Complement Receptor Type 1 (CD35) Mediates Inhibitory Signals in Human B Lymphocytes[1]

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The complement system—particularly component C3—has been demonstrated to be a key link between innate and adaptive immunity. The trimolecular complex of complement receptor type 2 (CR2), CD19, and CD81 is known to promote B cell activation when coligated with the B cell Ag receptor. In the present study, we aimed to elucidate the role of human complement receptor type 1 (CR1), the other C3-receptor on B cells. As ligand, aggregated C3 and aggregated C3(H_2O), i.e., multimeric "C3b-like C3", are used, which bind to CR1, but not to CR2. In experiments studying the functional consequences of CR1-clustering, the multimeric ligand is shown to inhibit the proliferation of tonsil B cells activated with a suboptimal dose of anti-IgM F(ab′)$_2$. Importantly, this inhibitory activity also occurs in the presence of the costimulatory cytokines IL-2 and IL-15. The anti-IgM-induced transient increase in the concentration of intracellular free Ca^{2+} and phosphorylation of several cytoplasmic proteins are strongly reduced in the presence of the CR1 ligand. Data presented indicate that CR1 has a negative regulatory role in the B cell Ag receptor mediated activation of human B lymphocytes. *The Journal of Immunology,* 2002, 168: 2782–2788.

I n recent years, several links have been revealed between innate and adaptive immunity (1, 2). One of the most important elements involved in such interactions is the complement system (3, 4). Several lines of evidence prove the role of the activation fragments of the third component (C3) and their receptors, namely complement receptor type 1 (CR1)[3] and type 2 (CR2), in directing and regulating adaptive immune responses. In mice, C3 has been shown indispensable to the development of an effective Ab response (5) and CR1/2-specific Abs were demonstrated to inhibit Ab production by B cells (6). In the germinal centers, follicular dendritic cells expressing CR1/2 can localize complement-coated Ags on their surface, thus, providing a positive signal for B cell survival and generation of memory B cells (7–9). Ag-bound C3d, the final cleavage product of C3 has been described as a molecular adjuvant which lowers the threshold of B cell activation by cross-linking CR2 to surface Ig (sIg) (10). A further link between innate and adaptive immunity was provided by experiments demonstrating that C3-deposition on APC enhances the proliferative response of Ag-specific naive T lymphocytes expressing CR1/2 (11). However, it is important to note that not all the results obtained from experiments with mice are applicable to the human system. Although mouse CR1 and CR2 are alternatively spliced gene products, in humans two different genes encode these cell membrane molecules, which differ not only in their extracellular domains, but in their intracellular part as well. Therefore, some

roles of human CR1 may exist that cannot be studied in mouse models.

Human B lymphocytes bear both CR1 and CR2 molecules. The C3d receptor, CR2 (CD21), appears as a member of a signaling complex (CR2/CD19/CD81) that transduces a positive activation signal upon coligation with surface IgM (sIgM) (9, 10, 12). A CR1/CR2 complex has also been described (13) whose role is yet unclear. A B cell Ag-receptor complex (BCR)-independent function of CRs is their participation in Ag uptake and Ag presentation (14–16). Using transfected fibroblasts, a cooperation between human CR1 and CR2 in internalization of ligands has been reported (17).

Whereas the role of human CR2 in B cell activation is relatively well-established, much less is known about the exact function of CR1 (CD35). CR1 is a single-chain glycoprotein, which exists in four allotypes, the most common of them having a molecular mass of ~220 kDa and consisting of 30 short consensus repeats. It binds the C3b fragment of C3 and, with lower affinity, iC3b and C4b (18). CR1 is long known to act as a cofactor for the factor I-mediated cleavage of C3 (19), and recently has also been described to interact with C1q (20) and mannose-binding lectin (21). Its role in B cell activation mediated by ligand-induced cross-linking, however, is controversial.

Daha et al. (22) reported that clustering CR1 with F(ab′)$_2$ anti-CR1 augments Ab production of human peripheral B cells stimulated with suboptimal amounts of PWM in the presence of T cells, while the natural ligand, monomeric C3b, was ineffective under the same conditions. Weiss et al. (23) demonstrated that Abs to CR1 enhance the differentiation of Ag-activated B cells in the presence of T cell-derived factors. However, in a different study, CR1-specific Abs did not influence the plasma cell formation induced by PWM (24). The inhibition of PWM-induced Ig production by C3b was also demonstrated (25, 26). As far as early signaling events are concerned, anti-CR1 Abs did not influence the anti-μ-induced change in the intracellular free Ca^{2+} level (27, 28). Regarding proliferation of human B cells, data are controversial as well. An inhibitory effect of CR1-sIg cross-linking was shown on peripheral blood B cells (28) and on resting splenic B cells (29), while others reported no role of CR1 in the anti-μ-induced B cell proliferation (23, 24, 27). The controversy found in literature regarding the

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function of CR1 on human B cells is most probably due to the different experimental conditions, the use of mixed cell populations and Abs that react with various epitopes of CR1.

