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Covalent binding of C3b to tetanus toxin: influence on uptake/internalization of antigen by antigen-specific and non-specific B cells

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SUMMARY

Antigen opsonization by the C3b fragment of complement is a significant event in the modulation of cell-mediated immune response, but its mechanism is still largely unknown. The structural characteristics of C3b allow it to act as a bifunctional ligand between antigen and cells via their membrane C3b receptors. It was thus of interest to study the influence of the covalent link between C3b and antigen on the fixation and internalization of this antigen by antigen-presenting cells. Tetanus toxin (TT) was used as antigen, either free or covalently linked to C3b (TT-C3b). The antigen-presenting cells were TT-specific (4.2) or non-specific (BL15) Epstein–Barr virus (EBV)-transformed B cells. C3b was found to play an important role in antigen fixation and internalization by both antigen-specific and antigen non-specific cells. Covalent binding of C3b on TT (1) permitted fixation and internalization of this antigen by non-specific cells via their complement receptors; (2) enhanced antigen fixation and resulted in cross-linking between membrane immunoglobulins and complement receptors on antigen-specific cells. The consequences of covalent C3b binding to TT were analysed using antigen-specific and antigen-non-specific cells. In both cases, a net increase in antigen fixation was observed. At the intracellular level, covalent C3b binding to TT resulted in a large TT incorporation in endosomes of non-specific cells, similar to that observed in antigen-specific cells. Thus, C3b covalently linked to antigen enlarges the array of B-cell types capable of presenting antigen, including non-specific cells.

INTRODUCTION

The modulation of the cell-mediated immune response by complement C3 and its fragments was described several years ago.[1] Increasing evidence from *in vivo* and *in vitro* experiments reveals the complexity of this modulation, which seems multiparametric, and affects different levels: inhibition of lymphoblastogenesis,[2] suppression of some T-lymphocyte functions,[3] modulation of interleukin-2 (IL-2)-dependent[4] and antigen-dependent[5] T-cell proliferation, increase in the antigen-presenting function of B cells[5] and of antibody-dependent cellular cytotoxicity[6] (reviewed recently by Erdei *et al.*[7] and Villiers[8]).

These effects are partly due to opsonization of antigens (Ag) with C3b: this protein acts as a bifunctional ligand between Ag and cells, involving covalent binding (C3b–Ag) and subsequent non-covalent interaction of C3b–Ag complexes with cellular C3b receptors.[5,9] Previous results demonstrated that C3b-binding on tetanus toxin (TT) used as Ag reduced, by a factor of 100, the amount of TT required for antigen-specific T-cell proliferation. This effect seems to be partly due to a delay in the intracellular proteolysis of TT in the TT–C3b complexes,[10] but other parameters, such as internalization, are also believed to play a role: the mechanism of Ag uptake by antigen-presenting cells (APC) may be different for TT alone (involvement of membrane immunoglobulin; mIg) or TT–C3b (additional involvement of complement receptors; CR1, CR2). A difference in the surface molecules involved in Ag uptake could lead to a change in intracellular signalling, thus using different processing and presentation mechanisms. This point has been analysed using purified TT–C3b complexes formed after C3 thioester disruption,[11] which are highly representative of those generated *in vivo* during the immune response.

This paper, specifically dedicated to the study of fixation and uptake/internalization of TT and TT–C3b complexes, reports data obtained using antigen-specific and non-specific lymphoblastoid B cells as APC. These experiments provide the first evidence for simultaneous involvement of mIg and CR in the internalization of an Ag covalently linked to C3b.

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Abbreviations: Ag, antigen; APC, antigen-presenting cell; C3b-Ag, C3b covalently linked to Ag; CR, complement receptor; DMEM, Dulbecco's modified Eagle's medium; FITC, fluorescein isothiocyanate; K_d, dissociation constant; mIg, membrane immunoglobulin; PBS-OVA-NaN_3, PBS containing ovalbumin and sodium azide; TT, tetanus toxin.

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MATERIALS AND METHODS

Cells

The TT-specific Epstein–Barr virus (EBV)-transformed B-cell clone 4.2 was kindly provided by Dr A. Lanzavecchia.[12] The non-specific EBV-transformed B-cell line BL15 was obtained as described previously.[13]

B-lymphoblastoid cells were maintained in culture at 0·5–2·0 × 10^6 cells/ml in RPMI-1640 (Gibco, Cergy-Pontoise, France) supplemented with 2 mM L-glutamine and 10% fetal calf serum (FCS; heat inactivated for 30 min at 56°). Cultures were routinely diluted every 3 days. Twenty hours before use, cells were centrifuged (5 min at 300 *g*) and resuspended in fresh medium at 10^6 cells/ml.

