

## EFFECTS OF C-REACTIVE PROTEIN ON THE LYMPHOID SYSTEM

### I. Binding to Thymus-Dependent Lymphocytes and Alteration of their Functions\*‡

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C-reactive protein (CRP)<sup>1</sup> was first described in 1930 by Tillet and Francis (2) who observed that sera from patients during acute febrile illnesses had the ability to precipitate the C-substance (later designated C-polysaccharide, CPS) of pneumococcal cell walls. The serum factor responsible for this reaction was defined as a "C-precipitin," and later designated "C-reactive protein" (3). This protein was found to appear in the blood during a variety of reactions of tissue destruction or inflammation (3), and has served as a useful clinical index of these processes (4-6). CRP was purified by MacLeod and Avery (7, 8) who also demonstrated the calcium dependency of its binding to CPS, and was later crystallized by McCarty (9). More recently, human CRP has been shown to be a gamma-migrating protein (10) (mol wt approximately 120,000 daltons [11-13]) consisting of six identical, noncovalently bound subunits (11).

CRP mediates many of the same *in vitro* reactions as the immunoglobulins, yet it differs from them in terms of antigenicity (8), tertiary structure (11), specificity of induction (3), and molecular heterogeneity (11, 13). In contrast to immunoglobulin the C-reactive protein molecules all share the same binding specificities which are directed toward phosphate esters (14, 15), especially phosphoryl choline and choline phosphatides (16); additional binding specificities have been reported for certain polyanions (14, 16) and polycations (17, 18), and even carbohydrate (19). Like the immunoglobulins CRP possesses the ability to initiate reactions of precipitation (2, 20), agglutination (21), capsular swelling (22), promotion of phagocytosis (23, 24), and complement consumption (17, 25). CRP and the immunoglobulins have a similar amino acid composition (11, 13) although the amino acid sequence of CRP is yet to be determined. Indeed, as a

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<sup>1</sup> *Abbreviations used in this paper:* ADL, antibody-dependent lymphocyte-mediated cytotoxicity; AHGG, aggregated human gamma globulin; BSA, bovine serum albumin; C, complement; Con A, concanavalin A; CPS, C-polysaccharide; CRL, complement receptor lymphocytes; CRP, C-reactive protein; E-R, spontaneous T-cell rosettes; EAC, complement coated erythrocytes; EAC-R, B-cell rosettes; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; F1, fluoresceinated; HBSS, Hanks' balanced salt solution; LD, lymphocyte defined; MLC, mixed lymphocyte culture; PBL, human peripheral blood lymphocytes; PBS, phosphate-buffered saline; PHA, phytohemagglutinin.

multivalent protein capable of reacting with and aggregating a variety of exogenous and endogenous substances on the one hand, and activating or influencing host effector mechanisms such as complement on the other, CRP is remarkably similar functionally to the immunoglobulins. These and related properties of CRP, such as enhancing the random motility of leukocytes (26), have implied a role for CRP in nonspecific resistance to infection (27) as well as in modulation of tissue injury and repair. It also has been suggested that CRP may be involved in the induction of specific immunity since CRP levels after adjuvant treatment correlated well with subsequent antibody titers (28). It therefore was of interest to define the reactivity of CRP with the cells of the lymphoid system.

Reports on the effect of CRP on lymphocytes have been limited and conflicting. Hornung and Fritschi (29) reported that CRP added to leukocyte suspensions was mitogenic at low concentrations (10  $\mu\text{g}/\text{ml}$ ) but toxic at higher concentrations. Hornung (30) later reported that CRP in the presence of lymphocytes inhibited the growth of human melanoma cells *in vitro*. By contrast, Hokama et al. (31) reported that purified CRP and CRP-containing sera were not mitogenic to lymphocytes but rather inhibited their mitogenic response to phytohemagglutinin (PHA); they suggested that CRP was one serum constituent responsible for the depression of the lymphoblastogenic response to mitogens that is observed in many disease processes. The present studies thus were undertaken to more clearly define the interactions between CRP and the lymphoid system, to determine whether CRP could interact with either T or B lymphocytes selectively, and to examine the consequences of any such interactions on lymphocyte functions *in vitro*.

We report here that CRP binds selectively to T lymphocytes, inhibits their ability to form spontaneous rosettes with sheep erythrocytes (E) and inhibits their response to allogeneic cells in mixed lymphocyte culture (MLC) reactions. By contrast CRP does not bind to B lymphocytes, and does not alter such B-cell functions as binding to activated complement components (C3b) or to the Fc portion of immunoglobulin, or the mediation of antibody-dependent cytotoxicity reactions. Furthermore, CRP fails to inhibit PHA or concanavalin A (Con A)-induced mitogenesis. It therefore seems that binding of CRP is a property of T lymphocytes or a subpopulation thereof, and can result in modulation of certain of the T-cell functional characteristics *in vitro*.

## Materials and Methods

*Purification of C-Reactive Protein (CRP).* CRP was purified from ascites or pleural fluids by affinity column chromatography based on the calcium-dependent binding of CRP to CPS (3, 7, 8). The CPS was extracted from a Cs-encapsulated pneumococcal mutant (generously provided by Dr. Gerald Schiffman, Department of Microbiology, Downstate Medical Center, Brooklyn, N. Y.) by the method of Anderson and McCarty (32), and attached to cyanogen bromide-activated Biogel-A-50 m beads (Bio-Rad Laboratories, Richmond, Calif.). CRP-rich ascites or pleural fluids were passed through this column in calcium-containing Tris-buffered saline, pH 7.4, and the CRP was eluted as a single protein peak with citrate-buffered saline. The protein-containing fractions were pooled and filtered through a Biogel-A-0.5 m (Bio-Rad Laboratories) column to remove CRP aggregates and subunits. The eluted CRP was dialyzed overnight against normal saline and sterilized by Millipore filtration. Analysis of these preparations by immunoelectrophoresis developed with antihuman whole serum/CRP revealed a single line having gamma mobility. A single protein band was also seen on

polyacrylamide gel electrophoresis. Monospecific antisera to this CRP have been raised in both goats and rabbits. The procedure for this purification will be published in detail elsewhere.<sup>2</sup>

*Separation and Identification of Peripheral Blood Lymphocytes (PBL).* Lymphocytes in heparinized blood from normal individuals were separated by centrifugation on Ficoll-Hypaque. The cells were washed twice in RPMI-1640 containing antibiotics, glutamine (2 mM), and 10% heat inactivated agammaglobulinemic fetal calf serum (FCS) (Baltimore Biological Laboratories, Cockeysville, Md.) and resuspended to 10<sup>6</sup>/ml. Cell viability as determined by dye exclusion was usually greater than 98% and contamination by neutrophils was always less than 4%.

