Inflammation, Vol. 12, No. 4, 1988

STIMULATION OF HUMAN NEUTROPHILS, MONOCYTES, AND PLATELETS BY MODIFIED C-REACTIVE PROTEIN (CRP) EXPRESSING A NEOANTIGENIC SPECIFICITY[1]

L. A. POTEMPA,[2] J. M. ZELLER,[2] B. A. FIEDEL,[3] C. M. KINOSHITA,[2] and H. GEWURZ[2]

[2] *Departments of Immunology/Microbiology and Medical Nursing*
Rush Presbyterian St. Luke's Medical Center
Chicago, Illinois

[3] *Division of Immunology, James N. Gamble Institute of Medical Research*
Cincinnati, Ohio

Abstract—C-reactive protein (CRP) can be structurally modified by heat, acid, or urea-chelation to express a neoantigen designated by us as neo-CRP. This antigen is also expressed on the in vitro primary protein translation products of both human and rabbit CRP. Unmodified CRP and CRP complexed with pneumococcal C-polysaccharide (CPS) do not express neo-CRP. Forms of CRP expressing neo-CRP but not native CRP antigenicity (even in the presence of CPS) consistently and in a dose-dependent manner potentiated the respiratory burst response of human polymorphonuclear leukocytes and peripheral blood monocytes to heat-modified IgG. Forms of CRP expressing neo-CRP antigenicity also induced reactions of aggregation and secretion from isolated platelets and potentiated platelet activation stimulated by ADP in platelet-rich-plasma, while native CRP alone or complexed with CPS again did not. Unlike CRP–CPS complexes, forms of CRP expressing neo-CRP were not able to activate the complement system. These data emphasize the biologic potential inherent in this humoral acute-phase reactant, particularly in the activation of the formed elements of the blood important in the inflammatory response. Since these cell-activating properties are preferentially observed when CRP is structurally modified to express the neo-CRP antigen, such a molecular conversion may be central to the structure–function relationships of CRP at local sites of inflammation and tissue injury.

[1] Supported in part by Biomedical Research Support Grant 97881 (Rush Presbyterian St. Luke's Medical Center), R23AI23030 (National Institute of Allergy and Infectious Diseases) and HL-23457 (National Heart, Lung & Blood Institute). B.A.F. was the recipient of an NIH Research Career Development Award HL-00614. H.G. holds the Thomas G. Coogan, Sr. Chair in Immunology established by Marjorie Lindheimer Everett.

0360-3997/88/0800-0391$06.00/0

INTRODUCTION

C-reactive protein (CRP) is an acute-phase reactant which markedly elevates in concentration in the sera of individuals undergoing reactions of inflammation and tissue degradation (1–4). Structurally, CRP is composed of five identical, noncovalently associated subunits arranged in cyclic symmetry (5, 6). Its amino acid and genomic sequences have been defined (7–9). Each subunit binds one to two calcium ions (10), which are known to regulate CRP structure (11–13) and binding reactivities for phosphate esters (10) [particularly phosphocholine (14)], polycations (15, 16), and chromatin (17, 18).

Native CRP, ligand-bound CRP, and modified and aggregated forms of CRP have been analyzed for a variety of in vitro and in vivo biologic effects. In general, native CRP alone is ineffective in stimulating human polymorphonuclear leukocyte (PMNL) (19, 20), monocyte (21, 22), platelet (23, 24), and lymphocyte (25, 26) reactivities (for reviews see references 3 and 4). In contrast, native CRP complexed with one of its defined ligands such as the pneumococcal C-polysaccharide (CPS), activates complement (C), (27–31) and stimulates PMNL phagocytic uptake in a manner apparently requiring cell activation and complement opsonization (20, 32–38). In other studies, CRP modified by heating (H-CRP) has been shown to induce aggregation and secretion in isolated platelets and to synergize with platelet activators to induce similar reactions in platelet-rich plasma (23, 24). H-CRP also potentiates the aggregated IgG-induced respiratory burst activity of human PMNLs and monocytes (19, 21).

In structure–function studies, our laboratory has observed that under certain adsorption or modification conditions, CRP undergoes conformational alterations which result in release of CRP subunits from the pentamer; these free subunits have reduced solubility, altered electrophoretic mobility, and unique surface characteristics hallmarked by the appearance of the neoantigen we termed neo-CRP (13, 37). The results of the present study indicate that forms of CRP that express neo-CRP are preferentially capable of activating the platelet, PMNL, and monocyte in vitro.

MATERIALS AND METHODS

Isolation and characterization of human CRP. Human CRP was isolated from pleural and ascites fluids by calcium-dependent affinity chromatography using PC-substituted Bio-gel as described (37). Final CRP preparations were concentrated to 1 mg/ml, exhaustively dialyzed in 10 mM Tris HCl buffered saline (pH 7.2), sterile filtered, and stored at 4°C. These preparations produced a single M_r 22,000 band on continuous SDS-PAGE electrophoresis stained with Coom-

assie brilliant blue (limit of detection ≥ 1 μg protein), were negative by radial immunodiffusion for SAP and Tri-partigen IgG (Calbiochem-Behring, San Diego, California) (limit of detection ≥ 2 μg/ml), and were nonreactive in double-diffusion assays in agarose using antisera directed against IgG, IgA, IgM, C1q, C1r, C1s, C3, normal human serum, serum amyloid P component (SAP), fibronectin, ceruloplasmin, albumin, α-lipoproteins, and β-lipoproteins (detection limits ≥ 30 μg/ml), respectively. By these criteria, final CRP preparations were $>99\%$ free of the SAP and IgG, and $>97\%$ free of other proteins.

Modification of CRP by Urea-Chelation, Heat, or Acid Treatment. Modified CRP expressing neo-CRP antigenicity was prepared from aliquots (1 mg/ml) of isolated CRP as follows. To prepare F-CRP (urea-chelated, fast migrating) ultrapure urea (Schwartz-Mann Inc., Spring Valley, New York) was added to CRP to a final concentration of 8 M prior to the addition of 10 mM EDTA; this mixture was incubated at 37°C for 1 h and dialyzed overnight at 4°C in 10 mM Tris-buffered saline (TBS) (pH 7.2). To prepare H-CRP (heat-modified C-reactive protein), CRP was heated at 63°C for 2 min in the absence of calcium chloride. To prepare A-CRP (acid-modified C-reactive protein), CRP was adjusted to pH 2.0 with HCl and incubated at ambient temperature for 1 min prior to neutralization with NaOH. These procedures utilized conditions identical to those previously defined as optimal for the preparation of F-CRP (13, 37), A-CRP (39), and H-CRP (40), respectively.