Proteins

Purified TT was generously supplied by Dr J.R. Cartier (Institut Mérieux, Lyon, France) and was further purified as described previously.[11] TT–C3b complexes (TT : C3b molar ratio = 1 : 1) were prepared according to Villiers *et al.*[11] C3b was prepared as described elsewhere.[11]

IgG–anti-TT (IgG–4.2) was purified from a 4.2 cell culture supernatant using protein A–agarose chromatography (ImmunoPure® Plus immobilized protein A; Pierce, Montluçon, France) as follows: 1 l culture supernatant was adjusted to pH 8·6 and applied to a column of protein A–agarose (12 × 60) equilibrated in 150 mM NaCl, 50 mM Tris-HCl, pH 8·6, at a flow rate of 20 ml/hr. The column was washed with the same buffer and IgG was eluted using 150 mM NaCl, 50 mM acetate buffer, pH 4.3. Fractions of 1 ml were collected in tubes containing 1 M Tris, pH 8·0 (50 μl), and their absorbance at 280 nm was monitored. IgG was dialysed against phosphate-buffered saline (PBS) and concentrated by ultrafiltration on Amicon (PM10; Amicon, Beverly, MA) before storage at −80°.

Mouse monoclonal anti-CR1 (J3.D3) and anti-CR2 (BL13) antibodies were purchased from Immunotech (Marseille, France), and anti-CR2 (OKB7) was purchased from Ortho Diagnostic Systems (Paris, France).

Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin $F(ab')_2$ was purchased from Jackson Immuno Research (West Grove, PA).

Ferri-transferrin was prepared from transferrin (Sigma, L'Isle d'Abeau, France) by incubation for 30 min at 37° in the presence of $FeCl_3$ (transferrin : $FeCl_3$ = 2 : 1 p/p) in 0·25 M Tris-buffer containing 10 mM $NaHCO_3$. Unbound Fe was removed by filtration through a PD10 column (Pharmacia, St Quentin Yvelines, France).

Protein labelling

Ferri-transferrin, purified TT and TT–C3b were labelled with $Na^{125}I$ (Amersham, Les Ulis, France) using the Iodogen® (Pierce) method.[14] Free iodine was eliminated by filtration through a Sephadex G50-fine column. Specific radioactivities were 11 × 10^6 c.p.m./μg ferri-transferrin, 7 × 10^6 c.p.m./μg C3b and 4 × 10^6 c.p.m./μg free or complexed TT.

Antibody assays

The reactivity of IgG–4.2 versus TT and TT–C3b was analysed using competition assays: IgG–4.2 (0·8 μg/ml) was preincubated for 15 min at room temperature in the presence of 0–3 μg/ml of TT alone or complexed to C3b before enzyme-linked immunosorbent assay (ELISA), as described elsewhere,[13] using TT-coated plates. Binding was revealed by peroxidase-conjugated goat anti-human IgG (Bio-Rad, Paris, France).

Quantification of receptors

CR1 and CR2 on BL15 and 4.2 cells were assayed by immunofluorescence. Cells were washed in ice-cold phosphate-buffer, referred as to PBS-OVA-NaN_3 buffer [PBS containing 1% chicken egg albumin (w/v) and 0·2% NaN_3 (w/v)]. 10^6 cells were distributed in each well of a 96-well U-bottomed plate (Falcon, Meylan, France) and centrifuged (3 min at 450 *g*, 4°). The cell pellets were preincubated for 15 min at 0° in the presence of 32 mg/ml non-immune rabbit IgG to saturate cell membranes. The cells were washed twice in PBS-OVA-NaN_3 buffer and were incubated for 45 min at 0° in the presence of 0·2 mg/ml mouse monoclonal anti-CR1 (J3.D3) or anti-CR2 (BL13). Cells were washed twice in PBS-OVA-NaN_3 buffer, and binding of monoclonal antibodies was revealed by 1 hr incubation at 0° in the presence of 60 μg/ml FITC-conjugated goat anti-mouse immunoglobulin $F(ab')_2$. A control with an irrelevant mouse antibody was run in parallel. The cells were analysed by cytofluorometry with a FACStar (Becton-Dickinson, Meylan, France).

C3b binding on BL15 and 4.2 was assayed as follows. Cells were washed and distributed in a 96-well plate as described above. The cell pellets were incubated for 1 hr at 4° in the presence of different concentrations of ^{125}I-C3b (0·5–10 μg/ml) alone, together with free TT (C3b : TT = 1 : 1) or complexed to TT, in 50 μl. The cell suspension was then centrifuged on an oil cushion [dinonyl phtalate (Fluka, L'Isle d'Abeau, France)/di-*n*-butyl phtalate (Siccap-Emmop, Marseille, France) 1 : 4, v/v][15] and the radioactivity of the pellet and supernatant fractions was counted. Binding constants were determined according to Scatchard.[16]

Antigen binding: competition assays

7 × 10^5 cells were washed in PBS-OVA-NaN_3, resuspended in the same buffer (100 μl) containing the competitor (competitor : Ag = 4 : 1, mole : mole), and incubated for 15 min at 0°. Then, 0·3 μg of radiolabelled antigen (TT alone or complexed to C3b) was added and cells were further incubated for 1 hr at 0°. The cell suspension was then centrifuged on an oil cushion as described above[15] and the radioactivity of the pellet and supernatant was counted. A control, where the first incubation was made in the absence of competitor, determined 100% fixation.

