*Brief Definitive Report*

# C-REACTIVE PROTEIN IS PRODUCED BY A SMALL NUMBER OF NORMAL HUMAN PERIPHERAL BLOOD LYMPHOCYTES

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C-reactive protein (CRP) is a major human acute phase protein composed of five identical, 21,500 mol wt subunits (1, 2). CRP is detectable on the surface of ~4% of normal PBL. CRP binds its physiological ligands in a $Ca^{++}$-dependent manner; removal of $Ca^{++}$ does not alter the presence of CRP on the lymphocyte surface (3). Recently, investigators in this laboratory have reported (4) substantial inhibition of NK activity when effector cells were treated with anti-CRP. Although the liver is the site of CRP production contributing to the acute phase response (5), data presented here suggests that some lymphocytes produce small amounts of CRP and express it on their surface. Surface CRP (S-CRP) is not acquired exogenously from trace levels of CRP in normal serum.

## Materials and Methods

*Isolation of PBMC.* PBMC were separated from whole blood on Ficoll-Hypaque. They were then washed and resuspended in balanced salt solution (BSS) containing 0.05% gelatin.

*Enrichment of PBL and Large Granular Lymphocytes (LGL).* PBL were incubated in RPMI containing 20% FCS and 10 mg/ml carbonyl iron with rotation at 37°C for 45 min. Phagocytic cells were removed with a magnet. Mononuclear phagocyte–depleted PBL were enriched for LGL by layering onto discontinuous Percoll gradients (6). The exact percentage of lymphocytes obtained from each Percoll layer varied with the donor.

*Anti-CRP Antibodies.* Immunoprecipitations were performed with sheep anti-CRP (lot 20557; Cappel Laboratories, Malvern, PA). Other experiments were performed with purified goat anti-CRP IgG (American Biosystems Laboratories, Marine of St. Croix, MN).

*Labeling PBL with $^{125}$I-conjugated Antibodies.* Goat anti-CRP was iodinated using the lactoperoxidase method (7). Monocyte-depleted PBL were incubated with $^{125}$I–anti-CRP for 30 min at 37°C. Antibody-labeled cells were centrifuged on Percoll gradients; lymphocytes were harvested and counted on a gamma counter.

*Anti-CRP Pretreatment of PBL.* PBL, LGL or target cells were resuspended in RPMI containing 0.05% gelatin and incubated for 30 min at 37°C in the presence or absence of various dilutions of anti-CRP.

*K562 Target Cells.* K562 cells used as target cells were labeled with $^{51}$Cr by incubating $5 \times 10^6$ cells/ml in RPMI containing 100 $\mu$Ci of $^{51}$Cr at 37°C for 1 h.

*$^{51}$Cr-release Assay.* A 4-h $^{51}$Cr-release assay using $5 \times 10^5$ effector cells and $10^4$ $^{51}$Cr-labeled K562 were incubated in 0.2 ml RPMI containing 0.05% gelatin at 37°C. After 4

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h, half (100 μl) of the supernatant was harvested, and percent specific release was calculated as 100 × [(experimental release) − (spontaneous release)]/[(detergent release) − (spontaneous release)].

*Radiolabeling of S-CRP.* Cells were biosynthetically labeled with [$^{35}$S]met (New England Nuclear, Boston, MA). Cells were washed three times with met-free DMEM (M. A. Bioproducts, Walkersville, MD) and resuspended in met-free DMEM containing 250 μCi/ml [$^{35}$S]met, 2 mM glutamine, 100 μg/ml gentamycin, 5% IL-1, and 5% IL-2. ~3.5 × $10^6$ cells per 200 μl were cultured in 96-well culture plates for 4 h. Plates were centrifuged at 200 *g* for 10 min; supernatants were collected and stored at −70°C. Cells were lysed and frozen at −70°C.

*Immunoprecipitation and SDS-PAGE.* Immunoprecipitations were performed as described by Sackstein and Colton (8). Radiolabeled cells were lysed with 200 μl ice-cold lysis buffer, and incorporation of isotope into total protein was assayed by cold TCA precipitation.

