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Agglutination of intravenous lipid emulsion ('Intralipid') and plasma lipoproteins by C-reactive protein

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SUMMARY

Isolated human C-reactive protein (CRP), or CRP in acute phase serum, produced in-vitro agglutination ('creaming') of the intravenously administered lipid suspension 'Intralipid'. CRP also produced similar agglutination of isolated normal very low density lipoproteins (VLDL). Agglutination in both cases was calcium-dependent and inhibitable by phosphoryl choline. These findings have important implications for patients receiving intravenous lipid suspensions and may be relevant to the pathogenesis of the fat embolism syndrome following trauma.

Keywords C-reactive protein lipoproteins Intralipid

INTRODUCTION

C-reactive protein (CRP) is the classsical acute phase reactant in man, the circulating concentration of which can rise up to a thousand-fold following most forms of tissue damage (Tillett & Francis, 1930; Pepys & Baltz, 1983). The CRP molecule has calcium-dependent binding sites for phosphoryl choline residues (Volanakis & Kaplan, 1971; Gotschlich, Liu & Oliveira, 1982) through which it can bind to a variety of lipids and liposomes of different composition *in vitro* (Pepys, Rowe & Baltz, 1985). CRP aggregated *in vitro* can bind low density lipoprotein (LDL) and very low density lipoprotein (VLDL) from autologous serum (de Beer *et al.*, 1982b; Rowe *et al.*, 1984a). Native, non-aggregated CRP can also form soluble complexes with certain abnormal VLDL particles (Rowe *et al.*, 1984b)) but CRP in normolipoproteinaemic acute phase serum is in a free non-complexed form (de Beer *et al.*, 1982b; de Beer, Shine & Pepys, 1982a).

Hulman *et al.* (1982) studied the phenomenon of agglutination of the intravenously administered lipid emulsion 'Intralipid' by sera of acutely ill patients and established a correlation between the serum CRP concentration and the ability of the sera to cream Intralipid. If such agglutination occurs *in vivo* it may lead to lipid microembolism (Hulman *et al.*, 1983).

The fat embolism syndrome occurs as a complication of major trauma and classically presents 2 days after injury (Gurd & Wilson, 1974) at a time when patients might be expected to have developed a peak CRP response. The origin of the fat, whether from bone marrow, injured soft tissue or circulating lipoproteins is not known (Gossling & Pellegrini, 1982). Other aspects of the pathophysiology of the condition are also poorly understood, in particular whether it is genuinely embolism or whether a significant element of inflammation is involved.

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We have studied further the phenomenon of creaming of Intralipid by acute phase sera, establishing that this is indeed mediated by CRP. We have also tested whether CRP may under certain circumstances cause agglutination of autologous lipoproteins or bone marrow fat.

MATERIALS AND METHODS

Serum. Fresh human serum was from laboratory volunteers, from patients during an acute phase response 48 h following cardiothoracic or orthopaedic surgery, from patients immediately following total hip replacement and from fasting patients with different hyperlipoproteinaemias. Fresh rabbit serum was from Half Lop rabbits. Acute phase rabbit serum was obtained 48 h after casein injection (Rowe *et al.*, 1984a).

Proteins. Human CRP was isolated and purified as previously described (de Beer & Pepys, 1982) and was used in solution in 0·01 M Tris-buffered 0·14 M NaCl, pH 8·0, containing 0·002 M $CaCl_2$ (Tris-saline-Ca).

Lipoproteins and lipids. VLDL (density $< 1{\cdot}006$ g/ml) and LDL (density 1·019–1·063 g/ml) were isolated by sequential ultracentrifugation in solutions of potassium bromide (KBr) (Havel, Eder & Bragden, 1955). Each lipoprotein fraction was washed once by recentrifugation at the higher density limit, and then dialysed against Tris-saline-Ca to remove the KBr. The isolated human lipoprotein fractions gave a single major band in non-denatured 4–30% gradient PAGE (Rowe *et al.*, 1984a); there was trace contamination with albumin in some preparations but no CRP was detected by electroimmunoassay (sensitivity 1 mg/l). Bone marrow fat was obtained from bone removed at the time of total hip replacement and separated by centrifugation in Tris-saline-Ca at room temperature (as the supernatant) from contaminants (infranatant). Intralipid emulsion (Kabi Vitrum Ltd, Bilton House, London) contained 200 g fractionated soybean oil, 12 g fractionated egg phospholipids and 22·5 g glycerol per 1,000 ml.