Antigen and ferri-transferrin internalization

Cells were washed in Dulbecco's modified Eagle's medium (DMEM; Gibco) and resuspended at 20–40 × 10^6 cells/ml in 500 μl DMEM containing radiolabelled ferri-transferrin (5 μg), TT alone (4·2 μg) or complexed to C3b (3·0 μg). After incubation at 37° for different times in the presence of Ag, or for 30 min in the presence of ferri-transferrin, cells were diluted in 10 ml cold DMEM, washed and resuspended in 1 ml homogenization buffer (1 mM HEPES, pH 7·2, 250 mM sucrose and 1 mM EDTA).

Subcellular fractionation

Cells (20–40 × 10^6 cells in 1 ml homogenization buffer) were disrupted by 30 passes through a 8·02 ball-bearing homogenizer (EMBL, Heidelberg, Germany) to obtain more than 80% lysis,

as monitored by phase-contrast microscopy. Nuclei and plasma membranes were removed by centrifugation at 2800 *g* (5 min, 4°) and the supernatant was fractionated by centrifugation on a Percoll gradient as follows: 11 ml Percoll (17%, v/v) (Pharmacia) in homogenization buffer was layered on a 1-ml 60% (w/v) sucrose cushion in 16 × 76-mm Quick-Seal tubes (Beckman, Gagny, France). The post-nuclear supernatant (1 ml) fraction was layered on top. After centrifugation for 90 min at 22 000 *g* in a 70.1 Ti rotor (Beckman) at 4°, gradients were recovered in 0·6-ml fractions from the bottom of the tubes, using an Auto Densi Flow IIC apparatus (HBI; Roucaire, Courtaboeuf, France) connected to a 200 PVP collector (ISCO; Roucaire).

Enzymatic activities

Galactosaminidase activity was determined using paranitrophenyl-*N*-acetylgalactosaminide (5 mM in 10% DMSO; Sigma) as substrate.[17] Twenty microlitres of each fraction collected from the gradient was mixed with 50 μl 0·5 M acetate buffer (pH 5·0) containing 0·1% (w/v) triton-X-100 in microtitre plates (Dynatec, Billingshurst, UK). After addition of 75 μl substrate and a 1-hr incubation at 37°, the reaction was stopped with 100 μl 0·1 N NaOH. Release of paranitrophenol was quantified at 405 nm using a Titertek Multiskan (Flow Laboratories, Irvine, UK).

Cathepsin B and D activities were determined using benzyloxycarbonyl-Arg-Arg-2-naphthylamide and benzoyl-Arg-Gly-Phe-Phe-Pro-4-methoxy-2-naphthylamide (20 mM in DMSO; Interchim, Montluçon, France), respectively, as substrates.[18] Twenty microlitres of each fraction collected from the gradient was mixed with 75 μl 50 mM acetate buffer (pH 5·0) containing 0·1% (w/v) triton-X-100 and 6 mM cysteine. After addition of 5 μl substrate and a 1-hr incubation at 37°, the reaction was stopped with 950 μl 1 mM iodoacetamide for cathepsin B and 1·5 μM pepstatin A for cathepsin D. Release of 2-naphthylamide was quantified from its fluorescence at 410 nm (excitation = 335 nm) using a F-2000 Fluorescence Spectrophotometer (Hitachi, Tokyo, Japan).

Characterization of endosomes and lysosomes

After subcellular fractionation on Percoll gradients, endosomes and lysosomes were localized using transferrin incorporation and measurement of galactosaminidase activity, respectively.

A single peak of radioactivity was observed after incorporation of radiolabelled transferrin; this peak was localized at the top of the gradient and the corresponding tubes were assessed to be endosome-containing fractions.[19] Galactosaminidase activity was seen in a single peak at the bottom of the gradient and the corresponding tubes were assessed to be lysosome-containing fractions.[20]

Cathepsin B and D activities were measured, as these proteases are known to play a role in Ag processing.[21] Both enzymes colocalized with galactosaminidase; weaker cathepsin B activity was observed for 4.2 compared with BL15, but in both cases cathepsin B activity was greater than cathepsin D activity.

