Cross-linking of at least three binding sites mediates signal transduction in a CR2-positive Burkitt lymphoma derived cell line (Raji)

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Abstract In this study we demonstrate that Raji cells, a CR2-positive Burkitt lymphoma-derived cell line, during cell growth, need the cross-linking of multiple OKB7 binding sites or C3d determinants to mediate signal transduction. The loss of one of these affects the cellular response. Moreover, OKB7, the anti-CR2 MoAb, recognizes C3d determinants on the cell surface and inhibits signal transduction induced by anti-C3d polyclonal antibody. Since Raji cells are always CR2 positive during cell growth, we suppose that at least another protein, along with CR2, may be involved in setting up a cell surface complex able to receive and transduce the signal triggered by OKB7. In our experimental system the protein that offers a third binding site to OKB7, may be represented by a 33 kDa protein bearing C3d determinants.

Key words: Complement receptor type 2; Anti-CR2 MoAb; Polyclonal antibodies for C3c and C3d fragments of human complement (C3); Inositol-(1,4,5)trisphosphate; Anti-C3d reactive binding site

1. Introduction

Human hepatocytes, monocytes, epithelial and endothelial cells synthesize C3 [1]. This seems to be true also for Raji cells, a CR2 positive Burkitt lymphoma derived cell line, which produce a 5.2-kb message and a truncated message of approximately 1.8-kb in size (W. Lernhardt, personal communication, [1]). Northern blot analysis revealed that the truncated message corresponds to the C3 α chain, and it is probably analogous to the truncated C3 message produced by murine T-cell hybridoma, lymphoma and macrophage cell lines [2]. Therefore, Raji cells seem to be able to produce C3 and use it. It has been already reported that hydrolyzed C3 ($C3_{H_2O}$) can be cleaved by cell surface proteins [3], and the derived C3 fragments can substain cell growth [3,4]. The cleavage of $C3_{H_2O}$ or iC3b is mediated by the cofactor activity that Complement Receptor type 2 (CR2) fulfils on the surface of CR2 positive cells [3,5].

CR2, a 140–145 kDa membrane protein, is the C3d receptor [6]. It also acts as a cellular receptor for Epstein-Barr virus (EBV) [7,8] and interferon α (α-IFN) [9]. It can be phosphorylated following interaction with its ligands [10,11] and it is involved in important immunoregulatory processes, including activation and growth of B lymphoblastoid cells [12–16]. CR2 has also been found associated with other cell surface proteins in mediating multiple signals [11,17–19]. Moreover, CR2 positive cells bind hemolytic inactive C3 and its fragments C3b, iC3b, C3dg and C3d [20–26].

Recently, the C3d homologous regions described on different CR2 ligands (α-IFN, EBV) [9,27,28] have been considered in the CR2 function. However, little is known about the expression of anti-C3d reactive binding sites (hereafter referred as C3d determinants) on CR2 or on proteins of CR2 positive cells, even if it is demonstrated that anti-C3D antibody inhibits the binding of α-IFN to CR2 [9].

We used CR2 positive Raji cells to investigate whether they could express C3d determinants. We found that: (a) Raji cells can be C3d positive; (b) cross-linking of multiple C3d determinants by anti-C3d polyclonal antibody mediates an increase of inositol-(1,4,5)trisphosphate ($InsP_3$), and (c) OKB7, an anti-CR2 MoAb that has been already reported to cross-link two CR2 epitopes [29], to stimulate B cell activation [30] and to mobilize intracellular C^{2+} in CR2 positive T cells [31], mediates signal transduction by $InsP_3$ and inhibits $InsP_3$ release induced by anti-C3d. Furthermore, we found that OKB7 recognizes also a 33 kDa protein bearing C3d determinants.

