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Research Article

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Lymphocytes Binding C-Reactive Protein during Acute Rheumatic Fever

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ABSTRACT Lymphocytes binding C-reactive protein (CRP) were studied in 31 patients with acute rheumatic fever and 30 controls who were children. Marked elevations in both proportions and absolute numbers of CRP-binding lymphocytes were recorded in rheumatic fever ($P < 0.001$). No clear correlation was noted between plasma CRP as quantitated by radioimmunoassay and proportions or numbers of CRP-binding cells. Double-labeling experiments indicated that 60–80% of CRP-binding lymphocytes also showed Fc receptors reacting with fluorescein-conjugated IgG aggregates. Passage of lymphocytes over Ig—anti-IgG columns, removed cells bearing surface Ig but not CRP-binding lymphocytes. Studies of T-cell subpopulations indicated no overlap between $T\mu$- and CRP-binding cells; however about half of $T\gamma$-cells showed concurrent CRP binding. "Active" T-cell rosetting cells did not bind CRP. A 12–15-h incubation of lymphocytes at 37°C in 5% CO_2-air showed persistence of CRP binding in substantial proportions of cells particularly in acute rheumatic fever. CRP-binding lymphocytes may represent a marker for immunologically committed cells in acute rheumatic fever.

INTRODUCTION

C-reactive protein (CRP)[1] constitutes a material which exists in low concentrations in normal serum but which is often elevated during the course of acute inflammatory reactions such as infection, trauma, or acute rheumatic fever (1–6). Recent comparative analysis of amino acid sequences as well as electron microscopic structure suggest that CRP may share evolutionary sources with C1[t] related to the C1q component of complement (7). Previous reports by Mortensen et al. (8–10) have indicated that CRP may bind to lymphocytes and in particular, may actually adhere to T cells activated by antigens but not by mitogens such as phytohemagglutinin. Our previous studies have indicated a marked increase in proportions of "active" rosette-forming T cells among children with acute rheumatic fever (11). It was not clear whether active (or rapid) rosette-forming cells constituted a population of immunologically activated T cells that somehow increased during the acute rheumatic process. Recent reports have related increments in proportions of active T-cell rosettes related to delayed type hypersensitivity reactions after in vivo skin testing (12). Previous studies had appeared to indicate that proportions or numbers of active rosettes might correlate with immune competence (13, 14). The purpose of the present study was to examine lymphocyte subpopulations in acute rheumatic fever patients with particular attention to what distinct populations of lymphocytes showed CRP binding. It was felt that such cells might represent immunologically committed or possibly antigen-activated cells called forth in the host response to the disease process.

METHODS

Patients studied. 31 patients with acute rheumatic fever were studied during the first 3 wk of April 1977. All of the patients were hospitalized at the Free Rheumatic Heart Center in Cairo, Egypt (11, 15), and were between the ages of 3 and 14 with active acute rheumatic fever. The diagnosis was established using the modified Jones Criteria as suggested by the American Heart Association (16). The majority of patients studied were subjects in the relatively acute stages of rheumatic pancarditis, 1–8 wk after the onset of symptoms. Of these patients, 11 were experiencing their first attack, whereas the other 20 children were studied in an exacerbation having had two to seven previous episodes of carditis or active chorea. 10 patients with acute chorea and associated carditis, or evidence on physical examination of active mitral valve involvement were included in the study; the remainder were studied during the progress of active carditis.

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[1] *Abbreviations used in this paper*: CRP, C-reactive protein; EA, erythrocyte antibody.

9 of the 21 patients with active carditis had received corticosteroids as therapy for their flagrant carditis. Concurrent dosage of prednisone ranged from 5 to 30 mg/day. Nine patients with active carditis were also studied who were receiving salicylates alone, and three were studied before the administration of any therapy. The 10 patients with chorea were receiving salicylates as well as reserpine preparations and phenobarbital for the control of their movement disorder.

The average age of patients with carditis was 9.1 years (13 females and 8 males). The chorea patients averaged 10.3 years of age (8 females and 2 males).

Six normal control subjects were studied from control Egyptian children hospitalized at Cairo University Hospital on the general pediatric service for correction of congenital deformities or treatment of noninflammatory conditions. In addition, a group of 24 normal children, ages 5–15 and matched for age and sex, were studied in Egypt and Albuquerque.

Special studies. CRP was determined in serum samples using radial diffusion and the technique of Kindmark (17), and all results expressed as nanograms per deciliter of serum. Serum samples which could not be used for CRP determination because of the limits of sensitivity of the radial diffusion method were assayed using the radioimmunoassay modified slightly from the original technique described by Claus et al. (6). In this assay, highly purified CRP was labeled with ^{131}I using the Bolton-Hunter reagent. Labeled CRP was repurified after labeling by elution from C-polysaccharide columns to insure specificity and biologic reactivity. The range of values for normal serum (50 samples) was similar to that previously recorded (60–8,000 ng/dl) (6). Serum samples assayed in parallel by the modified radial diffusion assay and radioimmunoassay, generally showed good agreement with not more than a 15% difference when the two methods were run in parallel on the same samples.