RESULTS

TT and TT–C3b uptake/internalization

BL15 and 4.2 cells were incubated for different times at 37° in the presence of radiolabelled TT or TT–C3b; internalization of these Ag was analysed on Percoll gradients after cell disruption, as described in the Materials and Methods. The radioactivity recovered on the gradient corresponded to Ag internalized during the incubation. As shown in Fig. 1, similar kinetic patterns were obtained in the various cases; the biphasic aspect of the curves observed in the different uptake experiments probably corresponds with a net uptake (first phase) followed by partial exocytosis. BL15 cells (non-specific for TT) incorporated weak TT compared with 4.2 cells (specific for TT). Covalent binding of C3b on Ag increased its incorporation during the initial phase for both cell lines. This effect was reduced after 1 hr incorporation by 4.2 cells, whereas it persisted for at least 2 hr of incubation with BL15, thus allowing these non-specific cells to incorporate the Ag as well as antigen-specific cells.

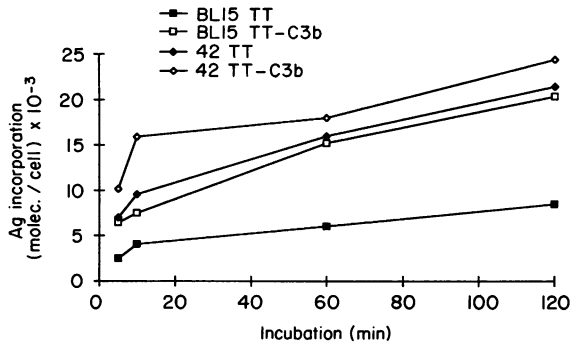

Figure 1. TT and TT–C3b internalization. BL15 and 4.2 cells (20×10^6) were incubated at 37° for various times in the presence of radiolabelled TT (4·2 μg) or C3b–TT (3·0 μg) and analysed on Percoll gradients after cell disruption, as described in the Materials and Methods. Total Ag incorporation by cells was determined by measuring the total radioactivity recovered on the gradient.

CR expression on BL15 and 4.2 cells

As the enhancement of TT internalization due to its binding to C3b was weaker for 4.2 cells compared with BL15 cells, we estimated the expression of complement receptors CR1 and CR2 on BL15 and 4.2 cells using fluorescent-activated cell sorter (FACS) analysis, as described in the Materials and Methods (Fig. 2). In both cases the majority of the cells expressed CR2, as 65% of BL15 and 74% of 4.2 cells were labelled using anti-CR2 (BL13) and a FITC-labelled second antibody. Expression of CR1 was determined with anti-CR1 (J3.D3) used as first antibody; similar results were obtained for both cell lines, indicating a lower expression of CR1 compared with CR2, as 35% (BL15) and 24% (4.2) of the cells were labelled in this case.

TT and TT–C3b binding to IgG–4.2

To analyse interactions between TT–C3b and 4.2 cells, we compared the binding of TT alone or complexed to C3b on mIg expressed on 4.2 cells. This study was performed using a competition assay where radiolabelled TT binding on 4.2-secreted immunoglobulin was measured after preincubation of these immunoglobulins with TT or TT–C3b, as described in the