2. Materials and methods

2.1. Experimental procedures

Raji cells were cultured in RPMI-1640 (Flow Lab. Ltd., Irvine, Ayrshire, Scotland) supplemented with 10% heat inactivated fetal calf serum (FCS) (Flow). Human serum, obtained from a healthy donor, was stored at −20°C and thawed before use. C3d [32] was kindly supplied by Prof. J.D. Lambris, University of Pennsylvania, Philadelphia, PA, USA. The presence of C3 fragments on the cell surface was analyzed by flow cytometry using fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human C3d (17 μg/ml) or anti-human C3c (34 μg/ml) polyclonal antibodies (Dakopatts, Copenhagen, Denmark). In some experiments the fate of CR2 was monitorized by phycoerythrin (PE)-conjugated mouse anti-CR2 MoAb (OKB7-PE) (5 μg/ml) (Ortho Pharmaceutical Corp., Raritan, NJ, USA). Reactivity with mouse IgG-PE (Ortho) served as negative control. The samples were analyzed in FACScan (Beckton Dickinson, Mountain View, CA, USA).

2.2. $InsP_3$ mass assay

Raji cells were collected few seconds after seeding in complete medium ($t = 0$), or after 5 h ($t = 5$), 18 h ($t = 18$) and 24 h ($t = 24$). The washed cells were resuspended in ice-cold phosphate buffered saline (PBS) at the concentration of 1×10^7 cells/ml and aliquots of 0.1 ml were incubated for 1 min or 20 min at 4°C with PBS, anti-C3c polyclonal antibody (6 μg/ml), anti-C3d polyclonal antibody (1.7 μg/ml), anti-mouse IgG (5 μg/ml) or OKB7 (5 μg/ml). For some experiments, Raji cells were incubated for 30 min at 4°C with OKB7. The washed cells (OKB7-coated) were treated for 1 min at 4°C with PBS, anti-C3c, anti-C3d or anti-mouse IgG. Treatments were stopped by adding 30 μl

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Abbreviations: CR2, Complement receptor type 2; OKB7, Anti-CR2 MoAb; anti-C3c and anti-C3d, MoAbs or polyclonal antibodies for C3c and C3d fragments of human complement (C3); $InsP_3$, inositol-(1,4,5)trisphosphate; C3d determinants, anti-C3d reactive binding sites.

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of cold perchloric acid (20%, v/v) (PCA). The samples were transferred on ice for 30 min. Acid extracts were centrifuged and supernatants were neutralized with 5 M KOH, 0.7 M HEPES and 10 mM EDTA. The precipitated salts were removed by centrifugation and $InsP_3$ content in the cellular extracts was determined by a specific radioreceptor assay, TRK 1000 (Amersham, Buckinghamshire, UK).

2.3. *NaDodSO$_4$/polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis*

2×10^6 cells were washed and treated for 30 min at 37°C with PBS, OKB7 (5 μg/ml), HB5 (5 μg/ml) or anti-HLA-DR (5 μg/ml) (Beckton Dickinson). The pellets were stored for 10 days at −80°C. 0.1 ml of cell culture supernatants were collected after three days, lyophilised and stored for 10 days at −80°C. The thawed samples were dissolved in 30 μl of Laemmli's buffer [33], sonicated (Soni Prep) and boiled for 3 min, before loading the SDS-PAGE. For each run prestained standard proteins (Bio-Rad, Richmond, CA, USA) were included. Proteins were transferred onto nitro-cellulose strips (120 min at 75 V) and incubated overnight at 4°C with PBS/non-fat milk (5%, w/v). The strips were then incubated with anti-C3d polyclonal antibody (1:500, PBS-BSA 1%) (Dako), anti-C3c MoAb (1:150, PBS-BSA 1%) or anti-C3d MoAb (1:300, PBS-BSA 1%) (Quidel, San Diego, CA, USA) for 90 min at room temperature. Afterwards, the strips were incubated with the respective second antibody (Sigma, Chemical Co, St. Louis, MO, USA) (1:1000, PBS/BSA 1%) for 60 min at room temperature. Proteins were visualized by the ECL prcedure (Amersham).