The E-rosette test for SRBC binding by T lymphocytes (33) was carried out by mixing 0.5 ml lymphocytes ( $2 \times 10^6$ /ml) in Hanks' balanced salt solution (HBSS) with 0.5 ml of 0.5% SRBC and 0.1 ml inactivated FCS (absorbed with SRBC). The mixture was centrifuged at 150 g for 5 min at room temperature and kept at 4°C for 2-18 h before counting. The EAC rosette test was used to measure a B-cell subset having receptors for C3b or C3d (34, 35). EAC were prepared by incubating hemolysin (rabbit 19S anti-SRBC)-sensitized E with a 1:25 dilution of normal human serum for 1 h at 37°C and washing the unlysed cells five times in HBSS. A mixture of 0.5 ml of 0.5% EAC and  $2 \times 10^6$  lymphocytes were incubated on a rotary wheel for 30 min at 37°C as described by Ross et al. (35). Both E and EAC rosettes were scored as positive when binding three or more SRBC; 100-200 lymphocytes were examined in each sample.

B cells were purified by removing T cells which had formed rosettes with SRBC (36). T-cell rosette formation was carried out as described above except volumes of all reactants were multiplied by 4. The mixture was layered on Ficoll-Hypaque and centrifuged for 20 min at 400 g at 4°C. Lymphocytes at the interface were collected and stained with polyvalent fluorescein isothiocyanate (FITC)-conjugated antisera to IgG, IgA, and IgM (anti-GAM) (Hyland Div., Travenol Laboratories, Costa Mesa, Calif.) to determine the fraction of B cells present. This procedure enriched the fraction of surface immunoglobulin (Ig)-bearing cells from 20-30% to 75-90%.

T cells were enriched from PBL suspensions by removing B cells by filtration through a nylon wool column (37). The column was packed with 300 mg of nylon fiber (Fenwall Leukopak, Downers Grove, Ill.) to the 5 ml mark in a syringe and preincubated with RPMI-1640 containing 10% FCS at 37°C for 10 min. PBL ( $10^7$ ) were added and the column was incubated for 60 min at 37°C. T cells were eluted with 15-20 ml medium at 1 ml/min. The number of Ig-bearing cells was reduced to 5% or less.

*Immunofluorescent Staining for CRP.* PBL ( $10^6$  cells in 1.0 ml) were incubated with CRP in RPMI-1640 containing 10% FCS at 37°C in a 5% CO<sub>2</sub> atmosphere. The cells were washed three times with 5 vol of RPMI-1640 containing 10% FCS. Fluoresceinated (Fl)-rabbit antihuman CRP (Behring Diagnostics, Somerville, N. J.) was added to a 0.1 ml suspension of lymphocytes in phosphate (0.01 M)-buffered saline (PBS), (pH 7.2) which contained 1.0% bovine serum albumin (BSA) and 0.01 M sodium azide. After staining for 30 min at 4°C the cells were washed three times in PBS-BSA, and examined using a Leitz Orthoplan photomicroscope (E. Leitz, Inc., Rockleigh, N. J.) under 400 × magnification by incident light fluorescence with either FITC or BG38 excitation filters. The percentage of cells binding CRP was determined by relating the number of fluorescent cells to the total number of cells in each field as visualized by dark-field illumination. Routinely at least 200 lymphocytes/sample were examined. Specificity of fluorescence was established with controls consisting of both cells incubated without CRP and cells treated with an excess of unconjugated anti-CRP followed by incubation with conjugated anti-CRP. Indirect fluorescent staining was performed by adding unconjugated rabbit anti-CRP followed by Fl-goat antirabbit gamma globulin (Cappel Laboratories, Dowington, Pa.). The purity of the Fl-anti-CRP rabbit antiserum was tested by immunoelectrophoresis against acute phase serum, normal serum, and purified CRP. A single fluorescent precipitation arc was seen when acute phase sera or CRP were analyzed.

*Lymphocyte Cultures.* Human lymphocyte suspensions ( $10^6$  in 0.1 ml) in RPMI-1640 containing 10% FCS were cultured in microplates (Microtest II, Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) in the presence of varying dilutions of the mitogens, PHA (PHA-P, Difco Laboratories, Detroit, Mich.) and Con A (Nutritional Biochemical Co., Cleveland, Ohio). 50 μl of the appropriate dilution of PHA or Con A was added to the cultures. CRP diluted in media was also added. Cells were

<sup>2</sup> Osmand, A. P., R. F. Mortensen, J. Siegel, and H. Gewurz. Interactions of C-reactive protein with the complement system. III. Complement-dependent passive hemolysis initiated by CRP. Manuscript in preparation.

cultured for 96 h, pulsed with 0.5  $\mu$ Ci of [ $^3$ H]thymidine (1.9 Ci/mmol; Schwartz Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.) for 18 h, and harvested on a multiple sample harvester as described by Hartzman et al. (38).

Unidirectional MLC reactions were conducted according to the method of Hartzman et al. (38). Responding cells ( $10^5$  in 0.1 ml) were added to  $2 \times 10^5$  mitomycin-C (Calbiochem, San Diego, Calif.) (25  $\mu$ g/ml, 30 min)-inactivated stimulating cells in a total vol of 0.2 ml in microculture plates. Cells were suspended in medium 199 (M-199) with antibiotics (Pen-Strep 100 U/ml, Difco Laboratories) containing 20% heat-inactivated AB human serum (Grand Island Biological Co., Grand Island, N. Y.). CRP was diluted in M-199 containing 20% AB serum before addition to the cultures. The plates were incubated for 5 days, pulsed with 0.5  $\mu$ Ci [ $^3$ H]thymidine/well and harvested 18 h later.

