TISSUE-SPECIFIC PHOSPHORYLATION OF COMPLEMENT RECEPTORS CR1 AND CR2

By PAUL S. CHANGELIAN and DOUGLAS T. FEARON

From the Department of Medicine, Harvard Medical School, and the Department of Rheumatology and Immunology, Brigham and Women's Hospital, Boston, Massachusetts 02115

Complement receptor type 1 (CR1),[1] the C3b/C4b receptor (1), and CR3, the iC3b receptor (2), of myelomonocytic cells have been shown to be involved in the phagocytosis of particles bearing the C3b and iC3b cleavage fragments of C3. Two states of activity exist for these receptors, a resting state, in which CR1 and CR3 only bind ligand-coated particles, and an activated state, in which these receptors also mediate the phagocytosis of such particles. The activated state was first described (3) when complement receptors on murine peritoneal macrophages were shown to acquire phagocytic capability after treatment of cells with soluble products of activated T cells. Subsequently, PMA was bound to impart phagocytic function on CR1 and CR3 of human monocytes and neutrophils (4, 5), and also to induce ligand-independent internalization of CR1 by these cells (6). Finally, the capacity of three proteins of the extracellular matrix, fibronectin (FN) (5, 7, 8), serum amyloid P (5), and laminin (9), and activate CR1 and CR3 suggests that this alteration of receptor function may occur physiologically when cells migrate to the extravascular space.

The biochemical reactions accounting for the transition of CR1 and CR3 from the resting to the activated state are not known, but the ability of PMA to induce this transition suggests that protein phosphorylation by protein kinase C (10) may be an important event. Therefore, we have assessed the phosphorylation of these proteins by phagocytic and nonphagocytic cells. CR1 of myelomonocytic cells became phosphorylated following treatment of cells with PMA. In contrast, PMA did not induce phosphorylation of CR1 in tonsilar cells containing B lymphocytes, B lymphoblastoid cells, or erythrocytes, although relatively intense phosphorylation of CR2 (11), the C3d/EBV receptor (12), was observed in both tonsilar B cells and B lymphoblastoid cells.

Materials and Methods

Chemicals. BSA (fatty acid–free, fraction V; Miles Laboratories, Kankakee, IL), SDS (BioRad Laboratories, Richmond, CA), NP-40 (BDH Chemicals, Ltd., Poole, England),

[1] *Abbreviations used in this paper:* CR1, C3b/C4b receptor; CR2, C3d receptor; CR3, iC3b receptor; FN, fibronectin.

CNBr-activated Sepharose 4 B and protein A–Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) were purchased. All other chemicals were purchased from Sigma Chemical Co., St. Louis, MO.

Antibodies.   YZ-1 is an IgG1 monoclonal anti-CR1 (6). 3G8.10 monoclonal anti–human neutrophil FcR (13) was a gift of Dr. Jay Unkeless, The Rockefeller University, New York. HB-5 is an IgG2a monoclonal anti-CR2 (11). W6/32, an IgG2a mAb, recognizes a common determinant on the HLA-A, -B, and -C heavy chain (14). MMA, a monoclonal IgM that recognizes a determinant on myelomonocytic cells (15), was a gift of Dr. Thomas Tedder, Dana-Farber Cancer Institute, Boston, MA. B1, an IgG2a mAb that recognizes a determinant on human B lymphocytes (16) (Coulter Immunology, Hialeah, FL); OKM1, an IgG2b mAb directed at human CR3 (2); OKT3, an mAb specific for human T cells (17) (Ortho Pharmaceuticals, Raritan, NJ); UPC10, an IgG2a myeloma with specificity for levan; MOPC141, an IgG2b myeloma with no known specificity (Bionetics, Kensington, MD); and FITC-labeled goat F(ab')₂ anti–mouse F(ab')₂ (Jackson ImmunoResearch Labs, Inc., Avondale, PA), were purchased. Antibodies were coupled to CNBr-activated Sepharose 4 B at a ratio of 1–2 mg protein per milliliter gel.

Flow cytometric analysis for membrane protein expression was performed using the Ortho Systems 50 H Cytofluorograf (Ortho Diagnostics, Westwood, MA) as described (6).

Cell Preparations.   Human erythrocytes were obtained from citrated blood after repeated washings and removal of buffy-coat cells. Neutrophils were purified from peripheral blood by dextran sedimentation and centrifugation through Ficoll-Paque (18). Tonsils were obtained after routine tonsillectomy. Single-cell suspensions were prepared, and contaminating neutrophils and erythrocytes were removed by centrifugation through Ficoll-Paque. Adherent cells were depleted during a 1-h incubation at 37°C of tonsil cells in plastic tissue culture plates (Costar, Cambridge, MA). Contaminating macrophages in the final lymphocyte preparation were counted by fluorescent flow cytography of MMA-stained cells. Peripheral blood monocytes were purified (19) by elutriation using a J2-21 centrifuge (Beckman Instruments, Inc., Palo Alto, CA) equipped with a JE-6 elutriator rotor with one standard separation chamber. The purity of the monocytes was >85%, as assessed by MMA-staining. Eosinophils were purified to >98% purity on discontinuous gradients of hypertonic Metrizamide (20). Cells of the human B lymphoblastoid line SB (21) were maintained in RPMI-1640 medium with 10% FBS.

Preparation of ³²PO₄-labeled Cells.   Cells were labeled with ³²P by a modification of a previously described procedure (22). 10⁷ cells/ml were initially depleted of intracellular phosphate by incubation with buffer lacking PO₄⁻³ (30 mM Hepes, pH 7.4, 110 mM NaCl, 10 mM KCl, 10 mM glucose, 1 mM CaCl₂, 1 mM MgCl₂, 2 mM L-glutamine, and 2 mg/ml BSA) for 60 min at 37°C. Cells were resuspended to 5 × 10⁷ cells/ml in buffer with ³²P-labeled phosphoric acid (orthophosphoric acid, 50 mCi/ml in HCl-free water; New England Nuclear, Boston, MA) at 1 mCi/ml for 90 min at 37°C. After labeling, cells were washed twice, resuspended in buffer, and immediately subjected to appropriate stimulation.