In the present study, we examined the effect of the natural ligand. We show that aggregated C3 and aggregated $C3(H_2O)$, i.e., "C3b-like C3", which mimic multimeric C3b and bind to CR1, strongly and dose-dependently inhibit the anti-IgM-induced proliferation of human B cells, even in the presence of the costimulatory cytokines IL-2 and IL-15. Parallel to this, the anti-IgM-induced transient increase of intracellular free Ca^{2+} level and phosphorylation of tyrosine residues of several cytoplasmic proteins are also inhibited by multimeric C3. Data presented indicate that CR1 expressed by human B lymphocytes mediates inhibitory signals, thus plays an opposite role to CR2 in the regulation of B cell activation.

Materials and Methods

Cell preparation and culture conditions

Tonsils from children undergoing routine tonsillectomy were passed through a sterile wire mesh, and mononuclear cells were isolated by centrifugation over Ficoll-Hypaque solution (Pharmacia Biotech, Uppsala, Sweden). After rosetting with 2-aminoethylisothiouronium bromide-treated sheep RBC, the remaining cell suspension was treated with 5 mM L-leucine methyl ester (Sigma Aldrich, Budapest, Hungary) for depletion of phagocytic cells. Over 97% of the cells obtained were $CD19^+$ with no detectable $CD3^+$ and $CD14^+$ cells in the suspension. In some experiments, B cells were further fractionated on a Percoll (Pharmacia Biotech) gradient to yield low- and high-density populations.

Tonsillar B lymphocytes were cultured at 1×10^5 cells/well in 200 μl serum-free X-VIVO 10 medium (BioWhittaker, Walkersville, MD) in flat-bottom, 96-well microtiter plates (Costar, Zenon, Hungary) at 37°C in a humidified atmosphere containing 5% CO_2. Cells were stimulated with 3 μg/ml $F(ab')_2$ of goat anti-human IgM (Fc5μ) (Axell, Westbury, NY) and cultured in the presence of various amounts of $C3(H_2O)$, heat-aggregated C3, or C3d. To abrogate the effect of C3, $F(ab')_2$ of goat anti-human C3 (Cappel, Turnhout, Belgium) was added to parallel cultures at 25 μg/ml. Recombinant human IL-2 and IL-15 (Genzyme, Cambridge, MA) were added at 100 U/ml after 24 h. Cells were pulsed with 1 μCi/well [^{3}H]thymidine (NEN, Boston, MA) for the last 16 h of culture. Incorporated radioactivity was measured after 72 h using a Wallac 1409 liquid scintillation beta counter (Wallac, Allerod, Denmark). The results are expressed as mean cpm ± SD of triplicate samples.

Isolation of human C3 and recombinant human C3d; generation of C3b-like C3

Human C3 was isolated from fresh serum by fast protein liquid chromatography (FPLC) as described by Basta and Hammer (30). Purified C3 was concentrated, dialysed against PBS, followed by incubation with protein G beads (Pharmacia Biotech) to minimize the amount of contaminating IgG. The remaining IgG content was <1%, as assessed by ELISA. The purity of the C3 preparation was assessed by SDS-PAGE and Coomassie blue staining. Aliquots were stored at −20°C and aggregated at 63°C for 20 min before use. $C3(H_2O)$, i.e., C3b-like C3 was generated by repeated freezing and thawing of the C3 preparation (31). For binding experiments, C3 was conjugated with biotin (Zymed Laboratories, San Francisco, CA) according to the method provided by the manufacturer.

pET15b-C3d(C1010A) oligoHis⁻ plasmid encoding the C3d fragment of human C3 was kindly provided by D. E. Isenman (University of Toronto, Toronto, Canada). Protein expression was induced in transformed *Escherichia coli* strain BL21(DE3) as described (32). C3d was isolated from inclusion bodies after extensive sonication, then solubilized, oxidized, and renatured following the method of Kurucz et al. (33). C3d was further purified on a Mono Q HR 5/5 FPLC column (Pharmacia Biotech) at pH 8.3. Purity was assessed on a 12% SDS-polyacrylamide gel. Heat aggregation was done as described for C3.

Any possible toxic effect of various C3 preparations had been excluded by assessing the viability of treated cells, using trypan blue staining for microscopical and propidium iodide staining in FACS analysis.

ELISA

Duplicate wells of microtiter plates (Costar) were coated with different concentrations of nonaggregated and heat-aggregated C3 or $C3(H_2O)$, diluted in PBS. After washing five times with PBS containing 0.05% Tween 20, the plates were incubated with HRP-conjugated rabbit anti-human C3c (1/3000) or HRP-conjugated rabbit anti-human C3d (1/1000) Abs, both purchased from DAKO (Glostrup, Denmark). For visualization, tetramethylbenzidine (Sigma Aldrich) was used as a chromogen, OD values were measured at 450 nm.