*Detection of Lymphocytes with Fc Receptors.* Fl-aggregated human gamma-globulin (Cohn Fraction II) (Fl-AHGG) was prepared exactly as described by Dickler and Kunkel (39). Lymphocytes ( $10^6$ /ml) were incubated (16 h) with CRP or CRP-CPS complexes, washed with PBS-BSA (pH 7.2), incubated with the Fl-AHGG (1 mg/ml) for 30 min at 23°C and examined for fluorescent patches on the cell surface (40). Percentages were based on examinations of at least 200 lymphocytes.

*Antibody-Dependent Lymphocyte-Mediated Cytotoxicity (ADL).* Assessment of ADL was determined by using the assay system described by Zigelboim et al. (41). Antiserum to the EL-4 lymphoma of the C57BL/6 mouse strain was prepared in rabbits by injecting  $5 \times 10^8$  cells at multiple sites subcutaneously, boosting with three weekly intravenous injections of  $10^8$  cells, and bleeding the animals 10–14 days after the last injection. This antiserum had a cytolytic titer (50% lysis) of 1:2560 when tested against Cr $^{51}$ -labeled EL-4 cells ( $10^6$ ) in the presence of absorbed rabbit complement (1:10). EL-4 ( $10^7$ ) grown in ascites form were labelled with 100  $\mu$ Ci Cr $^{51}$  (sodium chromitope, E. R. Squibb, Chicago, Ill.) in RPMI-1640 at 37°C for 60 min. A dilution of the antiserum in 0.1 ml was added to  $5 \times 10^4$  EL-4 cells and  $2 \times 10^6$  lymphocytes (PBL) in a total reaction volume of 0.5 ml in a (Linbro Chemical, New Haven, Conn.) tissue culture tray. The tray was incubated on a rocking platform for 3 h at 37°C in 5% CO $_2$ , the cells were centrifuged, and 0.1 ml was counted for Cr $^{51}$  release. Percent specific lysis was calculated from the following equation: (cpm-background cpm/maximum releasable cpm-background cpm)  $\times$  100. The maximum releasable cpm were obtained by adding 0.2 ml rabbit complement (absorbed with EL-4) to a mixture of 0.1 ml of the anti-EL-4 antiserum and  $5 \times 10^4$  EL-4 cells in 0.5 ml. This release was usually 80–90% of the total number of counts incorporated.

*Mice, Anti- $\theta$  Antiserum.* Female DBA/2, C57BL/6 and BALB/c mice were purchased from Jackson Laboratories, Bar Harbor, Maine). Athymic nude (nu/nu) mice and heterozygous (nu/+) littermates were a gift from Dr. Jerry Winkelstein, Department of Pediatrics, Johns Hopkins Medical School, Baltimore, Md. Anti- $\theta$  antisera (C3H specificity) (Litton Bionetics, Kensington, Md.) was used to both determine the fraction of  $\theta$ -bearing cells and to lyse spleen T cells. The number of  $\theta$ -bearing spleen cells was determined by Cr $^{51}$  release from lymphocytes incubated with anti- $\theta$  antisera (1:10) for 30 min in PBS containing 1% BSA, then centrifuged and resuspended in a 1:5 dilution of absorbed rabbit complement (42). The same procedure with unlabeled cells in RPMI-1640 containing 10% FCS was used to remove  $\theta$ -bearing cells.

*Lymphoid Cell Lines.* A human B-cell line (HRIK) and a marmoset T-cell line (1022) were kindly provided by Dr. Larry Falk of the Department of Microbiology of this institution. The T- and B-cell characteristics of these cell lines have been previously documented (43).

## Results

*Binding of CRP to Human Peripheral Blood Lymphocytes.* We first sought to determine whether CRP could bind directly to human lymphocytes. Washed PBL were incubated with CRP and reacted with fluoresceinated anti-CRP. This consistently resulted in a patchy fluorescence on the surface of many small and medium size lymphocytes (Fig. 1). There was no apparent "capping" or redistribution of the bound CRP, even in the absence of azide. Indirect immunofluorescent staining with Fl-goat antirabbit Ig did not noticeably change the staining pattern nor did it induce redistribution.

The fraction of normal human PBL that could bind CRP was proportional to

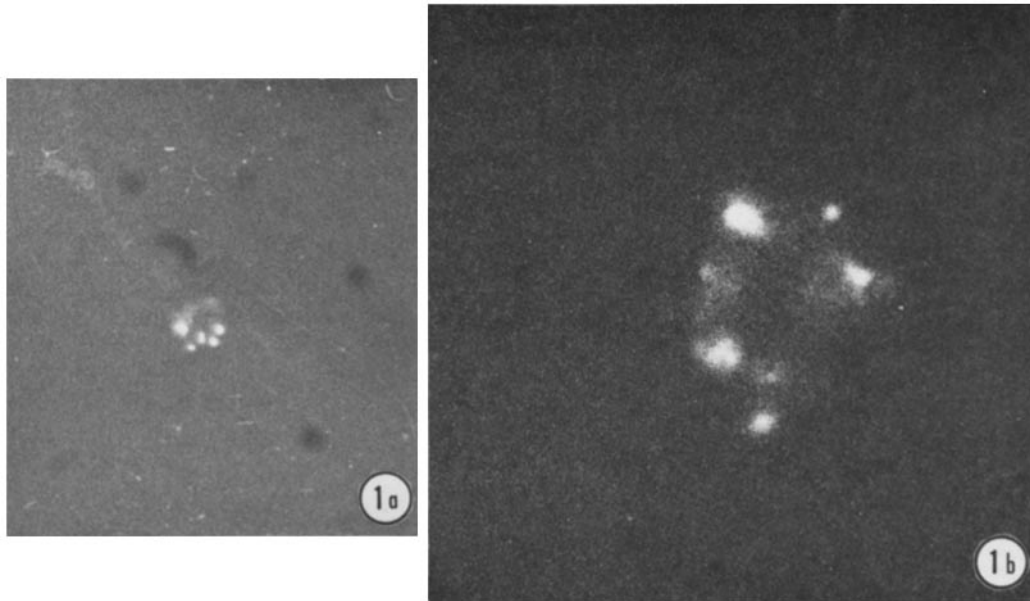


FIG. 1. Photomicrographs of lymphocytes showing typical patchy staining pattern on the cell surface after incubation with CRP ( $50 \mu\text{g}/\text{ml}$ ) and reacting with fluoresceinated anti-CRP. Approximate magnification: (a)  $\times 450$ . (b)  $\times 900$ .

the amount of purified CRP added to the lymphocyte cultures up to a concentration of  $10 \mu\text{g}/\text{ml}$ ; a maximum of 35–40% of the cells were stained (Fig. 2). The binding of CRP was specifically inhibited if the CRP substrate, CPS, was added at the time the cultures were initiated. Normal PBL showed background staining of less than 2–3% when FI-anti-CRP was added in the absence of preincubation with CRP. Although the cultures of lymphocytes and CRP were usually incubated for 16 h, an equal fraction of cells were stained after 3 h of incubation and a smaller fraction if incubated 2 h or less. Since PBL usually contains less than 30% cells staining for membrane Ig (B cells) it was apparent that CRP was binding to at least some T cells.