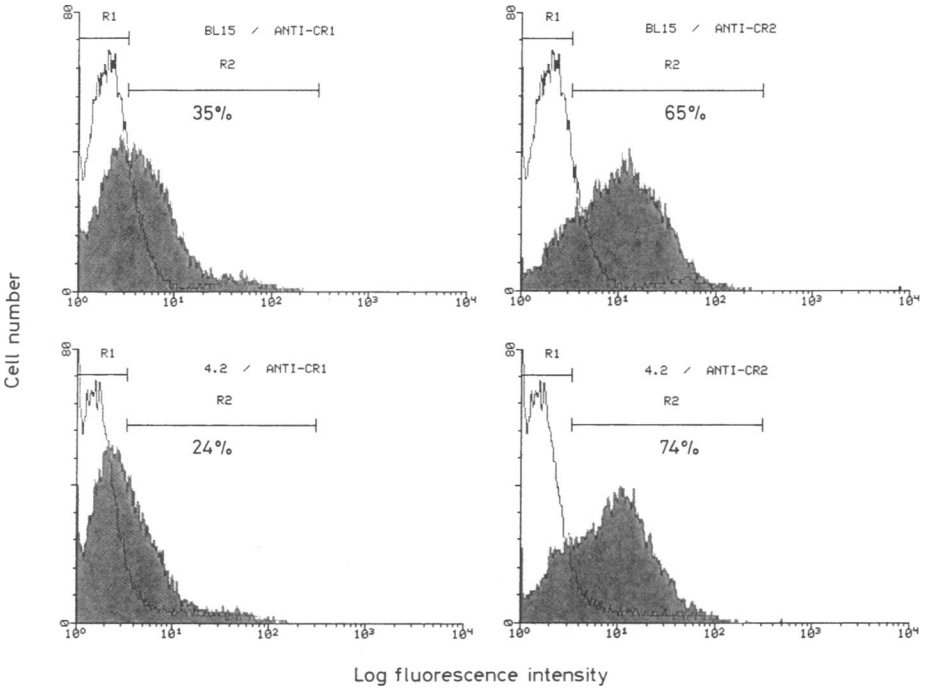

Figure 2. CR1 and CR2 expression by BL15 and 4.2 cells. Cell-surface expression was determined by FACS analysis as described in the Materials and Methods, using J3.D3 (anti-CR1) and BL13 (anti-CR2) as first antibody (shaded area). Controls using an irrelevant mouse antibody as the first antibody were performed (unshaded area) to evaluate the non-specific fixation of the FITC-labelled second antibody (R1 window). The percentages of positive cells were determined on the R2 window.

Materials and Methods. As shown in Fig. 3, the same patterns of inhibition were obtained using TT or TT–C3b as competitor, indicating equivalent access and affinity of TT for 4.2 antibody.

C3b binding on BL15 and 4.2 cells

To assess the binding of TT–C3b to cell CR, C3b fixation on BL15 and 4.2 cells was quantified. Scatchard analysis of radiolabelled C3b binding was performed as described in the Materials and Methods, in different conditions: C3b alone, in the presence of free TT (C3b + TT), or complexed to TT (TT–C3b). Typical saturable binding curves were obtained, allowing the number of binding sites/cell and the K_d to be determined. The results, summarized in Table 1, showed an enhancement of the total amount of C3b bound to the cells when covalent complexes (C3b–TT) were used. The affinity constant was about 8×10^{-8} M in all cases, except for the binding of C3b–TT complexes on 4.2 cells ($2{\cdot}5 \times 10^{-8}$ M).

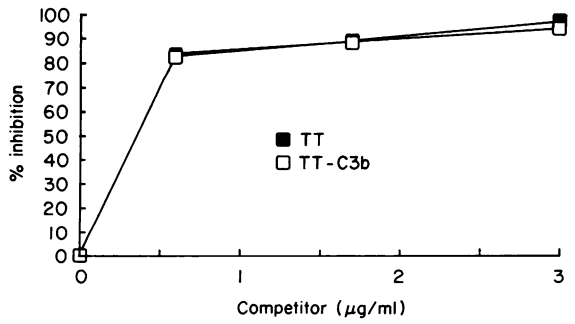

Figure 3. Binding of secreted IgG–4.2 to TT and TT–C3b. IgG–4.2 were preincubated in the presence of either TT alone or complexed to C3b before testing for TT binding in ELISA, as described in the Materials and Methods. 0% inhibition was determined using IgG–4.2 preincubated in the presence of PBS.

TT and TT–C3b binding on BL15 and 4.2 cells: competition assays

To determine which type of cell-surface receptor was involved in TT and TT–C3b binding on BL15 and 4.2 cells, competition assays were performed as described in the Materials and Methods. Before the addition of radiolabelled TT or TT–C3b, cells were preincubated with various competitors used alone or in different combinations: TT, TT–C3b, anti-CR1 (J3.D3) and anti-CR2 (BL13 or OKB7). Only the two monoclonal antibodies J3.D3 and OKB7 are known to block the binding of C3 fragments to CR1 and CR2, respectively.

The results are summarized in Fig. 4; fixation of TT on BL15 was not shown due to its low level, as expected with this TT non-specific B-cell line (no effect of the different competitors).

Fixation of TT–C3b on BL15 cells was not influenced by TT or anti-CR2 BL13, which was unable to block the binding of C3 fragments to CR2. In contrast, TT–C3b, anti-CR1 (J3.D3) and anti-CR2 (OKB7) were good competitors for TT–C3b binding to BL15. These results confirmed the involvement of CR in TT–C3b fixation to BL15.

Fixation of TT and TT–C3b to 4.2 cells was significantly inhibited by TT or TT–C3b as competitor. These observations

Table 1. Scatchard analysis of binding on BL15 and 4.2 cells of radiolabelled C3b under different conditions

	BL15		4.2	
	Sites/cell	K_d (M)	Sites/cell	K_d (M)
C3b	21 000 ± 3000	$7{\cdot}5 \times 10^{-8} \pm 0{\cdot}7$	20 300 ± 2700	$9{\cdot}5 \times 10^{-8} \pm 1{\cdot}1$
C3b+TT	20 500 ± 1500	$6{\cdot}6 \times 10^{-8} \pm 0{\cdot}6$	ND	ND
C3b–TT	41 000 ± 4000	$8{\cdot}7 \times 10^{-8} \pm 0{\cdot}5$	45 000 ± 3500	$2{\cdot}5 \times 10^{-8} \pm 0{\cdot}2$