Heat Modification of Human IgG (H-IgG). Human IgG (Cutter Laboratories, Inc., Berkeley, California) at a concentration of 10 mg/ml in saline, was aggregated by incubation at 63°C for 60 min, cooled, and centrifuged (180,000*g*) for 60 min. The supernatant containing unaggregated IgG was discarded. The pellet was resuspended in saline and further centrifuged at 600*g* for 15 min to remove the large insoluble aggregates. The protein concentration of the supernatant was adjusted to 1 mg/ml as determined by Lowry protein measurement (41). Samples containing soluble IgG aggregates were aliquoted and stored at −70°C until use.

Pneumococcal C-Polysaccharide and CRP–CPS Complexes. Pneumococcal C-polysaccharide (CPS), prepared as described by Liu and Gotschlich (42), was the generous gift of Dr. C. Mold, University of New Mexico, Albuquerque, New Mexico. CPS was prepared at 0.5 mg/ml in isotonic saline and stored at −70°C. Complexes of CRP–CPS were prepared at ratios of 100:1 to 2:1 CRP–CPS (w/w) during incubation in TBS (pH 7.3) containing 2 mM calcium chloride at 22°C or 37°C. In control experiments, the degree of precipitation of the CRP–CPS complexes was analyzed by nephelometry as described (16). The ratio of reagents used in leukocyte and platelet assays described herein was chosen based on previously published experiments (23, 25, 40).

Monospecific Anti-neo-CRP and Anti-native-CRP Antisera. Antiserum to the human CRP neoantigen was prepared in the goat using F-CRP as described (37). Anti-native-CRP also was prepared in the goat using the following procedure. Isolated CRP [5 ml at 200 μg/ml in 10 mM TBS (pH 7.3) containing 2 mM $CaCl_2$] was emulsified with an equal volume of complete Freund's adjuvant for multiple subcutaneous, unilateral paraspinal injections. Four booster inoculations of identical concentration were given in incomplete Freund's on opposite sides of the spine at three-week intervals. Immune serum was collected and fractionated with 45% ammonium sulfate and passaged through a Biogel A 1.5-m column (Bio-rad, Richmond, California) (1.5 × 100 cm). The IgG peak was pooled, adjusted to 1 mg/ml, dialyzed into 10 mM PBS (pH 7.4), and maintained at 4°C in the presence of 0.02% NaN_3 until used.

Biotinylated Anti-neo-CRP. Affinity-purified anti-neo-CRP (1 mg/ml) was dialyzed into 0.1 M sodium bicarbonate (pH 9.0). *N*-Hydroxy succinimidobiotin (NHS-d-Biotin) (Sigma Chemical Co., St. Louis, Missouri), dissolved in dimethyl sulfoxide (DMSO) at 1 mg/ml, was then added at a ratio of 1:8 (v/v) (reagent:antibody). This mixture was left at room temperature for 4 h, dialyzed into 10 mM phosphate, 0.3 M NaCl (pH 7.3), and maintained at −20°C until used.

Enzyme-Linked Immunosorbent Assays (EIA). One hundred microliters of F-CRP (10 μg/ml) in 0.05 M sodium carbonate (pH 9.6) were added per well to Nunc polystyrene EIA plates

(Vangard International Inc., Neptune, New Jersey) and incubated overnight at 22°C. The plates were washed with 10 mM Tris HCl buffer (pH 7.3) containing 0.3 M NaCl, 2 mM $CaCl_2$, and 0.05% Tween-20 (wash buffer), back-coated with 1% BSA (in water) for 30 min at 37°C, and rinsed with wash buffer. In separate tubes, CRP samples modified or complexed as described, were serially diluted, and a constant amount of biotinylated anti-neo-CRP was added to each tube. The CRP–anti-neo-CRP mixture was incubated for 1 h at 37°C and 1 h at 4°C. One hundred microliters of each mixture dilution then either were directly incubated on immobilized F-CRP or plated after particulate complexes were removed from solution by centrifugation. After an additional 1 h incubation at 37°C and multiple washing steps, 100 μl of avidin-peroxidase conjugate, diluted in 0.02 M TBS (pH 7.3) containing 1% BSA, were added and incubation was continued for 1 h at 37°C. After washing, 100 μl of peroxidase substrate solution [prepared by dissolving 150 μl of 2,2′-azino-di(ethyl benzthiazoline sulfonic acid (ABTS, Sigma Chemical Company) (44 mg/ml) into 10 ml 0.05 M citrate (pH 4.0), and 40 μl 3% H_2O_2 immediately prior to use] were added per well, and samples were incubated for 10 min at 22°C. Wells were read for absorbance at 414 nm on a Titertek multiscan plate reader (Flow Laboratories, Helsinki, Finland).

Isolation of Peripheral Blood Mononuclear and Polymorphonuclear Leukocytes. Peripheral venous blood samples were collected from healthy human volunteers, heparinized, and diluted 1 : 1 with saline containing 1 mM EDTA. Mononuclear leukocytes were separated from PMNLs by layering diluted blood onto Lymphocyte Separation Medium (Litton Bionetics, Kensington, Maryland), and centrifuging at 400*g* for 20 min. PMNLs were further purified from contaminating erythrocytes by dextran sedimentation followed by hypotonic lysis. Cells were washed twice with Hanks' balanced salt solution (HBSS) containing 1% BSA. The mononuclear leukocyte fraction consisted of 30% monocytes and 70% lymphocytes as determined by latex ingestion (43). The PMNL fractions consisted of greater than 98% PMNLs as determined by routine hemocytometry. Cell viability, as assessed by trypan blue dye exclusion, was routinely greater than 95%.

Measurement of Monocyte and PMNL Chemiluminescence Responses. Monocyte and PMNL respiratory burst activities were assessed in luminol-enhanced chemiluminescence (CL) assay (19, 21). Cells (0.5×10^6 monocytes or PMNLs) were suspended in veronal-buffered saline (VBS) containing 0.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1 mg/ml dextrose, 0.25% BSA, and 0.5 μM luminol (pH 7.4), added to siliconized glass vials and allowed to dark adapt for at least 30 min.