Immunoprecipitation and SDS-PAGE Analysis of Membrane Proteins.   Phosphorylation reactions were stopped and cells were lysed by the addition of an equal volume of ice-cold 2× RIPA buffer (2% NP-40, 0.2% SDS, 0.15 M NaCl, 10 mM NaH₂PO₄, 10 mM Na₂HPO₄, 0.68 M sucrose, 5 mM EDTA, 10 mM NaF, 5 mM sodium pyrophosphate, 1 mM sodium vanadate, 1 mM ATP, 10 mM DFP, 2 μM leupeptin, 2 μM peptstatin, 100 μg/ml DNase, 5% FCS, pH 7.2). This lysis buffer was designed to inhibit maximally phosphatase (23) and protease activities, as well as to disrupt intact cytoskeletal components (24) with which membrane proteins may be associated. Cell lysates were incubated on ice for 30 min, and insoluble material was removed by centrifugation at 25,000 g for 20 min at 4°C. Lysates were precleared by incubation overnight with protein A–Sepharose at 4°C. After centrifugation to remove the beads, lysates were sequentially incubated for 90 min with the control antibody UPC10-Sepharose, followed by YZ-1, HB5, 3G8.10, or W6/32-Sepharose to immunoprecipitate CR1, CR2, FcR, or HLA class I heavy chain, respectively. For the immunoprecipitation of CR3, cell lysates were first incubated for 90

min with the nonspecific control antibody, MOPC141 IgG, followed by addition of protein A–Sepharose. After centrifugation to remove the beads, CR3 was immunoprecipitated by sequential incubation with OKM1 IgG and protein A–Sepharose. The beads were washed five times with ice-cold RIPA buffer, and adsorbed proteins were eluted by incubation with 1% SDS for 5 min at 100°C. The eluates were lyophilized, redissolved in sample buffer without SDS, and subjected to SDS-PAGE on 5–15% polyacrylamide slab gels (25). Autoradiographs were prepared by exposing dried gels to XAR X-Omat film (Kodak) with Cronex Xtra Life Intensifying Screens (Dupont) at −70°C for 1–5 d.

Results

Analysis of Phosphorylation of CR1 by Neutrophils, Monocytes, and Eosinophils. To determine if stimulation of neutrophils with PMA was associated with the phosphorylation of CR1, $^{32}PO_4$-labeled neutrophils were prepared from two individuals who differed in their structural CR1 allotypes, having the FF and FS phenotypes, respectively (26, 27). Replicate samples of 5×10^7 cells from each donor were incubated for 2 min at 37°C in 3 ml of buffer alone or containing 100 ng/ml PMA. The reaction was ended and CR1 was immunoprecipitated from the cell lysates and analyzed by SDS-PAGE and autoradiography (Fig. 1). CR1 was not constitutively phosphorylated in neutrophils, despite incubation for >90 min in the presence of $^{32}PO_4$. The addition of PMA led to the phosphorylation of both the F and S allotypes of the receptor.

The kinetics of PMA-induced phosphorylation of CR1 by neutrophils was examined by incubating replicate samples of 5×10^7 cells in 3 ml of buffer alone or with 100 ng/ml PMA at 37°C for 3, 6, 12, and 24 min, respectively. After lysis of the cells, CR1 and the heavy chain of HLA class I, an endogenously phosphorylated protein (28), were immunoprecipitated and analyzed by SDS-PAGE and autoradiography (Fig. 2). Phosphorylation of CR1 was not detectable in the absence of PMA, peaked 3 min after addition of this agent, and declined during the subsequent 21 min of incubation. In contrast, the phosphorylation of the HLA heavy chain was constitutive and only slightly increased after addition of PMA to the neutrophils. As occurred with CR1, detection of HLA phosphorylation was greatly reduced by 24 min. The decline in labeling of these proteins observed in neutrophils may have been secondary to depletion of the intracellular pool of [^{32}P]ATP, because subsequent experiments with B lymphocytes (Fig. 6) demonstrated persistent labeling of HLA and complement receptors.

The chemotactic peptide FMLP has been shown to cause translocation of intracellular CR1 to the plasma membrane (29) and to activate CR1 for phagocytosis in concert with FN (8). Replicate samples of 10^8 $^{32}PO_4$-labeled neutrophils in 6 ml buffer were incubated at 37°C with 10^{-7} M FMLP for 20, 60, and 180 s, respectively. Additional samples of 5×10^7 labeled neutrophils in 3 ml buffer were incubated with 100 ng/ml PMA for the same intervals. The cells were lysed and CR1 was immunoprecipitated and analyzed. FMLP rapidly induced the phosphorylation of CR1, which peaked within 20 s of addition of the peptide, was less at 60 s, and was almost absent at 180 s (Fig. 3). In contrast, PMA-induced phosphorylation of CR1 was undetectable at 20 s, and was greatest at 180 s (Fig. 3). Since twice as many cells were employed for the FMLP stimulation, it is apparent that the maximal extent of CR1 phosphorylation induced by FMLP was substantially less than that caused by PMA.
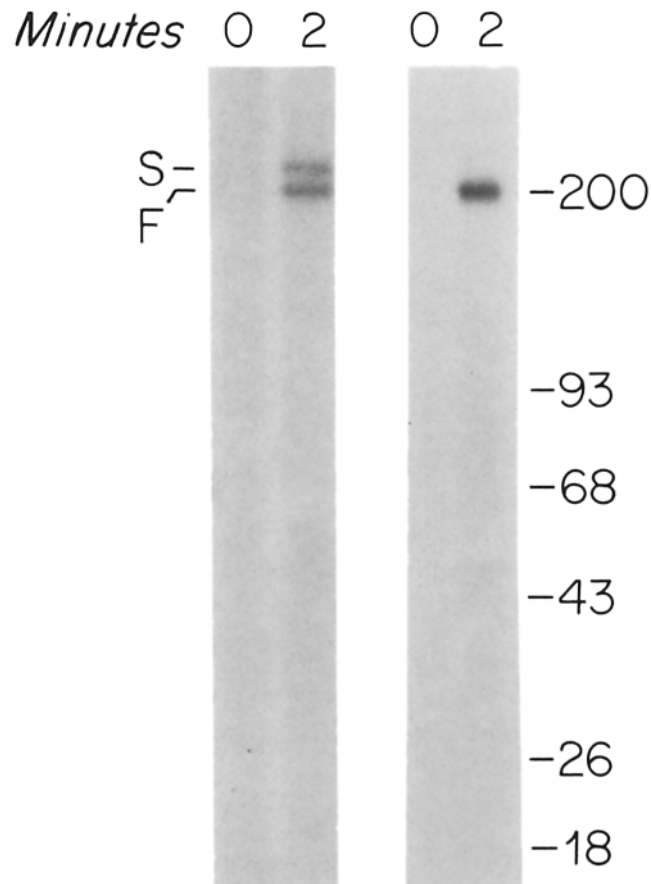
FIGURE 1. PMA-induced phosphorylation of the S and F allotypes of neutrophil CR1. Autoradiograph of SDS-PAGE analysis of CR1 from $^{32}PO_4$-labeled neutrophils that had been treated with buffer alone (0 min) or with 100 ng/ml PMA for 2 min. Neutrophils were from individuals who were heterozygous (*left*) and homozygous (*right*) for the CR1 allotypes. Numbers on the right refer to the positions of standard proteins having the indicated M_r × 10^{-3}.