Lymphocytes were isolated from 12-ml samples of heparinized blood using Ficoll-Hypaque and gradient separation (18). After harvesting the lymphocytes from the gradient, mononuclear cells were depleted of adherent cells and monocytes using adherence to glass petri dishes at 37°C for 30 min (19). This 37°C incubation also served to allow shedding of adherent immunoglobulin, thus depleting cell surfaces of the latter as noted by Horwitz et al. (20). Nonadherent cells were then utilized for assay of CRP binding. In preliminary assays, whole mononuclear cell preparations in which adherent cells had not been depleted and adherent cells, recovered from petri dishes by gentle scraping with rubber policeman were studied, to ascertain whether such cells constituted a major fraction showing CRP binding. These experiments did not indicate any appreciable contribution by monocytes or other adherent cells to CRP binding because <1% of such cell populations showed surface CRP.

CRP binding to lymphocytes utilized two parallel techniques. CRP antisera made in rabbits were obtained from N. L. Cappel Laboratories Inc., Cochranville, Pa., as well as through generous gifts from Dr. Osmand and Dr. Gewurz, Department of Immunology, Rush Medical College, Chicago, Ill., and later from Dr. E. Gotschlich, Rockefeller University, New York. In view of a recent report indicating that anti-CRP reagents might show inadvertent specificity for P-component as described by Pepys et al. (22), anti-CRP antiserum was tested for possible anti-P specificity using P component affixed to Sepharose 4B beads (Pharmacia Fine Chemicals Inc., Uppsala, Sweden) in the presence of calcium ions (23). Sepharose 4B beads coated with P component were strongly positive with specific anti-P-component antiserum but negative with our anti-CRP antisera. These experiments ruled out inadvertent specificity for P component in the antisera used. All anti-CRP antisera used in the cell-surface marker studies showed complete absorption of activity in the systems studied using CRP isolated from capsular (Cs) polysaccharide columns (8) and freed of contaminants by subsequent gel filtration. IgG fractions from rabbit anti-CRP antiserum were digested by pepsin as previously described (21) to obtain $F(ab')_2$ fragments and were then labeled with either fluorescein or rhodamine. Such labeled $F(ab')_2$ preparations showed F/P ratios ranging between 1.5 and 2.0. Completeness of pepsin digestion was assayed using inhibition of precipitation between rabbit Fc and specific goat anti-rabbit Fc antiserum in agar gel and by acrylamide gel electrophoresis. In several instances, pepsin-digested material was also absorbed with whole Cowan I protein A-containing *Staphyloccus aureus* to insure removal of whole IgG or material containing Fc determinants. Mononuclear cell preparations were incubated with fluorescein-labeled $F(ab')_2$ of anti-CRP and, after washing and resuspension, examined under the Zeiss fluorescence microscope using epifluorescence, a mercury HBO 200 W lamp with BG12 primary filter, 53 Barrier filter, and the fluorescein isothiocynate filter (Carl Zeiss, Inc., New York). Double labeling for CRP and other cell surface markers employed fluorescein-labeled aggregates of IgG to detect Fc receptors (11, 24) in conjunction with rhodamine-labeled $F(ab')_2$ fragments of anti-CRP. In addition, erythrocyte antibody (EA) rosettes prepared from bovine cells coated with optimal sensitizing dilution of isolated rabbit anti-bovine IgG were also used for the detection of Fc receptor-bearing cells (25). IgG, coating bovine erythrocytes, was titered to provide a plateau of optimal dilution for producing reproducible Fc receptor rosettes. Preliminary studies were conducted to determine whether cells binding fluorescein-labeled aggregates were identical to those bearing Fc receptors as detected by the IgG sensitized ox-cell technique. Comparative data illustrating these findings are presented in Table I. It can be seen that virtually all the cells showing Fc receptor-binding fluorescent aggregates were eliminated after removal by the rosetting of cells binding to ox cells sensitized with rabbit IgG. Since these experiments utilized lymphocyte populations already depleted of monocytes and previously incubated at 37°C to allow for shedding of cytophilic Ig on L cells, Fc receptor-bearing cells detected by these techniques constituted T cells bearing Fc receptors, B cells, and possibly L cells with Fc receptors. Double-labeling experiments were also conducted on cells showing Fc receptors either by EA or fluorescent aggregate-binding technique. This was performed using rhodamine-conjugated goat anti-human IgG. In all experiments given in Table I, cells showing EA or positive fluorescent aggregate binding showed no more than 1–2% concordance with cells bearing surface Ig as detected by rhodamine labeled goat anti-human IgG. In many instances, simultaneous double labeling of cells utilized incubation of fluorescein or rhodamine $F(ab')_2$ anti-CRP and formation of EA rosettes.

