis of lipid particles could, therefore, contribute to the increased generation of PCA described here. Alternatively,

Table 3. Characterization of Mononuclear Cell PCA

Cell Preparation	PCA in Normal and Coagulation Factor Deficient Human Plasma Substrate (U/10 ⁵ Mononuclear Phagocytes)			
	Normal	Factor IX Deficient	Factor VII Deficient	Factor X Deficient
Blood cells from endotoxemic rabbits				
Saline	10.8 ± 2.1	10.9 ± 2.4	<1	<1
Intralipid	81.3 ± 18.9	83.8 ± 19.4	1.8 ± 0.7	<1
Spleen cells from endotoxemic rabbits				
Saline	29.2 ± 4.3	28.9 ± 4.7	<1	<1
Intralipid	164.5 ± 19.0	159.9 ± 18.3	2.9 ± 0.8	<1

Blood and spleen mononuclear cells were obtained from animals infused with saline or intralipid after challenge with endotoxin (see Table 2).

Intralipid may have an effect on the cell membrane of mononuclear phagocytes. Intralipid may alter the lipid composition of the cell membrane and change some properties, such as fluidity and surface charge.⁵ These changes could either trigger the procoagulant response or render the cells more responsive to other stimuli (eg, endotoxin).

Lymphocytes reportedly provide "help" for monocyte PCA induction by various agents, including endotoxin,^{7-9,11} although the requirement for lymphoid cells is not absolute.^{7,10} In our study the number of lymphocytes was unchanged after Intralipid infusion. Although Intralipid does not seem to affect the function of lymphocytes,¹ the possibility that it may have exerted an indirect, lymphocyte-mediated effect on monocytes-macrophages in our experiments cannot be excluded.

The pathophysiologic significance of our findings remains to be established. Patients in need of Intralipid

administration are often at increased risk for thromboembolic complications or disseminated intravascular coagulation because of malignant disease, surgery, burns, and infections. Recent evidence indicates that the PCA of mononuclear phagocytes could play a major role in the activation of intravascular or extravascular coagulation observed in these pathologic processes,¹⁵⁻²⁰ thus contributing to the thrombotic risk. Our findings suggest that Intralipid might further accentuate the thrombotic tendency as a result of increased monocyte-macrophage PCA. In preliminary experiments we found that peripheral blood mononuclear cells from patients receiving a single Intralipid infusion generated more PCA than their preinfusion control samples.²⁹

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