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Evidence for three binding sites for C3 (hemolytically inactive), C3b and C3d on a CR2-positive Burkitt lymphoma-derived cell line (Raji)

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The present investigation shows that C3 (hemolytic inactive) as well as C3b and C3d bind Raji, a CR2-positive Burkitt lymphoma-derived cell line. Pretreatment of the cells with OKB-7 inhibited the binding of C3, whereas pretreatment with HB-5 inhibited the binding of C3b. Furthermore, the cells coated either with OKB-7 or HB-5 bound high amounts of C3d. TPA-treated cells showed binding for C3b and weak binding for C3 and C3d. Taken together, the data suggest that Raji cells may express three binding sites for C3, C3b and C3d which can be differently modulated by anti-CR2 MoAbs and TPA.

C3; C3b; C3d; CR2; Anti-CR2 MoAbs; TPA

1. INTRODUCTION

It has been reported that Burkitt lymphoma cell lines and certain human cultured tumor cell lines activate complement through the alternative pathwy (ACP) [1–4]. Mold et al. have shown that ACP activation by Raji cells occurs on CR2 molecules which also contain the C3 fragments acceptor sites [5]. CR2-positive cells bind C3 fragments [6–11] but not native C3. Native C3 contains an intramolecular reactive thioester bond which allows it to attach covalently, following a transacylation reaction, to receptive surfaces [12,13]. This thioester bond may undergo hydrolysis at a very slow rate, as during prolonged storage, or following treatment with chaotropes or ammonium [14–17], the resulting C3 molecules are uncleaved but devoid of hemolytic activity. In contrast to native C3, hemolytic inactive C3 was found to have markedly enhanced affinity for the C3b receptor [18].

We started the present investigation employing Raji cells, which in repeated experiments was shown to only possess CR2 from the complement receptors (reviewed in [19,20]), in order to answer the question whether hemolytic-inactive C3 would bind to them. The next question we addressed was to elucidate whether the binding sites on Raji cells for C3, C3b and C3d would be the same or not. For this purpose we used human C3, C3b and C3d preparations, two anti-CR2 MoAbs (OKB-7 and HB-5) and TPA. It has been shown that OKB-7 and HB-5 recognize different CR2 epitopes [21] and that the pretreatment of the cells with anti-CR2 MoAbs does not inhibit C3-fragments fixation [5,22]. On the other hand, TPA is a strong cellular activator [23–26] which causes CR2 phosphorylation [27,28]. It was worth to use these compounds in order to examine their role in modulating the binding of C3 preparations to Raji cells.

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Abbreviations: Complement (C3), third component of human complement; C3b, C3c and C3d, proteolytic fragments of C3; CR1, complement receptor type 1; CR2, complement receptor type 2; CR3, complement receptor type 3; OKB7, HB5, monoclonal antibodies anti-CR2; TPA: 12-*O*-tetra-decanoylphorbol-13-acetate.

2. MATERIALS AND METHODS

2.1. *C3 components*

Hemolytic active-C3 ($C3_{H50}$ 118 units/mg protein) was from Sigma Chemical Co, St. Louis, MO, USA; C3b, C3c and C3d [29,30] were kindly supplied by Dr J. Lambris, Basel Institute for Immunology, Basel, Switzerland. Active-C3 was stored for ten months at −80°C before use (C3). The hemolytic activity of C3 was determined by a standard assay [31].

2.2 *Iodination of C3 preparations*

C3 and C3 derived fragments were radiolabeled by the iodogen method [32]. Briefly, C3 (20 μg), C3b (6.5 μg), C3c (7 8 μg) and C3d (14 μg) were incubated with the same respective amounts of dry 1,3,4,6-tetrachloro-3α-6α-diphenylglycouryl (Sigma) and 0.1 mCi ^{125}Iodine (^{125}I) (NEN, Frankfurt, Germany) for 10 min at room temperature. Afterwards, the samples were diluted with 1 ml of phosphate-buffered saline (PBS) and dialyzed against PBS at 4°C for 18 h. The specific activity was for C3, 2.5 μCi/mg; for C3b, 7.01 μCi/mg; for C3c 10 μCi/μg; and for C3d, 7.0 μCi/μg. In repeated labeling the specific activities of C3-preparations were different. In order to explore whether C3 was cleavable by physical treatment, radiolabeled C3 was stored for 24 h at −20°C, during which time it was thawed and frozen twice.