The second parallel technique used for detecting lymphocytes bearing CRP utilized Cowan I strain of *S. aureus* containing surface protein A and suspensions of lightly formalinized bacteria. Such suspensions could be stored at 4°C for 2–3-wk periods with retention of equivalent activity as indicator particles in the assay (26).

Suspensions of bacteria standardized at uniform optical density (560) in the Coleman spectrophotometer (Coleman Systems, Irvine, Calif.) were utilized. 50 μl of anti-CRP was added to 5 × 10^6 lymphocytes in 0.25 ml of minimal essential medium, pH 7.4 plus 10% fetal calf serum. After being washed twice in the medium, 50 μl of standard bacterial suspension was added, the volume was brought to 2.5 ml, and the mixture was then centrifuged at 1,000 *g* to remove nonadherent bacteria. Lymphocyte suspensions were gently distributed on

TABLE I

Relative Percentages of Whole Unfractionated Lymphocytes Bearing Fc Receptors for IgG Using Parallel and Simultaneous Assays

Patient studied	Fc Receptors by fluorescent aggregates	Cells with Fc receptors also positive for surface Ig	Fc Receptors by EA ox cell rosettes	Cells forming EA also positive for surface Ig	Cells showing fluorescence with labeled aggregates after EA ox cell depletion
	%	%	%	%	%
1	8	0	12	1	0
2	9	1	14	1	0
3	10	2	12	2	0
4	8	1	14	1	1
5	11	2	13	1	0

glass slides, fixed with absolute methyl alcohol, and stained with Wright's-Giemsa stain. Lymphocytes binding CRP were then identified by counting 200 cells. Controls included cells coated with normal rabbit serum as well as cells and bacteria alone.

Before use in the respective assays, anti-CRP antiserum as well as normal rabbit sera were absorbed with lymphocytes using 10×10^6 cells per ml of serum. Background controls with normal rabbit serum or bacteria and cells alone never showed >1% apparent binding.

Specificity for both the indirect immunofluorescence as well as *S. aureus* protein A methods was tested by absorption of anti-CRP antisera with highly purified preparations of human CRP prepared from pneumococcal C-polysaccharide immunoabsorbent columns and CRP-rich pleural or ascitic fluids as previously described by Mortensen et al. (8).

Cell subpopulation separation. Cells depleted of monocytes or adherent cells were passed over Ig—anti-IgG Degalan columns to further deplete cells of those bearing surface Ig (19, 21). Cells were studied for CRP binding before and after such column passage.

In addition, the technique of Moretta et al. (27, 28) was utilized in an attempt specifically to prepare T cells bearing Fc receptors for IgM as well as those with receptors for Fc of IgG. This technique utilized enrichment for T-cell rosetting cells with neuraminidase-treated sheep erythrocytes followed by overnight incubation in 5% CO_2 air at 37°C in Hanks' balanced salt solution. Cells undergoing this selective T-cell enrichment and overnight shedding were then examined for T cells bearing IgM ($T\mu$) and IgG receptors ($T\gamma$) as well as for presence of CRP using direct immunofluorescence and $F(ab)_2$ anti-CRP in conjunction with bovine cell rosettes coated with rabbit IgM or IgG. These experiments also served to provide some evidence as to stability of surface binding to CRP.

Attempts were made to determine if active T-cell rosetting cells contained a population capable of binding CRP. In these experiments, active rosettes were prepared as previously described (11), and after rosetting at 1 h, actively rosetting cells were separated on Ficoll-Hypaque and adherent erythrocytes were lysed using 0.83% ammonium chloride for 10 min at 37°C. Cells sedimenting to the bottom of the gradient and, therefore, lymphocytes making active or rapid rosettes, were studied immediately or, in some instances, incubated overnight at 37°C in 5% CO_2 air in 20% fetal calf serum—Eagle's minimum essential medium, and then stained by direct immunofluorescence using $F(ab')_2$ fragments of anti-CRP. In parallel, the lymphocytes not forming active rosettes were processed and subsequently examined for immunofluorescent CRP binding.

A final control involved studies of five patients with chronic myelogenous leukemia and eight patients with acute lymphocytic leukemia. Small proportions (1–5%) of CRP-binding cells in peripheral blood samples from some of these subjects were always associated with T lymphocytes as monitored by concomitant E rosetting. No binding of CRP was detected with immature granulocytes or blast cells from patients with various forms of leukemia.