Two other neutrophil membrane proteins involved in phagocytic reactions, CR3 and the FcR, were also analyzed for phosphorylation induced by PMA. Two replicate samples of 5 × 10^7 $^{32}PO_4$-labeled neutrophils in 3 ml buffer were incubated at 37°C for 3 min in the presence or absence of 100 ng/ml PMA. The detergent lysates were prepared and sequentially immunoprecipitated with UPC10, YZ-1, 3G8.10, and OKM1. The immunoprecipitated proteins were analyzed by SDS-PAGE and autoradiography. In the absence of PMA, the phosphorylation of CR3 and FcR was not apparent, indicating that these membrane proteins differed from HLA class I antigen (28) in not being constitutively phosphorylated (Fig. 4). In addition, these receptors differed from CR1 by exhibiting no PMA-induced phosphorylation (Fig. 4).

The phosphorylation of CR1 by two other phagocytic cell types, monocytes and eosinophils, was examined. Replicate samples of 2.5 × 10^7 monocytes and
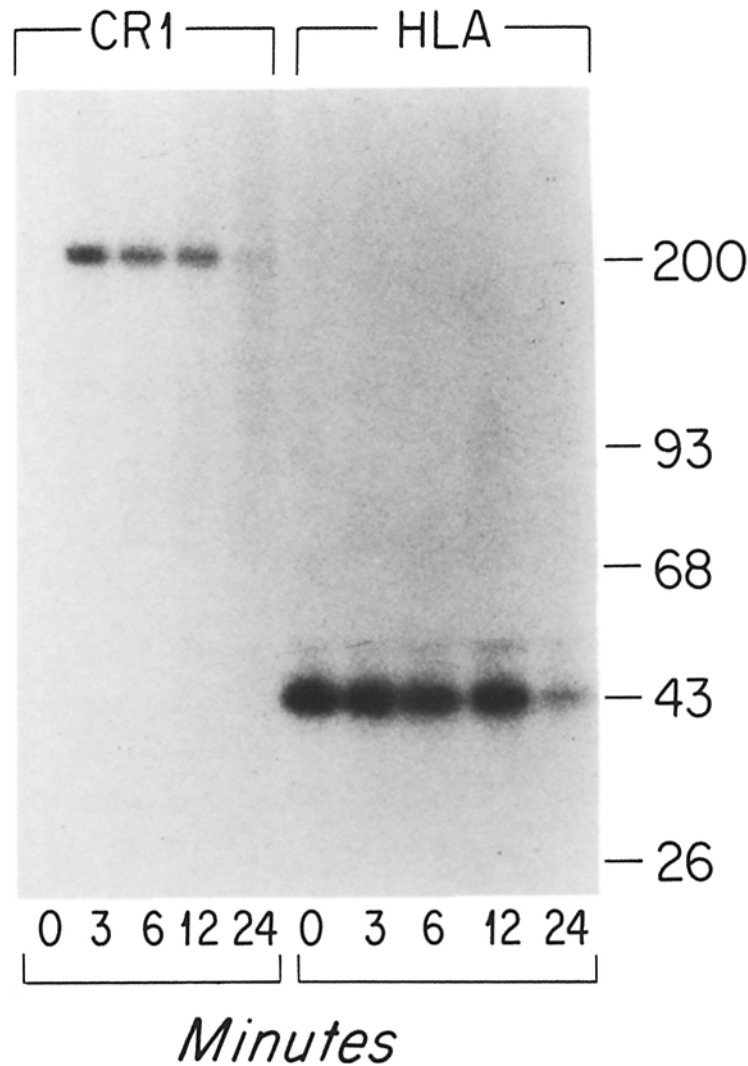
FIGURE 2. Kinetics of PMA-induced phosphorylation by neutrophils of HLA class I heavy chain and CR1. Membrane proteins were immunoprecipitated from $^{32}PO_4$-labeled neutrophils that had been treated with buffer or with 100 ng/ml PMA for timed intervals, and were analyzed by SDS-PAGE and autoradiography. Numbers on the right refer to the positions of standard proteins having the indicated $M_r \times 10^{-3}$.

eosinophils that had been prelabeled with $^{32}PO_4$ were incubated for 3 min at 37°C in 3 ml buffer alone or containing 100 ng/ml PMA. CR1 and CR3 were immunoprecipitated from detergent lysates of the cells and assessed by SDS-PAGE and autoradiography. As was found for neutrophils, CR1 of monocytes was not constitutively phosphorylated but became modified in this manner upon stimulation of the cells with PMA for 3 min (Fig. 5 *left*). CR3 of monocytes was not phosphorylated, constitutively or after stimulation (Fig. 5 *left*). Analysis of CR1 from eosinophils that had been treated for three min with PMA indicated
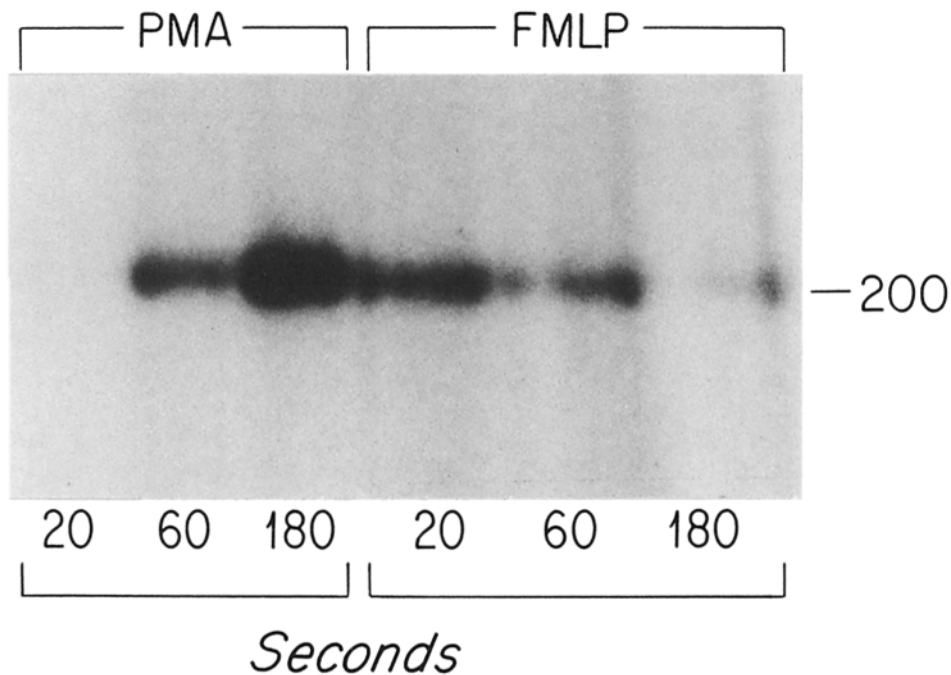
FIGURE 3.   Kinetics of neutrophil CR1 phosphorylation induced by PMA or FMLP. CR1 was immunoprecipitated from 5×10^7 ^{32}PO$_4$-labeled neutrophils that had been treated with 100 ng/ml PMA for 20, 60, or 180 s, or from 10^8 ^{32}PO$_4$-labeled neutrophils treated with 10^{-7} M FMLP for the same timed intervals, and were analyzed by SDS-PAGE and autoradiography. The number on the right refers to the position of a standard protein having the indicated M_r $\times 10^{-3}$.