2.3. *Cells and experimental procedure*

Raji cell were cultured in RPMI-1640 (Flow Lab. Ltd. Irvine, Ayrshire, Scotland) supplemented with 10% heat inactivated fetal calf serum (FCS) (Flow). 0.1 ml of 1×10^7 cells/ml, previously washed with cold PBS, were incubated for 30 min at 4°C or 37° with anti-CR2 MoAbs (10 μg/ml) or with TPA (30 ng/ml) (Sigma) The washed cells, resuspended in 0 1 ml PBS, were incubated with radiolabeled C3 (10 μl), C3b and C3d (5 μl) at 4°C and 37°C (TPA-treated cells) or 4°C (MoAb-coated cells) for 30 min in a final volume of 0.12 ml. Afterwards, the cells were washed and the radioactivity counted in a γ counter and dissolved in 50 μl of Laemmli's buffer [33] containing 2-mercaptoethanol (1 mM), (Sigma)

2.4. *Immunofluorescence*

Reactivity of the membrane bound molecules with HB-5 (Beckton Dickinson, Mountain View, CA, USA), OKB7 (Ortho Pharmaceutical Corp.,Raritan, NJ USA), anti-human C3b-receptor (14 μg/ml) and anti-human C3bi-receptor (7 μg/ml) (Dakopatts, Copenahagen, Denmark) was detected by indirect immunofluorescence using fluorescin isothiocyanate (FITC)-conjugated rabbit anti-mouse Ig (Cappel, West Chester, PA, USA). Reactivity with this reagent alone served as negative control. For some experiments direct FITC was performed by using anti-C3d-FITC (17 μg/ml) (Dakopatts). The samples were analyzed in Facscan (Beckton Dickinson)

2.5. *SDS-PAGE*

SDS-PAGE was carried out with slab gels of 10.5% or a 7.5–20% gradient of polyacrylamide. For each run, radiolabeled molecular weight markers (NEN) or prestained standard proteins (Bio-Rad, Richmond, CA, USA) were included. Before loading, the samples were sonicated (Soni-Prep) for 10 s and boiled for 3 min. After electrophoresis, the dried gels were exposed for 2–3 days at −80°C to New X-Ray film (Fuji Photo film Co Ltd, Japan).

3. RESULTS

3.1. *Characteristics of C3, C3b and C3d preparation*

Hemolytic active C3 was stored for ten months at −80°C. During this period the hemolytic titer ($C3_{H50}$) declined from 118 U/mg to 5 U/mg. C3 showed the typical α and β chains of 115 and 75 kDa, respectively (Fig. 1a). The radiolabeled preparation following freezing and thawing showed its intact chains and two not well defined fragments (Fig. 1b). C3b showed the α′ and β chains of 105 kDa and of 75 kDa, respectively (Fig. 2, lane a). The C3d preparation, consisted of a 35 kDa band (Fig. 2, lane c). C3c was used as internal marker (Fig. 2, lane b). The binding of C3 to Raji cells was dose-dependent as detected by cytofluorimetric analysis and saturation was achieved at a concentration of 2.6×10^{-7} M C3 (Fig. 3).

3.2. *Detection of CR1, CR2 and CR3 on Raji cells*

Cytofluorimetry showed that Raji cells were CR2-positive (99%), but not CR1- (0.8%) and CR3-positive (6%) (data not shown).

3.3. *Binding of C3, C3b and C3d to Raji cells is temperature-sensitive*

The radioiodinated C3 preparations were incubated with Raji cells for 30 min at 4°C or at 37°C. C3, C3b and C3d bound to the cells at 4°C (Fig. 4, lanes a,c,d) and in larger amounts at 37°C (Fig. 4, lanes b,d,f). The amount of the C3 preparations bound to Raji cells at 37°C is shown, in terms of cpm, in Fig. 5A. The temperature sensitive binding of C3 was also detectable by cytofluorimetry as shown in Fig. 6.