2.4. *Immunoprecipitation by MoAb or polyclonal anti-C3d of [^{35}S]Met-labeled secreted proteins*

[^{35}S]Met-labeled cells were washed three times with PBS and treated for 30 min at 37°C with PBS, OKB7 (5 μg/ml) or HB5 (5 μg/ml). The supernatants were diluted with RIPA (10 mM Tris, 1 mM EDTA, 50 mM NaCl, 10 mM KCl, 0.05% Tween 20, 0.1% SDS) (1:1 v/v), containing 1 mM PMSF (Sigma), aprotinin (10 mg/ml) (Sigma), and leupeptin (10 mg/ml) (Sigma). Anti-C3d MoAb (40 μg) (Quidel), anti-C3d polyclonal antibody (40 μg) (Dako) or mouse IgG (40 μg) (Sigma) were respectively adsorbed to 30 μl of Protein-A-Sepharose (20%, w/v) (Pharmacia Fine Chemicals, Uppsala, Sweden) for 3 h at 4°C. The [^{35}S]Met-labeled supernatants were subsequently incubated for 16 h at 4°C with packed IgG-Protein-A-Sepharose. The immunocomplexes were washed with RIPA, sonicated, boiled for 3 min and separated by SDS-PAGE.

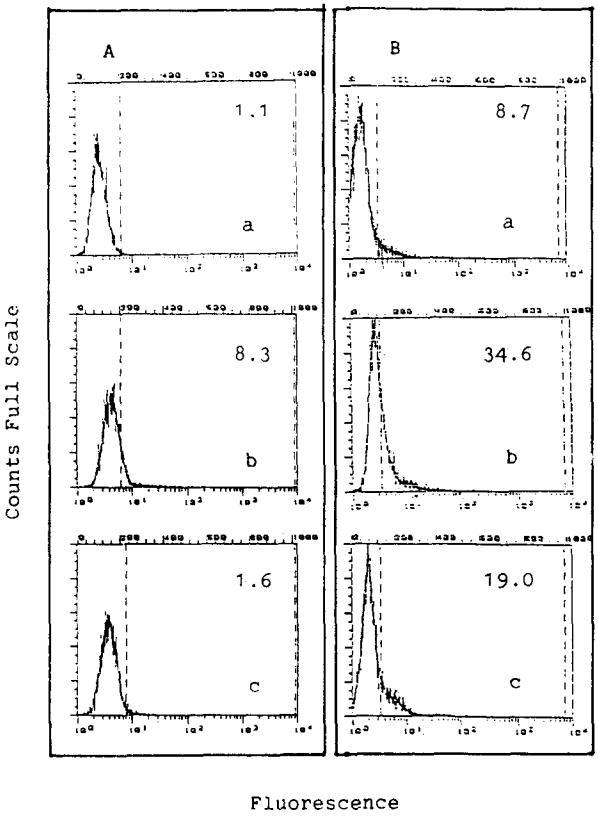

Fig. 1. Recognition of C3d determinants by OKB7 or monomeric C3d. (A) Raji cells were incubated with PBS (a,b) or OKB7 (c) for 30 min at 4°C. The washed cells were further incubated with rabbit IgG-FITC (a) or anti-C3d-FITC (b,c) for 30 min at 4°C and then analyzed in FACScan. (B) Raji cells were incubated for 30 min at 37°C with PBS (a,b) or with 0.05 μg of monomeric C3d (c). The washed cells were incubated for 30 min at 4°C with rabbit IgG-FITC (a) or anti-C3d-FITC (b,c) and analyzed in FACScan. The percent of positive cells is reported in each panel.