RESULTS

A marked increment in both proportions and total numbers of CRP-binding cells was noted in peripheral blood lymphocytes obtained from patients with acute rheumatic fever when compared with values obtained using normal child controls. These data are presented in Table II. Values for proportions of cells bearing CRP were slightly higher using direct immunofluores-

TABLE II

Lymphocytes Binding CRP in Normal Controls and Children with Acute Rheumatic Fever

	CRP-binding lymphocytes		Total no. of CRP	
	%		lymphocytes/ mm^3	
Acute rheumatic fever (30)	16.3±8.3	$P < 0.0001$	821.8±509.6	$P < 0.0001$
Normal children controls (30)	3.3±2.9		137.6±61.5	

cence and $F(ab')_2$ fragments of anti-CRP than those obtained in parallel using the Cowan I binding method. However, in general, excellent agreement in values for CRP-binding cells was noted using the two methods in parallel (Table III). Specificity of anti-CRP antiserum used in immunofluorescence and Cowan I S. *aureus* binding was confirmed by absorption using highly purified CRP obtained from C-polysaccharide affinity columns. After such absorption, no positive cells were detected using either method.

Because the high proportions of CRP-binding cells occurred in blood samples obtained from acutely ill subjects many with severe rheumatic pancarditis, it seemed possible that lymphocytes binding CRP might directly reflect the amounts of CRP concurrently present in plasma since CRP is known to be markedly elevated during the acute rheumatic process. No direct relationship was apparent between proportions or total numbers of CRP-binding cells and quantitative estimations of CRP in plasmas collected concurrently in subjects with rheumatic fever or normal children controls. Representative data illustrating this point are given in Table IV. It can be seen that the highest three concentrations of plasma CRP in carditis patients 2 (550,000 ng/dl), 7 (440,000 ng/dl), and 5 (305,000 ng/dl), were associated with 795, 993, and 624 CRP cells/mm^3, respectively, whereas in two chorea patients, 12 and 14, much lower plasma concentrations of CRP (640 ng/dl and 3,700 ng/dl) were associated with 1,247 and 853 CRP cells/mm^3. In addition, a series of overnight incubations were performed and cells were reexamined after a 12–15-h incubation at 37°C in 5% CO_2 air incubator. Of great interest was the apparent persistence and, in some instances, slight increase in proportions of CRP-binding cells after such incubation. Examples of such representative experiments using acute rheumatic fever subjects' cells as well as normal controls are shown in Fig. 1. Cell viability as measured by trypan blue exclusion remained between 95 and 98% after such incubations. It was clear that lymphocytes binding CRP did not undergo extensive elution under the circumstances of incubation, although in some instances a slight decrease or increase in proportions of CRP-binding cells was apparent. In the case of experiments where slight increments in proportions of CRP-binding cells were noted after overnight incubation, immunoassay of the supernates did not provide any clear evidence for synthesis of CRP by cultured lymphoid cells.

If, as originally suggested by Mortensen et al. (8–10),

TABLE III

Representative Determinations Using Immunofluorescence and Cowan I S. Aureus Methods for Determination of CRP-Binding Lymphocytes

Patient studied		CRP-binding cells	
Patient no.	Condition	Immunofluorescence	*S. aureus* binding
		%	
85	Carditis	9	5
70	Carditis	19	12
63	Carditis	35	28
73	Carditis	28	24
64	Carditis	14	10
30	Carditis	8	10
49	Carditis	13	10
329	Carditis	5	6
93	Carditis	10	11
92	Chorea	18	10
83	Chorea	23	24
75	Chorea	32	24
154	Chorea	11	11
69	Chorea	13	10

TABLE IV

Relationship between Numbers and Proportions of CRP-Binding Lymphocytes and Quantitative Estimations of Plasma CRP

Patient no.	CRP-binding cells	Absolute no. of CRP-binding cells	Plasma CRP
	%		ng/dl
Rheumatic carditis			
1	9	314	2,050
2	19	795	550,000
3	32	1,442	3,100
4	35	2,041	125,000
5	28	624	305,000
6	27	2,547	265,000
7	17	993	440,000
8	14	464	3,650
9	8	509	115,000
10	8	494	2,050
Chorea			
11	18	753	240,000
12	28	1,247	640
13	23	1,269	3,850
14	32	853	3,700
15	13	593	2,050
Normal controls			
1	2	156	56,000
2	2	147	4,000
3	3	140	1,170
4	4	206	640
5	2	74	1,050
6	1	48	3,900
7	3	85	750
8	4	150	3,200
9	2	52	3,350
10	2	100	1,050