Flow cytometry

Immunofluorescence measurements were performed using a FACSCalibur flow cytometer and the CellQuest software (BD Biosciences, Mountain View, CA). A total of 2×10^5 B cells were incubated with 100 μg/ml anti-human CR1 (clone To5, purchased from DAKO) or mouse anti-human CR2 Ab (clone FE8, a kind gift of W. M. Prodinger, University of Innsbruck, Innsbruck, Austria) in PBS containing 1% FCS and 0.1% NaN_3 for 30 min on ice. Isotype-matched Ab was used as control. After washing, 3 μg of biotin-labeled, heat-aggregated human C3 or $C3(H_2O)$ was added to the samples for 30 min. Binding of C3 was revealed after washing and incubating with ExtrAvidin-FITC (Sigma Aldrich) for a further 20 min. Data of 10,000 cells were collected.

To assess the presence of cell-bound C3 fragments, lymphocytes activated with anti-IgM $F(ab')_2$ and cultured with different amounts of aggregated C3 for 24 h were recovered, washed twice, and incubated with FITC-conjugated rabbit anti-C3c or anti-C3d (DAKO). Cells cultured without C3 were used as control. Data of 5,000 cells were collected.

For detection of changes in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), high-density tonsil B cells were loaded with 5 μM Fluo-3/AM indicator and 30 μg/ml Pluronic F-127 (both from Molecular Probes, Eugene, OR) for 30 min at 37°C at 1×10^7 cells/ml. After washing, the cells were resuspended at 5×10^5 cells/ml and kept on ice until use. All studies were conducted in RPMI 1640 medium (Sigma Aldrich). After incubating at 37°C for 5 min, cells were activated with 2.5 μg of anti-IgM $F(ab')_2$ in the presence of aggregated C3. Data were collected for 208 s. Nonviable cells were excluded by dyeing with 7-amino actinomycin D (Molecular Probes). Results shown are relative mean fluorescence values as the function of time.

Tyrosine phosphorylation

Resting tonsil B cells were washed and incubated for 15 min at 37°C at a concentration of 1×10^8 cells/ml RPMI 1640 medium. Then, 250-μl samples were activated with 2.5 μg of anti-IgM $F(ab')_2$ in the presence or absence of heat-aggregated C3 for 2 min at 37°C, centrifuged, and immediately frozen in liquid nitrogen. Cells from each sample were solubilized in 250 μl lysis buffer containing 50 mM HEPES (pH 7.4), 1% Triton X-100, 100 mM NaF, 10 mM EDTA, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 10% glycerol, 2 μg/ml aprotinin, 2 μg/ml pepstatin, 5 μg/ml leupeptin, and 1 mM PMSF (the enzyme inhibitors were all purchased from Sigma Aldrich). After incubation for 45 min on ice, cell lysates were centrifuged at $15,000 \times g$ for 20 min at 4°C. Samples were boiled in reducing sample buffer, electrophoresed on 10% SDS-PAGE gel, and the proteins were transferred to nitrocellulose membrane. After blocking with 0.1% gelatin, the blots were developed by subsequent incubation with anti-phosphotyrosine mAb (PY20, 1/1000, Transduction Laboratories, Lexington, KY) and HRP-conjugated anti-mouse Ig (Sigma Aldrich). Specific bands were visualized by the ECL method (Pierce, Rockford, IL).

Statistical analysis

Differences between sample means were analyzed using Student's *t* test, and were considered statistically significant when $p < 0.05$.

Results

Heat-aggregated C3 binds to CR1 on human B cells

To establish whether aggregated human C3 is a ligand for CR1 and/or CR2 on human B lymphocytes, two approaches were applied. First, we examined the accessibility of different epitopes of aggregated C3 by ELISA. Plates were coated with various dilutions of nonaggregated and heat-aggregated C3, and binding of HRP-conjugated rabbit anti-human C3c or anti-human C3d Ab was assessed. As shown in Fig. 1, the accessibility of the C3d sequence is markedly reduced after aggregation of intact C3 (Fig. 1A), while C3b epitopes remain unchanged (Fig. 1B). Experiments with nonaggregated and aggregated $C3(H_2O)$, i.e., C3b-like C3 (31), led to similar results (Fig. 1, C and D). These data suggest that aggregation of intact C3 induces conformational changes that
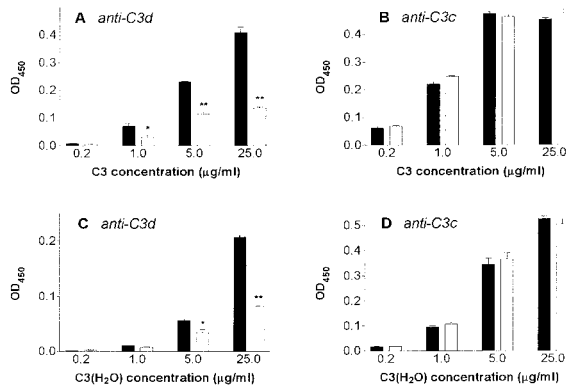
FIGURE 1. Aggregation of C3 generates C3b-like molecules. ELISA plates were coated with the indicated amounts of nonaggregated (■) and aggregated (□) human C3 (*A* and *B*), or C3(H$_2$O) (*C* and *D*). Binding of HRP-conjugated rabbit anti-human C3d (*A* and *C*) and anti-human C3c (*B* and *D*) was assessed as described in *Materials and Methods*. Data shown are average OD$_{450}$ ± SD of duplicate wells from a single experiment, and are representative of three independent experiments with similar results (∗, $p < 0.05$; ∗∗, $p < 0.01$, Student's *t* test).

bury C3d sequences inside the molecule, leaving exposed epitopes which are available mainly by C3c-specific Abs.