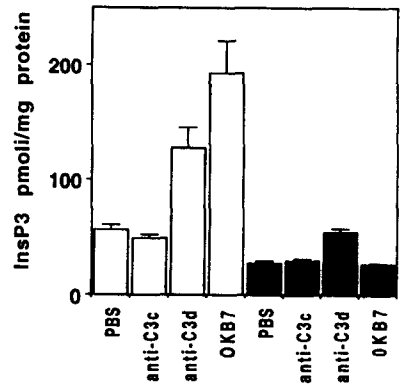

Fig. 2. Increase of $InsP_3$ levels in Raji cells following cross-linking of CR2 epitopes or C3d determinants. Raji cells, collected 72 h after seeding, were incubated with PBS, anti-C3c polyclonal antibody, anti-C3d polyclonal antibody or OKB7 for 1 min (open bars) or 20 min (black bars). The incubation was stopped adding cold PCA and the $InsP_3$ content determined by radioimmunoassay. The results derived from one representative experiment performed in triplicate. Data were reported as mean ± S.D.

3. Results

3.1. *Recognition of C3d determinants by OKB7 or monomeric C3d*

Raji cells could express C3d determinants. This expression was related to still unclear events, however, C3d positive Raji cells (Fig. 1b, panel A) might become C3d negative following preincubation with OKB7 (Fig. 1c, panel A).

C3d positive Raji cells (Fig. 1b, panel B) incubated with 0.05 μg of monomeric C3d had an unexpected decrease in the total amount of C3d detectable on the cell surface (Fig. 1c, panel B). At higher concentrations of the ligand, the binding of monomeric C3d to Raji cells was dose-dependent (data not shown).

3.2. *Increase of $InsP_3$ levels in Raji cells after cross-linking of CR2 epitopes or C3d determinants*

Raji cells treated with PBS or anti-C3c polyclonal antibody for 1 min (Fig. 2, open bars) or 20 min (Fig. 2, black bars) showed low levels of $InsP_3$. The $InsP_3$ levels increased following cell treatment with OKB7 or anti-C3d for 1 min (Fig. 2, open bars), while they dropped to basal levels after 20 min (Fig. 2, black bars). These data indicated that the increase of $InsP_3$ depended on cross-linking of multiple CR2 epitopes or C3d determinants during the early events of cell ligand interaction. However, a direct correlation between the presence of C3d determinants on the cell surface and their involvement in signal transduction was not observed. This because C3d determinants could be expressed to a very low extent on the cell surface or

that the transduction of the signal could involve the disclosing of hidden C3d determinants.