Agglutination ('creaming') experiments. To determine whether sera could produce 'creaming' of Intralipid, 10 μl of 20% Intralipid was mixed with 400 μl of serum in a LP3 tube (Luckham Ltd, Burgess Hill, Sussex) and incubated at 37°C for 24 h. 'Creaming' was judged to have occurred when a definite layer of Intralipid had formed above a clear infranatant layer. In 'creamed' samples, agglutinated Intralipid particles could be seen under high power microscopy. Quantification of the creaming phenomenon was made by adding different volumes of Intralipid to 50 μl acute phase serum (CRP 230 mg/l) in a microfuge tube (Beckman RIIC, High Wycombe, Bucks), the total volume being made up with 100 μl with Tris-saline-Ca. In other experiments 25 μl Intralipid was added to 75 μl of sera of known different CRP concentration. The thickness of the 'creamed' layer, if present, was measured in mm, and tubes were sectioned below the creamed layer to determine CRP concentration in the infranatant serum. Similar experiments were performed to determine whether agglutination of isolated lipoprotein particles of bone marrow fat could be produced by acute phase sera, or whether isolated CRP could provide agglutination of lipoproteins or bone marrow fat in sera from patients with different hyperlipoproteinaemias following major orthopaedic surgery.

Antisera. Monospecific sheep anti-human CRP and anti-rabbit CRP sera were raised by immunization with the pure proteins. Sheep anti-human CRP was covalently coupled to cyanogen bromide activated Sepharose 4B (Pharmacia Ltd, Milton Keynes) following manufacturers recommendations.

Protein assays. Concentrations of human and rabbit CRP were measured by electroimmunoassay and single radial immunodiffusion respectively, using pure CRP for standardization (Pepys *et al.*, 1978; Rowe *et al.*, 1984a).

Lipid composition. The following assays were used: triglycerides, Dow Diagnostics Kit (Uniscience Ltd, Cambridge); cholesterol, modification of procedure of Allain *et al.* (1974).

Gel filtration experiments. Samples of 0·7 ml were loaded on a 1 × 58 cm column of Sephacryl S300 (Pharmacia Ltd) eluted at 10 ml/h with Tris-saline-Ca and 0·35 ml fractions collected.

RESULTS

Agglutination of Intralipid by CRP. 'Creaming' occurred when Intralipid was added to six acute phase sera (with CRP levels > 200 mg/l) from patients 48 h following cardiothoracic surgery but not when added to six normal human sera (with CRP levels < 5 mg/l) from healthy volunteers. 'Creaming' was inhibited by addition of EDTA or phosphoryl choline to give a final concentration of 10 mM. Complete depletion of detectable CRP from acute phase serum (CRP 230 mg/l) by incubation of the serum with sheep anti-human CRP covalently conjugated to Sepharose abolished the ability of the serum to agglutinate Intralipid. Reconstitution of CRP depleted serum with isolated pure CRP in Tris-saline-Ca to give a concentration of 240 mg/l restored the ability of the serum to agglutinate Intralipid. Isolated pure CRP in Tris-saline-Ca also caused 'creaming' of Intralipid, and this was inhibited in the presence of 10 mM EDTA or 10 mM phosphoryl choline.

Similar experiments were performed by addition of Intralipid to acute phase rabbit serum (CRP 200 mg/l) and in no case was similar agglutination observed.

Agglutination of lipoproteins by CRP. No agglutination was observed when isolated human CRP was added to normal human serum or sera from patients with the following hyperlipoproteinaemias: type IIa (containing excess LDL), type III (containing an abnormal lipoprotein, β-VLDL), type IV (containing excess hypertriglyceridaemic VLDL) or type V (containing excess hypertriglyceridaemic VLDL and chylomicrons). Agglutination was also not observed after addition of isolated CRP to isolated LDL or β-VLDL in Tris-saline-Ca. However when isolated normal VLDL isolated separately from three pools of normal human sera was added to acute phase normolipoproteinaemic human serum to give triglyceride concentrations in the range found in patients with type IV or type V hyperlipoproteinaemia, the VLDL in each case agglutinated to form a creamed layer above the serum. The same effect could be observed after mixing isolated pure human CRP in Tris-saline-Ca with the isolated VLDL. This agglutination did not occur in the presence of 10 mM phosphoryl choline or 10 mM EDTA.