Next, we investigated whether this conformational change is also reflected in the receptor-binding activity of the complement protein. To this end, we studied whether aggregated C3 binds to CR1 and/or CR2. Binding of the biotin-labeled complement protein to human tonsil B cells was tested by flow cytometry after incubation of the cells with Abs specific to CR1 and CR2, respectively. As shown in Fig. 2, the interaction of aggregated C3 with B lymphocytes is completely inhibited by anti-CR1 Ab To5, which reacts with the C3b-binding site of the receptor. In contrast to this, the high-affinity anti-CR2 mAb FE8, which has been shown even to dissociate bound C3dg from CR2 (34), was ineffective under the
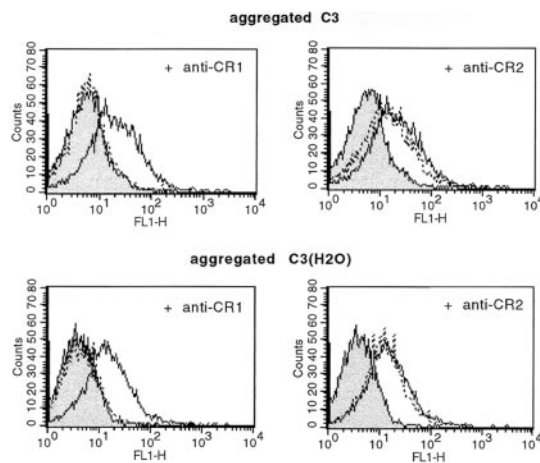
same conditions (Fig. 2). Experiments with aggregated C3(H$_2$O) led to similar results (Fig. 2). In the reverse experiments, aggregated C3 and aggregated C3(H$_2$O) prevented only the binding of the anti-CR1 Ab, but not that of mAb FE8 (not shown). These results clearly show that aggregated C3 behaves as aggregated C3b-like C3, and reacts preferentially with CR1 on the surface of human B cells.

Inhibition of anti-IgM-induced B cell proliferation by aggregated C3

Next, we studied the functional effect of the multimeric CR1 ligand on B cell activation. Low- and high-density B cells were isolated from human tonsils and activated with a suboptimal dose of anti-IgM F(ab′)$_2$ (3 μg/ml, as determined in preliminary experiments). Cells were cultured in the presence of different amounts of aggregated C3 for 72 h as described in the section of *Materials and Methods*. As shown in Fig. 3, the multimeric CR1-ligand exerts a dose-dependent inhibitory effect on the anti-IgM-induced proliferation of both high- and low-density tonsil B cells, with a more pronounced effect in the case of the former. (It must be noted that the proliferative response of the low-density blast cells is considerably reduced compared with that of the "resting" cells. This is reflected in the ~10-fold difference in the [^{3}H]thymidine uptake of the two cell populations, as seen in Fig. 3, *A* and *B*). Aggregated C3(H$_2$O) applied under the same conditions exerted an inhibitory effect as well (Fig. 3*D*). As a control, the proliferation of the CR1$^-$ B cells of Raji line has also been tested and found to be not inhibited by aggregated C3 (not shown). In good agreement with earlier data (35), aggregated C3d, the CR2 ligand, dose-dependently enhanced the proliferation of human B cells, as shown in Fig. 3*C*. Similar results were obtained using B cells isolated from peripheral blood (not shown).

The CR1-mediated inhibition of B cell activation is C3-specific

To exclude the possibility that contaminating proteins are involved in the action of C3, the proliferation assay has been conducted in the presence of anti-C3 F(ab′)$_2$ as well. As shown in Fig. 4, blocking the interaction between C3 and CR1 suspends the inhibitory effect of the complement protein.
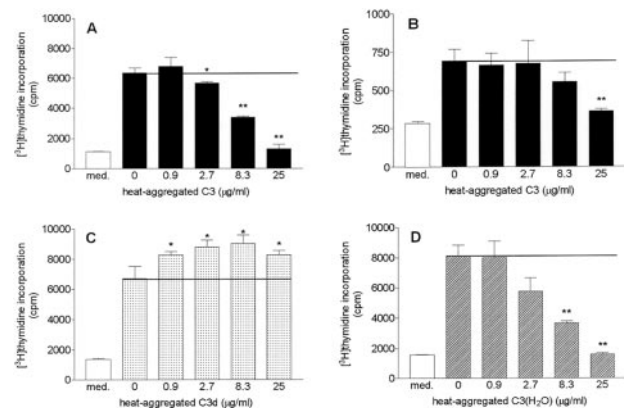

FIGURE 2. CR1-specific Ab blocks the binding of aggregated C3 to B lymphocytes. A total of 2×10^5 B cells were incubated with 100 μg/ml To5 anti-CR1 or 100 μg/ml FE8 anti-CR2 Ab for 30 min on ice. After washing the cells, 3 μg biotin-labeled heat-aggregated C3 or aggregated C3(H$_2$O) was added for 30 min. Binding of the complement protein was evaluated after incubation with ExtrAvidin-FITC (dotted lines). Shaded histograms show background fluorescence, while thin lines show the reactivity of cells preincubated with control, isotype-matched Ab followed by C3 and ExtrAvidin-FITC. The *x*- and *y*-axes correspond to the mean fluorescence intensity and the relative cell number, respectively. Histograms are representative of three experiments performed.

