Activation of Human Monocyte Tumoricidal Activity by C-reactive Protein

Barbara P. Barna, Karen James, and Sharad D. Deodhar

ABSTRACT

We examined the effect of purified human C-reactive protein (CRP) on induction of human peripheral blood monocyte (Mo)-mediated cytoxicity (CTX) and oxidative metabolism. Exposure of Mo to acute phase serum levels of CRP in vitro resulted in dose-dependent expression of CTX against human tumor cell lines. Nonneoplastic human fibroblasts and glial cells were not affected by CRP-exposed Mo, and treatment of Mo monolayers with anti-Leu 11b (a natural killer marker) and complement did not abrogate or diminish CTX. Tumoricidal activity was observed after 20–44 h of Mo exposure to CRP, and after 48–72 h of coculture with radiolabeled target tumor cells. Mo exposed to CRP for 48 h also demonstrated elevated superoxide anion production when challenged with phorbol myristate acetate. Unlike CTX induced by lipopolysaccharide, CRP-induced CTX was completely inhibited by preincubation of CRP with phosphorylcholine, a CRP ligand, at a concentration of 5.5 molecules phosphorylcholine per molecule CRP. Further, when Mo medium (which contained 5% human AB serum) was preincubated with immobilized CRP, exposure of Mo to CRP in such medium did not result in CTX. In contrast, LPS-induced CTX was not affected. CRP-induced Mo CTX was observed, however, when Mo were exposed to CRP in medium preincubated with phosphorylcholine-treated immobilized CRP, suggesting that an active serum component which complexed with CRP was not removed. These findings indicate that one of the functions of the acute phase protein, CRP, may be to activate Mo and that the process may require a CRP-binding serum component.

INTRODUCTION

During acute inflammation, the concentration of CRP, a plasma protein with a molecular weight of 110,000, becomes dramatically elevated from trace amounts to levels of 1000-fold or more (1, 2). Although CRP was first recognized in 1930 by Tillet and Francis (3), the biological functions of the molecule have not yet been firmly defined. That CRP has biological significance is suggested by its appearance and remarkable conservation of structure in a wide range of animal species, including invertebrates (4). Several types of activities associated with CRP are similar to those mediated by IgG, as for example, the binding and activation of C1q (5), and the precipitation of pneumococcal C-polysaccharide (1). CRP, when complexed with a ligand such as C-polysaccharide of pneumococcus, has been shown to bind to Fc-receptor-bearing monocytes (6, 7) and to enhance phagocytosis (8, 9). Some investigators have suggested that CRP can interact with lymphocytes and modulate immune responses (10).

In previous studies, we found that treatment of tumor-bearing mice with human CRP delivered in liposomes or red blood cell ghosts, inhibited lung metastases and prolonged survival (11, 12). Although the mechanism of this CRP-induced tumor-inhibitory effect has not been fully clarified, in vitro studies suggest macrophage involvement. Murine macrophages exposed to liposomal or soluble CRP were found to develop characteristics associated with an activated state; i.e., enhanced SA production and elevated tumoricidal activity (13). The purpose of this study was to determine if CRP would have a similar effect on human Mo. Results indicate that human Mo are indeed activated by CRP and that the effect is dependent upon a CRP-binding serum factor that can be blocked by PC.

MATERIALS AND METHODS

Reagents. Human C-reactive protein was purified to homogeneity from serous fluids as previously described (11). Preparations of CRP yielded one band (22,000 subunit weight) on silver-stained polyacrylamide gel electrophoresis. The endotoxin content of CRP preparations as determined by Limulus Amoebocyte Lysate assay (Woods Hole, MA) was less than 0.002 ng endotoxin per mg CRP.

Preparation of Peripheral Blood Mo. MNL were purified from Ficoll-Hypaque centrifugation of heparinized blood and allowed to adhere to microtiter plates for 1 h. Nonadherent MNL were then removed by vigorous washing. By nonspecific esterase cytochemistry, over 95% of adherent mononuclear cells were characterized as Mo. All incubations were carried out at 37°C in crPMe. Endotoxin contents of culture reagents did not exceed 0.3 ng/ml.