*Specific Binding of CRP to T lymphocytes.* The question arose as to whether there is a specificity of CRP for either the T- or B-lymphocyte populations. Recently devised marker systems and separation methods for human T and B cells provide a direct approach to establishing selective binding.

Two different methods were used to separate human PBL into T- and B-cell enriched fractions before testing each population for the presence of CRP-binding cells. In the first, B cells were enriched by removing T cells as E rosettes on Ficoll-Hypaque gradients and using the cells collected at the interface. The data from several such separations indicated that a single cycle of purification increased by threefold the number of Ig-bearing and complement receptor-bearing lymphocytes (CRL). This procedure correspondingly lowered the fraction of cells able to bind CRP (Table I). In the second approach B cells were removed on nylon wool columns, thus enriching the effluent lymphocytes in T cells as shown

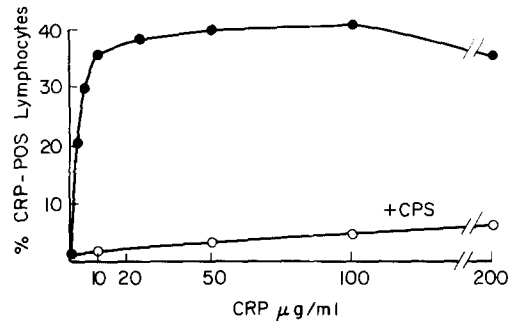


FIG. 2. The binding of purified C-reactive protein (CRP) to peripheral blood lymphocytes (PBL) in vitro. PBL ( $10^6$ /ml) were incubated for 16 h with various concentrations of CRP (●—●), washed and stained with FITC-conjugated rabbit-anti-CRP; up to 40% of the cells showed patchy fluorescence (CRP-POS). CPS ( $10 \mu\text{g/ml}$ ) was added in duplicate cultures with CRP (○—○) and prevented CRP binding.

TABLE I

*Binding of Purified CRP by Peripheral Blood Lymphocytes and B-Cell and T-Cell Enriched Populations*

Lymphocyte preparation*	E-R $\pm$ SE	POS surface Ig $\ddagger$	EAC-R $\pm$ SE	CRP-POS after incubation with CRP $\S$
	%	%	%	%
Peripheral blood lymphocytes	$58.5 \pm 4.4$	$27.0 \pm 2.8$	$21 \pm 6.1$	$34.4 \pm 4.0$
B-enriched, single cycle (E-rosette)	—	$80.6 \pm 7.6$	$55.3 \pm 6.8$	$10.8 \pm 2.3$
T-enriched (nylon wool column)	$69.0 \pm 6.6$	$3.8 \pm 1.8$	1-2	$49.8 \pm 6.7$

\* Determinations performed simultaneously with unfractionated and fractionated lymphocytes from each of eight healthy donors.

$\ddagger$  Cells stained with FITC-conjugated polyvalent anti-GAM antisera. Mean  $\pm$  SE.

$\S$  Cells incubated with CRP for 16 h after the separation procedures and stained with FITC-conjugated anti-CRP sera. Mean  $\pm$  SE.

by the increase in rosette-forming cells and the decrease of Ig-bearing cells (Table I). Upon incubation with CRP a significantly larger fraction of cells were stained for CRP than in the original population. Taken together these results indicate that CRP binds preferentially to the T-cell population.

Additional experiments testing the ability of CRP to bind to a human B-cell line and a marmoset T-cell line were conducted. A human B-cell line, HRIK, transformed by Epstein-Barr virus displayed minimal (10–15% stained cells) binding of CRP. However, a transformed marmoset T-cell line that formed rosettes (90–100%) with SRBC (43) displayed a high degree of CRP-binding (60–80% of the cells). These results lend further support to the T-cell specificity of CRP-binding.

Experiments were undertaken to examine the binding of human CRP to mouse spleen lymphocytes. The fractions of  $\theta$ -bearing cells in spleens of DBA/2, C57BL/6, and BALB/c mice as determined by  $\text{Cr}^{51}$  release with anti- $\theta$  antiserum

and complement were similar to those reported by others (44). The removal of mouse spleen T cells by anti- $\theta$  sera plus complement led to a decreased percentage of cells able to bind CRP upon subsequent incubation (Table II). The residual number of CRP-binding cells following treatment with anti- $\theta$  plus complement may reflect unlysed T cells. In addition, spleen lymphocytes from congenitally athymic (nu/nu) nude mice contained only a small number of CRP-binding cells, whereas spleens from heterozygous littermates (nu/+) had a fraction of CRP-binding cells similar to that of the other three strains tested. These manipulations indicate that human CRP can also bind to the T cells of a heterologous species.

TABLE II  
*Binding of Purified Human CRP to Mouse  $\theta$ -Bearing Spleen Lymphocytes*

Strain	$\theta$ -positive*	CRP-POS after treatment with:	
		Media	Anti- $\theta$ + complement‡
	%	%	%
DBA/2	42	30	7
C57BL/6	46	33	11
BALB/c	51	41	14
nu/nu§	2	11	ND
nu/+	43	35	ND

\* Percent  $\theta$ -positive determined by Cr<sup>51</sup> release after treatment with anti- $\theta$  (1:10) and rabbit complement.

‡  $\theta$ -bearing spleen cells ( $10^6$ ) were lysed with anti- $\theta$  and rabbit complement in RPMI-1640 containing 10% FCS. The remaining cells were incubated with CRP and stained for CRP as in Fig. 1.