Cell activation was initiated with stimulus addition, samples were mixed, and CL was measured at ambient temperature for 60 min in a 6895 Beta Trac Liquid scintillation counter (TM Analytic, Elk Grove Village, Illinois), set in the out-of-coincidence mode. Data are expressed as counts per minute (cpm) at the peak of the CL response. Alternatively, light emission was plotted as a function of time, and the area under the curve was measured by planimetry between the time of stimulus addition and 60 min (relative light emission, RLE). Light emission from stimulated samples of mononuclear leukocytes was the result of monocyte activation, since purified lymphocytes did not elicit a CL response using this assay.

Isolation of Platelets and Platelet Activation. Washed human platelets were prepared from platelet-rich plasma by washing in Tris–EDTA buffer (pH 7.5) and resuspended at a concentration of 3×10^8 platelets/ml in a diluent consisting of three parts 0.5% glucose in saline and five parts 0.09 M Tris, 30 mM KCl, 30 mM NaCl, and 0.8 mM $CaCl_2$ adjusted to pH 7.5 (final platelet buffer) (23). The aggregation of washed platelets was assessed using a Payton model 300 BD aggregometer (Payton Associates, Buffalo, New York) by equilibrating 100 μl final platelet buffer with 450 μl isolated platelets for 1 min at 3°C (1000 rpm), adding the test agent, and monitoring aggregation for 4 min. To assess aggregation in platelet-rich plasma, 100 μl test agent in buffer or buffer alone were added to 450 μl platelet-rich plasma and the reaction mixture was equilibrated as above. The platelet agonist was then added, and aggregation was recorded for 4 min.

Assay of Hemolytic Complement Activity. Consumption of total hemolytic complement activity was assayed in normal human serum exactly as previously described (44). In these assays, 0.1 ml native CRP, CRP–CPS complexes, or modified CRP was incubated for 60 min at 37°C

with 0.1 ml normal human serum plus 0.3 ml VBS, and residual complement activity was titrated utilizing antibody-sensitized sheep erythrocytes.

Statistical Evaluation. Data were compared using the two-tailed paired Student's t test. $P \leq 0.05$ was considered to be significant.

RESULTS

Detection and Quantitation of neo-CRP Antigen on CRP Treated with urea-chelation, acid and heat. An EIA inhibition assay was adapted in order to compare the relative ability of native CRP, CRP-CPS complexes, F-CRP, A-CRP, and H-CRP to express neo-CRP antigenicity. Since all indicated forms of CRP except for native CRP consisted of precipitated as well as soluble protein, we initially preincubated a constant amount of affinity-purified anti-neo-CRP with dilutions of suspended mixtures adjusted to contain known amounts of CRP. Residual free antibody activity was detected by binding to immobilized F-CRP.

As shown in Figure 1, suspensions of A-CRP, F-CRP, and H-CRP all

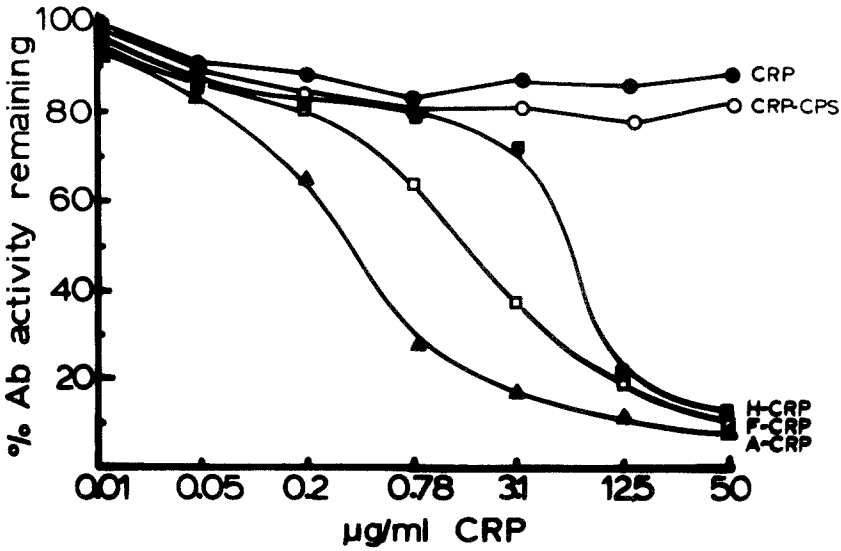

Fig. 1. EIA binding assay of residual affinity-purified, biotinylated-anti-neo-CRP reactivity after fluid-phase adsorption with various forms of CRP. A constant amount of biotinylated-anti-neo-CRP was preincubated for 1 h at 37°C with indicated amounts of native-CRP (●—●), H-CRP (■—■), F-CRP (□—□), A-CRP (▲—▲), or CRP-CPS complexes (○—○) at a 25:1 w/w ratio before being assayed for ELISA binding reactivity to F-CRP immobilized on polystyrene plates at 1 μg/well. Results are expressed as percent of uninhibited antibody binding.

effectively bound anti-neo-CRP with 50% inhibition observed at 0.4 μg protein/ml A-CRP, 1.5 μg/ml F-CRP, and 6.0 μg/ml H-CRP. When the precipitates were first separated from soluble protein by centrifugation and subsequently incubated with anti-neo-CRP, similar results were obtained, indicating that the neoantigen was expressed on the precipitated protein. In contrast, at all protein concentrations tested, neither native CRP nor CRP–CPS complexes bound anti-neo-CRP. No significant differences were observed when these studies were performed in the absence of calcium, indicating that anti-neo-CRP binding is not sensitive to calcium effects on CRP.

Effect of F-CRP and A-CRP on CL Response of Human PMNLs. Our laboratory has reported that while H-CRP alone was ineffective in eliciting a measurable CL response upon incubation with human PMNLs, H-CRP did potentiate the CL response induced by H-IgG in a dose-dependent manner (19). In order to determine whether the other forms of CRP presenting neo-CRP antigenicity could similarly influence the PMNL CL response, we compared the ability of F-CRP and A-CRP to stimulate similar reactivity as observed with H-CRP. At all dilutions tested, A-CRP and F-CRP when added alone, like H-CRP, native CRP or CRP-CPS complexes, failed to stimulate PMNL CL.