that the receptor on this third phagocytic cell type was also capable of being phosphorylated (Fig. 5 *right*).

Analysis of Phosphorylation of CR1 and CR2 by B Lymphocytes.   To determine if phosphorylation of CR1 was restricted to phagocytic cell types, tonsilar cells were examined for this reaction. In addition, because these cells also express CR2, the C3d/EBV receptor, this membrane protein was also assessed for phosphorylation. Although the B lymphocyte would be the major source of CR1 in this cell population, two other potential sources are present, T lymphocytes and macrophages. Only 10% of T lymphocytes express CR1 (30), and the number of receptors present on these cells is <10% that present on B lymphocytes. Therefore, T lymphocytes were not removed from the preparation of cells assessed for phosphorylation of CR1. Conversely, macrophages express large amounts of CR1 and three methods were used to effect their removal from the tonsilar cells. Each method, adherence to plastic culture dishes, adherence to Sephadex G-10, or elutriation, reduced the proportion of MMA$^+$ macrophages by 50% indicating that it was not possible to totally remove this cell type. Therefore, replicate samples of 5×10^7 ^{32}PO$_4$-labeled tonsilar cells, which had been depleted of plastic-adherent cells and which were comprised of 45% B1$^+$ lymphocytes, 48% T3$^+$ lymphocytes, and 5% MMA$^+$ macrophages, were incubated at 37°C in 3 ml of buffer alone or in buffer containing 100 ng/ml PMA for 2 or 20 min. CR1,
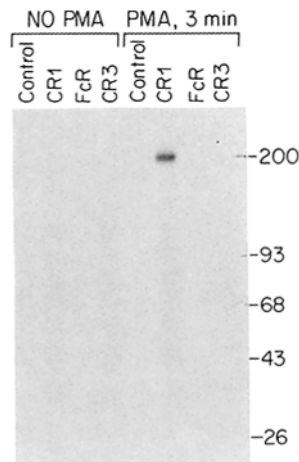
FIGURE 4. Analysis of CR1, FcR, and CR3 for PMA-induced phosphorylation by neutrophils. Membrane proteins were immunoprecipitated from $^{32}PO_4$-labeled neutrophils that had been treated with buffer or with 100 ng/ml PMA for 3 min, and were analyzed by SDS-PAGE and autoradiography. Control refers to the immunoprecipitate obtained with monoclonal UPC10 antilevan. Numbers on the right refer to the positions of standard proteins having the indicated $M_r \times 10^{-3}$.
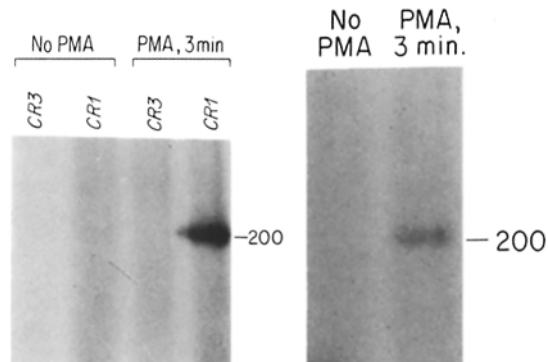

FIGURE 5. Analysis of PMA-induced phosphorylation of CR1 and CR3 in monocytes (left) and of CR1 in eosinophils (right). Membrane proteins were immunoprecipitated from 2.5 × 10^7 $^{32}PO_4$-labeled monocytes and eosinophils that had been treated with buffer or with 100 ng/ml PMA for 3 min, and were analyzed by SDS-PAGE and autoradiography. Numbers on the right refer to the positions of standard proteins having the indicated $M_r \times 10^{-3}$.