Cells were incubated in the presence of various concentrations of ^{125}I-C3b alone (C3b), together with free TT (C3b+TT) or complexed to TT (C3b–TT), and C3b binding was quantified using Scatchard's plot as described in the Materials and Methods (K_d, constant dissociation).
ND, not done.

suggest that both TT and TT–C3b fixation to antigen-specific cells involve mIg. As for BL15, but to a lesser extent, blocking anti-CR2 OKB7 competed for TT–C3b binding on 4.2 cells and enhanced the inhibitory effect of TT, confirming CR involvement in TT–C3b fixation to antigen-specific cells. On the other hand, anti-CR1 had a weak effect both on the direct binding of TT–C3b on 4.2 cells and on the inhibition induced in this case by anti-CR2 OKB7 or by TT. It must be stressed that experimental conditions such as the low amount of TT–C3b available limited the scope of this study.

Subcellular distribution of TT and TT–C3b

As C3b binding on Ag leads to modifications in Ag fixation on cells, we decided to analyse some of the intracellular consequences of such modifications. We therefore studied the subcellular distribution of TT and TT–C3b after their internalization by antigen-specific or non-specific cells. BL15 and 4.2 cells were incubated for different times at 37° in the presence of radiolabelled TT or TT–C3b, as described in the Materials and Methods, and the subcellular distribution of the Ag was analysed on Percoll gradients. Two peaks of radioactivity were observed in all cases (a typical radioactivity distribution is shown in Fig. 5a), corresponding to the endosomes (fractions 14–18) and lysosomes (fractions 4–8) characterized as described in the Materials and Methods. Increasing the incubation time in the presence of Ag led to an increase in the intensity of both peaks. The distribution of the radioactivity between endosome and lysosome fractions was calculated from these experiments (Fig. 5b, c). The radioactivity in endosomes after TT internalization by BL15 cells was weak compared with 4.2 cells, but increased when TT was complexed to C3b and reached the same level as in 4.2 cells. Direct quantification of the fragments present in each fraction was not possible due to intracellular proteolysis and non-homogeneous Ag labelling.

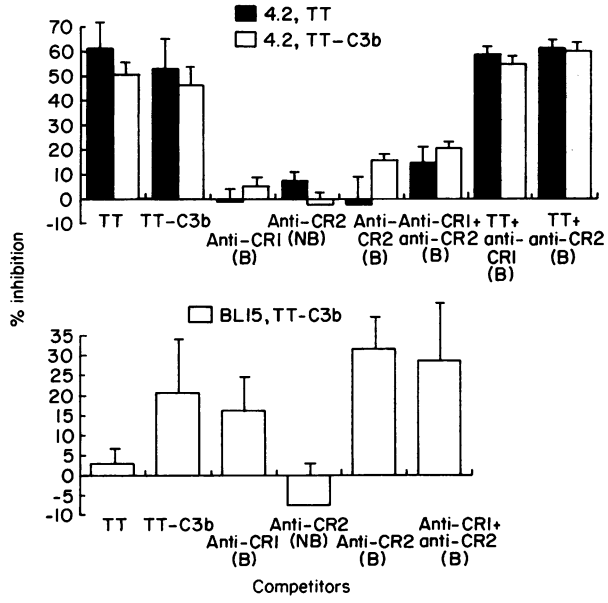

Figure 4. TT and TT–C3b binding on BL15 and 4.2 cells: competition assays. Cells (25×10^6) were preincubated for 15 min at 0° in the presence of TT, TT–C3b, anti-CR1 (J3.D3) or anti-CR2 (BL13 or OKB7) and further incubated for 1 hr at 0° after addition of 0·3 μg radiolabelled TT alone or complexed to C3b ([competitor]/[ligand] = 4). Ag fixation was determined as described in the Materials and Methods. Results are expressed as a percentage of inhibition of Ag binding. A control without competitor was used to determine 0% inhibition.

DISCUSSION

As expected, very weak Ag (TT) internalization by non-specific B-cell line (BL15) cells was observed, whereas covalent binding of complement component C3b to this antigen greatly increased its incorporation. These results suggest an effective Ag internalization via C3b or C3b fragments, involving antigen-specific complement component receptors (CR1, CR2, DAF, MCP, etc.). The presence of such receptors on lymphoblastoid cells has been described[22] and is confirmed for CR1 and CR2 on the cells used in this paper. The analysis of TT–C3b fixation on BL15 using competition assays confirmed CR involvement, since the blocking monoclonal antibodies anti-CR1 and anti-CR2 partially inhibited TT–C3b fixation on these cells.