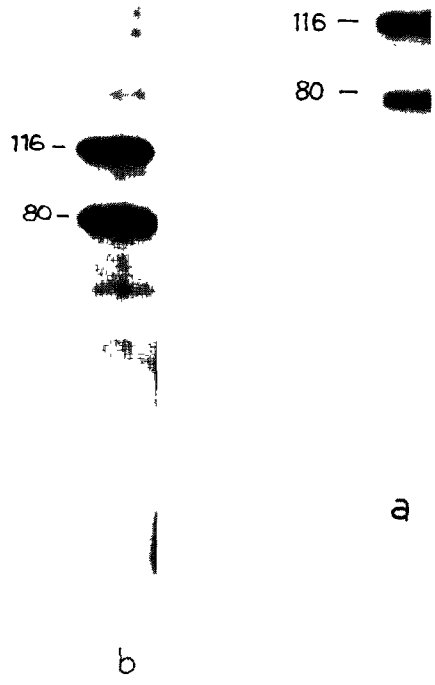

Fig. 1. SDS-PAGE autoradiography of C3. [^{125}I]-C3 (lane a), and [^{125}I]-C3 frozen and thawed twice (lane b) were run under reducing conditions on a 7.5–20% SDS-PAGE gradient.

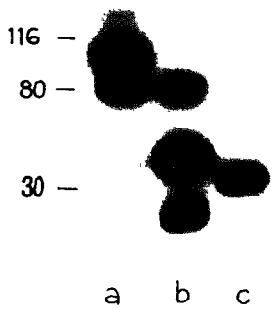

Fig. 2. SDS-PAGE autoradiography of C3b, C3c and C3d Radiolabeled C3b (lane a), C3c (lane b) and C3d (lane c) were run under reducing conditions on a 10.5% SDS-PAGE (mini gel).

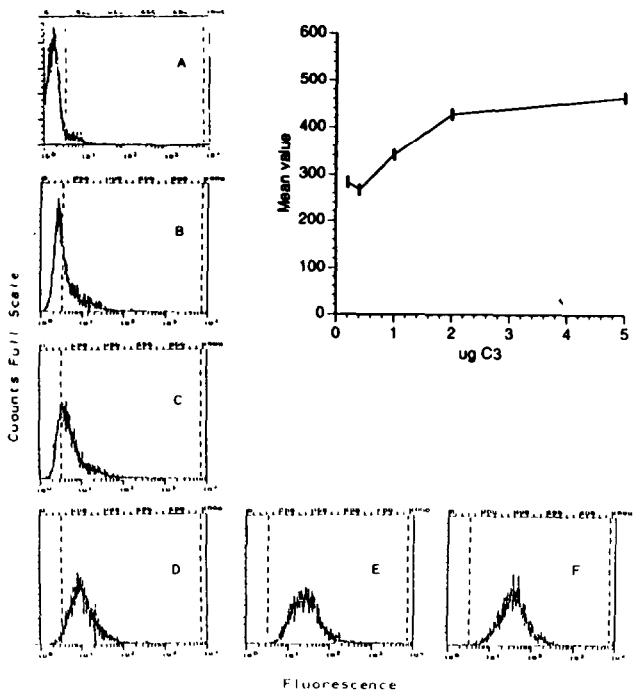

Fig. 3. Dose-dependent binding of C3 to Raji cells. 1×10^6 Raji cells were incubated with PBS (A) or with 0.1 μg (B), 0.4 μg (C), 1 μg (D), 2 μg (E) and 5 μg (F) of C3 for 30 min at 4°C. Afterwards, the washed cells were incubated with anti-C3d-FITC for 30 min at 4°C, washed and analyzed in Facscan. The mean value of the fluorescent peaks plotted versus the μg of C3 added to the incubation system is also reported.

3.4. *Anti-CR2 MoAbs, OKB-7 and HB-5, modulate the binding sites on Raji cells for C3, C3b and C3d*

Treatment of Raji cells with OKB7 and HB5 was performed both at 4°C and 37°C, whereas the relative binding with the radiolabeled preparations was done at 4°C. This experimental approach allowed us to evaluate eventual differences of binding of the C3 preparations.

