INFECTION AND IMMUNITY, Dec. 1993, p. 5388–5391
0019-9567/93/125388-04$02.00/0

Vol. 61, No. 12

Regulation of Inflammation-Primed Activation of Macrophages by Two Serum Factors, Vitamin D_3-Binding Protein and Albumin

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Received 4 March 1993/Returned for modification 25 June 1993/Accepted 25 August 1993

A very small amount (0.0005 to 0.001%) of an ammonium sulfate [50% saturated $(NH_4)_2SO_4$]-precipitable protein fraction of α_2-globulin efficiently supported inflammation-primed activation of macrophages. This fraction contains vitamin D_3-binding protein essential for macrophage activation. Comparative macrophage activation studies with fetal calf serum, α_2-globulin fraction, 50% $(NH_4)_2SO_4$ precipitate, and purified bovine vitamin D_3-binding protein revealed that fetal calf serum and α_2-globulin fraction appear to contain an inhibitor for macrophage activation while ammonium sulfate precipitate contains no inhibitor. This inhibitor was found to be serum albumin. When bovine serum albumin (25 μg/ml) was added to a medium supplemented with 0.0005 to 0.05% $(NH_4)_2SO_4$ precipitate or 1 to 10 ng of vitamin D_3-binding protein per ml, activation of macrophages was inhibited.

Microbial infections cause inflammation. Inflamed lesions attract and activate phagocytes and induce immunity against the infectious agents, implying that biochemical signals should be radiating from the inflamed lesions. The inflamed tissues release lysophospholipids and alkylglycerols, which are potent chemotactic and macrophage-stimulating agents (10, 11, 17, 19). Administration of lysophospholipids or alkylglycerols to mice activates macrophages to phagocytize target antigens or cells via the Fc receptor (10, 11, 17, 19) and to generate superoxide (18). In vitro treatment of mouse peritoneal cells (mixture of adherent and nonadherent cells) with lysophosphatidylcholine (lyso-Pc) or dodecylglycerol (DDG) for a few hours produced a markedly enhanced Fc receptor-mediated phagocytic activity of macrophages (12, 18, 19). Treatment of adherent cells (macrophages) alone with lyso-Pc or DDG resulted in no activation of macrophages. Inflammation-primed activation of macrophages requires the participation of B and T lymphocytes (12, 18, 19) and vitamin D_3-binding protein (DBP; human DBP is known as group-specific component or Gc) (14–16). DBP can be converted to a potent macrophage-activating factor by stepwise interaction with β-galactosidase of lyso-Pc- or DDG-treated B cells and sialidase of untreated T cells (14). Thus, DBP is a precursor of macrophage-activating factor (14).

A comparative analysis of the precursor activities of purified human DBP (Gc protein) and adult human serum was performed. The highest level of macrophage activation can be achieved with 0.1% adult human serum-supplemented RPMI 1640 medium and with about 0.26 ng of Gc protein per ml in 0.1% egg albumin-supplemented RPMI 1640 medium (EA medium) (15, 16). Since the Gc protein concentration in 0.1% adult human serum is about 260 ng/ml, the concentration of Gc protein in whole serum required for efficient activation of macrophages is about 1,000-fold-higher than that of purified Gc protein. Thus, the macrophage-activating factor is more efficiently generated from purified Gc protein than from the same protein in whole serum, and generation of the macrophage-activating factor from Gc protein is less efficient in the presence of other serum proteins (15, 16). We propose that a serum factor(s) appears to be inhibitory to the macrophage activation process.

We fractionated fetal calf serum (FCS) by ammonium sulfate precipitation. Two milliliters of saturated ammonium sulfate was added to 2 ml of gamma globulin-free FCS (GIBCO, Gaithersburg, Md.), and the mixture was incubated for 1 h at room temperature. Supernatant and precipitate were separated by centrifugation (8,500 × *g*) for 10 min. The pellet was suspended in 1 to 1.5 ml of saline (0.85% NaCl) and dialyzed three times against a large volume (500 ml) of saline at 4°C. The dialyzed fraction was adjusted by adding saline to yield the same volume (2 ml) as the original volume of serum used for ammonium sulfate precipitation. Thus, the concentration of the protein of interest per volume corresponded to that in the original serum. Supernatant was also dialyzed against saline. α_2-Globulin was fractionated by starch block electrophoresis (7, 16). These fractions were filtered through a Millipore filter (type HA, 0.45-μm pore size) for sterilization.