§ Athymic nude (nu/nu) mice on a Swiss-NIH background as well as heterozygous (nu/+) littermates were tested.

*Effect of CRP on the Formation of Human T- and B-Cell Rosettes.* During attempts to show the presence of CRP on rosetted T cells (E rosettes; E-R), we found that preincubating human PBL with CRP inhibited rosette formation (Fig. 3). To study this reaction, separated lymphocytes were incubated with various concentrations of CRP, washed free of unbound CRP, and tested for ability to form rosettes with E. Inhibition of rosette formation proportional to the concentration of CRP added was seen; it was fully neutralized when small quantities of CPS were included in the incubation mixture with CRP. Efforts to stain residual E rosettes for CRP on their surface were unsuccessful; indeed, most of the staining cells had few or no attached E.

A marker for B cells expressing a receptor for C3b or C3d is the formation of stable rosettes with EAC (34, 35). Incubation of PBL with CRP did not alter the ability of CRL to form rosettes with EAC (Fig. 3). Thus, CRP inhibited rosette formation by T cells but not by CRL (B cells). Since it has been found that not all B lymphocytes express the C3 receptor (35), experiments to test the effect of

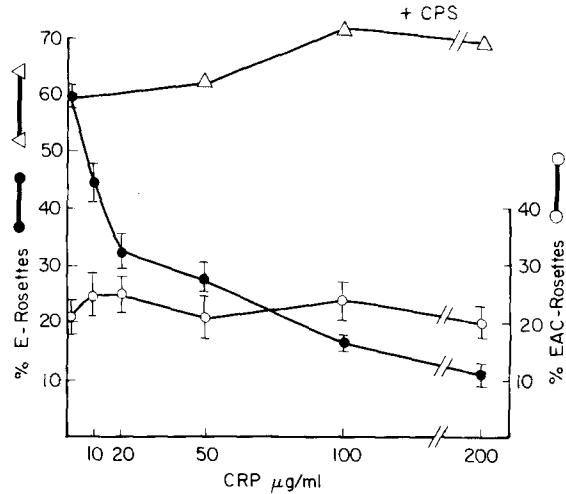


FIG. 3. The effect of CRP on the formation of T-cell rosettes (E-R) (●—●). Peripheral blood lymphocytes were incubated with increasing concentrations of CRP, washed, and mixed with sheep erythrocytes; the percent rosette-forming cells was determined. CPS (20  $\mu\text{g/ml}$ ) added at the same time as CRP neutralized the inhibition of E-R ( $\Delta$ — $\Delta$ ). The effect of CRP on the formation of rosettes with EAC by PBL (○—○) using the same culture conditions was also determined. The results are expressed as the mean  $\pm$  SE.

CRP on binding to other B-cell receptors were performed and are described in subsequent sections.

*Effect of CRP on the Response of Human PBL to Mitogens.* It seemed possible that binding of CRP to T lymphocytes might alter their response to the T-cell-specific plant lectins, PHA and Con A. The results of incorporating CRP into lymphocyte cultures along with PHA or Con A at the time of their initiation are shown in Figs. 4 and 5, respectively. A consistent but statistically insignificant (Student's *t* test) enhancement of the response was observed over a wide range of mitogen concentrations when CRP was added at doubling concentrations between 1.25 and 20.0  $\mu\text{g/ml}$ . Since enhancement was observed at each CRP concentration only the data for 10  $\mu\text{g/ml}$  CRP is shown in the figures. The lack of statistical significance may be a reflection of the variation in responsiveness of lymphocytes from different donors. CRP was not significantly mitogenic by itself, nor was it toxic for peripheral lymphocytes up to 100  $\mu\text{g/ml}$  since "background" [ $^3\text{H}$ ]thymidine incorporation was equal to that of the controls (Table III). When CRP toxicity was assayed directly by determining the percent viable cells in culture by trypan blue dye exclusion, no significant differences from the control cultures were observed. Comparisons by direct cell counting gave similar results. These results strongly indicate that the action exerted by CRP on T cells does not involve toxicity.

*Effect of CRP and CRP-CPS Complexes on the Binding of Aggregated Ig.* Since CRP can consume C after binding to its substrates (17, 18, 25), it may possess a region functionally analogous to the Fc portion of Ig. A receptor for the Fc portion of aggregated IgG or antibody complexed to antigen has been shown to exist on most or all human B cells (40). Because of the many shared



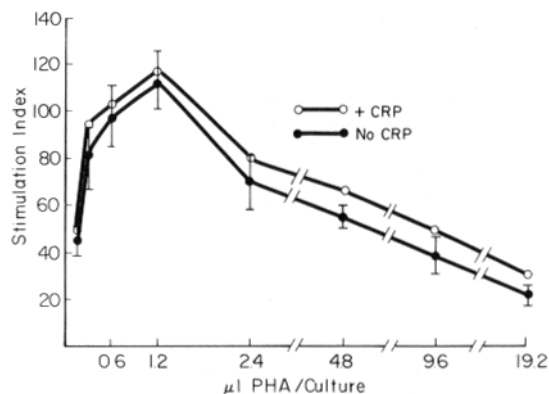


FIG. 4. The effect of CRP on the response of PBL to PHA. The abscissa represents the equivalent volume of undiluted stock PHA per culture. CRP ( $10 \mu\text{g}/\text{ml}$ ) was added 1 h before the addition of the appropriate PHA dilution. The stimulation index is expressed as the mean ( $\pm\text{SD}$ ) of cultures of lymphocytes from 7-10 healthy donors tested against the PHA concentrations shown.