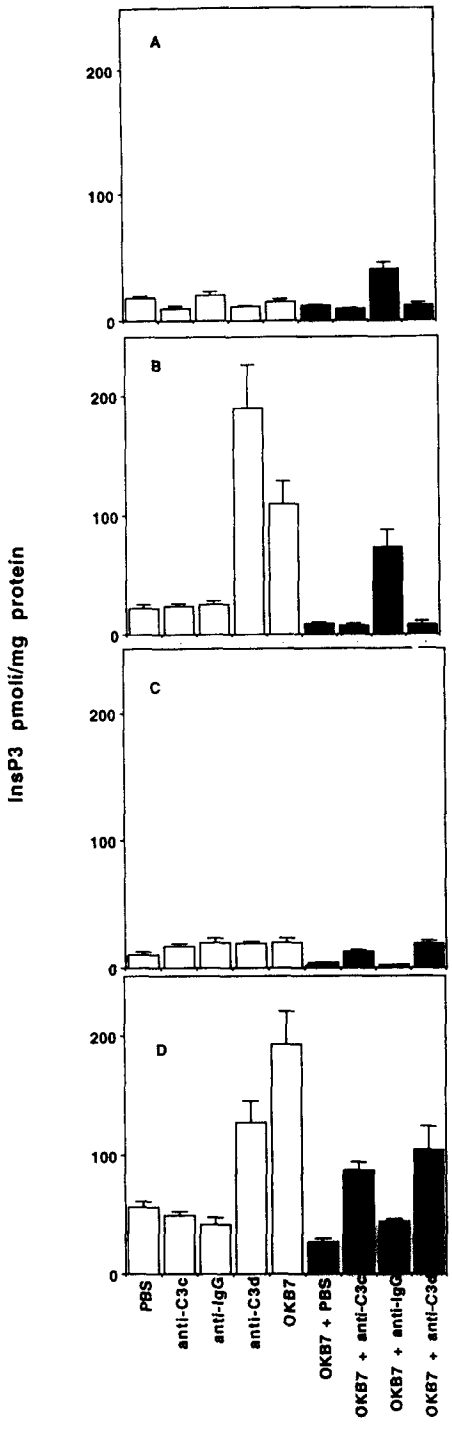

Fig. 3. Modulation of $InsP_3$ mediated signals during cell growth. Raji cells were collected at $t = 0$ (panel A), $t = 5$ h (panel B), $t = 18$ h (panel C) or $t = 72$ h (panel D) after seeding. The washed cells were incubated for 1 min with PBS, anti-C3c polyclonal antibody, anti-mouse IgG, anti-C3d polyclonal antibody, or OKB7 (open bars). OKB7-coated cells were incubated for the same period of time with PBS, anti-C3c polyclonal antibody, anti-mouse IgG or anti-C3d polyclonal antibody (black bars). The incubation was stopped adding cold PCA and the $InsP_3$ content determined by radioimmunoassay. The results derived from one representative experiment performed in triplicate. Data were reported as mean ± S.D.

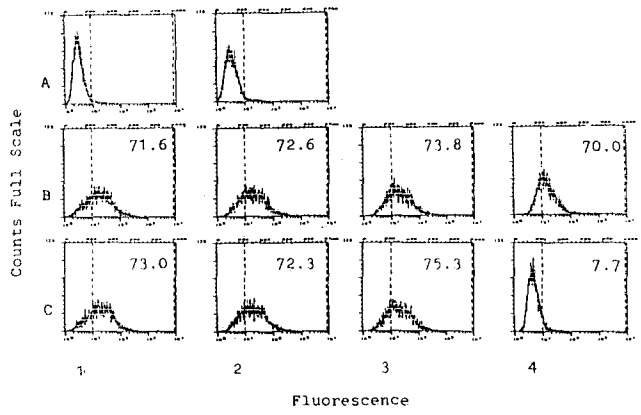

Fig. 4. Fate of OKB7-PE bound to CR2. Raji cells were incubated with PBS (A1), mouse IgG-PE (A2) or OKB7-PE (5 μg/ml) (B1–B4, C1–C4) for 30 min at 37°C. The washed cells were incubated with PBS at 37°C and after 0.5 h (B1,C1), 2 h (B2,C2), 8 h (B3,C3) or 24 h (B4,C4) aliquots were washed and incubated for further 30 min at 4°C with PBS (B1–B4), or anti-mouse IgG (C1–C4). The cells were then analyzed in FACScan. The percent of positive cells is reported in each panel.

3.4. Modulation of $InsP_3$ mediated signals during cell growth

Raji cells collected at $t = 0$ or 18 h of culture were unresponsive to the inductive signals triggered by anti-C3d or OKB7 (Fig. 3, panels A, C, open bars).

Cells collected at $t = 5$ or 72 h and treated with anti-C3d or OKB7 showed increased levels of $InsP_3$ when compared to PBS, anti-C3c or anti-mouse IgG treated cells (Fig. 3, panels B, D, open bars).

Similar experiments were performed with OKB7-coated cells (Fig. 4, black bars). Raji cells collected at $t = 0$ or 5 h and coated with OKB7 were responsive to inductive signals if the OKB7-coated binding sites were cross-linked by anti-mouse IgG (Fig. 3, panels A, B, black bars), whereas those collected at 18 h were unresponsive (Fig. 4, panel C, black bar). Moreover, the inductive signal mediated by anti-C3d polyclonal antibody was inhibited by cell pretreatment with OKB7 (Fig. 3, panels A, B, C black bars).

Cells collected at 72 h showed an increase of basal $InsP_3$ (Fig. 3, panel D). Cell pretreatment with OKB7 could increase the expression of C3c and C3d determinants, whose cross-linking enhanced $InsP_3$ levels (Fig. 3, panel D, black bars).