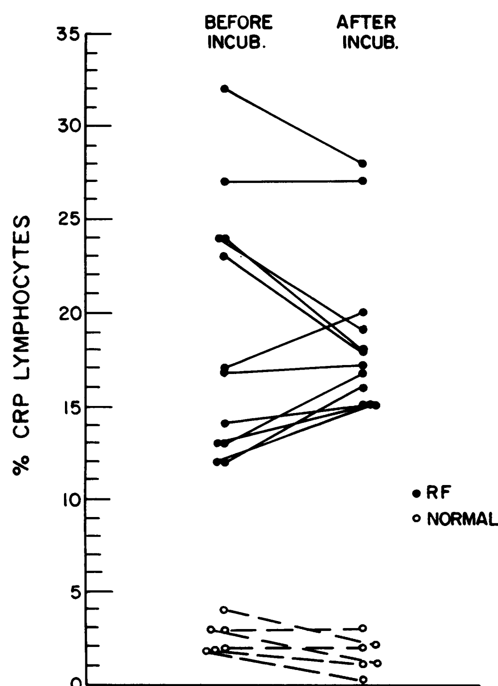

FIGURE 1 Results of changes in proportions of CRP-bearing cells as identified using fluorescein-labeled $F(ab')_2$ fragments of anti-CRP before and after 12–15-h incubations at 37°C in 5% CO_2-air incubator. RF, rheumatic fever.

CRP-binding cells represent antigen-activated T cells, their concomitant cell-surface markers might provide an indication as to what lymphocyte subpopulations were actually involved in such a process during the rheumatic fever episode. Accordingly, a variety of double-labeling experiments focused on this particular point. Among unfractionated lymphocytes depleted of monocytes and adherent cells, about one-half of all the cells reacting with fluorescein-conjugated aggregates showed concurrent staining for rhodamine-conjugated $F(ab')_2$ fragments of anti-CRP. Representative experiments illustrating these findings are provided in Table V*a*. Cells showing staining for both CRP and human aggregated IgG showed distinct and separate immunofluorescence. Thus, the staining for rhodamine-labeled anti-CRP appeared as red fluorescent spots and patches clearly distinct from the apple-green particulate surface immunofluorescence produced by the Fc receptor adherence to isothiocyanate-labeled IgG aggregates. Observation of cells after incubation at 37°C for 30–60 min showed a tendency for polarity or capping of anti-CRP-binding structures which was clearly separate from Fc receptors identified with the fluorescent green IgG aggregates. However, endocytosis and disappearance of CRP was not observed after observations up to 3 h. From these experiments, it was apparent that a considerable proportion of CRP-binding cells were lymphocytes which showed IgG Fc receptors.

A further attempt to define lymphocyte subpopulations bearing CRP was performed using Ig—anti-IgG Degalan column passage to deplete surface Ig containing cells. The results obtained after such immunoabsorbent column depletion are given in Table V*b*. The increase in proportions of CRP-binding cells after Ig—anti-Ig column passage along with virtually complete elimination of cells bearing surface Ig, indicated that B cells with surface immunoglobulin did not constitute a major population showing CRP binding, and that the residual population—presumably mainly T or L cells without surface Ig but possessing Fc receptors (29, 30), were largely responsible for surface CRP. To examine these findings further, Fc receptor-bearing cells were specifically enriched for using EA rosette formation with ox cells coated with rabbit IgG antibody and isolation of such rosette-forming cells by Ficoll gradients. Proportions of CRP-binding cells in non-EA rosette-forming lymphocyte cell preparations were compared to those enriched by rosetting for EA Fc receptor bearing cells. Again, as in the case of double-labeling studies described above, it was clear from

TABLE V

Double-Labeling Experiments to Determine Overlap of Cell Surface Markers with Lymphocytes Binding CRP

(*A*) Patient studied	Proportion of cells staining with fluoresceinated IgG aggregates	Proportion of cells staining with pepsin-digested anti-CRP (rhodamine-labeled)	Proportion of cells showing double staining
	%	%	%
82, Carditis	14	11	7
5105, Chorea	14	15	9
329, Carditis	28	16	13

Experiments before and After Passage of Cells over Ig—Anti-Ig Degalan Columns

(*B*) Patient studied	Proportion of cells staining with pepsin-digested anti-CRP	Proportion of cells with complement receptor EAC	Proportion of cells showing surface Ig*
	%	%	%
106 Before Ig—anti-Ig column	23	30	25
After Ig—anti-Ig column	40	5	1
114 Before Ig—anti-Ig column	17	0	23
After Ig—anti-Ig column	24	0	0

* Cells with surface Ig identified using fluorescein-labeled $F(ab')_2$ fragments of rabbit anti-human Ig.