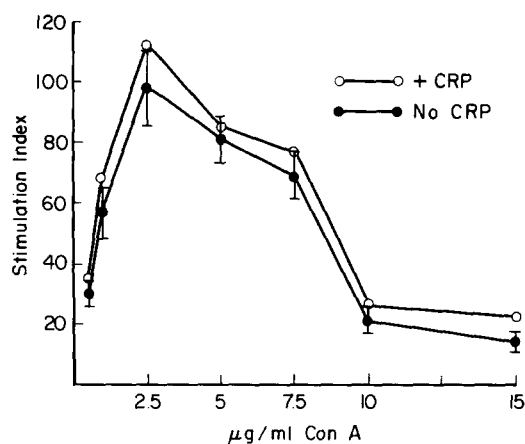


FIG. 5. The effect of CRP on the response of PBL to Con A. CRP was added at  $10 \mu\text{g}/\text{ml}$  1 h before the addition of the appropriate Con A dilution. The stimulation index is the mean ( $\pm\text{SD}$ ) of cultures of lymphocytes from 5-8 healthy donors.

characteristics of Ig and CRP, we tested the ability of CRP or CRP-CPS to inhibit the binding of F1-aggregated Ig. The results in Table IV indicated that neither CRP nor CRP-CPS interfered with the binding of aggregated Ig to B cells. The fraction of cells binding aggregated Ig correlated closely with the number of cells with membrane Ig. Thus, it appears that CRP does not bind to the Fc receptor of B lymphocytes.

*Effect of CRP and CRP-CPS Complexes on Antibody-Dependent Lymphocyte-Mediated Cytotoxicity.* An important function of lymphocytes with Fc receptors is antibody-dependent lymphocyte-mediated cytotoxicity (ADL) (42). This cytotoxicity can occur with as few as 10-100 IgG antibody molecules per target cell (41, 45) and therefore should be readily inhibited by substances binding to

TABLE III  
*The Effect of C-Reactive Protein on [<sup>3</sup>H]Thymidine Incorporation  
 and Viability of Lymphocytes*

CRP	No. of donors§	Stimulation index ( $\pm$ SD) +CRP/no CRP‡	Viability§	
			24 h	48 h
<i><math>\mu</math>g/ml</i>			<i>%</i>	<i>%</i>
—	19	1.00	95	90
2.5	17	1.17 $\pm$ 0.69	—	—
5.0	20	1.14 $\pm$ 0.64	96	91
10	19	1.26 $\pm$ 0.79	95	89
20	12	1.29 $\pm$ 0.77	95	92
30	12	1.40 $\pm$ 0.70	—	—
50	6	0.89 $\pm$ 0.35	93	90
100	6	1.07 $\pm$ 0.56	94	91

\* The range of CRP concentrations (2.5–100  $\mu$ g/ml) were tested with cells from each donor.

‡ Cultures were labeled on day 3 and harvested 18 h later.

§ Percent viability by trypan blue dye exclusion.

the Fc receptor. We therefore tested the inhibitory activity of CRP or CRP-CPS complexes on an ADL system consisting of rabbit antibody-coated EL-4 lymphoma target cells and PBL at a lymphocyte/target cell ratio of 40:1 (see Materials and Methods). As can be seen in Fig. 6, neither CRP nor CRP-CPS complexes inhibited cytolysis at even very high dilutions of anti-EL-4, where competition for Fc receptors would be most apparent. Since EL-4 is  $\theta$ -bearing (46) and binds CRP as shown by fluorescent staining, attempts were made to lyse CRP-coated EL-4 cells with the addition of lymphocytes. CRP alone was not able to mediate this cytolytic reaction even at very high lymphocyte to target cell ratios (100:1), whereas maximum cytolytic activity with a 1:10 dilution of anti-EL-4 occurred at a lymphocyte/target cell ratio of 40:1 (data not shown). These results imply that CRP and CRP-CPS complexes do not mediate lymphocytotoxicity or inhibit ADL via binding to the Fc receptor of B lymphocytes.

*Effect of CRP on the Mixed Lymphocyte Reaction.* Since CRP could bind to T cells without eliciting a blastogenic response or significantly influencing the response to T-cell mitogens, it seemed important to test the effect of CRP upon the one-way MLC reaction, a T-cell function involving recognition of allogeneic cells and readily measureable with human PBL. We found that CRP, added directly to the MLC reaction mixtures, markedly inhibited the proliferative response (Fig. 7). This inhibition was proportional to the CRP concentration over the dose range of 5–100  $\mu$ g/ml; at 100  $\mu$ g/ml the MLC response was only 10–20% of that of controls. These experiments have been repeated with eight different allogeneic combinations using each of the CRP concentrations, and similar results have been obtained. Parallel experiments measuring trypan blue exclusion by the responding cells in medium 199 containing 20% AB serum and CRP

TABLE IV  
*Failure of CRP Alone or CRP-CPS Complexes to Prevent Binding  
of Aggregated IgG to Human PBL*

Exp.	CRP	CPS	% Positive	
			Surface Ig $\ddagger$	Aggregated Ig $\ddagger$
	$\mu\text{g/ml}$	$\mu\text{g/ml}$		
A	—		26	23
	50			20
	100			20
	200			21
	100	5		24
B	—		20	18
	50			17
	100			16
	200			19
	100	5		15

\* Percentage of cells staining with polyvalent Fl-anti IgG, IgA, and IgM (anti-GAM).

$\ddagger$  Cells tested for binding of FITC conjugated aggregated Ig after incubation (16 h) with CRP or CRP-CPS mixtures. At least 200 cells were examined at each CRP concentration.

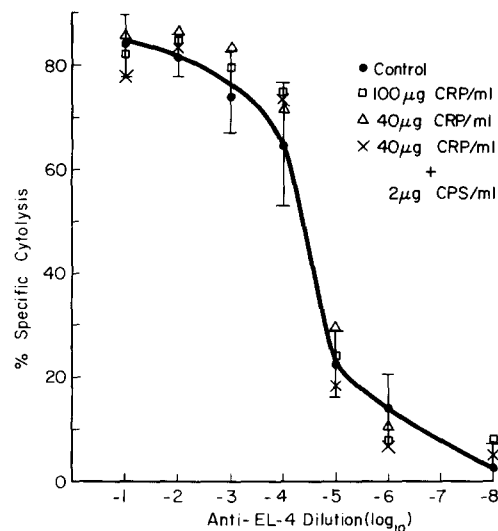


FIG. 6. The effect of CRP and CRP-CPS mixtures on antibody-dependent lymphocyte-mediated cytotoxicity. CRP at 100  $\mu\text{g/ml}$  ( $\square$ ) or 40  $\mu\text{g/ml}$  ( $\Delta$ ), or CRP (40  $\mu\text{g/ml}$ )-CPS (2  $\mu\text{g/ml}$ ) complexes (X) were added to assay mixtures of EL-4 target cells coated with increasing dilutions of rabbit anti-EL-4 (IgG) and PBL at a lymphocyte/target cell ratio of 40:1. The percent specific cytolysis ( $\pm$ SD) was calculated as described in Materials and Methods.