The ability of A-CRP and F-CRP to potentiate the H-IgG-induced CL response was further examined. As shown in Figure 2A, where results obtained with doses of 100 μg/ml CRP are compared, F-CRP and A-CRP were similar to H-CRP in effectively potentiating the CL response. F-CRP contributed to a 218 ± 45% increase (Student's t test $P < 0.05$), and A-CRP contributed to a 69 ± 20% increase ($P < 0.05$). Native CRP or CRP complexed with CPS did not augment PMNL H-IgG-induced CL. CPS alone, when added at the 4 μg/ml dose used in CRP–CPS complexes, also was ineffective. The level of enhancement elicited by 100 μg/ml H-CRP (110 ± 28%; $P < 0.01$) is shown for reference.

The dose-dependent enhancing effects of F-CRP on H-IgG-induced PMNL CL are shown in time-course analyses in Figure 3A. H-IgG elicited a fivefold increase in CL relative light emission over saline control when integrated over 60 min. When 25, 50, and 100 μg of F-CRP were added simultaneously with H-IgG, the PMNL CL response was potentiated 77, 101, and 170%, respectively. Similar dose-dependent enhancing effects were observed using A-CRP in place of F-CRP (range of enhancement from 100 to 136%). In all experiments described thus far, F-CRP, A-CRP, and H-CRP were added as suspended mixtures of aggregated and soluble protein. When aggregates were removed from soluble protein, the potentiating activity was always found in the aggregate fraction, suggesting either that the protein remaining soluble after treatment was not active in potentiating PMNL CL or that the concentrations remaining soluble were too low to enhance the H-IgG response.

Effect of F-CRP and A-CRP on CL Response of Human Peripheral Blood

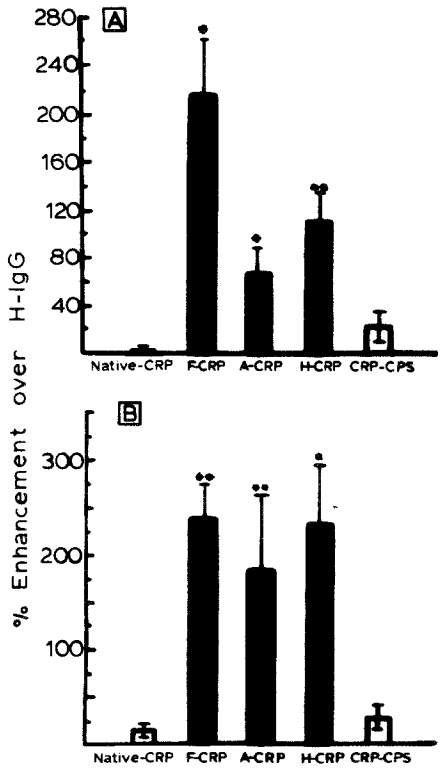

Fig. 2. Enhancing effect of CRP reagents on the H-IgG-induced CL response of human PMNLs (panel A) and human monocytes (panel B). CL was measured by liquid scintillation technology for 60 min following the addition of 20 μg/ml H-IgG to PMNLs or 75 μg/ml H-IgG to monocytes together with the indicated CRP reagents (100 μg/ml) to 0.5 $\times$ 10^6 cells suspended in veranol-buffered saline containing 0.5 μM luminol. F-CRP: urea-chelated (fast) CRP; A-CRP: acid-treated CRP; H-CRP: heat-treated CRP; CRP-CPS: CRP complexed with C-polysaccharide at a 25 : 1 w/w ratio. Data are presented as percent enhancement of peak CL over the H-IgG control. Similar results were observed when results were expressed as integrated relative light transmission over the 60-min incubation. Data represent the mean $\pm$ SEM of 4–11 separate experiments done on different days and with different donors. *P* values were calculated by paired Student's *t* test. * $P < 0.05$; ** $P < 0.01$.

Monocytes. In experiments identical to those described for PMNLs, the effect of CRP reagents on human monocyte oxidative metabolism was tested. As seen with PMNLs, F-CRP and A-CRP alone, in a manner comparable to H-CRP, native CRP, and CRP–CPS complexes, were unable to directly stimulate monocyte CL response. However, when added with H-IgG, A-CRP and F-CRP potentiated the monocyte CL response, while native CRP, CRP–CPS complexes, and CPS controls lacked this activity. Figure 2B illustrates the results

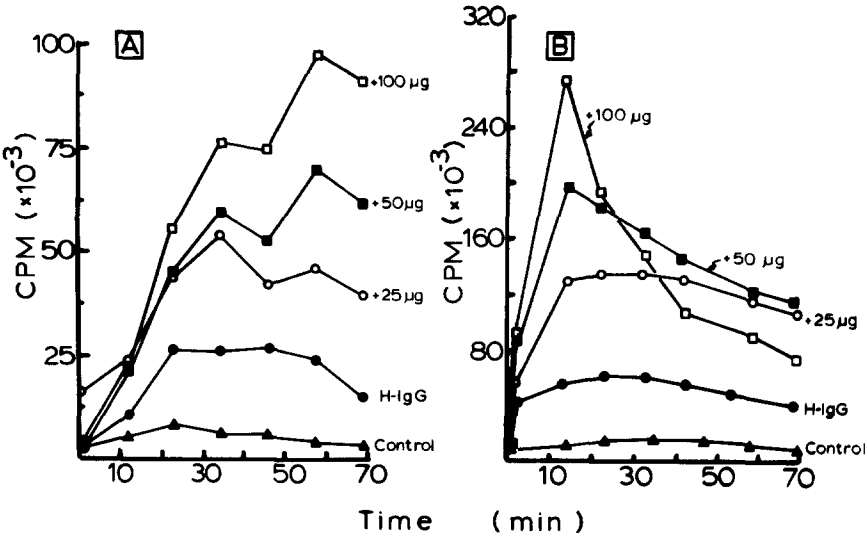

Fig. 3. Dose-dependent and kinetic effects of F-CRP on the H-IgG-stimulated CL response of human PMNLs (panel A) and human monocytes (panel B). 0.5 × 10^6 cells, suspended in veronal-buffered saline containing 0.5 μM luminol, were stimulated with saline (▲—▲), or with 20 μg/ml (to PMNLs) or 75 μg/ml (to monocytes) H-IgG alone (●—●), or H-IgG in combination with 25 (○—○), 50 (■—■), or 100 μg/ml (□—□) F-CRP. Relative light emission was measured as described in Materials and Methods. Data presented here are representative of those observed using three different donors.

obtained using 100 μg/ml doses of CRP. F-CRP potentiated the H-IgG-induced peak CL response 236 ± 39% ($P < 0.01$) while A-CRP potentiated the H-IgG response 179 ± 89% ($P < 0.01$). Figure 3B compares the dose-dependent enhancing effects of F-CRP in a time course analyses. When the relative light emission was integrated over 60 min, F-CRP contributed to a 118, 188, and 190% potentiation over the H-IgG response when 25, 50, and 100 μg/ml of reagent, respectively, were added concomitantly with H-IgG. Similar results were observed using A-CRP (range of enhancement 65–132%).