Target Cells in Cytotoxicity Assays. Target cells included the CCF-STTG1 astrocytoma cell line previously established in our laboratory from a grade IV astrocytoma (now part of the ATCC), the CAKI 1 renal carcinoma (ATCC), and SK-MEL 28 melanoma (ATCC). Nonneoplastic glial cells were derived from biopsies of epileptogenic tissues, and identified as astrocytes by immunoperoxidase detection of glial fibrillary acidic protein as previously described (14). Nonneoplastic fibroblasts were cultured from skin biopsies. Target cells were cultured in RPMI 1640 medium supplemented with 10% FBS, l-glutamine (292 μg/ml), penicillin (100 units/ml), and streptomycin (100 μg/ml), and were free of mycoplasma or other microbial contamination.

Measurement of Cytotoxic Activity. The method for determination of human Mo-mediated CTX was a modification of that described in detail elsewhere (15). Adherent Mo were incubated with or without test reagents in microtiter plates at a concentration of 100,000 Mo/200 μl crPMe/well. After 20 h, Mo were gently washed with warm (37°C) RPMI 1640 and 10,000 tritiated thymidine-labeled human tumor cells were added in crPMe. After an additional 24–72 h, plates were washed, and residual cells were lysed with 0.1 N NaOH. Aliquots of lysis were placed in scintillation fluid for determination of radioactivity. Percentage of reagent-induced CTX was calculated by the following formula:

\[
\% \text{ Cytotoxicity} = \frac{\text{mean cpm in: control} - \text{test}}{\text{control} \times 100}
\]
Measurement of Superoxide Anion. The microassay for measurement of superoxide anion has been previously described in detail (13, 16). Briefly, MNL were plated in Microtest III plates (B-D Labware) to yield 1 x 10⁵ Mo/well. After 60 min, nonadherent MNC were washed away and remaining adherent Mo were incubated in 200 µl/well CRPMI with or without CRP or LPS for 24 to 96 h. After incubation, Mo were gently washed with phenol red-free Hanks' balanced salt solution and each well received 100 µl Hanks' balanced salt solution containing 16 nmol ferricytochrome c, with or without 1 nmol PMA as stimulant, or 600 units SOD as inhibitor. Production of SA was detected by measuring absorbance of reduced ferricytochrome c at 550 nm in an automated enzyme immunoassay reader. The reaction was monitored sequentially every 30 min for up to 3 h. Readings from wells containing SOD were automatically subtracted from non-SOD-treated wells. Data were obtained for both unstimulated and PMA-stimulated Mo after subtracting baseline readings at time 0, and final results were expressed as net nmol SA/10⁶ Mo obtained by subtracting mean nmol SA in unstimulated Mo from nmol SA in PMA-stimulated Mo.

Absorption of Culture Medium with Immobilized CRP. CRP (100 µg/ml of Tris-buffered saline + 10 mM Ca ++, pH 7.8) was incubated in plastic 24-well culture dishes at 1.0 ml/well for 18 h at 4°C. Wells were then washed with the above buffer. Nephelometric quantitation of CRP solutions removed from wells indicated that each well was coated with approximately 28 µg of CRP. No CRP was detectable in buffer washes. In some experiments, CRP-coated wells were also incubated with 1.0 mM PC + 10 mM Ca ++ at 25°C for 60 min. Following additional washes, 1.0 ml cRPMI was incubated in each well for 60 min at room temperature. This procedure was repeated and the absorbed cRPMI was passed through micropore filters and used for tumoricidal assays.

Assay for Natural Killer Cell Activity. NK activity was measured as described elsewhere (17). K562 myeloid leukemia cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 15 mM 4-(2-hydroxyethyl)-l-piperazineethanesulfonic acid buffer, 2 mM l-glutamine, penicillin (100 units/ml), and streptomycin (100 µg/ml). Target cells were labeled with Na2 51CrO4 (New England Nuclear, Boston, MA) for 1.5 h, then washed three times in RPMI 1640 containing 5% FBS, 15 mM 4-(2-hydroxyethyl)-l-piperazineethanesulfonic acid buffer, 2 mM l-glutamine, penicillin (100 units/ml), and streptomycin (100 µg/ml). Results were expressed as percentage of cytotoxicity by the following formula:

\[
\% \text{cytosis} = \frac{\text{mean cpm in test} - \text{spontaneous release}}{\text{maximum} - \text{spontaneous release}} \times 100
\] (B)

NK Antibody Studies. Unfractionated MNL or adherent Mo were treated with RPMI 1640 with or without 0.3 µg anti-Leu 11b (Becton-Dickinson, Mountain View, CA) per 1 x 10⁶ MNL for 30 min at room temperature. The antibody was removed and RPMI 1640 medium with or without 50% fresh rabbit serum (PelFreeze, Rogers, AZ) as a source of C' was added. After an additional hour of incubation at 37°C, cells were washed and fresh medium was added. Treated and untreated MNL were used for NK cytotoxicity assays, and for determination of proportions of Leu 11 positive cells by flow cytometry using a fluorescence-activated cell sorter (FACS 440; Becton-Dickinson). Surface marker analysis by flow cytometry indicated a mean reduction of 50% (n = 3) in percentage of Leu 11 positive MNL after treatment with anti-Leu 11b and C'.