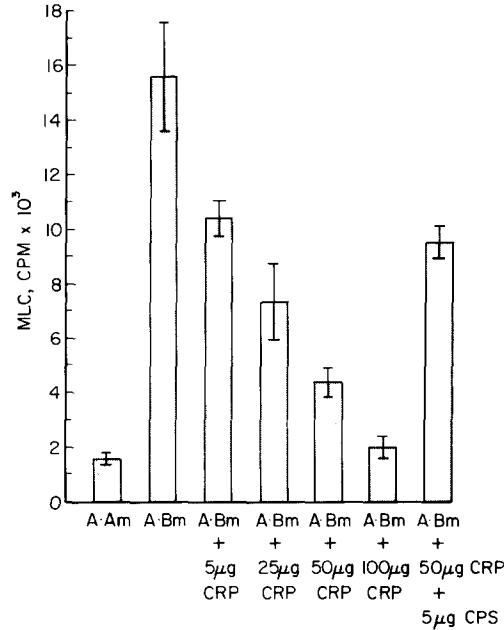


FIG. 7. The effect of CRP on the response of human lymphocytes to allogeneic lymphocytes in a one-way mixed leukocyte culture reaction. Responding lymphocytes ( $1 \times 10^6$ ) were stimulated with  $2 \times 10^5$  mitomycin-treated allogeneic lymphocytes (designated "m") in the presence of various concentrations of CRP, or CRP and CPS. The data shown is from a single allogeneic combination and expressed as mean cpm ( $\pm$ SD).

at the various concentrations revealed no significant alteration of cell viability. Furthermore, cells failing to respond in the MLC could respond to PHA after they were removed from the CRP-containing medium. The addition of CPS at the time the cultures were initiated could prevent at least partially the inhibitory effect exerted by CRP (Fig. 7). The ratio of CPS to CRP was chosen on the basis of optimal proportions for both precipitation and complement consumption, which were found to be approximately 1:20 (15, 25). CPS alone did not significantly alter the MLC response.

Since CRP could conceivably inhibit the MLC response by binding to either the stimulating or responding cells or both, we briefly (3 h) exposed the stimulating and responding cells, respectively, to CRP before adding each to the MLC. As shown in Table V, preincubation of the responding lymphocytes with CRP in medium 199 containing 20% AB serum, followed by the removal of unbound CRP, resulted in a lowered MLC response. However, considerably higher concentrations of CRP were required in the preincubation mixture to obtain a degree of suppression equivalent to that observed when CRP was added directly to the cultures (Fig. 7). Similar experiments in which the mitomycin C inactivated stimulating cells were preincubated with CRP did not lower the MLC response (Table V). Thus, these observations clearly indicate that CRP is capable of modifying the recognition and/or proliferative phases of the response to allogeneic cells.

TABLE V  
*The Effect of Preincubating the Responding or Stimulating Cells with CRP on MLC Responsiveness*

Cells preincubated*	CRP	Cell combination	[ <sup>3</sup> H]-thymidine	% of control
	<i>μg/ml</i>		<i>cpm</i>	
Responding cells (A)	—	AA <sub>m</sub>	555	
	—	AB <sub>m</sub>	4,190	
	50	AB <sub>m</sub>	3,458	83
	100	AB <sub>m</sub>	2,928	69
	200	AB <sub>m</sub>	2,370	57
Stimulating cells (B <sub>m</sub> )‡	—	AA <sub>m</sub>	584	
	—	AB <sub>m</sub>	4,376	
	50	AB <sub>m</sub>	4,332	99
	100	AB <sub>m</sub>	5,937	136
	200	AB <sub>m</sub>	4,463	102

\* Responding or stimulating cells were incubated with the indicated concentration of CRP for 3 h at 37°C, washed twice, and added to the cultures.

‡ Cells were incubated with CRP after mitomycin C inactivation.

### Discussion

CRP is a homogeneous protein, which is structurally and antigenically distinct from the immunoglobulins, but shares several of their functional characteristics (2, 14-25). Its similarity to antibody, as well as the association of CRP with host functions involved in "nonspecific" resistance to infection (26, 27) and its potential influence on the induction of antibody formation (28), have led us to initiate studies to determine the relationship between CRP and the lymphoid system.

Several lines of evidence indicated that CRP binds selectively to T lymphocytes. Incubation of normal human peripheral blood lymphocytes with human CRP, followed by addition of FITC-conjugated anti-CRP, resulted in patchy staining of the surface of 30-40% of the cells. When B cells were removed on nylon wool columns, the remaining lymphocytes had an increased fraction of cells capable of binding CRP; conversely, when T cells were removed as E rosettes, a decreased percentage of CRP-binding cells remained. Similar results were obtained when murine lymphocytes were exposed to CRP. Approximately 40% of the splenic lymphoid cells from several mouse strains were able to bind human CRP; this fraction was reduced by more than 80% after treatment with anti- $\theta$  serum and complement. Furthermore, only 11% of the spleen cells of athymic nude mice were capable of binding CRP. Finally, a marmoset T-cell culture line displayed a high degree of CRP binding while only a small fraction of cells of a human B-cell line could bind CRP. Thus, human CRP binds preferentially to T lymphocytes. Since CRP reacts with T cells but not B cells, it may prove to be a marker for a subpopulation of T cells, but further experiments are needed to establish this point.

CRP also was found to selectively inhibit certain T-cell mediated functions.