In order to evaluate the specificity of the enhancing effect of altered CRP on Fc receptor-mediated CL, further studies were performed using native and heat-modified forms of bovine serum albumin and human IgM. In all cases, these control reagents failed to either directly stimulate or potentiate monocyte and PMNL respiratory burst activities (data not shown).

Effect of F-CRP and A-CRP on Aggregation Response of Human Platelets. Our laboratory had previously reported that H-CRP activated isolated human platelets to aggregate, secrete dense and α-granule constituents, and generate thromboxane A_2 (23). The present study was designed to evaluate whether F-CRP and A-CRP could similarly induce platelet activation.

As shown in Figure 4, F-CRP was similar to H-CRP on a weight basis in activating platelets to aggregate; A-CRP also activated platelets and was the most potent agonist of these three reagents. F-CRP, A-CRP, and H-CRP all induced monophasic platelet aggregation. As was seen with the PMNL and monocyte, the majority of platelet-active material was in the aggregate fraction of CRP. Native CRP alone or complexed with CPS at weight ratios varying from 0.5 : 1 to 100 : 1 was ineffective at stimulating platelet aggregation.

It has been observed that H-CRP functions in platelet-rich plasma as a synergist rather than as a direct agonist (24). In the present study, the ability of F-CRP, A-CRP, native CRP, and CRP–CPS complexes to similarly potentiate platelet aggregation to a suboptimal dose of ADP was examined. As shown in Figure 5, H-CRP, A-CRP, and F-CRP, like H-CRP as previously described, were observed to potentiate platelet aggregation; by contrast, and consistent with previous results, native CRP and CRP–CPS complexes did not act synergistically with ADP. Identical results were observed when collagen was used as the platelet stimulus.

Effect of F-CRP and A-CRP upon Hemolytic C Activity in Normal Human Serum. Suspensions of F-CRP and A-CRP (up to 250 μg/ml) were tested for the ability to activate hemolytic C activity during incubations in normal human serum. As illustrated in Figure 6, both F-CRP and A-CRP failed to deplete hemolytic C activity in this assay (100 μg/ml doses of each preparation of CRP

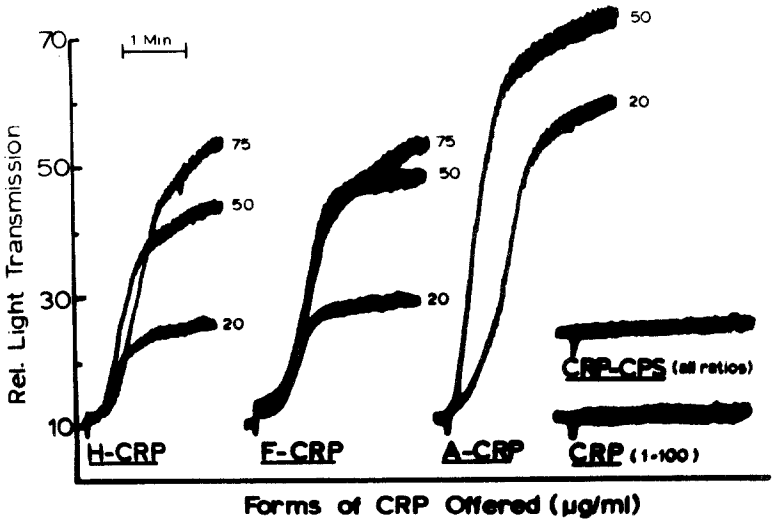

Fig. 4. Effect of various forms of CRP on the aggregation of washed human platelets. Platelets, prepared as described in Materials and Methods, were suspended at 3×10^8/ml before the addition of test reagent at the indicated concentrations (μg/ml). CRP–CPS complexes were formed at various weight ratios ranging from 0.5 : 1 to 100 : 1. Platelet aggregation was monitored for 4 min at 37°C.

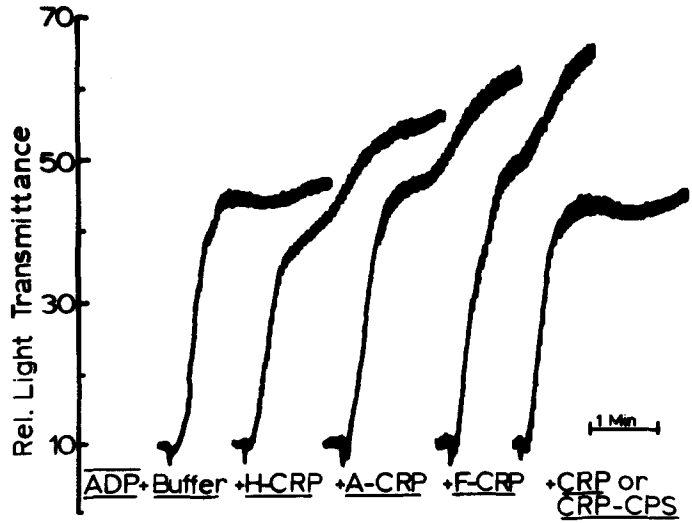

Fig. 5. Effect of various forms of CRP on the platelet synergistic response to adenosine diphosphate (ADP) in human platelet-rich plasma. A sufficient amount of ADP (8×10^{-6} M) was added to induce only a weak aggregation profile. CRP reagents were added at 75 μg/ml simultaneously with ADP. CRP–CPS reagents were prepared at weight ratios identical to those described in Figure 4. Identical results were observed using collagen as platelet stimulus.