CR2, and HLA class I were immunoprecipitated from each cell lysate, and analyzed by SDS-PAGE and autoradiography. Phosphorylation of CR1 was undetectable at t_0 and, although detectable at 2 and 20 min following stimulation with PMA (Fig. 6), it was markedly less than that observed with the phagocytic cell types (Figs. 1–5). In contrast, CR2, which also was not constitutively phosphorylated, became intensely labeled following B lymphocyte activation by PMA (Fig. 6). Thus, CR2, rather than CR1, is the predominant phosphorylated complement receptor of the B lymphocyte. Further, CR2, HLA, and CR1 all showed persistent labeling at 20 min, suggesting the intracellular ^{32}P-labeled
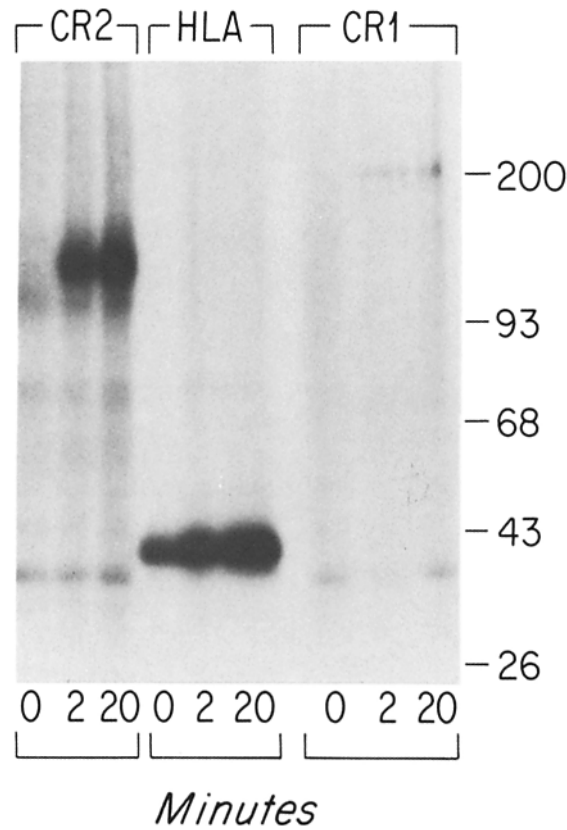
FIGURE 6.   Analysis of PMA-induced phosphorylation of CR1, CR2, and HLA class I heavy chain in tonsillar cells. Membrane proteins were immunoprecipitated from 5×10^7 ^{32}PO$_4$-labeled tonsil cells that had been treated with buffer or with 100 ng/ml PMA for 2 or 20 min, and were analyzed by SDS-PAGE and autoradiography. Numbers on the right refer to the positions of standard proteins having the indicated $M_r \times 10^{-3}$.

ATP pool had not been depleted in this cell population, as had probably occurred in neutrophils (Fig. 2).

To characterize further the potential of B lymphocyte CR1 for phosphorylation after activation of protein kinase C, the human B lymphoblastoid line, SB, was examined. Since monocytes and neutrophils express ~50,000 CR1 molecules per cell, and SB cells express only 5,000 (P. S. Changelian and D. T. Fearon, unpublished observations), it was necessary to use 10 times as many SB cells for a comparable analysis. Replicate samples of 2×10^8 ^{32}PO$_4$-labeled SB cells were incubated in 15 ml buffer alone or with 100 ng/ml PMA for 2 min at 37°C. Replicate samples of 2×10^7 ^{32}PO$_4$-labeled monocytes in 1.5 ml buffer were identically treated. After lysis, CR1 and CR2 from SB cells and CR1 from monocytes were immunoprecipitated, and the eluted proteins analyzed by SDS-PAGE and autoradiography (Fig. 7). Although phosphorylation of monocyte CR1 was readily detectable following stimulation with PMA for 2 min, an equivalent number of CR1 molecules from SB cells exhibited no phosphorylation. In contrast, CR2 from SB cells became intensely phosphorylated after PMA
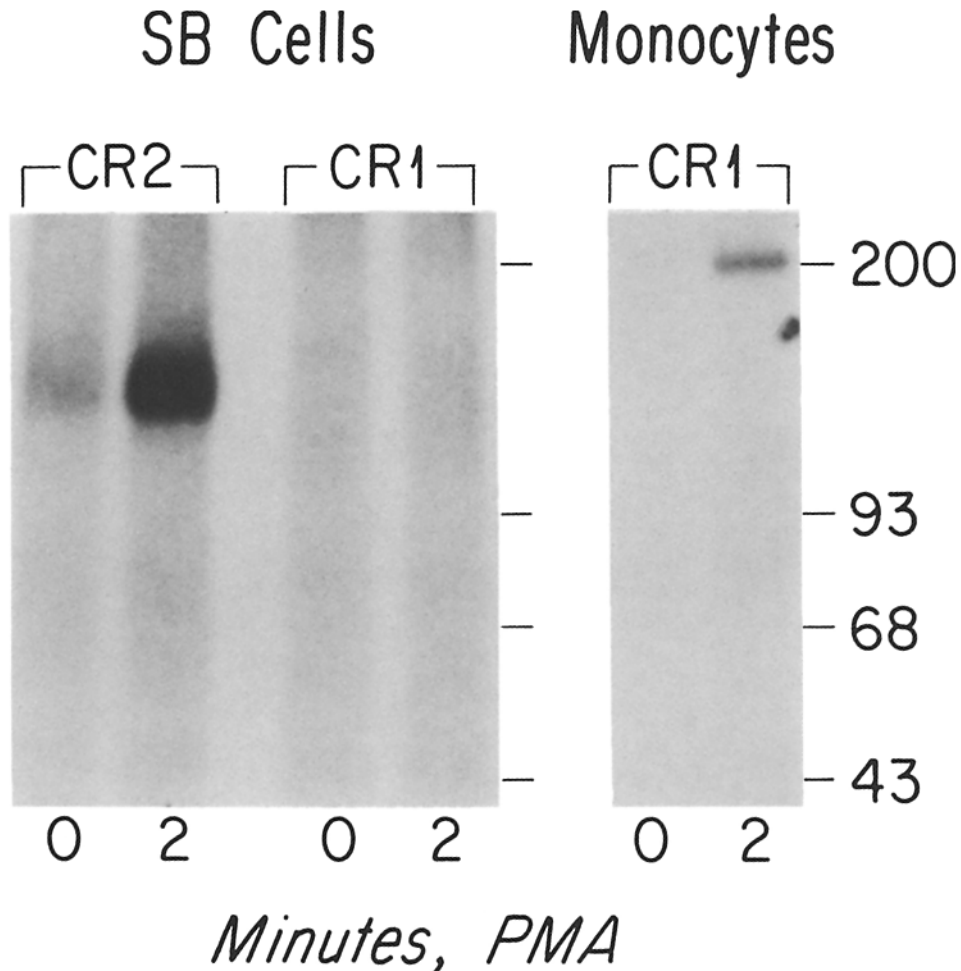
FIGURE 7.   Analysis of PMA-induced phosphorylation of CR1 and CR2 in SB cells and monocytes. Membrane proteins were immunoprecipitated from 2×10^8 $^{32}PO_4$-labeled SB cells or 2×10^7 $^{32}PO_4$-labeled monocytes that had been treated with buffer or with 100 ng/ml PMA for 2 min, and were analyzed by SDS-PAGE and autoradiography. Numbers on the right refer to the positions of standard proteins having the indicated $M_r \times 10^{-3}$.

stimulation, analogous to the findings in normal B lymphocytes (Fig. 6). Buffer-treated SB cells displayed low levels of CR2 phosphorylation, which is in contrast to the lack of constitutive phosphorylation of CR2 found in tonsilar B lymphocytes (Fig. 6). However, the immunoprecipitates from SB cells contained ~15-fold greater numbers of CR2 molecules than were present in the immunoprecipitates from tonsilar cells, perhaps accounting for detection of constitutive phosphorylation.