3.5. Fate of the OKB7-PE bound to CR2

Raji cells incubated with OKB7-PE retained this antibody for 24 h (Fig. 4, B1–B4). The cross-linking of the OKB7-PE binding sites with anti-mouse IgG, performed at 0.5, 2, or 8 h did not remove OKB7-PE from the cell surface (Fig. 4, C1–C3), while this happened at 24 h (Fig. 4, C4). Therefore, the Fc region of anti-mouse IgG could bind a third binding site, lately expressed on OKB7-PE coated-cells, and contribute to the internalization or shedding of the bound Ab to CR2.

3.6. Interaction of anti-CR2 MoAbs with a 33 kDa protein bearing C3d determinants

Immunoblotting of cellular lysates developed with anti-C3d polyclonal antibody showed four bands of 200, 97, 35 and 33 kDa (Fig. 5, lane b). OKB7 or HB5, two anti-CR2 MoAbs, interacted with the 33 kDa band, since the latter was absent in

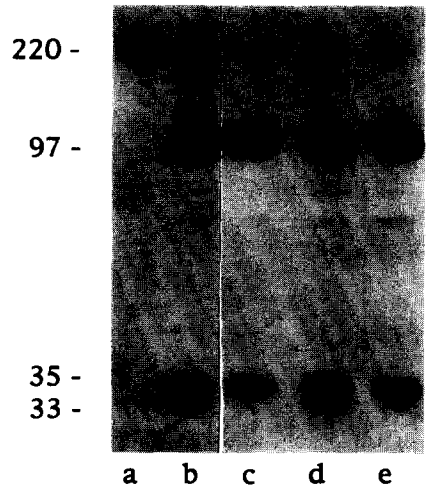

Fig. 5. Interaction of anti-CR2 MoAbs with a 33 kDa protein bearing C3d determinants. Raji cells were cultured in complete medium for three days, washed and treated for 30 min at 37°C with PBS (b), OKB7 (c), anti-HLA-DR (d) or HB5 (e). The cellular lysates were run on 9% SDS-PAGE, transferred onto nitro cellulose strips and developed by anti-C3d polyclonal antibody. 20 μl of human serum (1 : 500) (lane a).

the SDS-PAGE of Raji cells pretreated with these Abs (Fig. 5, lanes c, e). MoAb anti-HLA-DR did not interact with the 33 kDa protein (Fig. 5, lane d).

3.7. Secretion of a C3-like protein by Raji cells

Immunoblotting of three days-culture cell-derived supernatants showed some high molecular weight proteins and a 200 kDa protein recognized by anti-C3d and anti-C3c MoAbs (Fig. 6, lanes a, h). Anti-C3d also recognized a 145 kDa protein (Fig. 6, lane a), whereas anti-C3c recognized another protein with the molecular weight of 110 kDa (Fig. 6, lane h). Both MoAbs recognized C3 in human serum (Fig. 6, lanes d, e), and both cross-reacted with a low molecular weight protein of the complete medium (Fig. 6, lanes c, f), whereas they did not react with RPMI-1640 (Fig. 6, lanes b, g).

Since the 200 kDa protein was recognized by both anti-C3c and anti-C3d MoAbs and co-migrated with C3 of the human serum, we refer it as C3-like protein.

3.8. Immunoprecipitation of [^{35}S]Met-labeled secreted Raji proteins by MoAb and polyclonal anti-C3d

[^{35}S]Met-labeled Raji cells were incubated for 30 min at 37°C with PBS. The relative supernatant, immunoprecipitated with anti-C3d MoAb, showed two bands of 97 and 78 kDa (Fig. 7, panel A, lane a) which were not precipitated by mouse IgG (Fig. 7, panel A, lane b). For other experiments, [^{35}S]Met-labeled Raji cells were washed and then incubated with PBS, OKB7 or HB5. The relative supernatants, immunoprecipitated with anti-C3d polyclonal antibody, revealed two bands of 48 and 46 kDa (Fig. 7, panel B, lanes c, f, i). These bands were not detectable in the whole supernatants (Fig. 7, panel B, lanes a, d, g), suggesting that they could derive from the cleavage of parental proteins with molecular weights higher than 48 and 46 kDa. Moreover, these data indicated that the anti-C3d antibodies could recognize multiple and different binding sites on the parental protein(s), as they immunoprecipitated couples of proteins of different molecular weights (97/78 kDa or 48/46 kDa).