Mouse peritoneal cells (5×10^5 cells per ml) were placed in 16-mm wells and treated with 1 μg of lyso-Pc or 50 ng of DDG per ml in EA medium for 30 min. To remove residual lyso-Pc or DDG, the nonadherent and adherent cells were separately washed with phosphate-buffered saline and admixed or the treated nonadherent cells were added to untreated adherent cells and cultured in FCS medium or EA medium supplemented with various amounts of α_2-globulin fraction, ammonium sulfate precipitate, or purified bovine DBP (bDBP). After 3 h of cultivation, phagocytic activity (2, 8, 19) and superoxide production (1, 6, 13) of macrophages were assayed.

As shown in Table 1 (lines 4 and 5), the ammonium sulfate precipitate supported macrophage activation for greatly en-

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TABLE 1. Requirement of ammonium sulfate-precipitable serum factor for macrophage activation by cocultivation of lyso-Pc- or DDG-treated nonadherent cells and untreated adherent cells[a]

Posttreatment culture condition	Ingestion index[b]		
	Untreated cells	Lyso-Pc-treated cells	DDG-treated cells
EA medium	69 ± 11	66 ± 9	68 ± 13
10% FCS medium	55 ± 9	215 ± 45	263 ± 42
1% FCS medium	74 ± 20	365 ± 63	333 ± 77
EA medium containing FCS-$(NH_4)_2SO_4$ supernatant (1%, vol/vol)	58 ± 10	69 ± 12	72 ± 18
EA medium containing FCS-$(NH_4)_2SO_4$ pellet (0.001%, vol/vol)	72 ± 21	362 ± 61	352 ± 68
EA medium containing α_2-globulin fraction (0.05%, vol/vol)	68 ± 13	322 ± 48	315 ± 55
EA medium containing α_2-globulin-$(NH_4)_2SO_4$ pellet (0.001%, vol/vol)	70 ± 25	358 ± 72	374 ± 75

[a] Nonadherent cells were treated with 1 μg of lyso-Pc or 50 ng of DDG per ml in EA medium for 30 min. After being washed, the nonadherent cells were admixed with untreated adherent cells and cultured in FCS-supplemented RPMI 1640 medium (FCS medium) or EA medium supplemented with α_2-globulin fraction or 50% saturated ammonium sulfate precipitate and α_2-globulin fraction. After 3 h of cultivation, the phagocytic activity of macrophages was assayed and expressed as an ingestion index. The concentrations (percent, by volume) indicated were required for maximal activation with the indicated fractions. Values represent means ± standard errors of triplicate assays. The results were reproduced at least three times.

[b] Ingestion index = percentage of macrophages with ingested sheep erythrocytes × average number of erythrocytes ingested per macrophage (2,8).

TABLE 2. Lyso-Pc-primed activation of macrophages with a culture medium containing bDBP-depleted FCS or α_2-globulin fraction with or without purified bDBP[a]

Lyso-Pc treatment[b]	Posttreatment culture condition	Ingestion index[c]
−	1% FCS	56 ± 12
+	1% FCS	325 ± 45
+	bDBP-depleted FCS	75 ± 22
+	bDBP-depleted FCS + 2.6 μg of bDBP per ml	285 ± 47
+	bDBP-depleted FCS + 10 ng of bDBP per ml	68 ± 17
+	0.05% α_2-globulin	257 ± 55
+	bDBP-depleted α_2-globulin	77 ± 20
+	bDBP-depleted α_2-globulin + 130 ng of bDBP per ml	283 ± 48
+	bDBP-depleted α_2-globulin + 10 ng of bDBP per ml	59 ± 15
+	10 ng of bDBP per ml only	312 ± 69
−	10 ng of bDBP per ml only	62 ± 13

[a] Lyso-Pc-treated peritoneal cells were cultured in EA medium containing 1% FCS or α_2-globulin fraction (0.05%) or in bDBP-depleted FCS or α_2-globulin fraction. After 3 h of cultivation, macrophage phagocytic activity was assayed and expressed as an ingestion index. Values represent means ± standard errors of triplicate assays.

[b] Peritoneal cells were treated with 1 μg of lyso-Pc per ml in EA medium for 30 min. Nonadherent and adherent cells were washed separately and admixed.

