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B Cell Receptors and Complement Receptors Target the Antigen to Distinct Intracellular Compartments

Laure A. Perrin-Cocon,* Christian L. Villiers,2* Jean Salamero,† Françoise Gabert,* and Patrice N. Marche*

The processing of exogenous Ags is an essential step for the generation of immunogenic peptides that will be presented to T cells. This processing relies on the efficient intracellular targeting of Ags, because it depends on the content of the compartments in which Ags are delivered in APCs. Opsonization of Ags by the complement component C3 strongly enhances their presentation by B cells and increases their immunogenicity in vivo. To investigate the role of C3 in the targeting of Ags, we compared the intracellular traffic of proteins internalized by complement receptor (CR) and B cell receptor (BCR) in B lymphocytes. Whereas both receptors are able to induce efficient Ag presentation, their intracellular pathways are different. CR ligand is delivered to compartments containing MHC class II molecules (MHC-II) but devoid of transferrin receptor and Lamp-2, whereas BCR rapidly targets its ligand toward Lamp-2-positive, late endosomal MHC-II-enriched compartments through intracellular vesicles containing transferrin receptor. CR and BCR are delivered to distinct endocytic pathways, and the kinetic evolution of the protein content of these pathways is very different. Both types of compartments contain MHC-II, but CR-targeted compartments receive less neosynthesized MHC-II than do BCR-targeted compartments. The targeting induced by CR toward compartments that are distinct from BCR-targeted compartments probably participates in C3 modulation of Ag presentation. The Journal of Immunology, 2004, 172: 3564–3572.

The efficiency of Ag presentation depends not only on the efficiency of Ag internalization but also on the intracellular transport of Ags toward compartments equipped for processing and loading of antigenic peptides on MHC class II molecules (MHC-II).1 It was previously shown that B cell receptor (BCR)-mediated uptake rapidly targets Ags toward multilamellar or multivesicular compartments (1, 2), rich in neosynthesized class II molecules and specialized for efficient peptide loading, which were named MHC-II loading compartments (MIIC) (3–6). Neosynthesized class II molecules are targeted from the secretory trans-Golgi network to early endosomes before reaching MIIC where they accumulate (7, 8). Therefore, in B cells, class II molecules are present throughout the endocytic pathway (9). The addressing of neosynthesized class II molecules is monitored by their association with the invariant chain (Ii), which chaperones the newly formed complex to the endocytic pathway (10–12). Once in the endocytic compartments, the invariant chain is degraded by proteases into class II-associated invariant chain peptide, which is exchanged with an antigenic peptide under HLA-DM (DM) catalysis, a protein highly enriched in MIIC (13–15). MHC-II-peptide complexes are then exported to the plasma membrane. Surface class II molecules can be reinternalized into early endosomes (16), where they may exchange their peptide and then recycle to the plasma membrane (17). Some peptides rapidly generated in the endocytic pathway are presented by these recycling class II molecules (18).

The BCR is internalized via clathrin-coated pits and delivered to early endosomes. Signals in the cytoplasmic tails of surface Ig and side-chains Igα and Igβ control the internalization and addressing of this receptor to the appropriate compartment. The mutation of one tyrosine (Y587) in the cytoplasmic tail of surface IgM does not affect the efficiency of Ag capture but alters Ag presentation to T lymphocytes (19, 20), highlighting the importance of the addressing role of the receptors. Furthermore, Igα and Igβ control the transport to late endosomes, leading to efficient Ag presentation (21). Analysis of the addressing motifs of these chains has revealed that Igα targets the Ag to compartments containing neosynthesized class II molecules, resulting in presentation of numerous epitopes, whereas Igβ targets the Ag toward recycling class II molecules, resulting in presentation of a restricted number of epitopes (22). Because the internalization capacities of these receptors are quite similar, the targeting of the Ag thus appeared to be crucial for their presentation.

Evidence from in vivo and in vitro experiments demonstrate that the complement component C3 plays a critical role in the specific immune response. For instance, mice deficient in C3 show severely impaired Ab responses to T cell-dependent Ags (23). Under...
activation, at a site of inflammation, for example, C3 is cleaved into C3b, which can covalently bind to proximal Ags, leading to the formation of C3b-Ag complexes (24). C3b acts as a natural adjuvant of the secondary immune response. When C3b is coupled to hen egg lysozyme (HEL) (HEL-C3b), 200 times less HEL is required to immunize mice and elicit a B cell response compared with HEL alone (25), and humoral immunity to HEL persists much longer than when immunization is performed with CFA (26). Moreover, a recombinant fusion protein containing three copies of C3d coupled to HEL is highly immunogenic, inducing a maximal Ab response at doses a thousand times lower than native HEL Ag (27). In vitro, the covalent binding of C3b to Ags such as tetanus neurotoxin (TeNT) enhances their presentation to specific T cells (28). One part of this effect can be explained by increased internalization of C3b-TeNT complexes mediated by complement receptors (CR) (29). Moreover, C3b induces some modifications of processing of the Ag linked (30), resulting in increased formation of highly stable MHC-II-peptide complexes (31). Thus, the binding of C3 to Ags modifies not only their capture but also their processing and presentation to T cells, resulting in enhanced specific immune response in vivo.