Preincubation of human peripheral blood lymphocytes with small noncytotoxic amounts of CRP resulted in their inability to show characteristic binding to sheep erythrocytes; yet similar amounts of CRP had no effect upon B-cell binding of complement-coated erythrocytes (EAC) or aggregated IgG. CRP also was effective in inhibiting blastogenesis in unidirectional MLC reactions. The inhibition of both T-cell rosette formation and MLC reactivity was neutralized by adding the CRP substrate, CPS. Preincubation of CRP with stimulating cells had no effect on subsequent MLC reactivity, while preincubation with responding cells inhibited the proliferative response to allogeneic cells. This specificity for the responding cells was not altogether unexpected since recent reports have shown that stimulating cells in human MLC reactions consist largely of B cells whereas the responding cells are largely or entirely T cells (47, 48). Since proliferation in the MLC reaction is a prerequisite for the generation of cytotoxic cells (49) it seemed likely that the generation of effector T cells would be inhibited by CRP as well; preliminary studies suggest that this is the case.<sup>3</sup>

CRP shares with certain antibodies the ability to inhibit the MLC reaction, but CRP does so exclusively at the level of the responding cells. Anti-HL-A sera have been shown to inhibit at the level of both stimulating and responding cells (50-52) and may be directed not only to HL-A but also to receptors for the products of the MLC loci (reviewed in 53). The CRP-induced inhibition of the MLC response was similar to that seen by Wernet and Kunkel using certain sera from patients with systemic lupus erythematosus (54). These sera were found to inhibit the responding but not the stimulating cells, possibly by combining with the T-cell receptor for lymphocyte defined (LD) antigens. Whether CRP interferes by binding to recognition units for LD antigens is not yet clear. Since CRP binding resulted in a patchy surface staining without redistribution, even when unconjugated anti-CRP followed by fluoresceinated goat antirabbit gamma globulin was used, the CRP-binding sites seem to be distantly spaced and/or fixed, perhaps in the choline phosphatide-containing lipid component of the membrane (13, 15, 25). However, we do not yet know the chemical nature of the CRP receptor, its distribution on T-cell subpopulations, or its relative availability during the life cycle of either intact or damaged T lymphocytes. The binding of CRP could inhibit T-cell functions in several ways, including steric hindrance of interactions with other cell surface components, elevation of cyclic AMP levels which already have been correlated with inhibition of T-cell rosette formation (55) or activation of "suppressor T cells" which regulate the response to allogeneic cells (56, 57). We do not yet have experimental support to favor any of these several hypotheses.

CRP did not inhibit all T-cell-mediated functions. It had no inhibitory effect on stimulation of lymphocytes by PHA or Con-A over a wide range of CRP and mitogen concentrations; indeed, a minimal but distinct enhancing effect on response to these T-cell specific mitogens was observed. It did not have toxic or mitogenic activities of its own. CRP had been reported by one group (29) to be mitogenic to lymphocytes at low doses (<10  $\mu\text{g/ml}$ ) and cytotoxic at higher doses, and by another group (31) to have an inhibitory effect upon PHA-induced mitogenicity at higher concentrations. The disparity between our results and those previously reported is not clear; perhaps it pertains to the nature of the C-reactive protein preparation, the presence of granulocytes in lymphocyte preparations, culture conditions or other factors. The slight enhancing effect of CRP on PHA- or Con A-induced mitogenesis, concomitant with inhibition of formation of T-cell rosettes and proliferation in the MLC reaction, suggests that CRP either has no effect

<sup>3</sup> Mortensen, R. F., D. Claus, A. P. Osmand and H. Gewurz Manuscript in preparation.

upon subpopulations of T cells involved in the response to these mitogens, or that it acts in events of the recognition phase which are not required for PHA or Con A responsiveness. The effects on other T-cell functions such as lymphokine release and soluble antigen-induced blastogenesis have not yet been determined.

In contrast to the reactivity between CRP and T cells, multiple lines of evidence indicated that CRP has minimal, if any, effect on B lymphocytes. No binding of CRP was detected on separated B lymphocytes incubated with concentrations of CRP up to 200  $\mu\text{g/ml}$ . Preincubation with these concentrations of CRP failed to influence the binding to B lymphocytes of either FITC-conjugated AHGG or sensitized sheep cells bearing complement components. CRP failed to initiate or inhibit antibody-dependent lymphocytotoxicity reactions, a function dependent upon cells with Fc receptors (45). Finally, CRP failed to inhibit the stimulating cells in the human MLC reaction which are considered to be primarily B cells (47, 48). We therefore conclude that CRP has minimal, if any, direct effect upon B lymphocytes, and that if CRP influences these cells, it is apt to do so indirectly. We had thought that since CRP shares with immunoglobulins the ability to activate complement at the level of C1 (17, 25), it might share a structural homology with the Fc fragment of IgG, which could result in interference with lymphoid cell functions dependent on the presence of Fc receptors. This obviously was not the case. Since the complement reacting region on IgG with C1q involves the CH<sub>2</sub> domain (58, 59), whereas the region on IgG required for binding to the Fc receptor on B lymphocytes is in the CH<sub>3</sub> region (45), a structural similarity between the CH<sub>2</sub> region of IgG and CRP still seems possible. Further interpretation awaits determination of the amino acid sequence and structure of C-reactive protein.

The relationship between the acute phase reactants including CRP and the control of immunologic mechanisms is not yet fully known. We do not know the effects of elevated levels of CRP *in vivo* on T-cell distribution and function. The spectrum of cells of both lymphoid and nonlymphoid varieties with which CRP binds, as well as the conditions and consequences of these interactions, seem likely to be of great importance in the inflammatory process. Perhaps an examination of the effect of acute phase serum reactants such as CRP on immune mechanisms will help explain certain phenomena which accompany the induction of the inflammatory response, including elevated levels of natural antibodies, enhanced resistance to infection, enhanced antibody responses, and in some instances decreased cell-mediated immune responses.

### Summary

C-reactive protein (CRP) is an acute phase protein which shares with the immunoglobulins the ability to induce precipitation and agglutination reactions and activate the complement system. We report here that purified human CRP binds selectively to human T lymphocytes, inhibits their ability to form spontaneous rosettes with sheep erythrocytes and inhibits their response to allogeneic cells in mixed lymphocyte culture reactions; it fails to inhibit phytohemagglutinin- or concanavalin-A-induced mitogenesis. CRP does not bind to human B lymphocytes, nor does it alter the following B-cell functions: binding to activated complement components or the Fc portion of immunoglobulins, mediation of antibody-dependent cytotoxicity reactions or the ability of allogeneic cells to stimulate a mixed lymphocyte culture reaction. Human CRP shows similar selective binding with murine T lymphocytes. It therefore seems

that binding of CRP is a property of T lymphocytes or a subpopulation thereof, and can result in modulation of certain of the T-cell functional characteristics in vitro. We suggest that CRP may play a role in modulating T-cell functions during the inflammatory state.

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