[c] See Table 1, footnote *b*.

hanced phagocytic activity, while the supernatant did not. A small amount (0.001%, vol/vol) of the precipitate efficiently supported lyso-Pc- or DDG-primed activation of macrophages, while 1% FCS was required for the same extent of macrophage activation (Table 1, lines 3 and 5). Thus, the strikingly increased activity of the serum factor after ammonium sulfate fractionation suggests that this procedure separates the precursor of macrophage-activating factor from the inhibitor(s). In separate studies with electrophoretic serum fractionation, we found that a serum factor in the α_2-globulin fraction is essential for the activation process of macrophages after lyso-Pc or DDG treatment of peritoneal cells and that a precursor of the macrophage-activating factor exists in the α_2-globulin fraction (7, 16). When lyso-Pc- or DDG-treated nonadherent cells were cocultured with untreated adherent cells in EA medium supplemented with the α_2-globulin fraction (0.05%, vol/vol) of FCS, a greatly enhanced activation of macrophages was observed (Table 1, line 6). When the α_2-globulin fraction was treated with 50% saturated ammonium sulfate, a small amount (0.001%, vol/vol) of the pelleted fraction of α_2-globulin efficiently supported macrophage activation (Table 1, line 7). Although 50% saturated ammonium sulfate ordinarily precipitates larger serum proteins, particularly the entire gamma globulin fraction, this procedure appeared to precipitate the precursor of macrophage-activating factor from the α_2-globulin fraction.

There are a few major components in the α_2-globulin fraction. The serum factor required for macrophage activation can be identified by the use of antisera against these components. Gc protein (human DBP) is one of the major components of α_2-globulin of human serum and was found to be essential for the activation of macrophages (14, 16). bDBP can be depleted from FCS and α_2-globulin fraction by treatment with anti-bDBP immunoglobulin G, as described previously (16). The bDBP-depleted FCS and α_2-globulin fraction did not support lyso-Pc-primed activation of macrophages, as shown in Table 2. Thus, DBP is the sole serum component required for macrophage activation. When we purified bDBP from FCS by the method of Link et al. (9) and added various concentrations of bDBP to the bDBP-depleted FCS and α_2-globulin fraction, lyso-Pc-primed activation of macrophages could be supported. Although 10 ng of purified bDBP per ml produced a greatly enhanced phagocytic activity, a large amount of bDBP (2.6 μg/ml), equivalent to that found in 1% FCS, was required for the bDBP-depleted FCS to activate macrophages (Table 2). bDBP-depleted α_2-globulin fraction also required about 130 ng of bDBP per ml for lyso-Pc-primed activation of macrophages. These results imply that a large amount of inhibitor is present in FCS while some inhibitor is contained in the electrophoretic α_2-globulin fraction.

Human Gc protein was shown to be precipitable by treatment of whole serum with 50% saturated ammonium sulfate (5, 16). Although 50% saturated ammonium sulfate ordinarily precipitates larger serum proteins, but not smaller proteins such as albumin, this procedure appeared to precipitate bDBP. As shown in Table 3, a very small amount (as low as 0.0005%, vol/vol) of 50% ammonium sulfate precipitate in culture medium efficiently supported lyso-Pc-primed macrophage activation, as demonstrated by a greatly increased superoxide-generating capacity. This level of macrophage activation cannot be achieved by a medium supplemented with 1 to 10% FCS (Table 4). Thus, ammonium sulfate precipitation greatly increases the macrophage-activating capacity of the serum factor. Therefore, it is possible that the supernatant fraction may contain an inhibitor of

TABLE 3. Inhibition of macrophage activation by BSA during cocultivation of lyso-Pc-treated nonadherent cells with untreated adherent cells in an ammonium sulfate precipitate-supplemented medium[a]

Posttreatment culture condition (precipitate concn)	nmol of superoxide/min/10^6 cells	
	50% $(NH_4)_2SO_4$ only	50% $(NH_4)_2SO_4$-BSA (25 μg/ml)
0.5%	22.46 ± 0.3	1.86 ± 0.30
0.05%	21.26 ± 0.4	1.09 ± 0.02
0.005%	13.01 ± 2.1	0.76 ± 0.01
0.0005%	12.66 ± 0.25	0.62 ± 0.02
0.5% ± supernatant	1.25 ± 0.05	

[a] Mouse nonadherent cells were treated with 1 μg of lyso-Pc per ml in EA medium for 30 min. The nonadherent cells were washed and admixed with untreated adherent cells and cultured in EA medium supplemented with various concentrations of 50% $(NH_4)_2SO_4$ precipitate in the presence or absence of BSA (25 μg/ml). After 3 h of cultivation, the superoxide-generating capacity of macrophages was determined. The BSA concentration corresponds to that in 0.1% FCS (see Table 4). Values represent means ± standard errors of triplicate assays. The results were reproduced at least three times.

macrophage activation. When the supernatant was added to the precipitate, a greatly reduced superoxide generation of macrophages was observed (Table 3). Since DBP and serum albumin share extensive similarity in three-dimensional configuration (3, 4, 20, 21), various amounts of 50% saturated ammonium sulfate precipitate and 25 μg of bovine serum albumin (BSA) per ml were added to EA medium for 3 h of cocultivation of lyso-Pc-treated nonadherent cells and untreated adherent cells. As shown in Table 3, the capacity of 50% saturated ammonium sulfate precipitate to support macrophage activation was inhibited by the presence of BSA.