The mechanism of action of C3 is poorly understood; nevertheless, CR have been largely implicated. Knockout mice for CR1 and CR2 show impaired primary and secondary T-dependent humoral responses (32, 33), similarly to C3-deficient mice. Saturation of CR by injection of soluble CR2 or Abs against CR1/2 strongly inhibits Ab response to immunizations (34–37). In vitro, coligation of CR2 and BCR results in enhanced release of intracellular Ca2+ (38) and lowers the threshold for B cell activation (27, 29), leading to significant augmentation of B cell responses.

Therefore, BCR and CR both sustain efficient Ag presentation. To investigate the role of C3 in the intracellular traffic of Ags, we analyzed the intracellular targeting induced by C3 CR and compared it with the trafficking induced after BCR internalization.

Materials and Methods

Materials

4.2 (HLA DR3/DR1101) is B cell clone specific for TeNT (generous gift from Dr. A. Lanzavecchia (Basel Institute, Basel, Switzerland)); SWEIG (HLA DR1101) is an EBV-transformed B cell line and was obtained from American Type Culture Collection (Manassas, VA). TDR11-I2V is a T cell clone specific for the peptide p30 (947-967) of TeNT, obtained from a homozygous HLA DR1101 donor as described (28). All were grown in RPMI 1640 medium with Glutamax I/10% heat-inactivated FCS (all from Life Technologies, Cergy Pontoise, France). mAb against transferrin receptor (TfR; clone DF1513) was purified from a hybridoma (American Type Culture Collection). 5C1 is an anti-HLA-DM Ab (34). Rabbit polyclonal antiserum against Rab7 was generously provided by Dr. P. Chavrier (Institut Curie, Paris, France) (40); mAb against cytoplasmic tails of MHC-II α-chain (DA6-147) and Ii (Pin1) have been previously described (41, 42). L243 Ab was purified from HBSS hybridoma (American Type Culture Collection). 5C1 is an anti-HLA-DMA Ab (43). HRP-conjugated polyclonal anti-rabbit IgG was from Jackson ImmunoResearch Laboratories (West Grove, PA), and HRP-conjugated polyclonal anti-mouse IgG was from Sigma-Aldrich (St. Quentin-Fallavier, France). mAb against transferrin receptor (TIR; clone DF1513) was purchased from Sigma-Aldrich; mouse anti-lysosomal-associated membrane protein-2 (Lamp-2) (CD107b) was from BD Pharmingen (San Diego, CA); Texas Red-labeled donkey anti-mouse IgG and donkey anti-rabbit IgG were from Jackson ImmunoResearch Laboratories.

TeNT was from Institut Mérieux (Marcy L’Etoile, France). Human C3 was purified as described (44). TeNT-C3b complexes were prepared as described (25).

T cell proliferation assay

4.2 or SWEIG cells were incubated for 300 min at 37°C with TeNT or TeNT-C3b, washed, and further incubated for 0–40 h at 37°C. After fixation (0.15% paraformaldehyde for 10 min), 5 × 105 cells were incubated in 96-well flat-bottom microtiter plates with the T cell clone TDR11-I2V (2 × 104 cells). After 20 h, supernatants (100 μl) were assayed for the presence of IL-2 by incubation with the IL-2-dependent CTLL-2 cell line (1 × 104 cells/well) for 20 h. Then, 1 μCi of [3H]thymidine (Applied Biosystems, Courtaboeuf, France) was added to the culture for 6 h, and proliferation was measured by counting [3H]thymidine incorporation into the cells.

Microbeads coupled to receptor ligand

Microbeads of 10-μm diameter (iron concentration, 6 mg/ml) were prepared as described previously (45). TeNT or C3b (240 μg/ml) were covalently linked to microbeads in 0.1 M phosphate buffer (pH 7.5), by addition of 0.025% (w/v) glutaraldehyde. Excess glutaraldehyde was inactivated by ethanolamine (0.1 M; pH 7.5). Seventy-five to 90% of the protein was covalently bound to microbeads.