4. Discussion

In this study we demonstrate that the cross-linking of C3d determinants on Raji cells surface with anti-C3d polyclonal antibody mediates signal transduction. Moreover, at least one C3d determinant is involved in signal transduction by OKB7, as this Ab can recognize C3d determinants on the cell surface.

The C3d positivity might depend on the binding to the cell surface of the secreted C3-like protein or on the disclosing of the C3d homologous region of CR2 (it is known that twenty amino acids in CR2$^{247-266}$ share a 23.8 percent homology with those in the C3d region of C3$^{1201-1220}$, and the homology reaches 80 percent if it is restricted to the first five). The resulting coating of Raji cells could be requested for the neutralization of high reactive binding sites, while the resulting stripping could disclose binding site involved in signal transduction mechanisms.

OKB7, as the anti-C3d polyclonal antibody, mediated signal transduction in cells collected only at 5 or 72 h; no signal transduction was mediated at $t = 0$ or 18 h. Since Raji cells are always CR2 positive during cell growth, we conclude that at least another protein, along with CR2, should be involved in setting up a cell surface complex able to receive and transduce the signal triggered by OKB7. This protein might expose specific C3d determinants, which could be internalized/neutralized or shedded following interaction with OKB7 or monomeric C3d.

Monomeric C3d, unlikely from its multimeric form, has an inhibitory effect on the cellular activation [12]. Therefore, inductive signals via CR2 should need the involvement of multiple, at least three, binding sites. The loss of one of them would fail cellular response [12,14,27,34] and consequently signal transduction. We support this hypothesis as: a) three binding sites for native C3-derived proteins, bearing C3d determinants, are expressed on Raji cells surface [26], and b) the cross-linking of three binding sites by a C3 synthetic peptide, C3$^{1187-1214}$, produces stimulatory effect, whereas other peptides, able to bind two binding sites, are uneffective [35].

In our experimental system, two binding sites are offered by

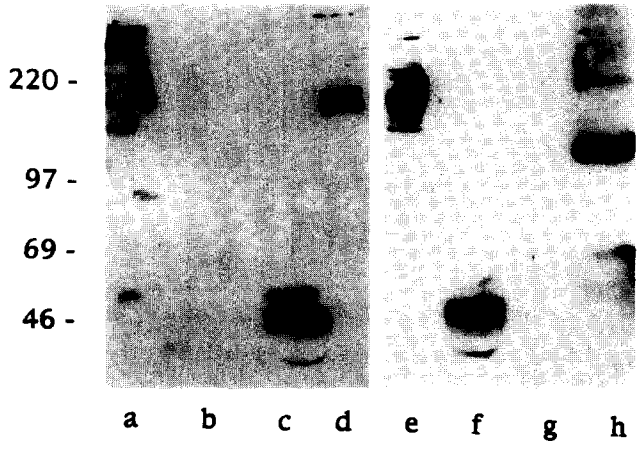

Fig. 6. Secretion of a C3-like protein by Raji cells. 0.1 ml of three days-culture Raji cells supernatant (lanes a, h), 0.1 ml of RPMI-1640 (lanes b, g), 0.1 ml of complete medium (lanes c, f) or 20 μl of human serum (1 : 500) (lanes d, e) were lyophilised, resuspended in Laemmli's buffer, run on 7.5% SDS-PAGE, transferred onto nitro cellulose strips and developed by anti-C3d (lanes a–d) or anti-C3c (lanes e–h) MoAbs.