Statistical Analysis. Statistical significance of data was determined by comparing results of test and control groups in the various assays by two-tailed Student's t test.

RESULTS

Generation of Tumoridicial Activity. Adherent Mo incubated in vitro with human CRP in doses ranging from 5 to 100 µg/ml developed tumoricidal activity in a dose-dependent fashion against the allogeneic human astrocytoma line CCF-STTG1 (Fig. 1). Significant (P < 0.05) tumoricidal activity was observed in monocytes treated with 50 to 100 µg/ml CRP in assays of 19 out of 24 (79%) normal individuals tested throughout the course of this study. Monocytes of these 24 donors were also activated by LPS which was included in assays to represent a more well-recognized Mo and macrophage activating agent (15).

To determine if CRP-induced CTX was directed only against tumor cells, Mo were incubated with an activating dose of CRP (50 µg/ml) and CTX was evaluated against two nonneoplastic and three neoplastic human allogeneic cell lines (Fig. 2). No CTX was observed against nonneoplastic target cells after exposure to either LPS or CRP.

Kinetics of CRP-generated Tumoridicial Activity. Tumoridicial activity was examined in Mo exposed to CRP for 4 to 44 h before coculture with radiolabeled target cells for 48 h (Fig. 3). Exposure of Mo to CRP for only 4 h did not result in generation of CTX, but 20 or 44 h exposure was effective. Similar findings were seen in Mo exposed to LPS.

When Mo were exposed to CRP for 20 h, and length of coculture period with target cells was varied from 24 to 72 h, results again indicated similarities between CRP and LPS (Fig. 4). Tumoricidal activity with either LPS or CRP was highest after 72 h of coculture.

NK Cell Activity. In order to determine whether the presence of NK cells within adherent Mo populations contributed to Mo tumoricidal activity, Mo were treated with anti-Leu 11b and complement before and after exposure to CRP or LPS (Table 1). Results indicated no abrogation or decrease in tumoricidal activity of treated adherent Mo. Similar treatment of unfractionated MNL resulted in total abrogation of NK cell cytotoxicity against K562 cells. Untreated MNL displayed 85.7 ± 10.4 (SD%) (n = 3) cytosis at a 25/1 effector/target cell ratio while anti-Leu 11b and C'-treated MNL showed only 4.3 ± 5.9% cytosis.
CRP-INDUCED MONOCYTE ACTIVATION

Fig. 2. Tumor cell-directed cytotoxicity of CRP-activated monocytes. Adherent human monocytes of 10 normal donors were incubated for 20 h in control medium, with purified human C-reactive protein (50 μg/ml) (CRP), or with S. typhimurium lipopolysaccharide (5 μg/ml) (LPS), then washed and cocultured for 48 h with tritiated thymidine-labeled target cells indicated. Not all donors were tested against each target cell. Data represent mean values of percentage cytotoxicity of monocytes not treated with CRP as the medium control. Mean cpm in target cells cultured alone was: SK-MEL-28, 5074; fibroblasts, 678; CCF-STTG1, 8248; nonneoplastic astrocytes, 2145; and CAKI-1, 2416.

Superoxide Anion Production. Because our previous studies had indicated that CRP-enhanced superoxide anion production in murine macrophages, we examined human Mo for evidence of similar activity. Culture of Mo with LPS for up to 4 days resulted in elevated SA production compared to control Mo cultured in medium alone (Fig. 5). Significant (P < 0.05) differences between LPS-treated and control Mo were found after 3 and 4 days in culture with LPS, while SA production of CRP-treated and control Mo were significantly (P < 0.05) different after 2 days in culture with CRP and responses diminished thereafter.

Effect of Phosphorylcholine on Tumoricidal Activity. Because of the similarities in patterns of tumoricidal activity induced by CRP and LPS, we sought to determine if we could detect differences by using PC, a CRP ligand (1), to block CRP activity. Exposure of Mo to CRP (50 μg/ml) in cRPMI containing 1.0 mM PC (a ratio of 5.5 PC molecules per molecule CRP) totally inhibited CRP-induction of tumoricidal activity while 0.5 mM PC (a ratio of 2.8 PC molecules per molecule CRP) was not inhibitory in all experiments (Table 2). LPS-induced Mo tumoricidal activity was completely unaffected by either concentration of PC.