Quantification of binding of CRP to Intralipid and VLDL. Experiments were performed to attempt to measure the quantity of CRP binding to Intralipid or VLDL. Intralipid or VLDL had to be present in sufficient concentration for agglutination by CRP to be observed ($\geqslant 1$ μl Intralipid; $\geqslant 12{\cdot}5$ μl VLDL in total volume of 100 μl). Similarly CRP had to be present in sufficient concentration (> 80 mg/l) for agglutination of Intralipid to occur. Even in the presence of agglutination, however, no significant depletion of CRP from the infranatant layer could be detected (Tables 1–3).

Investigation of interaction of CRP with isolated bone marrow fat or sera from patients following orthopaedic surgery. Isolated human CRP or acute phase human serum was added to isolated bone

Table 1. 'Creaming' of Intralipid by acute phase serum – A

Intralipid (μl)	Acute phase serum: 'Creaming' (thickness, mm)	Acute phase serum: CRP remaining in infranatant (mg/l)	Normal serum 'Creaming'
0	—	126	—
0·5	—	130	—
1·0	1	126	—
2·5	1	124	—
5·0	2	130	—
12·5	3	110	—
25·0	4	120	—

Different volumes of Intralipid were added to 50 μl of normolipoproteinaemic acute phase serum (CRP 230 mg/l) or normal human serum (CRP < 5 mg/l) as control.

Table 2. 'Creaming' of Intralipid by acute phase serum – B

CRP in serum offered (mg/l)	'Creaming' (thickness, mm)	CRP remaining in infranatant (mg/l)	% of CRP in starting serum remaining in infranatant
108	2	110	102
98	1	95	97
83	1	79	95
67	—	65	97
39	—	38	97
21	—	22	105
14	—	9	(65)
0	—	0	—

25 μl Intralipid added to 75 μl of sera of known CRP concentration.

Table 3. 'Creaming' of isolated normal VLDL by acute phase serum

VLDL (1 μl)	Acute phase serum 'Creaming' (thickness, mm)	Acute phase serum CRP remaining in infranatant (mg/l)	Normal serum 'Creaming'
0	—	116	—
0·5	—	113	—
2·5	—	116	—
5·0	+/–	109	—
12·5	+(<1)	110	—
25·0	+(<1)	105	—
50·0	+(1)	113	—

Different volumes of VLDL (cholesterol 10 mmol/l, triglyceride 25 mmol/l) added to 50 μl of normolipoproteinaemic acute phase serum (CRP 2a30 mg/l) or normal human serum (CRP <5 mg/l) as control.

(+), presence of creaming.

marrow fat in the presence of calcium. No agglutination was observed and there was no evidence of soluble complexes detectable as 'heavy' CRP after gel filtration of the mixture in Tris-saline-Ca. Similarly when isolated CRP was added to sera (CRP < 10 mg/l) taken from two post-operative patients immediately following hip replacement or when isolated bone marrow fat was added to acute phase sera (CRP > 150 mg/l) from two patients taken 48 h after hip replacement no evidence of lipid agglutination or soluble complexes involving CRP could be demonstrated.

DISCUSSION

The results presented demonstrate that CRP in acute phase serum is responsible for the in-vitro phenomenon of creaming of Intralipid, and that creaming can also occur if isolated CRP and

Intralipid are mixed in a calcium-containing buffer. Creaming is inhibited by EDTA or phosphoryl choline suggesting that it is the calcium-dependent phosphoryl choline binding site on the CRP molecule which is involved. Rabbit CRP has binding properties subtly different from those of human CRP (Oliveira, Gotschlich & Liu, 1980), probably due at least in part to differences in amino acid sequence at the phosphoryl choline binding site (Wang *et al.*, 1982), and this appears to be reflected in the ability of rabbit CRP to agglutinate Intralipid. Agglutination similar to the creaming phenomenon was observed when normal VLDL in excess of the level in normolipoproteinaemic individuals was mixed with acute phase serum or isolated human CRP in the presence of calcium. Agglutination of other classes of lipoprotein by CRP could not, however, be demonstrated. CRP has previously been shown to form soluble complexes with βVLDL in sera from patients with type III hyperlipoproteinaemia and with hypertriglyceridaemic VLDL in patients with types IV and V hyperlipoproteinaemia (Rowe *et al.*, 1984b). In β-VLDL there is a marked increase in the amount of apolipoprotein E (apo E) present compared with normal VLDL (Frederickson, Goldstein & Brown, 1978), and it is possible that apo E in hypertriglyceridaemic type IV or V VLDL may be more exposed on the surface of the lipoprotein particle than in normal VLDL (Gianturco *et al.*, 1982). The ability of CRP to agglutinate normal VLDL particles may therefore be due to difference in lipoprotein composition compared with the other VLDL particles studied.