Scatchard analysis of C3b binding on BL15 and 4.2 cells gave higher K_d values than those previously published,[23] which may be partly due to the radiolabelling of C3b. This could lead to a partial aggregation of the protein, enhancing its interaction with CR, as it has been shown that dimerization of C3b considerably increases its affinity for CR.[23] The enhancement of binding sites observed on BL15 when C3b is complexed to

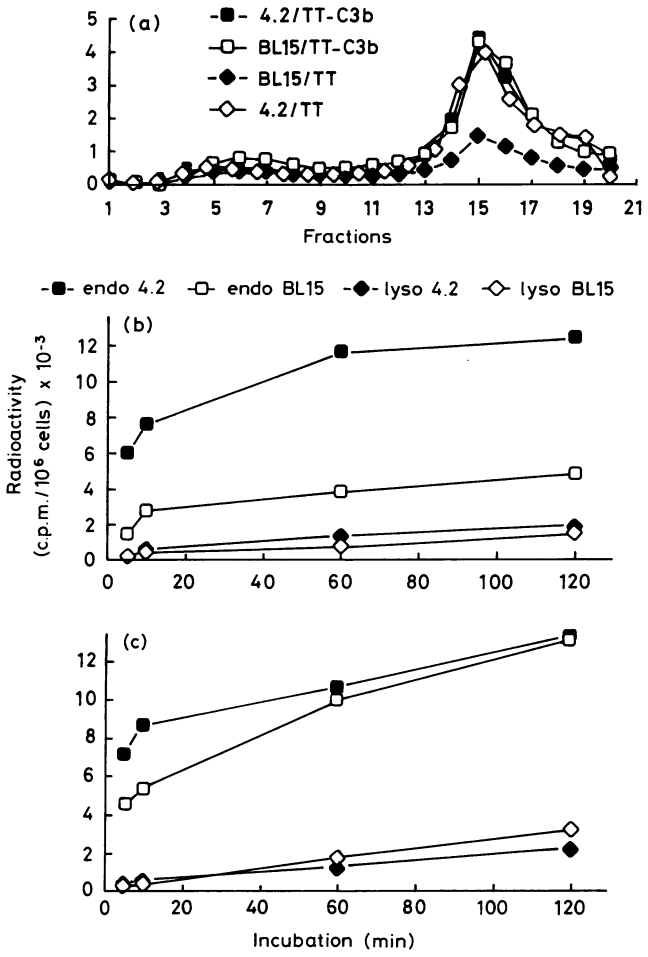

Figure 5. Subcellular distribution of Ag after internalization by cells. Cells (30×10^6) were incubated at 37° for different times in the presence of radiolabelled TT (4·2 μg) or TT–C3b (3·0 μg) and analysed on Percoll gradients after disruption as described in the Materials and Methods. (a) Localization on the gradient of TT and TT–C3b after 2 hr internalization by BL15 and 4.2 cells. The radioactivity recovered in each fraction was measured. (b, c) The radioactivity recovered in endosomal 14–18 and lysosomal 4–8 fractions was measured; results are expressed after TT (b) or TT–C3b (c) internalization by BL15 and 4.2 cells.

TT may be related to structural modifications of C3b inside the physiological complexes, allowing its binding on other(s) C3b-binding site(s) on CR1 (the same results were obtained using physiological C3b–C3b complexes; data not shown).[24] The equivalent number of C3b binding sites found on BL15 and 4.2 cells fits well with FACS analysis of CR1 and CR2 using indirect labelling (Fig. 2). These results were confirmed using direct labelling (^{125}I-J3.D3 and ^{125}I-BL13; data not shown). Results dealing with binding of C3b–TT to 4.2 cells are discussed below.

Uptake of TT by 4.2 cells was significant, in agreement with the antigen-specificity of this cell clone,[12] but the amount of Ag internalized by these cells was even higher when C3b was covalently bound to the Ag, especially during the first 30 min of incubation. Nevertheless, the increase of TT internalization following C3b binding on this Ag was less for 4.2 cells than for BL15 cells, although CR expression was the same for the two cell lines. This result was due neither to a limiting amount of Ag (only 2% of added Ag was internalized by the cells after 120 min incubation at 37°) nor to a defect in recognition of TT in the TT–C3b complexes by mIg, as suggested by Garred *et al.* in the case of C3b binding to immune complexes,[25] because the affinities of IgG–4.2 for TT and TT complexed to C3b were similar. This absence of an additive effect could be due to a saturation of the internalization system; another hypothesis is that both mIg and CR are simultaneously involved in TT–C3b internalization. This was confirmed by (1) the increased affinity of C3b–TT for 4.2 cells compared with BL15, without modification of the number of binding sites (Table 1), and (2) the analysis of TT–C3b fixation on 4.2 cells using competition assays: blocking monoclonal antibodies anti-CR1 and especially anti-CR2 enhanced the competitive effect of TT for TT–C3b fixation. The incomplete inhibitions obtained may be due to the experimental conditions (small amount of competitor available) and/or the involvement of other CR (CR4, MCP, DAF, etc.).