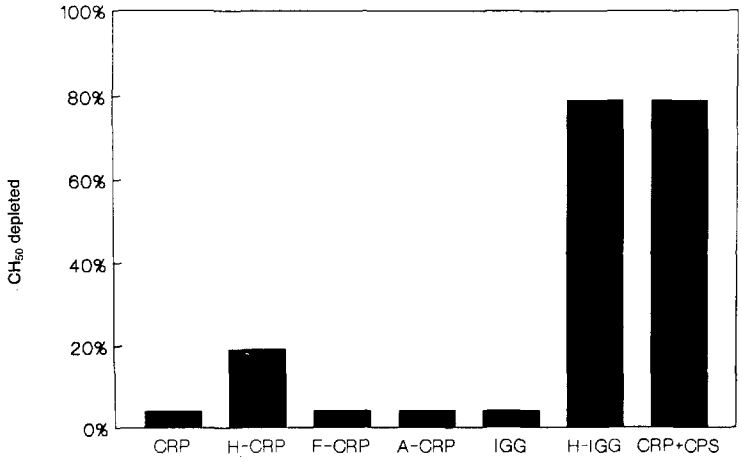

Fig. 6. Effect of various forms of CRP on hemolytic complement (CH_{50}) activity. A constant amount (100 μg/ml) of native CRP, CRP–CPS complexes, F-CRP, A-CRP, or H-CRP was reacted with 20% human serum in veronal-buffered saline at 37°C for 60 min, and the residual CH_{50} was determined as described in Materials and Methods. C-depletion by identical amounts of human IgG and heated IgG (H-IgG) are shown for comparison.

are compared in this figure). H-CRP also lacked significant capacity to induce complement depletion. These results are in marked contrast to the depletion of hemolytic C activity observed when either H-IgG or CRP–CPS complexes were incubated with normal human serum. F-CRP and A-CRP also lacked the ability to consume C in the presence of CPS. The inability of F-CRP and H-CRP to activate C stands in contrast to the capacity of these preparations to active monocytes, neutrophils, and platelets.

DISCUSSION

Previous studies have shown that in the presence of certain ligands, a low level of native CRP binds to resting cells with no or minimal biological consequence (19, 21, 26, 40). However, under appropriate conditions, certain forms of complexed or aggregated CRP both bind to and stimulate or modify biological activities of human perpheral blood lymphocytes (25, 26, 40, 45), platelets (23, 24), monocytes (21, 22, 38) and PMNLs (20, 21, 35, 36). In the present study, CRP modified by acid or urea-chelation was examined for in vitro effects on PMNL and monocyte oxidative metabolism, platelet aggregation and release, and complement consumption. These forms of CRP were chosen in light of the observation that CRP modified by acid or urea-chelation is similar to CRP modified by heat in expressing a unique antigenicity, designated neo-CRP, which is not expressed on native CRP or CRP complexed with CPS (13, 37).

Using an EIA inhibition assay, the neo-CRP antigen on aggregated insoluble fractions of A-CRP and F-CRP as well as on H-CRP was detected and quantified. It was previously shown that modifications of CRP described here result in release of the CRP subunit with a concomitant structural change as determined by intrinsic fluorescence, decreased solubility in saline buffers, self-aggregation, and a shift in electrophoretic mobility (13, 37). The current experiments confirmed that the neo-CRP antigen is expressed not only on the modified CRP which remains soluble (i.e., the free CRP subunit), but on the aggregated fractions as well. It is important to point out that conditions described here for H-CRP resulted in substantial amounts of soluble material which retained the size (as determined by chromatographic procedures) and antigenicity of the native molecule, while conditions described for preparations of A-CRP and F-CRP resulted in essentially a total loss of these native CRP characteristics (37). CRP complexed in the fluid phase with CPS via its calcium-dependent phosphorylcholine binding site did not express the neo-CRP antigen, confirming our previous report comparing native and neo-CRP antigenicity on direct binding and capture EIAs (37).

Forms of CRP expressing neo but not native antigenicity were found to

potentiate the IgG-induced respiratory burst activity of both PMNLs and monocytes. No form of CRP tested was able to directly stimulate respiratory burst activity in the absence of H-IgG. The potentiating effect of A-CRP, F-CRP, and H-CRP on IgG-induced CL was related to expression of CRP neoantigenicity and was not the direct result of protein aggregation, as neither heat-modified bovine serum albumin nor heat-modified IgM mimicked CRP in these assays.

Our laboratory previously reported that the H-CRP potentiation of PMNL and monocyte CL was selective for H-IgG-mediated cell activation, since modified CRP was ineffective in enhancing respiratory burst activity induced by phorbol myristate acetate or serum-opsonized zymosan (19, 21). Potentiation of Fc-receptor-mediated phagocyte CL by H-CRP required that IgG be presented in an aggregated form (19, 21, 46), suggesting that cross-linking of IgG receptors may be necessary for CRP-induced cell activation. The mechanism of CRP enhancement of Fc-receptor-mediated CL has not yet been established. It is plausible that CRP and IgG bind to related membrane structures or share common pathways of cell activation. While previous studies have suggested a relationship between the binding site for CRP and IgG Fc receptors (25, 26, 36, 40, 46), the identity of a putative CRP receptor and its relationship to an identified IgG Fc receptor have not been established.

Forms of CRP expressing neo-CRP but not native CRP antigenicity were also effective in directly activating platelets and serving as synergists in platelet activation. These studies utilized H-CRP or CRP adsorbed onto latex beads (23) and indicated that an actual activation process requiring metabolic energy was involved. Consistent with the findings reported here, we have reported that CRP adsorbed onto plastic also expresses neoantigenicity (37). Since H-IgG and forms of CRP expressing neoantigenicity each independently activate isolated platelets, the platelet receptor for modified CRP has been proposed to be distinct from the Fc receptor for IgG (47).

Interestingly, the three forms of CRP expressing neo-CRP were unable to consume complement. This is in contrast to CRP complexed with CPS which is a potent activator of the primary complement pathway (3, 27–31). Our data thus support the contention that forms of CRP expressing the neo-CRP antigen can preferentially activate the formed element of the blood important in the inflammatory response, while native CRP complexed with CPS shows preferential activity with the humoral complement system. In this aspect, preliminary studies have shown that A-CRP and F-CRP can stimulate glass-adherent monocytes to secrete IL-1 and PGE_2 and to act as better accessory cells in a phytohemagglutinin-stimulated autologous lymphoblastogenesis assay (22). In addition, A-CRP and F-CRP can suppress the development of cytotoxicity in glass-adherent monocytes from normal individuals (48).