Attempts were made to quantify the interaction between CRP and Intralipid or VLDL but although CRP had to be present in sufficient concentration for agglutination to occur, no detectable depletion of CRP could be detected in the infranatant below the creamed layer. These findings argue against a firm binding of CRP to phosphoryl choline residues in the lipid or lipoprotein particles, but the presence of sufficient CRP may alter the surface properties of such particles so as to promote agglutination. In this respect it is of interest that soluble CRP-lipoprotein complexes previously demonstrated in rabbit sera (Rowe *et al.*, 1984a) and produced *in vitro* in certain human sera (Rowe *et al.*, 1984b) appear to be due to a relatively weak interaction routinely demonstrable only by gel filtration, immunoelectrophoresis and sometimes by sucrose density ultracentrifugation.

Interaction between CRP and lipids or lipoproteins may be of considerable importance in the pathogenesis of lipid embolism. Patients who receive Intralipid are usually suffering from serious acute or chronic illness and would often be expected to have significantly raised circulating CRP concentrations. Lipid emboli have been observed post-mortem in pulmonary arterioles and capillaries of seriously ill patients who had received Intralipid before death as well as in other organs ncluding brain, kidney and spleen (Hulman *et al.*, 1983). Further work is required to demonstrate whether CRP-Intralipid agglutination is responsible for these emboli but it would seem prudent before clinical administration of Intralipid to measure the patient's serum CRP concentration. If CRP concentration can not be readily measured the ability of the patient's serum to produce creaming of Intralipid *in vitro* should be determined.

The fat embolism syndrome may occur as a complication of major trauma and classically presents clinically with cerebral signs, including confusion, with respiratory distress and with petechiae of skin mucosae. Pathological fat globules may be demonstrated in blood samples (Gurd & Wilson, 1974). A notable feature is a latent period between injury and onset of symptoms with an average time of 46 h. The syndrome may present with circulatory collapse, sometimes fatal, in young patients after an 'untroubled interval' of 24 to 48 h following an uncomplicated fracture of he pelvis or femur (Fuchsig *et al.*, 1967). The syndrome therefore occurs at a time of peak CRP production 24–48 h following an acute phase stimulus (Kushner, Broder & Karp, 1978).

Reports have suggested that bone marrow fat or fat derived from soft tissue damage may be nportant in the pathogenesis of fat embolism (Gossling & Pellegrini, 1981). In our studies we could nd no evidence of interaction between isolated bone marrow fat and CRP *in vitro*, nor could we emonstrate agglutination of bone marrow fat or any evidence of complexed CRP by gel filtration fter addition of isolated bone marrow fat to acute phase serum obtained 48 h following total hip replacement. Similarly no interaction could be demonstrated between CRP and lipid material expected to be in the circulation immediately following total hip replacement.

Circulating lipoproteins have also been implicated in the pathogenesis of the fat embolism yndrome. Lipoprotein electrophoresis in a series of patients following fractures has shown a eduction in VLDL for up to 48 h in uncomplicated cases and for 8–10 days in two patients with the

fat embolism syndrome (Treiman, Waisbrod & Waisbrod, 1981). VLDL was undetectable for 7 days in another patient with multiple fractures and fat embolism syndrome (Hillman & Lequire, 1968). This is in contrast to the usual increase in circulating VLDL concentrations observed during acute phase response (Pepys & Baltz, 1983; Coombes, Shakespeare & Batstone, 1980). We have demonstrated agglutination of excess VLDL by CRP *in vitro* and it is therefore possible that CRP-VLDL agglutinates may occur *in vivo* and contribute to the development of fat emboli and the reduction in VLDL observed in patients with fat embolism. In addition to the purely physical effects of aggregation of VLDL particles any interaction with CRP might also have inflammatory sequelae since bound CRP is a potent activator of the classical complement pathway (Kaplan & Volanakis, 1974) and this might contribute to the circulatory collapse often observed in patients with the fat embolism syndrome. Further studies will however be required to elucidate the precise nature of the fat emboli and the possible role of CRP in their formation *in vivo*.

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