The binding of C3 and C3d to 4°C-OKB7-coated cells was weak (Fig. 7, lanes a,c). The binding of C3 to 37°C-OKB7-coated cells was undetectable, whereas that for C3d was high (Fig. 7, lanes b,d). The binding of C3b to OKB7-coated Raji cells was low (data not shown).

The results with HB5-coated Raji cells were partly similar (Fig. 7, lanes e–j). 4°C HB5-coated cells showed binding to C3, C3b and C3d (lanes e,g,i). 37°C-HB5-coated cells bound C3 (lane f), to a high extent C3d (lane j), whereas C3b was undetectable (lane h).

The amount of the C3 preparations bound to 37°C anti-CR2 MoAb coated-Raji cells is shown, in terms of cpm, in Fig. 5B,C.

The results indicate that C3 and C3b may bind different binding sites and that treatment of the cells with anti-CR2 MoAbs increased the binding of monomeric C3d.

3.5. *TPA modulates the binding sites on Raji cells for C3, C3b and C3d*

37°C TPA-treated-Raji cells bound C3 and C3d to a low extent (Fig. 8, lanes b,f) and showed high binding capacity for C3b (Fig. 8, lane d). The amount of the C3 preparations bound to TPA-treated Raji cells is shown, in terms of cpm, in Fig. 5D. The results demonstrate that C3 and C3d binding sites are different from those of C3b.

Fig. 5 clearly shows that the binding sites for the C3 preparations may be differently expressed following the interaction of the cells with anti-CR2 MoAbs and TPA.

3.6. *Binding of C3 to CR2*

Raji cells incubated with C3 showed an anti-C3d-FITC peak (Fig. 9a) that was shifted toward the left when OKB7-coated cells were incubated with the same amount of C3 (Fig. 9c) indicating that OKB7 inhibited the binding of C3 to CR2. HB5 did not affect the binding of C3 (Fig. 9d).

4. DISCUSSION

In the present study we have investigated the capacity of Raji cells to bind C3 and C3-derived fragments.

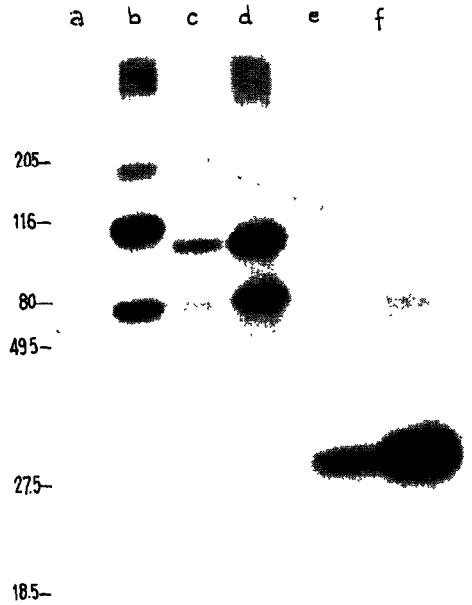

Fig. 4. SDS-PAGE autoradiography of C3, C3b and C3d bound to Raji cells. Raji cells were incubated with [^{125}I]-C3 (lanes a,b); [^{125}I]-C3b (lanes c,d) and [^{125}I]-C3d (lanes e,f) for 30 min at 4°C (lanes a,c,e) or at 37°C (lanes b,d,f). The washed cells were dissolved in Laemmli's buffer and run under reducing conditions on a 7.5–20% SDS-PAGE gradient