FIGURE 3. Inhibition of B cell proliferation by aggregated C3. Tonsillar high- (*A*, *C*, and *D*) and low- (*B*) density B cells (1×10^5/well) were activated with 3 μg/ml of goat F(ab′)$_2$ of anti-human IgM, and cultured in the presence of various amounts of heat-aggregated C3 (*A* and *B*), C3d (*C*), or C3(H$_2$O) (*D*) for 72 h as described in *Materials and Methods*. □, Proliferation of cells cultured in medium only. Data showing [^{3}H]thymidine incorporation are mean cpm ± SD of triplicate cultures and are representative of 12 (*A* and *B*) and 3 (*C*) experiments performed with similar results. (∗, $p < 0.05$; ∗∗, $p < 0.01$, Student's *t* test).

To further investigate the role of C3, it has been tested whether CR1-bound C3 can be detected on B lymphocytes recovered from the cultures with anti-IgM and aggregated C3 after 24 h. Cells were subjected to FACS analysis using FITC-labeled C3c- and C3d-specific Abs. In agreement with data shown in Figs. 1 and 2, all the cells were stained with the anti-C3c Ab, and the extent of positivity corresponded to the concentration of the ligand added to the cultures. Using the C3d-specific Ab, no staining could be observed (Fig. 5).

To rule out any role of IgG contamination present in our C3 preparation (which was found to be <1%, as assessed by ELISA), heat-aggregated IgG was added to the cells in different concentrations. IgG, even if applied in a 10-fold higher concentration than the possible contamination, had no effect at all on the anti-IgM-induced B cell proliferation (not shown).

All these data strongly support the assumption that the inhibition of B cell proliferation is mediated by CR1 clustered by its multimeric natural ligand.

Aggregated C3 inhibits the IL-2- and IL-15-dependent proliferation of B cells

Similar to the effect of the T cell-derived cytokine IL-2, the macrophage-derived IL-15 is also known to costimulate the proliferation and augment the Ab production of B lymphocytes (36). Therefore, we aimed to investigate whether cytokine-dependent B cell growth is also affected by the engagement of CR1. The effect of aggregated C3 on the IL-2- and IL-15-dependent proliferation of tonsil B cells was studied using low- and high-density cells activated by anti-human IgM F(ab')$_2$. Fig. 6 shows that heat-aggregated C3 dose-dependently inhibits the anti-IgM-induced proliferation of B cells in the presence of either cytokine. In agreement with earlier data (36), IL-2 or IL-15 alone (shown as controls in Fig. 6) induced the proliferation of low-density B cells only.

CR1 clustering results in the inhibition of the anti-IgM-induced transient increase of intracellular free Ca^{2+} level

One of the early events of cellular activation is the elevation of cytosolic free Ca^{2+} concentration. It is known that cocross-linking CR2 and sIgM enhances the transient increase of $[Ca^{2+}]_i$ induced by BCR alone (27, 28, 37). Based on our results shown in Figs. 3, 4, and 6, we assumed that the inhibitory effect of multimeric C3 on B cell proliferation is caused by interfering with the sIgM-mediated activation signal. To test this possibility, we examined the effect of aggregated C3 on the anti-IgM-induced Ca^{2+} response of resting tonsillar B cells. Fig. 7 shows that the multimeric ligand dose-dependently reduces the anti-IgM-induced elevation of $[Ca^{2+}]_i$ both in the first phase (release of Ca^{2+} from intracellular

FIGURE 5. Detection of CR1-bound C3 fragments on tonsil B lymphocytes cultured in the presence of aggregated C3. Resting tonsil B cells were cultured with 3 μg/ml F(ab')$_2$ of goat anti-human IgM and different amounts of heat-aggregated C3. After 24 h, cells were recovered from the cultures, washed twice, and incubated with FITC-labeled rabbit anti-human C3c or anti-human C3d, respectively. Histograms show the binding of Abs to the cells cultured without C3 (control sample, shaded), with 1 μg/ml (dotted line), 5 μg/ml (thin line), and 25 μg/ml C3 (thick line). The *x*- and *y*-axes correspond to the mean fluorescence intensity and the relative cell number, respectively. Data shown are representative of three experiments.

pools) and in the late phase (Ca^{2+} influx from the extracellular space) of the Ca^{2+} response. When added alone, C3 has no effect at all. In agreement with the proliferative response, the effect of CR1 cross-linking was more pronounced when the cells were activated with a suboptimal concentration of anti-IgM F(ab')$_2$, while optimal BCR stimulation overcame the inhibitory effect of C3. We obtained similar results using aggregated $C3(H_2O)$ (data not shown).