Antibodies were added to cell lysates at a 1:20 final dilution, and mixtures were incubated for 1 h at room temperature or overnight at 4°C. To each sample, 100 μl of fixed *Staphylococcus aureus* (IgGsorb; The Enzyme Center, Boston, MA) was added and incubated for 1 h at 4°C. The IgGsorb had been prepared as described by Auerbach et al. (9). In all experiments, samples were precleared with 50 μl of a 10% IgGsorb suspension. A preprecipitation reaction was also run with irrelevant antisera (sheep IgG fraction) to minimize nonspecific binding to relevant immunoprecipitates. In some experiments, a 1:20 dilution of anti-CRP was allowed to react with unlabeled human serum CRP. Blocked antibody was then used in the immunoprecipitation reactions. Immunoprecipitates were washed six times in ice-cold lysis buffer and prepared for SDS-PAGE under reducing conditions as described by Laemmli (10). Gels were fixed, stained, and treated with Amplify (Amersham Corp., Arlington Heights, IL) and dried. Dried gels were exposed to x-ray film (Kodak XAR-5) at −70°C.

## Results

*S-CRP Is Not Bound to NK Cells in a $Ca^{++}$-dependent Manner.* Since earlier studies (4) have shown that NK activity could be inhibited by anti-CRP, the effect of EDTA on S-CRP and NK activity was tested. Normal blood was collected in either EDTA or heparin and examined for NK activity; cells collected in EDTA and those collected in heparin had identical NK activities. Cells collected in either EDTA or heparin were tested for their reactivity with anti-CRP antibodies in a standard $^{51}$Cr-release assay. As shown in Fig. 1, NK activity of both groups was significantly abrogated by anti-CRP, suggesting that S-CRP is not removed from the lymphocyte membrane upon removal of $Ca^{++}$. These results are in agreement with those of James et al. (3), who showed that S-CRP is still detectable on lymphocytes after removal of $Ca^{++}$.

*S-CRP Is Present on LGL.* The above and previous data (4) suggest that S-CRP may be expressed on NK effectors. NK activity is attributed to LGL isolated from the low-density fractions of discontinuous Percoll gradients (6). Monocyte-depleted PBL were treated with $^{125}$I-labeled anti-CRP antibodies ($^{125}$I–anti-CRP). $^{125}$I–anti-CRP–treated cells were then layered on Percoll gradients consisting of five layers ranging from 34 to 50%. As seen in Table I, the majority of $^{125}$I was bound to cells in the uppermost low-density fractions.

*S-CRP Produced by Lymphocytes.* Although S-CRP could not be removed with EDTA, the possibility remained that S-CRP was acquired exogenously, and that its binding to lymphocytes was $Ca^{++}$ independent. PBMC, PBL, and LGL were incubated for 4 h in met-free DMEM supplemented with [$^{35}$S]met. Fig. 2 shows