TABLE VI

Relative Proportions and Total Numbers of T-Cell Subpopulations in Patients with Rheumatic Carditis or Chorea and Normal Controls

Patient group	T cells bearing receptors for Fc IgG (Tγ) %		T cells bearing receptors for Fc IgG (Tγ) Absolute no.		T cells bearing receptors for Fc IgM (Tμ) %		T cells bearing receptors for Fc IgM (Tμ) Absolute no.
Normal controls (30)	10.5±4.5*	$P < 0.01$	313.1±184.5	$P < 0.01$	49.5±10.4	$P < 0.01$	1,577±721.6‡
Rheumatic fever and carditis (16)	15.9±6.6		811.4±742.4		33.4±10.3		1,654.9±1,348‡
Rheumatic fever and chorea (8)	10.9±3.5		392.8±148.5		30.1±7.3		1,158.1±560‡

* Numbers refer to mean±SD.
‡ Difference between absolute no. of lymphocytes bearing Fc IgM receptors NS.

several experiments that Fc receptor-bearing cells and CRP binding were occurring together. In these experiments, cell populations specifically enriched for lymphocytes bearing Fc receptors in four experiments showed 21–25% concurrent double labeling for CRP. However, fluorescence CRP identification in the presence of concurrent EA rosettes was technically judged more difficult.

During various physiologic states, particularly those related to activation, T cells are known to show Fc receptors (31, 32). Accordingly, a series of experiments which focused on specific T-cell populations were conducted. T cells bearing specific IgM or IgG Fc receptors were prepared by the techniques of Moretta et al. (27, 28). Results of Tμ-and Tγ-cell determinations on children with acute rheumatic fever as well as normal controls are given in Table VI. It was clear that Tμ cells predominated and that Tγ cells constituted only a small fraction of the total T cells that were specifically enriched by neuraminidase sheep cell rosetting and overnight incubation to allow shedding and membrane stabilization. Of interest were the findings of significant elevation of proportions and numbers of Tγ cells in rheumatic children with decrease in proportions of Tμ cells as compared to controls. After isolation of T cells using rosetting with neuraminidase-treated sheep erythrocytes and overnight incubation at 37°C in IgM-free medium, concurrent labeling of Tμ and Tγ cells was performed using fluorescein-labeled $F(ab')_2$ fragments of anti-CRP. These parallel-labeling experiments showed that no Tμ cells bearing receptors for Fc of IgM showed CRP binding, whereas 25–60% (mean 46%) of Tγ cells bearing IgG Fc receptors bound CRP. Thus, when specific T-cell populations were examined for CRP, only Tγ cells showed surface staining. Representative data illustrating these findings are given in Table VII.

Experiments were also conducted on cells remaining at the Ficoll-Hypaque gradient interface after neuraminidase-rosetting of E-binding T cells. In five experiments, 12–16% of cells remaining in this B-cell layer showed CRP binding—even after overnight incubation at 37°C. Cell surface-marker studies in this presumptive B-cell layer showed 50–65% cells with surface Ig and 20–26% cells with Fc receptors by bovine EA rosettes. In two experiments where enough cells were present for accurate double-labeling studies on these isolated B cells after overnight incubation, 40 and 38% showed concurrent binding of pepsin-digested fluorescein-labeled anti-$F(ab')_2$ and rhodamine-labeled $F(ab')_2$ of anti-CRP. This clearly established that there was a definite overlap between cells bearing

TABLE VII

CRP-Binding T-Cell Populations in Acute Rheumatic Fever

Patient studied: Patient no.	Patient studied: Condition	Unfractionated lymphocytes binding CRP*	Total isolated T cells positive for CRP*	Tγ cells	Tγ + CRP*	Tμ cells	Tμ + CRP*
		%	%	%	%	%	%
73	Acute carditis	26	12	16	25	20	0
92	Chorea	18	20	10	40	37	0
56	Chorea	28	17	13	50	37	0
65	Carditis	24	20	23	60	47	0
83	Chorea	26	18	12	58	35	0

* Unfractionated cells and isolated T cells studied by indirect immunofluorescence using $F(ab')_2$ anti-CRP.

surface Ig and those capable of binding CRP. In addition, double-labeling experiments using EA rosettes and anti-CRP $F(ab')_2$ showed concomitant occurrence of these surface markers in 40 and 52% of isolated B-cell fractions. T-cell contamination as monitored by E rosettes was <2%. These data are given in Table VIII.

Experiments were next designed to examine whether active rosette-forming cells participated in CRP binding. Very little overlap in these two populations occurred. Separation of active or rapid rosette-forming cells and comparison with remaining whole lymphocyte populations either immediately before or after 12–14-h incubations at 37°C showed that very few (0–1%) of the active rosette-forming cells bound CRP. By contrast, a considerable fraction of cells which did not form early rosettes were shown to bind CRP (Table IX). Subsequent double-labeling experiments with this latter population again confirmed the presence of frequent concurrent Fc receptors and CRP binding in cells not participating in active T-cell rosette formation.