In subsequent experiments, we attempted to determine

Fig. 3. Kinetics of CRP-induced monocyte activation: length of monocyte incubation with CRP. Adherent human monocytes were incubated for the times shown in medium with or without purified human CRP (50 μg/ml) (O) or S. typhimurium LPS (5 μg/ml) (●), then washed and cocultured with tritiated thymidine-labeled human tumor cells for 48 h. Data represent mean percentage of cytotoxicity values (±SD) of CRP or LPS-treated monocytes in three experiments.

Table 1 Cytotoxic activity of monocytes after treatment with anti-LEU-11b and C

<table>
<thead>
<tr>
<th>Treatment of monocytes</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
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<tbody>
<tr>
<td>None</td>
<td>9468 ± 886</td>
<td>8483 ± 522</td>
</tr>
<tr>
<td>CRP</td>
<td>4397 ± 466 (54)</td>
<td>4419 ± 669 (48)</td>
</tr>
<tr>
<td>Anti-Leu-11b + C, then CRP</td>
<td>3163 ± 114 (67)</td>
<td>ND</td>
</tr>
<tr>
<td>CRP, then anti-Leu-11b + C'</td>
<td>ND</td>
<td>4238 ± 727 (50)</td>
</tr>
<tr>
<td>LPS</td>
<td>6256 ± 492 (34)</td>
<td>3297 ± 853 (61)</td>
</tr>
<tr>
<td>Anti-Leu-11b + C' then LPS</td>
<td>4149 ± 146 (56)</td>
<td>ND</td>
</tr>
<tr>
<td>LPS, then anti-Leu-11b + C'</td>
<td>ND</td>
<td>4332 ± 232 (49)</td>
</tr>
</tbody>
</table>

* Monocytes were treated with medium or anti-Leu-11b antibody (0.3 μg/106 cells) with or without 50% rabbit C'. In experiment 1, medium with or without CRP (50 μg/ml) or LPS (0.5 μg/ml) was added after antibody + C' treatment. Following an additional 20 h, monocytes were washed and tritiated thymidine-labeled target cells were added. In experiment 2, monocytes were treated with antibody and C' after 20 h incubation with CRP or LPS and before addition of tritiated thymidine-labeled target cells. Two experiments of each type were done.

Fig. 4. Kinetics of CRP-induced monocyte activation: length of monocyte-tumor cell co-culture. Adherent human monocytes were incubated for 20 h in medium with or without purified CRP (50 μg/ml) (●) or S. typhimurium LPS (5 μg/ml) (O), then washed and cocultured with tritiated thymidine-labeled human tumor cells for the times shown. Data represent mean percentage cytotoxicity values (±SD) of CRP or LPS-treated monocytes in three experiments.

Fig. 5. Kinetics of monocyte superoxide anion production. Adherent monocytes were challenged with 1 nmol phorbol myristate acetate after culture for times indicated in medium alone (●), CRP (5 μg/ml) (△), or LPS (5 μg/ml) (○). Data represent mean nanomoles superoxide anion accumulated at 2 h/106 monocytes (SA) (±SD) from three experiments. Monocyte production of SA after CRP or LPS treatment was compared by Student's t test to SA production in medium alone. * P < 0.05; **, P < 0.001.
whether PC blocked CRP binding to components of CRPMI, the standard culture medium in which the CTX assay was performed. CRP was immobilized by absorption to plastic macrowell plates, and CRPMI was incubated with immobilized CRP before assay. Exposure of Mo to CRP in preincubated CRPMI eliminated tumoricidal activity while Mo exposed to LPS in similarly treated medium demonstrated significant (P < 0.001) CTX (Table 3). When CRPMI was incubated with immobilized CRP that had been previously exposed to 1 mM PC, Mo tumoricidal activity was again generated by CRP.

**DISCUSSION**

Our findings demonstrate that purified human CRP induces tumoricidal activity in human Mo. The specificity of this activity for neoplastic cells and the kinetics of activity induction and expression are similar to characteristics of CTX induced by LPS, an established macrophage-activating agent (15). The total inhibition of CRP effects by a CRP ligand, PC (19), and the lack of PC effect on LPS, clearly indicated a specific interaction between CRP and Mo that was independent of soluble CRP. The rationale for examining CRP binding to medium components rather than to Mo was based on previous work showing that soluble, uncomplexed CRP was not bound by Mo (6, 7). Further, human CRP, when aggregated, has been reported to bind serum components, specifically low density and very low density lipoproteins (22, 23). Human CRP has also been found to bind to abnormal very low density lipoproteins in a PC-inhibitable manner (24). These reports also suggest that CRP activation of Mo CTX in our studies may be associated with CRP binding to a component of human serum present in culture medium.