Taken together, these results give rise to a 'double anchorage' mechanism. This mechanism, which leads to a stronger Ag binding on the cells, could also account for the weaker competitive effect of (1) anti-CR on TT–C3b binding on 4.2 cells compared with the same experiments carried out with BL15, and (2) TT alone on TT–C3b binding to 4.2 cells compared with TT binding on the same cells. Moreover, FITC or ^{125}I-labelled IgG–4.2 could interact with TT–C3b bound to BL15 but not with complexes bound to 4.2 cells under the same conditions (data not shown). The fact that the TT epitope recognized by IgG–4.2 was not available on TT–C3b bound to antigen-specific cells confirmed the interaction of the TT moiety of the complexes with mIg on 4.2 cells in addition to the C3b interaction with CR.

These results are important as they could account for previous observations on the role of covalent C3b binding to antigen: Jacquier-Sarlin *et al.*[10] showed that the amount of TT required for antigen-specific T-cell proliferation was reduced by a factor of 100 when TT–C3b was used. A similar effect was described by Carter & Fearon,[26] who observed that co-ligation of CD19 with mIgM decreased the concentrations of anti-IgM required to induce B-cell proliferation. It is now well known that all these surface molecules (CR2, mIg, CD19) are closely related and co-operate: interactions between mIg–CD19,[27] CR2–mIg[28] and CR2–CD19[29] have been described. Thus, cross-linking of CR2 and mIg induces an increase in intracellular Ca^{2+} probably via CD19 (involvement of CR2–CD19 complex), which activates a phospholipase C by a protein tyrosine kinase-dependent pathway.[30] Covalent C3b-binding to Ag, by inducing a CR–mIg bridging, may enhance signal transduction and, consequently, stimulation of B lymphocytes. Thus, if an inhibition of Ag proteolysis could partly account for the enhanced presentation of Ag–C3b complexes, as demonstrated previously,[10] the effect described above (co-operation of several surface molecules) also plays an important part in the increased efficiency of Ag–C3b presentation.

The difference between anti-CR1 (J3.D3) and anti-CR2 (OKB7) competitive activity reflects the preponderance of CR2 over CR1 on BL15 and 4.2 cells and, perhaps, a difference in the affinity of monoclonal antibodies used. Moreover, the role of CR2 over CR1 (also reported by Thornton *et al.*[31] in the case of

responses to primary immunization) is in favour of a rapid cleavage of C3b bound to Ag leading to C3dg–Ag complexes that preferentially interact with CR2. This cleavage could be due to membrane proteases present on the cell surface.[32] Moreover, there is 10 times as much CR2 as CR1 on BL15 and on 4.2 cells, as measured using ^{125}I-monoclonal antibodies J3.D3 and BL13 (data not shown).

The consequences of covalent C3b binding to TT were analysed at the intracellular level. The use of a Percoll gradient allowed us to separate endosome- and lysosome-containing fractions, which were characterized using radiolabelled ferritransferrin incorporation and enzymatic activities. Analysis of Ag subcellular repartition revealed that Ag is distributed among two peaks corresponding to endosome and lysosome compartments. The Ag distribution between these two subcellular fractions was studied using Ag alone or complexed to C3b, and antigen-specific (4.2) or non-specific (BL15) B cells.

The radioactivity content of endosome after TT internalization by BL15 cells was weak compared with 4.2 cells, reflecting the difficulty for these antigen non-specific cells to incorporate TT. However, the amount of radioactivity recovered in lysosome fractions was similar whether BL15 or 4.2 cells were used: the migration of Ag between extracellular space and lysosome seemed to be identical whatever the amount of Ag in the endosomes. There was no apparent accumulation of radioactive material in the lysosomes, probably due to exocytosis.

In the present study, no significant differences were detected in the subcellular distribution of Ag when complexed to C3b using antigen-specific and non-specific cells. Moreover, both B-cell lines presented Ag bound to C3b to antigen-specific T cells in a very efficient manner.[10] In conclusion, our results highlight the role of C3b in the uptake of Ag by APC: C3b modifies the entry of Ag even in specific APC. Our observations form the first experimental confirmation of a previous hypothesis[33] concerning the cross-linking of B-cell receptor (mIg) and CR2 via C3b–Ag complexes in the absence of immune complexes, and emphasize the simultaneous contribution in the immune response of components non-related (C3b) and related (Ag) to the infectious agent. This phenomenon could have many implications, not only at the Ag binding and signal transduction levels, but also in the Ag processing and presentation mechanisms. Moreover, C3b covalently linked to Ag allows non-specific cells to uptake and internalize Ag in a very efficient manner, quantitatively similar to antigen-specific cells, suggesting an important role for these non-specific cells in Ag presentation.

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