To substantiate these observations further, bDBP was purified from the 50% saturated ammonium sulfate precipitate of FCS. The dose effect of bDBP was studied for comparison with that of the ammonium sulfate precipitate. Lyso-Pc-treated nonadherent cells were cocultured for 3 h with untreated adherent cells in EA medium containing various concentrations (0.1 to 20 ng/ml) of bDBP. As can be seen in Table 5, experiment 1, purified bDBP at concentrations as low as 1 to 2 ng/ml produced greatly enhanced macrophage activation. As the concentration of bDBP was increased up to 20 ng/ml, lyso-Pc-primed activation of macrophages for superoxide generation increased. Since the bDBP concentration of the 0.0005% ammonium sulfate precipitate is about 1.3 ng/ml, the macrophages are as efficiently activated in the presence of the ammonium sulfate precipitate of FCS as with purified bDBP, as summarized in Table 6. These findings suggest that the generation of the macrophage-activating factor from bDBP is equally efficient in the presence of other serum proteins in the ammonium sulfate precipitate, suggesting that no inhibitor is detectable in the ammonium sulfate precipitate. When BSA (25 μg/ml) was added to EA medium supplemented with 1 or 10 ng of bDBP per ml for cultivation of lyso-Pc-treated peritoneal cells, the activation of macrophages was greatly inhibited (Table 5, experiment 2).

Therefore, we conclude that serum albumin inhibits inflammation-primed activation of macrophages, possibly competitively with DBP. The gene encoding human DBP (i.e., Gc protein) is located on human chromosome 4 and is linked to the albumin gene (4). These genes have evolved from a smaller ancestral gene by intragenic triplication and by intergenic duplication. Human, rat, and mouse DBPs share significant amino acid sequence homology and nucleotide sequence homology with the albumin of their respective species (3, 4, 20, 21). A conserved pattern of disulfide bridges forms triple-domain structures in DBP and albumin. Because of the structural similarity, serum albumin

TABLE 4. Effect of FCS concentration on superoside generation[a]

Concn of FCS (%)	nmol of superoxide/min/10^6 cells
10.0	6.48 ± 0.12
1.0	7.80 ± 0.05
0.3	1.13 ± 0.12
0.1	1.10 ± 0.02

[a] See footnote *a*, Table 3.

TABLE 5. Macrophage activation and inhibition by cocultivation of lyso-Pc-treated nonadherent cells and untreated adherent cells in the presence of various concentrations of purified bDBP with or without BSA[a]

Expt	Amt of bDBP (ng/ml)	BSA (25 μg/ml)	nmol of superoxide/min/10^6 cells
1	20		24.72 ± 0.89
	10		18.32 ± 0.76
	5		14.83 ± 0.47
	2		12.51 ± 0.56
	1		10.87 ± 0.35
	0.3		2.06 ± 0.21
	0.1		1.32 ± 0.23
	None		0.63 ± 0.14
	20, untreated[b]		0.85 ± 0.18
2	10	−	20.72 ± 1.23
	10	+	0.54 ± 0.07
	1	−	13.21 ± 1.45
	1	+	0.20 ± 0.08

[a] Nonadherent cells were treated with 1 μg of lyso-Pc per ml in EA medium for 30 min. After being washed, the treated nonadherent cells were admixed with untreated adherent cells and cultured for 3 h in EA medium supplemented with 0.1 to 20 ng of bDBP per ml or with 1 or 10 ng of bDBP plus 25 μg of BSA per ml prior to the superoxide generation assay. Values represent means ± standard errors of triplicate assays.

[b] Untreated nonadherent cells were cultured with untreated adherent cells.

TABLE 6. Stimulatory and inhibitory serum proteins involved in inflammation-primed activation of macrophages

Serum fractionation	Protein involved		Amt of fraction required (bDBP concn) for macrophage activation
	Stimulatory	Inhibitory	
FCS	bDBP	Albumin	1% (2.6 μg/ml)
α_2-globulin	bDBP	Albumin[a]	0.05% (130 ng/ml)
$(NH_4)_2SO_4$ precipitate	bDBP		0.0005% (1.3 ng/ml)
Purified bDBP	bDBP		1–2 ng/ml

[a] Electrophoretic α_2-globulin fraction is contaminated with albumin. Contamination of albumin in the α_2-globulin fraction can be seen in immunoelectrophoresis of the α_2-globulin fraction against anti-FCS (8).

appears to compete with DBP in the activation of macrophages.

This investigation was supported in part by National Science Foundation grant DMB-8511651 and Public Health Service grant AI-32140 to N.Y.

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