Our laboratory has reported that neo-CRP is naturally expressed in tissues at sites of inflammation and necrosis (49). Further, using fluorescence-activated

cell sorting, neo-CRP is naturally expressed on the surface of a subpopulation of human peripheral blood lymphocytes expressing large-granular lymphocyte morphology and, interestingly, on B lymphocytes (50). We therefore propose that a natural mechanism regulating CRP function may involve a conformational alteration associated with the dissociation of the CRP pentameric structure and hallmarked by the expression of neo-CRP antigenicity. When CRP expresses this neo-CRP, it differentially influences effector mechanisms of the immune response, losing its ability to active C, while gaining the capacity to influence various formed elements of the blood.

Acknowledgments—We thank Mrs. Mary Rolfe-Shaw for her expert secretarial assistance.

REFERENCES

1. KUSHNER, I., M. L. BRODER, and D. KARP. 1978. Control of the acute phase response. Serum C-reactive protein kinetics after acute myocardial infarction. *J. Clin. Invest.* **61**:235–242.
2. MCADAM, K. P., R. J. ELIN, J. D. SIPE, and S. M. WOLFF. 1978. Changes in human serum amyloid A and C-reactive protein after etiocholanolone-induced inflammation. *J. Clin. Invest.* **61**:390–394.
3. GEWURZ, H., C. M. MOLD, J. SIEGEL, and B. A. FIEDEL. 1982. C-reactive protein and the acute phase response. *Adv. Intern. Med.* **27**:345–372.
4. PEPYS, M. B., and M. L. BALTZ. 1983. Acute phase proteins with specific reference to C-reactive protein and related proteins (pentraxins) and serum amyloid A protein. *Adv. Immunol.* **34**:141–212.
5. OSMAND, A. P., B. FRIEDENSON, H. GEWURZ, R. H. PAINTER, T. HOFMANN, and E. SHELTON. 1977. Characterization of C-reactive protein and the complement subcomponent C1t as homologous proteins displaying cyclic pentameric symmetry (pentraxins). *Proc. Natl. Acad. Sci. U.S.A.* **74**:739–743.
6. GOTSCHLICH, E. C., and G. M. EDELMAN. 1965. C-reactive protein: A molecule composed of subunits. *Proc. Natl. Acad. Sci. U.S.A.* **54**:558–566.
7. LEI, K.-J., T. LIU, G. ZON, E. SORAVIA, T.-Y. LIU, and N. D. GOLDMAN. 1985. Genomic DNA sequence for human C-reactive protein. *J. Biol. Chem.* **260**:13377–13383.
8. WHITEHEAD, A. S., G. A. P. BRUNS, A. F. MARKHAM, H. R. COLTEN, and D. E. WOODS. 1983. Isolation of human C-reactive protein complementary DNA and localizing of the gene to chromosome 1. *Science* **221**:69–71.
9. WOO, P., J. R. KORENBERG, and A. S. WHITEHEAD. 1985. Characterization of genomic and complementary DNA sequences of human C-reactive protein and comparison with the complementary DNA sequence of serum amyloid P component. *J. Biol. Chem.* **260**:13384–13388.
10. GOTSCHLICH, E. C., and G. M. EDELMAN. 1967. Binding properties and specificity of C-reactive protein. *Proc. Natl. Acad. Sci. U.S.A.* **57**:706–712.
11. YOUNG, N. M., and R. E. WILLIAMS. 1978. Comparison of the secondary structures and binding sites of C-reactive protein and the phosphorylcholine-binding murine myeloma proteins. *J. Immunol.* **121**:1893–1898.
12. VOLANAKIS, J. E., and J. F. KEARNEY. 1981. Cross-reactivity between C-reactive protein and idiotypic determinants on a phosphocholine-binding murine myeloma protein. *J. Exp. Med.* **153**:1604–1614.
13. POTEMPA, L. A., B. A. MALDONADO, P. LAURENT, E. ZEMEL, and H. GEWURZ. 1983. Anti-

genic, electrophoretic and binding alterations of human C-reactive protein modified selectively in the absence of calcium. *Mol. Immunol.* **20**:1165–1175.

14. VOLANAKIS, J. E., and M. H. KAPLAN. 1971. Specificity of C-reactive protein for choline phosphate residues of pneumococcal C-polysaccharide. *Proc. Soc. Exp. Biol. Med.* **136**:612–614.
15. DICAMELLI, R., L. A. POTEMPA, J. SIEGEL, L. SUYEHIRA, K. PETRAS, and H. GEWURZ. 1980. Binding reactivity of C-reactive protein for polycations. *J. Immunol.* **125**:1933–1938.
16. POTEMPA, L. A., J. N. SIEGEL, and H. GEWURZ. 1982. C-reactive protein (CRP) reactivity with polycations. II. Modulatory effects of calcium and phosphocholine. *J. Immunol.* **127**:1509–1514.
17. ROBEY, F. A., K. D. JONES, T. TANAKA, and T.-Y. LIU. 1984. Binding of C-reactive protein to chromatin and nucleosome core particles. *J. Biol. Chem.* **259**:7311–7316.
18. SHEPARD, E. G., P. D. VANHELDEN, M. STRAUSS, L. BOHM, and F. C. DEBEER. 1986. Functional effects of CRP binding to nuclei. *Immunology* **58**:489–494.
19. ZELLER, J. M., A. L. LANDAY, T. F. LINT, and H. GEWURZ. 1986. Aggregated C-reactive protein binds to and potentiates Fc receptor-mediated chemiluminescence in human polymorphonuclear leukocytes. *J. Lab. Clin. Med.* **108**:567–576.
20. SHEPHARD, E. G., R. ANDERSON, A. F. STRACHAN, S. H. KUHN, and F. C. DEBEER. 1986. CRP and neutrophils: Functional effects and complex uptake. *Clin. Exp. Immunol.* **63**:718–727.
21. ZELLER, J. M., A. L. LANDAY, T. F. LINT, and H. GEWURZ. 1986. Enhancement of human peripheral blood monocyte respiratory burst activity of aggregated C-reactive protein. *J. Leukocyte Biol.* **40**:769–783.
22. POTEMPA, L. A., H. GEWURZ, J. E. HARRIS, and D. P. BRAUN. 1986. Stimulatory effects of the C-reactive protein subunit on monocyte function including release of IL-1. *Protides Biol. Fluids.* **34**:287–290.
23. FIEDEL, B. A., R. M. SIMPSON, and H. GEWURZ. 1982. Activation of platelets by modified C-reactive protein. *Immunology* **45**:439–447.
24. FIEDEL, B. A. 1985. Platelet agonist synergism by the acute phase reactant C-reactive protein. *Blood* **65**:264–269.
25. JAMES, K., B. HANSEN, and H. GEWURZ. 1981. Binding of C-reactive protein to human mononuclear cells. I. Requirement for a binding specificity. *J. Immunol.* **127**:2539–2544.
26. JAMES, K., L. BAUM, M. VETTER, and H. GEWURZ. 1982. Interactions of C-reactive protein with lymphoid cells. *Ann. N.Y. Acad. Sci.* **389**:274–285.
27. KAPLAN, M. H., and J. E. VOLANAKIS. 1974. Interaction of C-reactive protein complexes with the complement system. I. Consumption of human complement-associated with the reaction of C-reactive protein with pneumococcal C-polysaccharide and with choline phosphatides, lecithin and sphingomyelin. *J. Immunol.* **112**:2135–2147.
28. CLAUS, D. R., J. SIEGEL, K. PETRAS, A. P. OSMAND, and H. GEWURZ. 1977. Interactions of C-reactive protein with the first component of human complement. *J. Immunol.* **119**:187–192.
29. VOLANAKIS, J. 1982. Complement-induced solubilization of C-reactive protein-pneumococcal C-polysaccharide precipitates: Evidence for covalent binding of complement proteins to C-reactive protein and to pneumococcal C-polysaccharide. *J. Immunol.* **128**:2745–2750.
30. VOLANAKIS, J. E., and A. J. NARKATES. 1983. Binding of human C4 to C-reactive protein-pneumococcal C-polysaccharide complexes during activation of the classical complement pathway. *Mol. Immunol.* **20**:1201–1207.
31. VOLANAKIS, J. E. 1982. Complement activation by C-reactive protein complexes. *Ann. N.Y. Acad. Sci.* **389**:235–250.
32. MOLD, C., T. W. DUCLOS, S. NAKAYAMA, K. M. EDWARDS, and H. GEWURZ. 1982. C-reactive protein reactivity with complement and effects on phagocytosis. *Ann. N.Y. Acad. Sci.* **389**:251–262.