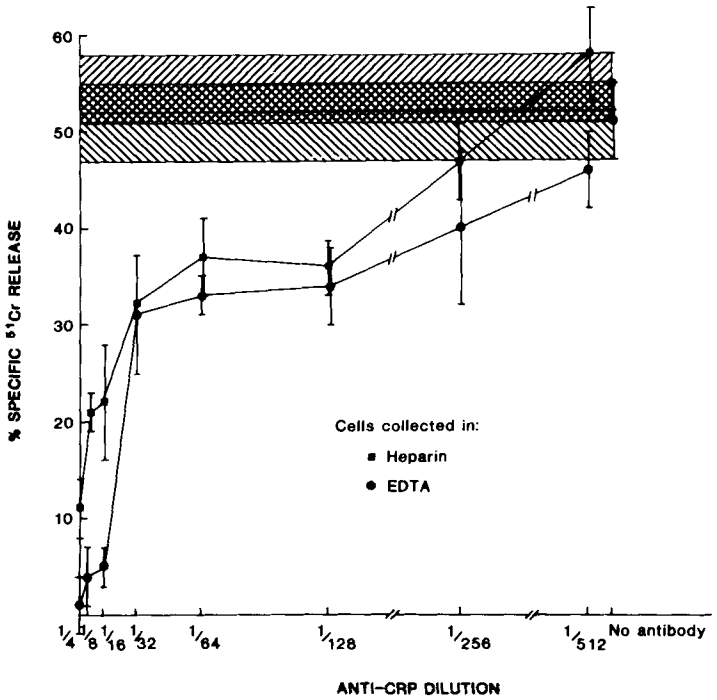


FIGURE 1. EDTA collection of lymphocytes does not affect S-CRP of NK effector cells. Although CRP-ligand interactions are $Ca^{++}$ dependent and fail to occur in the presence of EDTA, anti-CRP inhibited NK-mediated killing of K562 by effectors collected in EDTA or heparin.

TABLE I

*Separation of $^{125}I$-Labeled Cells on Percoll Density Gradients*

| Exp. | Percoll fraction (% Percoll) | $^{125}I$-Anti-CRP (% recovered) | Cells (% recovered) |
|---|---|---|---|
| 1 | 1 (34) | 87 | 6 |
| | 2 (38) | 3 | 26 |
| | 3 (42) | 2 | 41 |
| | 4 (46) | 2 | 16 |
| | 5 (50) | 1 | 10 |
| | 6 pellet | 5 | 2 |
| 2 | 1 (34) | 63 | 4 |
| | 2 (38) | 7 | 29 |
| | 3 (42) | 2 | 45 |
| | 4 (46) | 2 | 18 |
| | 5 (50) | 1 | 2 |
| | 6 pellet | 27 | 2 |

PBL were labeled with $^{125}I$-anti-CRP and centrifuged on discontinuous Percoll gradients.

an autoradiograph of [$^{35}S$]met-labeled CRP subunits at ~21,500 mol wt (PBMC, PBL, and LGL; lanes *A*, *C*, and *E*, respectively). Experiments using unlabeled human serum CRP to block the binding sites of anti-CRP antibodies before immunoprecipitation show that cold CRP can reduce the amount of labeled S-CRP recovered (PBMC, PBL, and LGL; Fig. 2, lanes *B*, *D*, and *F*, respectively).

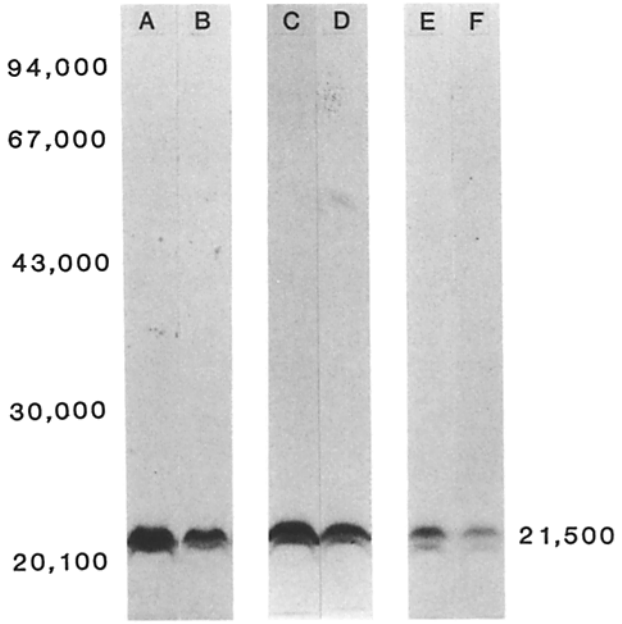


FIGURE 2. S-CRP production by PBMC, PBL, and LGL. After a 4-h incubation, biosynthetically labeled S-CRP was detectable in cultures of PBMC, PBL, and LGL (lanes *A*, *C*, and *E*, respectively). When anti-CRP antibodies were blocked with cold CRP at a 1:6 (wt/wt) ratio, the amount of [$^{35}$S]met-labeled S-CRP recovered was reduced (PBMC, PBL, and LGL; lanes *B*, *D*, and *F*, respectively). Each pair is from a separate experiment.