To determine whether PMA can induce phosphorylation of CR1 on another nonphagocytic cell type, replicate samples of 5×10^9 $^{32}PO_4$-labeled erythrocytes were incubated at 37°C in 3 ml buffer alone or containing 100 ng/ml PMA for 2 or 20 min. 100-fold more erythrocytes were used because this cell type expresses only 500 receptors/cell, whereas myelomonocytic cells and B lympho-
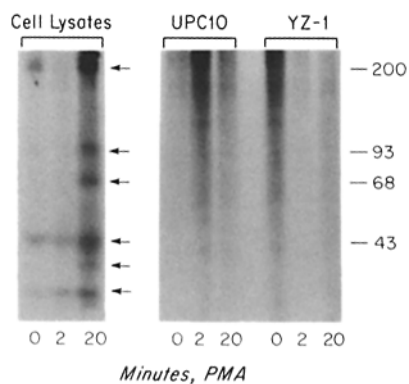
FIGURE 8.   Analysis of PMA-induced phosphorylation of CR1 in erythrocytes. Autoradi-
ograph of SDS-PAGE analyses of whole cell lysates or immunoprecipitates obtained with
UPC10 antilevan or YZ-1 anti-CR1 from 2×10^9 $^{32}PO_4$-labeled erythrocytes incubated in
buffer alone or containing 100 ng/ml PMA for 2 or 20 min. Arrows indicate erythrocyte
proteins that displayed increased phosphorylation after treatment with PMA. Numbers on the
right refer to the positions of standard proteins having the indicated $M_r \times 10^{-3}$.

cytes express 50,000 receptors/cell. Although PMA increased the phosphoryla-
tion of several erythrocyte proteins, phosphorylation of CR1 was not detected
(Fig. 8).

Discussion

The transition of CR1 on myelomonocytic cells from the resting to the
activated state, in which it can mediate phagocytosis of C3b-coated particles, can
be induced by a variety of agents. These agents, such as T cell–derived lympho-
kines (3), phorbol esters (4, 5), and extracellular matrix proteins (5, 7–9), have
pleiotropic effects on the cells they activate. A biochemical mechanism correlating
this cellular activation to the activation of CR1 has not been found. In this study
we have shown PMA-inducibility of CR1 phosphorylation that is specific for cells
of the myelomonocyte lineage, and phosphorylation of CR2 rather than of CR1
by B lymphocytes.

Phosphorylation is an important mechanism for regulation of cellular protein
function, examples being the enzymes involved in glycogen metabolism (31) and
the contractile proteins of muscle cells (32). A general role for phosphorylation
in the activation of human neutrophils has been suggested by studies (22, 33)
demonstrating enhanced phosphorylation of four to six proteins in cells stimu-
lated with PMA or FMLP. A protein of M_r 44,000, normally phosphorylated
after PMA induction, is absent in cells from patients with chronic granulomatous
disease (34). These human phosphoproteins, however, have not been identified,
in contrast to studies of rabbit neutrophils activated with FMLP, in which
enhanced phosphorylation of vimentin (35) and myosin light chain (36) was
shown. Thus, the phosphorylation of a specific protein in human neutrophils in
association with a change in that protein's function has not been demonstrated.

The evidence suggesting that phosphorylation of CR1 by stimulated myelo-
monocytic cells is the structural basis for the activated state of the receptor is
correlative. As has been found for phagocytosis by CR1, phosphorylation of the

receptor did not occur constitutively in any cell type. Incubation of neutrophils, monocytes, and eosinophils in the presence of $^{32}PO_4$ for up to 90 min was not associated with phosphorylation of CR1, despite intense labeling of HLA class I heavy chain (Figs. 1–5). The absence of phosphorylation of CR1 by unstimulated cells also is in contrast to the constitutive phosphorylation of other membrane proteins present on these cell types, such as the transferrin receptor (37) and the leukocyte common antigen (T200) (38). Therefore, the induction of phosphorylation of CR1 on myelomonocytic cells by PMA correlates with the acquisition of phagocytic function by CR1. The induction of CR1 phosphorylation by FMLP supports this conclusion because this chemotactic peptide also confers phagocytic activity on CR1 in the presence of FN (8). The more rapid kinetics of FMLP-induced phosphorylation is caused by the peptide inducing a rapid turnover of inositol phospholipids (39), with transient activation of protein kinase C by diacylglycerol. Phosphorylation of CR1 by PMA-treated monocytes and neutrophils was selective, with CR3 and FcR not being subject to this form of covalent modification. We do not have an explanation for the absence of phosphorylation of CR3, which has also been reported to be functionally activated by PMA (4).

The restriction of phosphorylation of CR1 to myelomonocytic cells is perhaps the most interesting finding in support of a causal relationship between phosphorylation of CR1 and phagocytosis. All three phagocytic cell types, neutrophils, monocytes, and eosinophils, were capable of phosphorylating CR1 (Figs. 1–5), whereas two nonphagocytic cell types, erythrocytes and normal and transformed B lymphocytes (Figs. 6–8), were not. This tissue-specificity of CR1 phosphorylation may be secondary to structural differences between receptors of different cell types. For example, minor differences have been detected in the glycosylation of CR1 on different cell types that do not, however, correlate with the occurrence of phosphorylation (40). Differences in the cytoplasmic domain of CR1 would be a more likely mechanism accounting for the tissue-specificity of CR1 phosphorylation. These differences might occur through alternative splicing of the CR1 primary transcript, by analogy to the generation of membrane and secreted forms of immunoglobulin (41), or by the presence of multiple CR1 genes. The latter possibility is unlikely because CR1 allotypes present on myelomonocytic cells are the same as those found on lymphocytes and erythrocytes (26, 42). Analysis of potential cell type–dependent variations in the cytoplasmic domain of the receptor will now be possible based on the recent cloning of a partial cDNA for CR1 (43). Two additional mechanisms that might account for the tissue-specific phosphorylation of CR1 are: different cytoplasmic environments regulating access of the protein kinase to CR1, and tissue-specificity of the kinase responsible for phosphorylation of CR1. Although PMA is known to activate protein kinase C, it cannot be concluded that protein kinase C phosphorylates CR1. For example, other studies (44) have shown that phosphorylation of ribosomal protein S6 in 3T3-L1 cells treated with PMA was mediated by S6 kinase rather than by protein kinase C. Whatever mechanism is involved in CR1 phosphorylation, its restriction to cell types capable of engaging in phagocytosis suggests that this covalent modification of the receptor is related to its activation.