CR1 clustering inhibits the anti-IgM-induced protein tyrosine phosphorylation in tonsil B cells

To investigate whether the engagement of CR1 influences the general phosphorylation pattern of intracellular proteins, resting tonsil B lymphocytes were isolated and stimulated with anti-IgM F(ab')$_2$ in the presence of aggregated C3, as described in the section of *Materials and Methods*. As shown in Fig. 8, the anti-IgM-induced tyrosine phosphorylation of several intracellular proteins is reduced in the presence of the multimeric CR1 ligand. The identification of cytoplasmic molecules (such as proteins with approximate molecular masses of 50–55 and 90 kDa) involved in this inhibition is in progress in our laboratory.

Discussion

In the present study, we have used aggregated human C3 to cross-link CR1 on human B lymphocytes and to further investigate the
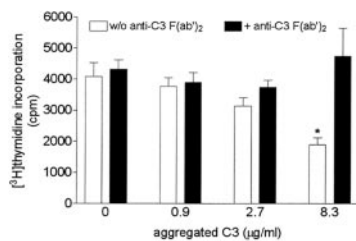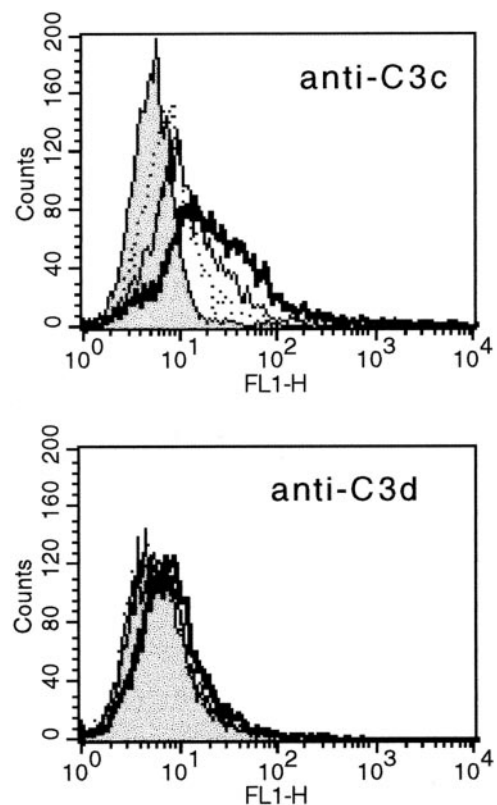

FIGURE 4. Abrogation of C3-mediated inhibition by anti-C3 F(ab')$_2$. Resting tonsil B cells were activated with 3 μg/ml anti-IgM F(ab')$_2$ and cultured with various amounts of heat-aggregated C3 in the presence (■) or absence (□) of 25 μg/ml anti-C3 F(ab')$_2$. Cells were harvested after 72 h. Data showing [^{3}H]thymidine incorporation are mean cpm ± SD of duplicate cultures and are representative of three independent experiments. (∗, $p < 0.05$, Student's *t* test).
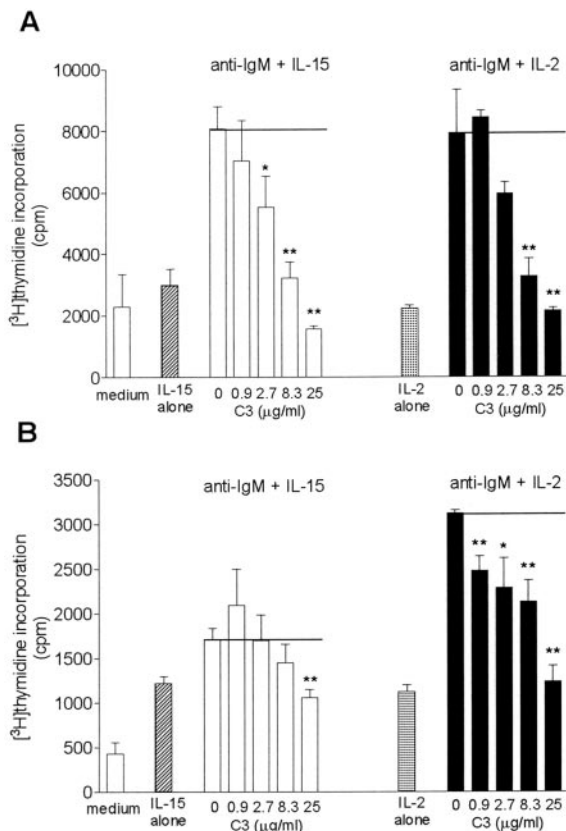
A

B

FIGURE 6. Aggregated C3 inhibits the IL-2- and IL-15-dependent proliferation of tonsil B cells. High- (*A*) and low- (*B*) density tonsil B cells were activated with 3 μg/ml F(ab')$_2$ anti-human IgM, and treated with the indicated amounts of heat-aggregated C3 for 72 h. IL-15 ($\square$) or IL-2 ($\blacksquare$) was added to the cells after 24 h at 100 U/ml. Samples indicated as "IL-2 alone" and "IL-15 alone" were treated with the ILs only; neither anti-IgM nor aggregated C3 were added. Data showing [^{3}H]thymidine incorporation are mean cpm $\pm$ SD of triplicate wells and are representative of six independent experiments. (*, $p < 0.05$; **, $p < 0.01$, Student's *t* test).