Our studies indicated that Mo tumoricidal activity generated in vitro by CRP or LPS did not involve NK cells, a finding supported by the report of Kleinerman and Herberman (25) who also found no evidence of NK participation in human Mo tumoricidal activity assayed by a similar procedure. A relationship between NK function and CRP has been described however by Baum et al., who detected expression of CRP on the surface membranes of NK cells. Antiserum to CRP inhibited NK activity, although the addition of soluble or complexed CRP did not enhance NK responses (26). Our use of antibody to the human NK marker, Leu 1b (18), together with C' clearly abrogated NK cytotoxicity against K562 cells, but had no effect on CRP or LPS-induced Mo tumoricidal activity, whether Mo were treated before or after exposure to activating agents.

The finding that CRP enhanced SA production by Mo confirmed data from our previous studies of murine macrophages (13). The kinetics of Mo development of SA responses to LPS and CRP differed, however. SA responses were detected after 2 days with CRP compared to 4 days with LPS exposure. The kinetics of SA response to LPS readily supported CRP-induced Mo CTX, suggesting that PC competed with this factor for binding to CRP. The rationale for examining CRP binding to medium components rather than to Mo was based on previous work showing that soluble, uncomplexed CRP was not bound by Mo (6, 7). Further, human CRP, when aggregated, has been reported to bind serum components, specifically low density and very low density lipoproteins (22, 23). Human CRP has also been found to bind to abnormal very low density lipoproteins in a PC-inhibitable manner (24). These reports also suggest that CRP activation of Mo CTX in our studies may be associated with CRP binding to a component of human serum present in culture medium.

**Table 2** Inhibition of CRP-induced monocyte tumoricidal activity by phosphorylcholine

<table>
<thead>
<tr>
<th>Treatment of monocytes</th>
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<tbody>
<tr>
<td>CRP (µg/ml)</td>
</tr>
<tr>
<td>None</td>
</tr>
<tr>
<td>50</td>
</tr>
<tr>
<td>None</td>
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<tr>
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<tr>
<td>50</td>
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<td>None</td>
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<tr>
<td>None</td>
</tr>
<tr>
<td>50</td>
</tr>
<tr>
<td>None</td>
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</tbody>
</table>

* Adherent Mo were exposed to medium with or without CRP, LPS, or phosphorylcholine (PC) in the concentrations shown for 20 h. Cells were washed and tritiated thymidine-labeled target cells were added. Two experiments were done.

**Table 3** Inhibition of CRP-induced monocyte tumoricidal activity by preincubation of culture medium with immobilized CRP

<table>
<thead>
<tr>
<th>Treatment of culture medium</th>
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<tbody>
<tr>
<td>CRP (µg/ml)</td>
</tr>
<tr>
<td>None</td>
</tr>
<tr>
<td>None</td>
</tr>
<tr>
<td>Immobilized CRP</td>
</tr>
<tr>
<td>Immobilized CRP bound with phosphorylcholine</td>
</tr>
<tr>
<td>None</td>
</tr>
<tr>
<td>Immobilized CRP</td>
</tr>
</tbody>
</table>

* Adherent monocytes (Mo) were exposed to medium with or without CRP or LPS in the concentrations shown for 20 h. Culture medium was untreated or had previously been incubated at room temperature for 2 h in plastic macrowell dishes coated with either CRP or PC treated with phosphorylcholine. Two experiments were done.

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exposure were similar to those reported with γ-interferon (27), but the differences between CRP and LPS in our SA assays cannot be adequately explained without further study. In all experiments, both CRP and LPS-induced SA responses were lower than that of freshly tested Mo, suggesting that these reagents maintained rather than elevated SA production.

The studies presented here indicate that purified human CRP, in the presence of a serum factor that provides binding specificity, enhances human Mo display of tumoricidal activity. CRP also enhanced Mo production of SA, a finding which emphasized the activating effect of CRP on Mo and confirmed earlier studies in murine macrophages. These data suggest that an important biological role for CRP may be as a modulator of Mo and macrophage function during inflammatory responses.

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REFERENCES

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