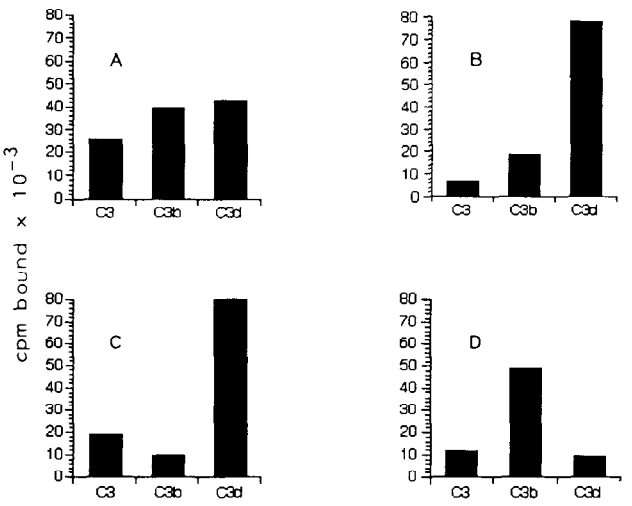

Fig. 5. Histograms of C3, C3b and C3d cpm bound to Raji cells. Panel A: Raji were incubated for 30 min with C3, C3b and C3d at 37°C. Panel B: Raji were incubated for 30 min with OKB-7 at 37°C. Afterwards, the washed cells were incubated with the radiolabeled preparations for 30 min at 4°C. Panel C: Raji were incubated for 30 minutes with HB-5 at 37°C. Thereafter, the washed cells were incubated with the radiolabeled preparations for 30 min at 4°C. Panel D: Raji were incubated for 30 min with TPA at 37°C. Afterwards, the washed cells were incubated with the radiolabeled preparations for 30 min at 37°C. Bound radioactivity was counted in a γ-counter. The results derive from one single experiment, but they are representative of three experiments performed with different radiolabeled preparations (Table I).

The C3 used was hemolytically inactive, due to the long storage of original active C3 at low temperature [14]. Raji cells bound this form of C3 in a dose-dependent fashion in the range of 10^{-9}–10^{-8} M and the molecular size of α and β chains of the reacting molecule did not change. Also physical treatment did not cause cleavage of this C3 preparation. These results confirm that hemolytic inactive C3 does not undergo autolytic cleavage [17] and that it can bind cell surfaces [18]. However, in the case of CR1-negative Raji cells the binding site for C3 has to be offered by another membrane protein.

It has been shown that the binding of C3b to CR2-positive cells is temperature-sensitive [10] and this characteristic can also be applied to C3 and C3d as demonstrated above. These and other unpublished data support the hypotesis that the temperature-sensitive binding of CR2 ligands to Raji cells may be due to a disclosing process of hidden or new binding sites on the cell surface similar to that described on mucins [34].

Multiple C3 binding sites for CR2 have been predicted [35]. We used the radiolabeled C3 preparations in the concentration range of 2–10 nM to reduce the possibility of cross-binding of each C3 preparation to different and multiple binding sites. Indeed, the binding of C3 fragments to CR2-positive cells is rather complex, depending on acceptor and receptor binding sites [5–11] and on the fact that CR2 is the C3d receptor and thus potentially able to bind any molecule bearing and exposing C3d. The aspect of multiple binding sites was then approached by treating the cells with anti-CR2 MoAbs or with TPA and thereafter with the radiolabeled C3 preparations at temperatures and concentrations that allowed to evaluate their modulation. TPA and anti-CR2 MoAbs, in fact, induce activation and different fate of CR2-positive cells [26–28,36], according to specific signaling of different triggers [37].

OKB-7 determined a decrease of the binding of C3, while HB-5 was inhibitory for C3b. These two MoAbs recognize different SCRs on CR2 [21] and some epitopes recognized by OKB7 [38] should also be recognized by C3 since OKB7 and not HB5 inhibited the binding of C3. These results, derived from autoradiographic analysis, were fully confirmed by cytofluorimetric studies. Therefore, it is evident that C3 binds CR2 on Raji cells.

The binding of C3d molecules to anti-CR2 MoAb-coated cells was the result of an active cellular metabolism since this result was obtained following incubation of the cells at 37°C with the MoAbs. These findings are