Although PMA-stimulated B lymphocytes did not mediate the phosphorylation of CR1, CR2 was phosphorylated by these cells. This contrast may indicate that

CR2, by analogy to CR1 of myelomonocytic cells, has resting and activated states, the latter caused by covalent modification by phosphorylation. The recent reports that simulation of B lymphocytes with antibody to CR2 requires the presence of T lymphocytes or soluble factors elaborated by T cells (45–47) would be consistent with a two-step process of B cell activation by this complement receptor. As CR2 also serves as the B cell receptor for EBV (12), it will be of interest to determine the effects of this herpesvirus on phosphorylation of CR2.

Summary

CR1 of neutrophils and monocytes may exist in a resting state, in which it only binds ligand-coated particles, or an activated state, in which it mediates phagocytosis. Because the activated state of CR1 can be induced by the stimulation of protein kinase C with PMA, CR1 was assessed for phosphorylation. Purified human neutrophils, monocytes, eosinophils, tonsilar lymphocytes, SB cells, and erythrocytes were labeled with $^{32}PO_4$ and incubated with buffer or 100 ng/ml PMA. Membrane proteins were immunoprecipitated and analyzed by SDS-PAGE and autoradiography. CR1, unlike HLA class I heavy chain, was not constitutively phosphorylated by any cell type. PMA induced phosphorylation of CR1 in three phagocytic cell types, but did not induce the phosphorylation of CR3 or FcR. FMLP also induced the phosphorylation of CR1 in neutrophils. In contrast, PMA did not induce phosphorylation of CR1 in tonsilar B lymphocytes, SB cells, or erythrocytes, indicating restriction of this reaction to phagocytic cell types. This may be due to differences in the structure or presentation of the cytoplasmic domain of CR1 in phagocytic vs. nonphagocytic cells. Phosphorylation of CR2, however, did occur in PMA-treated B lymphocytes and SB cells, suggesting that this receptor, rather than CR1, may be involved in regulation of B lymphocyte function.

References

1. Fearon, D. T. 1979. Identification of the membrane glycoprotein that is the C3b receptor of the human erythrocyte, polymorphonuclear leukocyte, B lymphocyte and monocyte. *J. Exp. Med.* 152:20.
2. Wright, S. D., P. E. Rao, W. C. Van Voorhis, L. S. Craigmyle, K. Iiade, M. A. Talle, E. F. Westberg, G. Goldstein, and S. C. Silverstein. 1983. Identification of the C3bi receptor of human monocytes and macrophages by using monoclonal antibodies. *Proc. Natl. Acad. Sci. USA.* 80:5699.
3. Griffin, J. A., and F. M. Griffin. 1979. Augmentation of macrophage complement receptor function in vitro. I. Characterization of the cellular interaction required for the generation of a T-lymphocyte product that enhances macrophage complement receptor function. *J. Exp. Med.* 150:653.
4. Wright, S. D., and S. C. Silverstein. 1982. Tumor-promoting phorbol esters stimulate C3b and C3b' receptor-mediated phagocytosis in cultured human monocytes. *J. Exp. Med.* 156:1149.
5. Wright, S. D., L. S. Craigmyle, and S. C. Silverstein. 1983. Fibronectin and serum

amyloid P component stimulate C3b- and C3bi-mediated phagocytosis in cultured human monocytes. *J. Exp. Med.* 158:1338.

6. Changelian, P. S., R. M. Jack, L. A. Collins, and D. T. Fearon. 1985. PMA induces the ligand-independent internalization of CR1 on human neutrophils. *J. Immunol.* 134:1851.

7. Pommier, C. G., S. Inada, L. F. Fries, T. Takahashi, M. M. Frank, and E. J. Brown. 1983. Plasma fibronectin enhances phagocytosis of opsonized particles by human peripheral blood monocytes. *J. Exp. Med.* 157:1844.

8. Pommier, C. G., J. O'Shea, T. Chused, K. Yancey, M. M. Frank, T. Takahashi, and E. Brown. 1984. Studies on the fibronectin receptors on human peripheral blood leukocytes. *J. Exp. Med.* 159:137.

9. Bohnsack, J. F., H. K. Kleinman, T. Takahashi, J. O'Shea, and E. J. Brown. 1985. Connective tissue proteins and phagocytic cell function. Laminin enhances complement and Fc-mediated phagocytosis by cultured human macrophages. *J. Exp. Med.* 161:912.

10. Castagna, M., Y. Takai, K. Kaibuchi, K. Sano, U. Kikkawa, and Y. Nishizuka. 1982. Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J. Biol. Chem.* 257:7847.

11. Weis, J. J., T. F. Tedder, and D. T. Fearon. 1984. Identification of a 145,000 M_r protein as the C3d receptor (CR2) of human B lymphocytes. *Proc. Natl. Acad. Sci. USA.* 81:881.

12. Fingeroth, J. D., J. J. Weis, T. F. Tedder, J. L. Strominger, P. A. Biro, and D. T. Fearon. 1984. Epstein-Barr virus receptor of human B-lymphocytes is the C3d receptor CR2. *Proc. Natl. Acad. Sci. USA.* 81:4510.

13. Fleit, H. B., S. D. Wright, and J. C. Unkeless. 1982. Human neutrophil Fc-gamma receptor distribution and structure. *Proc. Natl. Acad. Sci. USA.* 79:3275.

14. Barnstable, C. J., W. F. Bodmer, G. Brown, G. Galfre, C. Milstein, A. F. Williams, and A. Ziegler. 1978. Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell surface antigens—new tools for genetic analysis. *Cell.* 14:9.

15. Hanjan, S. N. S., J. F. Kearney, and M. D. Cooper. 1982. A monoclonal antibody (MMA) that identifies a differentiation antigen on human myelomonocytic cells. *Clin. Immunol. Immunopathol.* 23:172.

16. Stashenko, P., L. M. Nadler, R. Hardy, and S. F. Schlossman. 1980. Characterization of a human B lymphocyte specific antigen. *J. Immunol.* 125:1678.

17. Kung, P. C., G. Goldstein, E. L. Reinherz, and S. F. Schlossman. 1979. Monoclonal antibodies defining distinctive human T cell surface antigens. *Science (Wash. DC).* 206:347.

18. Boyum, A. 1968. Isolation of leukocytes from human blood: further observations. *Scand. J. Clin. Lab. Invest.* 21:759.