FIGURE 7. Anti-IgM-induced Ca^{2+} response is down-regulated by multimeric CR1 ligand. Resting tonsil B cells filled with Fluo-3/AM (2.5 $\times$ 10^5 cell/sample) were activated with 2.5 μg F(ab')$_2$ of goat anti-human IgM immediately after the addition 5 μg ($\blacklozenge$) and 30 μg ($\bigcirc$) heat-aggregated, C3b-like C3. As negative control, cells were treated with 30 μg C3 alone ($\blacksquare$). As positive control, the Ca^{2+} response of anti-IgM-stimulated B cells is shown ($\square$). The arrow indicates the addition of the reagents. The *x*- and *y*-axes correspond to the time and the mean fluorescence intensity, respectively. Data are representative of five independent experiments.

out that while in mice CR1 and CR2 are products of alternatively spliced mRNA from the *Cr2* gene, in humans these complement receptors are encoded by different genes (40, 41). Consequently, human CR1 may have different and/or additional roles which cannot be studied in mice.

Concerning the role of CR1 on human B lymphocytes, results of earlier studies are controversial, most probably due to the different experimental settings. Although in several studies PWM was used as polyclonal B cell activator, in other experiments a more physiologic stimulus, namely anti-μ, was applied. Tedder et al. (24) showed no effect of CR1-specific Ab on the anti-μ-induced proliferation and on Ig production of PWM-stimulated blood B cells, while other groups reported both CR1-mediated enhancement (22, 23) and inhibition (25, 26) of Ig synthesis. Using receptor-specific

role of CR1 in anti-IgM-driven B cell activation. Based on our results, we propose that human CR1 can also regulate B cell responses. However, in contrast to CR2, clustering of CR1 inhibits the proliferation of B cells activated via the BCR.

Complement has been shown to modulate and direct immune responses, thus, bridging the innate and the adaptive immune system (1–4). The important role of primate CR1 in several immunological processes was acknowledged long ago. CR1 is a multiligand receptor that binds the complement activation products C4b and C3b. This potential enables CR1 present in the membrane of erythrocytes to deliver opsonized immune complexes to the liver and the spleen, thus, eliminating them from the circulation. CR1 is long known to play an important role in the phagocytosis of opsonized particles by neutrophils and monocytes. It is also a regulator of the complement cascade as a decay accelerator and a cofactor for the enzyme, factor I (19). By its cofactor activity, together with other complement regulators in the cell membrane, CR1 promotes the conversion of C3b to iC3b and C3d(g).

Regarding the immunomodulatory role of C3 fragments and their corresponding receptors CR1 and CR2 on B lymphocytes, most of the studies were conducted in murine system (1, 3, 5–10, 38, 39). It had been shown that monovalent C3b and C3d inhibit, while the same ligands applied in polymeric form enhance the proliferation of mouse B cells (38, 39). Similar results were obtained with C3d on human B cells (35, 37). It is important to point
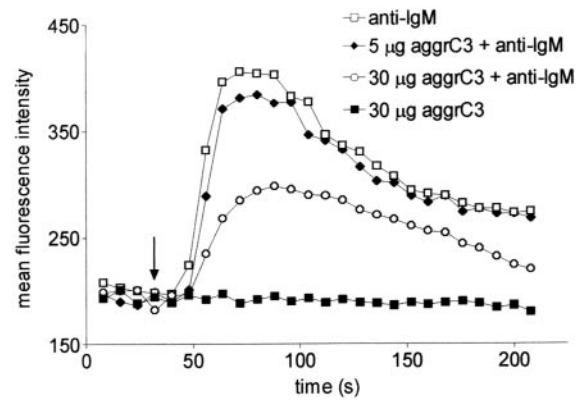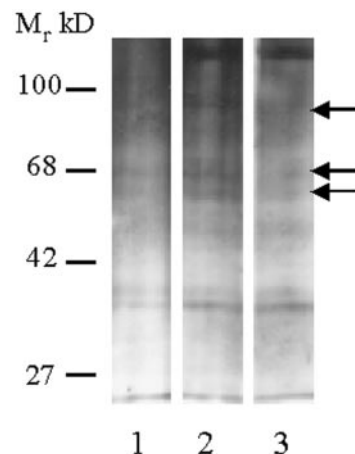

FIGURE 8. The effect of CR1 clustering on anti-IgM-induced tyrosine phosphorylation in human B lymphocytes. A total of 2.5 $\times$ 10^7 cells/sample were incubated with 2.5 μg anti-IgM F(ab')$_2$ (*lane 2*), 30 μg heat-aggregated C3 + 2.5 μg anti-IgM F(ab')$_2$ (*lane 3*) for 2 min. As negative control, nonstimulated cells were applied (*lane 1*). Cells were immediately frozen after treatment. After lysis, proteins were separated by SDS-PAGE on a reducing 10% gel and transferred to nitrocellulose sheet. The blot was developed by subsequent incubation with anti-phosphotyrosine mAb and HRP-anti-mouse Ig, followed by ECL visualization.