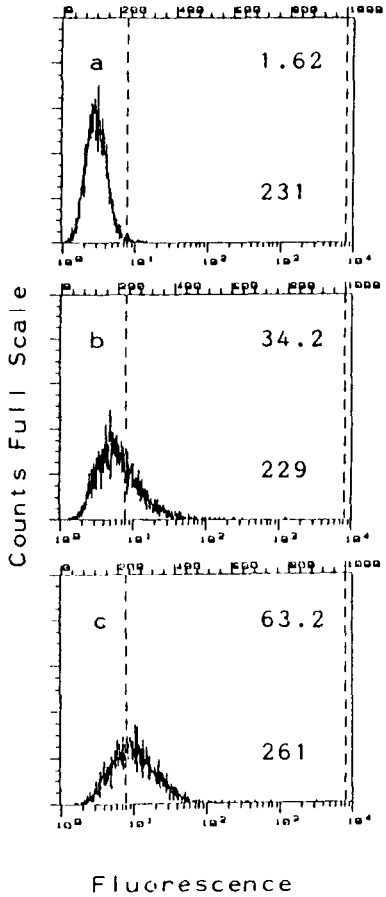

Fig 6. Temperature sensitive binding of C3 to Raji cells. Raji were incubated with PBS (a) and 1 μg of C3 (b,c) for 30 min at 4°C (a,b) or 37°C (c). The washed cells were incubated with anti-C3d-FITC for 30 min at 4°C, washed and analyzed in Facscan. The percent of positive cell (top value) and the fluorescent peak mode (bottom value) are also reported in each panel.

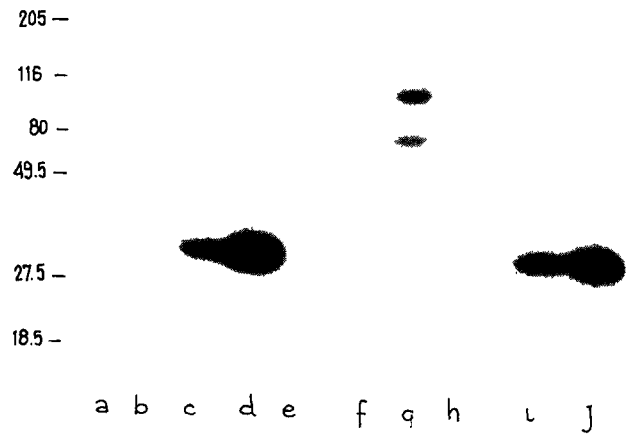

Fig. 7. SDS-PAGE autoradiography of C3, C3b and C3d bound to anti-CR2 MoAb coated-Raji cells. Raji cells were incubated with OKB7 (lanes a–d) or HB5 (lanes e–j) for 30 min at 4°C (lanes a,c,e,g,i) or at 37°C (lanes b,d,f,h,j). The cells were washed and then incubated with [^{125}I]-C3 (lanes a,b,e,f), [^{125}I]-C3b (lanes g,h) and [^{125}I]-C3d (lanes c,d,i,j) for 30 min at 4°C. The washed pellets were dissolved in Laemmli's buffer and run under reducing conditions on a 7.5–20% SDS-PAGE gradient.

Table I

Raji cells were incubated with PBS, OKB7, HB5 and TPA for 30 minutes at 37°C

Treatment		cpm bound C3	C3b	C3d
(a) PBS treated-Raji	*	25.907	39.002	42.537
	**	18.940	20.811	37.940
	***	21.354	18 673	26.539
(b) OKB7 coated-Raji	*	6 448	18.340	77 718
	**	9.340	19.870	53.739
	***	7.374	10.352	55.392
(c) HB5 coated-Raji	*	18.637	9.827	80.000
	**	26.352	6.025	49.818
	***	25.396	13.438	33.000
(d) TPA treated Raji	*	11.588	48.997	9.715
	**	8.320	16.400	20.341
	***	2.022	33 100	5 409

Washed cells were incubated with radiolabeled C3, C3b and C3d at 37°C (a,d) or at 4°C (b,c) for 30 min. Afterwards the cells were washed and the radioactivity counted in a γ counter. Single values of three different experiments whose specific activities were: *C3, 2.5 μCi/μg, C3b, 7.01 μCi/μg, C3d, 7.0 μCi/μg; **C3 1.9 μCi/μg, C3b 4.8 μCi/μg, C3d 6.7 μCi/μg, ***C3 2 2 μCi/μg, C3b 8.2 μCi/μg, C3d, 7.6 μCi/μg.

in contrast with data showing a competitive or common binding between OKB-7 and C3dg on CR2 [21,39]. Most likely, this is due to the fact that our data derive from experiments in which CR2-positive cells and not purified CR2 have been used. However, the C3d binding sites present on anti-CR2 MoAb-coated cells would be related to those responsible for the fixation of C3-related fragments on a cell surface which is not inhibited by anti-CR2 MoAbs [5,22].