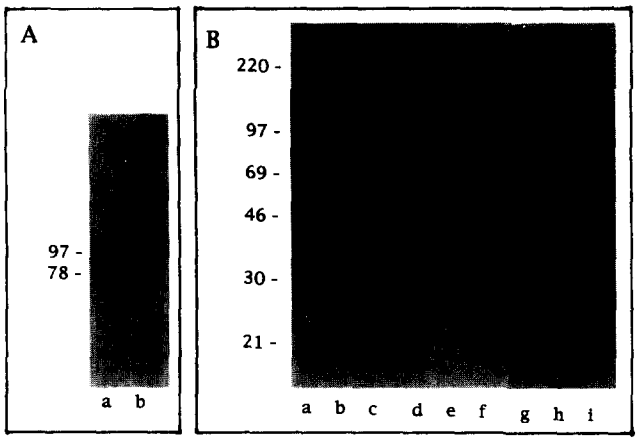

Fig. 7. Immunoprecipitation of [^{35}S]Met-labeled secreted Raji proteins by anti-C3d antibodies. Panel A: [^{35}S]Met-labeled secreted Raji proteins were incubated with anti-C3d MoAb (a) or mouse IgG (b), previously adsorbed to Protein-A-Sepharose. The immunocomplexes were washed and run on 7.5% SDS-PAGE. Panel B: [^{35}S]Met-labeled Raji cells were treated for 30 min at 37°C with PBS (a), OKB7 (d) or HB5 (g). Aliquots of the relative supernatants as such (a, d, g), treated with Protein-A-Sepharose (b, e, h) or immunoprecipitated with anti-C3d polyclonal antibody complexed to Protein-A-Sepharose (c,f,i), were run on 9% SDS-PAGE. The dry gels were developed by autoradiography.

CR2 [29], and the third might come from the 33 kDa protein recognized by OKB7. The 33 kDa protein was not secreted by Raji cells, since it was not immunoprecipitated from [^{35}S]Met-labeled supernatants by anti-C3d monoclonal or polyclonal antibodies. This protein is supposed to link CR2, through its own C3d determinants, to other molecules generating a high molecular weight complex. The consequential bindings to this complex, of repeated C3d/C3dg molecules or C3d homologous regions present on the surface of multimeric CR2 ligands [11–14, 27, 28, 34], should mediate signal transduction and the physical association of CR2, or part of it, to other cell surface proteins [11,17–19].

Other proteins expressed in Raji lysates and bearing C3d determinants had different behaviours. The 97 and 35 kDa proteins were not affected from cell–ligand interaction, whereas the C3-like protein was secreted and cleavable.

The anti-C3d polyclonal antibody immunoprecipitated, from [^{35}S]Met-labeled supernatants, a couple of 48 and 46 kDa bands which were absent in the controls. The parental proteins should correspond to the C3-like protein or to the high molecular weight proteins secreted by Raji cells and recognized by monoclonal and polyclonal anti-C3d. This protein(s) could interact, during its secretion, with the above mentioned cell surface complex and affect the cellular response to the inductive signals mediated by the two Abs.

During the late events of cell–ligand interaction, the cross-linking of OKB7 coated binding sites by anti-mouse IgG expressed an increase of $InsP_3$ similar to that induced by surface immunoglobulins in B lymphocytes [36]. This cross-linking, in specific conditions, induced the removal of OKB7-PE from the cell surface, as emphasized by our cytofluorimetry. Therefore, when the Fc region of anti mouse IgG bound a third binding site on the cell surface, the OKB7-PE was forced in a new conformational state and internalized or shedded following breakdown of CR2. This mechanism of action is consistent with previous data showing that: CR2 ligands may be internalized during the late events of cell-ligand interaction [28]; tumor cells undergo shedding [37]; CR2 is a clevable protein [38]; CR2 fragments are shedded by Raji cells [39–41].

In conclusion our data support the idea that signal transduction via CR2 needs the cross-linking of multiple, at least three, binding sites during both the early and late events of cell–ligand interaction.

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