33. NAKAYAMA, S., C. MOLD, H. GEWURZ, and T. W. DUCLOS. 1982. Opsonic properties of C-reactive protein in vivo. *J. Immunol.* **128:**2435–2438.
34. EDWARDS, K. M., H. GEWURZ, T. F. LINT, and C. MOLD. 1982. A role for C-reactive protein in the complement-mediated stimulation of human neutrophils by type 27 *Streptococcus pneumonia. J. Immunol.* **128:**2493–2496.
35. KILPATRICK, J. M., and J. E. VOLANAKIS. 1985. Opsonic properties of C-reactive protein. Stimulation by phorbol myristate acetate enables human neutrophils to phagocytize C-reactive protein-coated cells. *J. Immunol.* **134:**3364–3370.
36. MULLER, H., and J. FEHR. 1986. Binding of C-reactive protein to human polymorphonuclear leukocytes: Evidence for association of binding sites with Fc receptors. *J. Immunol.* **136:**2202–2207.
37. POTEMPA, L. A., J. N. SIEGEL, B. A. FIEDEL, R. T. POTEMPA, and H. GEWURZ. 1987. Expression, detection and assay of a neoantigen (neo-CRP) associated with a free, human C-reactive protein subunit. *Mol. Immunol.* **24:**531–541.
38. MORTENSEN, R. F., A. P. OSMAND, T. F. LINT, and H. GEWURZ. 1976. Interaction of C-reactive protein with lymphocytes and monocytes: Complement-dependent adherence and phagocytosis. *J. Immunol.* **117:**774–781.
39. FAIRBANKS, T. R. 1984. Influence of acid pH on structure, binding and biological reactivity of C-reactive protein. PhD dissertation. Rush University, Chicago, Illinois, 60612.
40. JAMES, K., B. HANSEN, and H. GEWURZ. 1981. Binding of C-reactive protein to human mononuclear cells. II. Interation with a subset of cells bearing the Fc receptor. *J. Immunol.* **127:**2545–2552.
41. LOWRY, O. H., H. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:**265–275.
42. LIU, T.-Y., and E. C. GOTSCHLICH. The chemical composition of pneumococcal C-polysaccharide. *J. Biol. Chem.* **238:**1928–1934.
43. ROSS, G. D., and R. J. WINCHESTER. 1980. Methods for enumerating lymphocyte populations. *In* Manual of Clinical Immunology. N. R. Rose and H. Friedman, editors. American Society of Microbiology, Washington, D.C. 213.
44. GEWURZ, H., and L. A. SUYEHIRA. 1980. Complement. *In* Manual of Clinical Immunology. N. R. Rose and H. Friedman, editors. American Society for Microbiology, Washington, D.C. 163–174
45. VETTER, M. L., H. GEWURZ, B. HANSEN, K. JAMES, and L. L. BAUM. 1983. Effects of C-reactive protein on human lymphocyte responsiveness. *J. Immunol.* **130:**2121–2126.
46. ZELLER, J. M., J. CALIENDO, and H. GEWURZ. 1986. Modulation of C-reactive protein and IgG binding to human monocytes by surface immobilized ligands. *Fed. Proc.* **45:**262a.
47. FIEDEL, B. A., J. N. SIEGEL, H. GEWURZ, R. M. SIMPSON, and J. M. IZZI. 1982. Comparison of the enzymatic sensitivities of the platelet receptor for human C-reactive protein and its functional relationship to the platelet IgG Fc receptor. *Clin. Exp. Immunol.* **50:**215–222.
48. CHU, E. B., L. A. POTEMPA, J. E. HARRIS, H. GEWURZ, and D. P. BRAUN. 1987. Modulation of monocyte cytotoxicity with urea-chelated and acid-modified C-reactive protein (CRP). *Proc. Am. Cancer Soc.* **28:**344a.
49. REES, R., H. GEWURZ, J. N. SIEGEL, and L. A. POTEMPA. 1986. An immunochemical study of the in vivo expression of a C-reactive protein neoantigen (neo-CRP) in the rabbit. *Fed. Proc.* **45:**263a.
50. BRAY, R. A., N. L. SAMBERG, A. L. LANDAY, H. GEWURZ, and L. A. POTEMPA. 1987. Identification of a novel antigenic specificity present on the surface of human natural killer cells and B-lymphocytes. *Clin. Immunol. Newsl.* **8:**137–140.