In this study we also investigated the differences of the binding of C3 preparations following cell activation by TPA which has been shown to be inhibited by monomeric C3dg [26]. Our results demostrate that TPA-treated cells bound C3b whereas the binding of C3 and C3d was strongly inhibited if compared to the control, thus demonstrating that C3b may bind different binding sites from those recognized by C3 and C3d.

Taken together these data indicate that Raji cells may express on their surface three binding sites, respectively for C3 (hemolytically inactive), C3b and C3d, which can be modulated by anti-CR2 MoAbs or TPA.

A final aspect to be taken into account is that under our experimental conditions the C3 preparations were never further cleaved, thus indicating that they may exist as stable cellular ligands.

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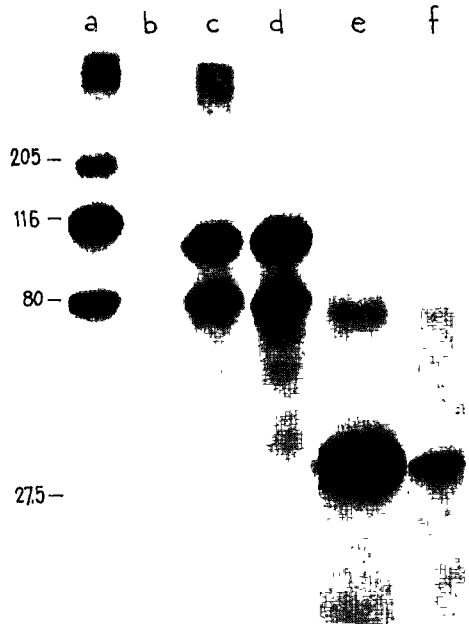

Fig. 8. SDS-PAGE autoradiography of C3, C3b and C3d bound to TPA treated-Raji cells. Raji cells were incubated with PBS (lanes a,c,e) or TPA (30 ng/ml) (lanes b,d,f)) for 30 min at 37°C. Afterwards the cells were washed and incubated with [^{125}I]-C3 (lanes a,b), [^{125}I]-C3b (lanes c,d), and [^{125}I]-C3d (lanes e,f) for 30 min at 37°C. The washed pellets were dissolved in Laemmli's buffer and run under reducing conditions on a 7.5–20% SDS-PAGE gradient.

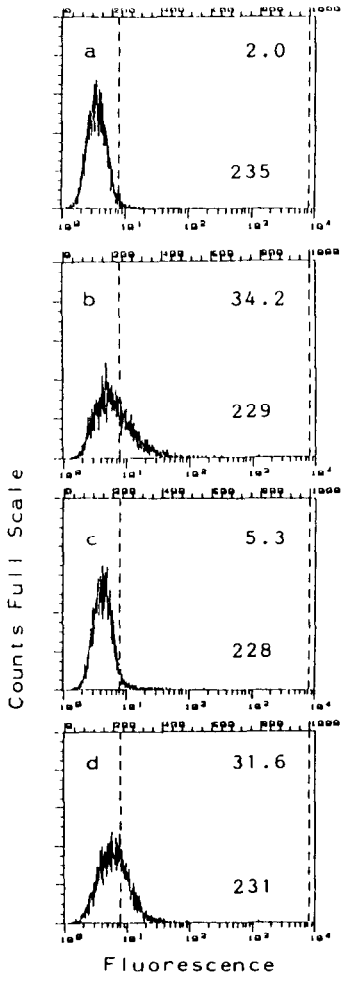

Fig. 9. Cytofluorimetry of C3 bound to Raji cells. 1×10^6 Raji were incubated with PBS (a), 1 μg of C3 (b), 1 μg of OKB7 (c), 1 μg of HB5 (d) for 30 min at 4°C. The washed cells were incubated with PBS (a,b) or 1 μg of C3 (c,d) for 30 min at 4°C. Afterwards the washed cells were incubated with anti-C3d-FITC for 30 min at 4°C, washed and analyzed in Facscan. The percent of positive cell (top value) and the mode of fluorescent (bottom value) are also reported in each panel.

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