DISCUSSION

The current findings indicate that a marked increase in cells binding CRP occurs during the course of acute rheumatic fever. This was demonstrated using two, parallel independent methods of lymphocyte-CRP binding, which included direct immunofluorescent labeling of cells using $F(ab')_2$ fragments of anti-CRP as well as binding of Cowan I *S. aureus* bacteria coated with anti-CRP through Fc-reactive sites on protein A of the staphlococcus. Use of pepsin-digested $F(ab')_2$ fragments of anti-CRP avoided inadvertent adsorption of reagent to cell surface Fc receptors (33). It is possible that other nonimmunologic changes may actually affect lymphocyte-binding to CRP. Availability of enough purified CRP at the time of the work in Cairo prevented studies aimed at this point, utilizing lymphocytes from rheumatic fever children and addition of exogenous CRP after appropriate shedding or incubation. We have, however, confirmed the fact that CRP added to normal lymphocytes does show membrane binding. This does not appear to be a function of P component and was observed using CRP free of the latter material.

CRP-binding lymphocytes could not be directly correlated with quantitative elevations of plasma CRP since high proportions and total numbers occurred both in patients with elevated as well as much lower quantitative amounts of plasma CRP. It is clear, however, from the data presented in Table IV that some limiting increase in plasma CRP or threshold may be involved in lymphocyte CRP binding. A majority of CRP-binding lymphocytes did not appear to be conventional B cells with surface Ig since depletion of B cells using Ig—anti-IgG columns resulted in relative increase and enrichment of CRP-binding lymphocytes. It must be recognized, however, that such procedures could also have depleted adherent T cells and other cells besides those with surface Ig. When isolated B cells were studied, however, a definite proportion (38–40%) showed concordance between CRP and cell surface Ig using $F(ab')_2$ reagents. Several approaches at double labeling showed that CRP-binding cells often occurred in association with cell surface IgG Fc receptors. In addition, studies of cell populations enriched for T cells by neuraminidase-sheep cell rosetting and subsequent overnight incubation showed that $T\mu$ cells did not bind CRP, but that about one-half of $T\gamma$ cells were associated with CRP binding. Thus, the CRP-binding

TABLE VIII
Studies of CRP Binding in Unfractionated and Isolated Lymphocyte Populations in Patients with Acute Carditis of Rheumatic Fever

Patient studied		Unfractionated lymphocytes binding CRP	CRP binding		Double-labeling methods on B-cell fraction	
No.	Condition		T cells*	B cells‡	Cells showing surface Ig plus CRP	Cells showing EA rosette plus CRP
		%	%		%	
103	Acute carditis	14	7	8	38	40
108	Acute carditis	16	7	11	40	52

* T cells isolated by neuraminidase E rosetting and allowed to shed or incubate in 5% CO_2-air at 37°C in medium for 12 h.
‡ B cells refer to the cells not separated by Ficoll-Hypaque as neuraminidase rosette-forming cells, but remaining at Ficoll-Hypaque interface. Cell-surface marker studies on these cell fractions showed 50–65% cells with surface Ig and 20–26% cells with Fc receptors by bovine EA rosette formation.

TABLE IX
Rapid Rosette-Forming Cells and Nonrapid Rosetting Cells Double-Labeled for CRP

Patient studied	Binding CRP: Rapid rosettes	Binding CRP: Nonrapid rosette-forming cells
	%	%
1	2	14
2	0	24
3	0	17
4	0	15

lymphocytes in rheumatic fever represent a definite proportion of B cells, but appear to be mainly associated with presence of cell surface receptors for Fc of IgG. Our findings in children with acute rheumatic fever are somewhat different from the CRP binding primarily to T cells in normal peripheral blood initially reported by Mortensen et al. (8, 9). Data shown in Table VIII indicate some binding to B cells as well as T cells in the subjects studied. It seems possible that CRP may bind to lymphocytes in diseased patients perhaps on a different basis from those of normal subjects. It is conceivable that CRP binding may, instead of reflecting an immunological mechanism, merely be related to lymphocyte membrane damage of some kind. Some of the cells bearing Fc receptors and CRP appear to be $T\gamma$ cells as defined by Moretta et al. (28). From the data currently available, it appears possible that other lymphocytes bearing Fc receptors—perhaps similar to L, null, or K cells (30)—may also bind CRP. Experiments are now in progress to define this possibility, using CRP-binding cells in an antibody-mediated cytotoxicity system (34, 35). CRP-binding cells did not overlap with T cells forming active rosettes. These findings appear to confirm recent observations by West et al. (36, 37) showing that cells active in T-cell rosette formation at temperatures of 32°C do not overlap with T cells bearing Fc receptors.