Although S-CRP production by PBL and LGL was detectable, it was possible that monocytes were contributing to the S-CRP produced by PBMC. Fig. 3 shows that, while PBL and LGL produce S-CRP (*middle* and *right* lanes, respectively), monocytes do not (left lane). No CRP production was detectable in monocyte cultures. In addition, biosynthetically labeled CRP could not be detected in 4-h supernatants of lymphocyte cultures, suggesting that lymphocytes may not secrete CRP (data not shown).

## Discussion

James et al. (3, 11) reported the presence of CRP on ~4% of human lymphocytes. We have previously described (4) substantial inhibition of NK activity when lymphocytes were treated with anti-CRP. This suggests that CRP is on the majority of lymphocytes that function as NK effector cells. The data presented here confirm the presence of S-CRP on certain lymphocytes and suggest that lymphocytes can produce CRP.

Great quantities of serum CRP are produced by the liver during the acute phase response (1, 5). We have shown that lymphocytes produce CRP or an immunologically crossreactive protein of ~21,500 mol wt under reducing conditions. This is the mol wt of CRP subunits (1). We suggest that the same cells that produce S-CRP express this protein. The production of serum proteins by both hepatocytes, and to a lesser extent, cells of hematopoietic origin, is well known (12–14). Gahmberg and Anderson (13) described a membrane form of

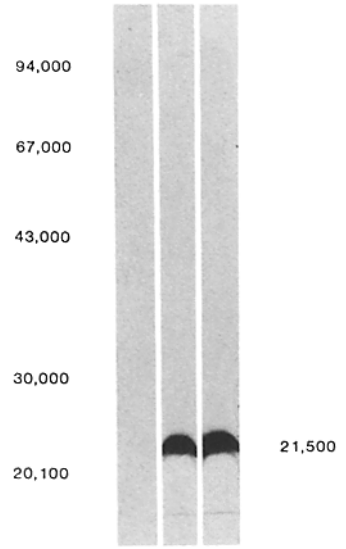


FIGURE 3. Lack of S-CRP production by monocyte/macrophages. PBMC as well as PBL produced CRP. Mononuclear phagocytes did not produce detectable S-CRP (*left*), PBL and Percoll-enriched LGL did produce S-CRP (*middle* and *right*, respectively).

the human acute phase protein $\alpha_1$-acid glycoprotein (AAG) on normal lymphocytes and showed that the membrane form of AAG is produced by lymphocytes. The complement componant C3, also an acute phase protein (1), is produced primarily by the liver (12, 14); however, considerable C3 is produced by monocytes and macrophages (12, 14).

Extrahepatic synthesis of CRP could only be attributed to lymphocytes; no CRP production by monocytes could be detected. This is in concert with the findings of Kushner and Feldman (15), who reported CRP production by hepatocytes only; no CRP synthesis could be attributed to the mononuclear phagocytes of the liver. In addition, when immunoprecipitation was performed on lymphocyte or monocyte culture supernatants, no CRP was found, which suggests that the lymphocytes that produce CRP may not secrete it.

Since the mol wt of CRP subunits is similar to that of immunoglobulin light chains, it was possible that anti-CRP antibodies were crossreacting with light chains produced by B cells in the cultures. If this were the case, immunoprecipitation of culture supernatants should detect secreted light chains, but none were detected. In addition, light chains on the stained, dried gels did not correspond to the labeled, 21,500 mol wt labeled proteins on the respective autoradiographs.

## Summary

Biosynthetic labeling with [$^{35}$S]met and immunoprecipitation with anti–C-reactive protein (CRP) antibodies and *Staphylococcus aureus* indicate that cell surface CRP is produced by lymphocytes. The ability of anti-CRP to reduce NK activity, and the demonstration that $^{125}$I–anti-CRP–labeled PBL are found in low-density Percoll fractions associated with large granular lymphocyte (LGL) and NK activity suggest that S-CRP-bearing cells are NK effectors. The produc-

tion of S-CRP by LGL supports this hypothesis. While lymphocytes were shown to synthesize S-CRP, monocytes produced no detectable S-CRP. The lymphocytes that produce S-CRP apparently do not secrete it; when lymphocyte culture supernatants were tested, no S-CRP was found. This is the first description of extrahepatic synthesis of CRP.

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