19. Lionetti, F. J., S. M. Hunt, and C. R. Valeri. 1980. Isolation of human blood phagocytes by countercurrent centrifugation elutriation. *In* Methods of Cell Separation. N. Castimpoolas, editor. Plenum Publishing Corp., New York. 141.

20. Vadas, M. A., J. R. David, A. Butterworth, N. T. Pisani, and T. A. Siongok. 1979. A new method for the purification of human eosinophils and neutrophils, and a comparison of the ability of these cells to damage schistosomula of *Schistosoma mansoni*. *J. Immunol.* 122:1228.

21. Royston, I., R. W. Smith, D. N. Buell, E. S. Huang, and J. S. Pagano. 1974. Autologous human B and T lymphoblastoid cell lines. *Nature (Lond.).* 251:745.

22. Schneider, C., M. Zanetti, and D. Romeo. 1981. Surface-reactive stimuli selectively

increase protein phosphorylation in human neutrophils. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 127:4.

23. Swarup, G., K. V. Speeg, S. Cohen, and D. L. Garbers. 1982. Phosphotyrosyl-protein phosphatases of TCRC-2 Cells. *J. Biol. Chem.* 257:7298.

24. Hartwig, J. H., and T. P. Stossel. 1974. Isolation and properties of actin, myosin and a new actin-binding protein in rabbit alveolar macrophages. *J. Biol. Chem.* 250:5696.

25. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680.

26. Wong, W. W., J. G. Wilson, and D. T. Fearon. 1983. Genetic regulation of a structural polymorphism of human C3b receptor. *J. Clin. Invest.* 72:685.

27. Dykman, T. R., J. L. Cole, K. Iida, and J. P. Atkinson. 1983. Polymorphism of the human erythrocyte C3b/C4b receptor. *Proc. Natl. Acad. Sci. USA.* 80:1698.

28. Pober, J. S., B. C. Guild, and J. L. Strominger. 1978. Phosphorylation in vivo and in vitro of human histocompatibility antigens (HLA-A and HLA-B) on the carboxy-terminal intracellular domain. *Proc. Natl. Acad. Sci. USA.* 75:6002.

29. Fearon, D. T., and L. A. Collins. 1983. Increased expression of C3b receptors on polymorphonuclear leucocytes induced by chemotactic factors and by purification procedures. *J. Immunol.* 130:370.

30. Wilson, J. G., T. F. Tedder, and D. T. Fearon. 1983. Characterization of human T lymphocytes that express the C3b receptor. *J. Immunol.* 131:684.

31. Cohen, P. 1982. The role of protein phosphorylation in neuronal and hormonal control of cellular activity. *Nature (Lond.).* 296:613.

32. Adelstein, R. S. 1983. Regulation of contractile proteins by phosphorylation. *J. Clin. Invest.* 72:1863.

33. Andrews, P., and B. Babior. 1983. Endogenous protein phosphorylation by resting and activated human neutrophils. *Blood.* 61:333.

34. Segal, A. W., P. G. Heyworth, S. Cockcroft, and M. M. Barrowman. 1985. Stimulated neutrophils from patients with autosomal recessive chronic granulomatous disease fail to phosphorylate a M_r-44,000 protein. *Nature (Lond.).* 316:547.

35. Huang, C., J. M. Hill, B. Bormann, W. M. Macklin, and E. L. Becker. 1984. Chemotactic factors induced vimentin phosphorylation in rabbit peritoneal macrophages. *J. Biol. Chem.* 259:1386.

36. Fechheimer, M., and S. H. Zigmond. 1983. Changes in cytoskeletal proteins of neutrophils induced by chemotactic peptides. *Cell Motil.* 3:349.

37. May, W. S., S. Jacobs, and P. Cuatrecasas. 1984. Association of phorbol ester-induced hyperphosphorylation and reversible regulation of transferrin membrane receptors in HL60 cells. *Proc. Natl. Acad. Sci. USA.* 81:2016.

38. Omary, M. B., and I. S. Trowbridge. 1980. Disposition of T200 glycoprotein in the plasma membrane of a murine lymphoma cell line. *J. Biol. Chem.* 255:1662.

39. Serhan, C. N., M. J. Broekman, H. M. Korchak, J. E. Smolen, A. J. Marcus, and G. Weissmann. 1983. Changes in phosphatidylinositol and phosphatidic acid in stimulated human neutrophils. *Biochim. Biophys. Acta.* 762:420.

40. Lublin, D. M., E. A. Jones, R. Griffith, and J. P. Atkinson. 1985. Carbohydrate structure of human CR1. *Fed. Proc.* 44:987 (Abstr.).

41. Early, P., J. Rogers, M. Davis, K. Calame, M. Bond, R. Wall, and L. Hood. 1980. Two mRNAs can be produced from a single immunoglobulin mu gene by alternative RNA processing pathways. *Cell.* 20:313.

42. Dykman, T. R., J. L. Cole, K. Iiada, and J. P. Atkinson. 1983. Structural heterogeneity of the C3b/C4b receptor (CR1) on human peripheral blood cells. *J. Exp. Med.* 157:2160.

43. Wong, W. W., L. B. Klickstein, J. A. Smith, J. H. Weis, and D. T. Fearon. 1985.

Identification of a partial cDNA clone for the human C3b/C4b receptor. *Proc. Natl. Acad. Sci. USA.* In press.

44. Tabarini, D., J. Heinrich, and O. M. Rosen. 1985. Activation of S6 kinase activity in 3T3-L1 cells by insulin and phorbol ester. *Proc. Natl. Acad. Sci. USA.* 82:4369.

45. Nemerow, G. R., R. Wolfert, and N. R. Cooper. 1984. Monoclonal antibody to the Epstein-Barr virus receptor blocks virus binding, inhibits complement receptor (C3d/CR2) function, and triggers B-lymphocyte activation. *Complement.* 1:163 (Abstr.).

46. Frade, R., M. C. Crevon, M. Barel, A. Vazquez, L. Krikorian, C. Charriaut, and P. Galanaud. 1985. Enhancement of human B cell proliferation by an antibody to the C3d receptor, the gp140 molecule. *Eur. J. Immunol.* 15:73.

47. Wilson, B. S., J. L. Platt, and N. E. Kay. 1985. Stimulation of B cell DNA synthesis by monoclonal antibodies to the CR2 (C3d) complement fragment receptor. *Fed. Proc.* 44:1876 (Abstr.).