Abs, Fingeroth et al. (29) reported that cocross-linking of sIg and CR1 on resting splenic B cells results in inhibition of the anti-IgM-induced proliferation. Carter et al. (28) obtained similar results with blood B cells. However, the use of receptor-specific Abs does not reflect the in vivo conditions. Moreover, these data do not describe the result of clustering sIg and CR1 independently.

For these considerations, we have used C3 in aggregated form to cluster CR1 molecules on B cells. We have demonstrated that heat-aggregated C3 behaves like aggregated $C3(H_2O)$ or C3b-like C3 in respect to its receptor-binding activity, i.e., it primarily binds to CR1 (Fig. 2). Because aggregation results in the reduced accessibility of the C3d regions of C3 molecules (Fig. 1), this aggregated form of the complement protein can be used for modeling C3b-like C3, and to study the cellular events induced by CR1-cross-linking. Our data presented in this study clearly show that clustering CR1 via its natural ligand on anti-μ-activated human B cells induces negative regulatory signals, a function which is particularly pronounced when the cell is activated suboptimally via sIg (Figs. 3 and 6–8). To confirm the role of C3 and CR1 in this process, additional experiments have been performed. Data obtained from FACS analysis of cells removed from culture revealed that bound C3 can be detected on their surface with C3c-specific Abs even after 24 h, although we were unable to show the presence of C3d fragments/epitopes (Fig. 5). Furthermore, we could abrogate the effect of aggregated C3 using anti-C3 F(ab′)$_2$ (Fig. 4). These results and the finding that the proliferation of Raji cells lacking CR1 is not inhibited by the same C3 preparation (not shown) support our assumption of CR1-mediated inhibition. To exclude the direct involvement of IgG and FcγRIIb, we have used C3 purified by FPLC method and incubation with protein G beads to minimize contamination. In addition, we found that aggregated IgG used in the concentration range in which it might contaminate our C3 preparation, did not affect the studied cellular events. We also studied the effect of aggregated C3 when further stimulus was provided for B cells. Our results show that the inhibition of anti-IgM-induced B cell proliferation is not abrogated in the presence of the costimulatory cytokines IL-2 and IL-15, added after 24 h (Fig. 6). The inhibitory effect involves early intracellular signaling events, such as changes in $[Ca^{2+}]_i$ (Fig. 7), and phosphorylation of certain cytoplasmic proteins (Fig. 8).

Ag-bound C3d(g) is known to promote B cell activation by binding to CR2, a constituent of the coreceptor complex CR2/CD19/CD81, thus, lowering the threshold of Ag-specific BCR for activation (10, 12, 35, 38, 42). In the cell membrane of human B lymphocytes, CR1 molecules appear not only in free form, but also in association with CR2 alone, i.e., not within the trimolecular complex of CR2/CD19/CD81 (13). Although the short cytoplasmic tail of CR2 includes potential protein kinase C and tyrosine phosphorylation sites, and the phosphorylation of CR2 in PMA-treated tonsillar B cells has been observed earlier (43), it is thought that the activatory signal is transduced via the associated CD19 molecule, and CR2 serves only as a ligand-binding subunit in the coreceptor complex (12). However, recent study provided evidence for CD19-independent signaling through CR2 (44). In contrast to CR2, the intracytoplasmic of human CR1 does not seem to have the ability of transducing signals. Yet data obtained earlier with antireceptor Abs (28, 29) suggest that CR1 has signal-mediating capacity, either itself or via associated molecules. This is clearly supported by the results of our experiments, since BCR-induced elevation of $[Ca^{2+}]_i$ is inhibited, and tyrosine phosphorylation of certain cytoplasmic proteins is strongly reduced upon clustering CR1 (Figs. 7 and 8). Regarding the mechanism, we assume that inhibitory molecules (such as FcγRIIb) cocluster with CR1 upon engagement of the latter by its multimeric ligand. However, because this process occurs in the absence of IgG, the possible involvement of lipid rafts containing CR1 and B cell inhibitory receptors should be taken into consideration.

The fate of a B lymphocyte is determined by the integration of signals transduced via several cell membrane molecules, including sIg, CR2, FcγRII, CD22, and CD45 (45, 46). The CR2-mediated enhancement of B cell function is important in limiting Ag doses (47), particularly at suboptimal activation, when modulatory effects of complement and FcR become more pronounced. Multimeric C3b-like C3 used in our experiments is a physiological ligand of CR1, which is able to cluster these receptors. Because several pathogens are known to fix C3b fragments on their surface in a protected state, i.e., inaccessible to factors H and I (48), multimeric C3b is available for a long period during infections.

Our data point to the importance of the local C3b:C3d ratio and the strength of the BCR-mediated stimulatory signal in determining the fate of human B cells. It is clearly demonstrated that human CR1, in contrast to CR2, is involved in the elevation of the activation threshold of B cells (Figs. 3, 6–8). This mechanism may ensure an additional level of regulation, which, depending on the composition of immune complexes and the degradation stage of C3, may reduce the possibility of nonspecific B cell activation.

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