If CRP-binding cells actually represent in part antigen-activated T cells, then such cells could provide an interesting possible source of cells activated towards streptococcal products or possibly cross-reacting cardiac antigens. Our current work has been specifically directed at this possibility. The use of anti-CRP affinity columns as a method to isolate CRP-binding cells and to study their specific reactions to mitogens as well as to a variety of streptococcal or cardiac antigens could provide direct data on whether or not such CRP-binding cells are indeed activated by identifiable antigens. The stability of CRP binding in lymphocytes incubated for 12–15-h periods is of considerable interest in this regard. Since data in Table VIII indicated that a proportion of CRP-binding cells appeared to bind to cells with Fc receptors which were not T cells as separated by neuraminidase rosetting, it is also possible that such cells might function as nonspecific effector cells in such systems as natural killing as described by Nelson et al. (38, 39).

CRP elevation is known to accompany many acute or subacute inflammatory conditions (1–6, 40, 41). The exact origin or sites of CRP synthesis as well as localization within inflammatory tissue previously have been studied by several groups (42–46). It is well known that marked CRP elevation in serum accompanies the acute process in rheumatic fever (4). No precise definition of the role of CRP in such disorders as acute rheumatic fever has yet been identified. Because of its elevation in so many inflammatory reactions, CRP has long been regarded merely as an acute-phase reactant similar perhaps to other serum proteins of this type such as ceruloplasmin, haptoglobin, or seromucoid (41, 47, 48). It is clear that CRP is present in small amounts in normal human serum (6) so that its presence does not necessarily reflect synthesis of an abnormal protein. The following hypothesis might be suggested by our own data as well as the previous reports of Mortensen et al. (8–10). If CRP is indeed capable of preferential binding to antigen-activated T, K, or natural killer cells, it is conceivable that such CRP binding might serve to defuse or attenuate self-directed cellular responses possibly harmful to the host.

The studies involving attempts to determine possible lymphocyte subpopulations binding to CRP bear some comment. Double-labeling techniques clearly indicated that a substantial proportion of CRP-binding cells were T cells. In addition, when T cells were selectively enriched using the neuraminidase erythrocyte-rosetting technique, T cells showing IgG Fc receptors ($T\gamma$) but not those bearing IgM Fc receptors ($T\mu$) were positive for CRP binding. These findings are of considerable interest since $T\mu$ cells have been suggested as putative markers for helper cells (49, 50). In the present study, proportions of $T\mu$ cells have been noted to be decreased during the acute rheumatic process, whereas $T\gamma$ cells were significantly increased. However, T cells binding CRP clearly appeared also to have IgG Fc receptors. Whether such $T\gamma$ cells carry a specific functional role such as suppressor or killer function remains to be determined. Recent data presented by Moretta et al. (50) appear to indicate a suppressor role for $T\gamma$ cells in some in vitro assay systems involving B-cell activation.

Our studies of CRP in supernates from lymphocyte cultures or merely incubation experiments have not provided clear evidence for active synthesis of CRP by isolated T cells. These possibilities have been recently pursued in view of the report of extensive sequence homologies between $C1^t$ and CRP and the P-amyloid component (7). It is important in this regard to point out that $F(ab')_2$ fragments obtained from anti-CRP antiserum generously provided by Dr. E. Gotschlich showed similar reactivities in the lymphocyte cell surface assays to those obtained from Dr. Osmand and Dr. Gewurz. The antiserum provided by Dr. Gotschlich was prepared against crystallized CRP whereas that obtained from Dr. Osmand and Dr. Gewurz was prepared from affinity column-purified material. Our testing for anti-P-component specificity in anti-CRP antisera proved negative (22, 23). If lymphocytes were under certain circumstances capable of CRP synthesis, they might under previous or current evolutionary circumstances be capable of $C1^t$ synthe-

sis as well. However, the vast bulk of evidence clearly implicates the liver as primary site of synthesis for CRP (42, 46).

Recent extensive studies (51–57) have described many of the important potential biological effects of CRP both in vitro and in vivo. Previous work has indicated that mixed leukocyte reactions are diminished when non-HLA-compatible lymphocytes from two patients with acute rheumatic fever are studied together in one-way stimulation (58). Gewurz et al. (8, 53) have also shown that mixed leukocyte reaction in normal subjects is diminished by CRP. In the latter instance, CRP effect was attributed to modulation of antigen-activated cells in the mixed leukocyte reaction. It now seems important to recognize that proportions of CRP-binding cells may directly influence several expressions of the immune response. These collective findings are of particular interest in the light of current reports indicating that streptococcal antigens produce an altered cell-mediated immune response in patients with acute rheumatic fever (59).

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