TeNT and C3b binding and internalization

TeNT and C3b were labeled with Alexa 488 (see Immunofluorescence and confocal microscopy) or iodinated using the iodogen method previously described (46), and covalently linked to microbeads as described above. To analyze the binding of C3b microbeads to cells, B lymphoblastoids were washed and incubated in 20 mM NaCl/1 mM Na2HPO4/1.5 mM KH2PO4/240 mM sucrose/1% OVA for 1 h on ice with various amounts of microbeads bearing fluorescent C3b. When mentioned, incubation was conducted in presence of unlabeled C3b microbeads in excess. After three washes in the same buffer, cells were analyzed by flow cytometry.

To compare internalization via BCR and CR, iodinated TeNT or C3b microbeads (3.6 μg of protein) were incubated with 105 4.2 B cells at 4°C for 1 h. Cells were washed three times and further incubated at 37°C for 30 min. The amount of radioactivity bound to the cells was quantified before (total binding) and after (amount internalized) incubation.

Radiolabeling and biotinylation of cells

Cells (5 × 105) in Met/Cys-free DMEM were pulse-labeled with 2 mCi 35S-Promix (Amersham Biosciences, Les Ulis, France) for 20 min at 37°C. After washing in cold PBS (pH 7.4), cells were incubated at 15 × 106 cells/ml with 0.5 mg/ml N-hydroxysulfoSuccinimide ester-long chain biotin (Pierce Chemicals, Rockford, IL) for 30 min on ice, under intermittent shaking. Then, cells were washed three times with DMEM before use.

Electron microscopy

Cells were incubated with TeNT or C3b microbeads at 4°C and internalized at 37°C for 10 or 60 min, as described for magnetic fractionation. Cells were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 1 h at room temperature. Pelleted cells were postfixed in 1% OsO4 for 1 h at 4°C and included in Epon. Ultrathin sections were stained with 5% uranyl acetate and lead citrate, and examined with a JEOL (Peabody, MA) 1200 EX II transmission electron microscope at 80 kV.

Subcellular fractionation on Percoll gradient

Centrifugation using self-forming Percoll density gradient was performed in 13-ml Quick-Seal tubes (Beckman Instruments, Piscataway, NJ) as recommended by the manufacturer. Fluorescent C3b beads (in green) and radiolabeled ferri transferrin (in red) were covalently linked to microbeads in 0.1 M phosphate buffer (pH 7.2). The amount of radioactivity bound to the cells was quantified before and after incubation with Percoll gradient, and several markers were quantified in the fractions. Early endosomes were localized using 125I-labeled transferrin labeling. Lysosomes were characterized by their high β-galactosaminidase and cathepsin B activity, which were determined as described (29), using paranitro-phenyl-N-acetylgalactosaminide (5 mM in 10% DMSO) and benzoylcarbonyl-Arg-Arg-2-naphthylamide (20 mM in DMSO), respectively, as substrate.

Immunofluorescence and confocal microscopy

TeNT or C3b were labeled with Alexa 488-protein labeling kit (Molecular Probes, Leiden, The Netherlands) or with Cy3 (Amersham Pharmacia BioTech, Piscataway, NJ) as recommended by the manufacturer. Fluorescent proteins were fixed on microbeads as described above.

For TeNT internalization, cells were washed in RPMI 1640 and incubated (106 cells/ml) with TeNT microbeads for 1 h on ice. After two washes, cells were further incubated at 37°C for the indicated chase time minus 10 min. Cells were then diluted to 5 × 108/ml and incubated for 10 min at 37°C on poly-L-lysine-coated lamellas. For C3b internalization, cells were washed three times in RPMI 1640, resuspended at 2 × 106 cells/ml, and further incubated at 37°C for 30 min. After washing, cells were incubated with TeNT or C3b microbeads at 4°C for 1 h, washed, and resuspended in 50 μl of RPMI 1640 containing 10% FCS and 10 μg/ml FcR blocking reagent.
cells/ml, and allowed to adhere on poly-L-lysine-coated lamellas for 10 min at 37°C. Lamellas were washed three times with cold C3b buffer (20 mM NaCl/8 mM Na 2 HPO 4 /1.5 mM KH 2 PO 4 /240 mM sucrose/1% OVA) and incubated with Alexa 488-C3b microbeads, for 1 h on ice. After two washes, cells were incubated in the same buffer at 37°C, for the indicated chase time.

For cocentrilization, cells were incubated in C3b buffer in presence of Alexa 488-TeNT microbeads and Cy3-C3b microbeads for 1 h on ice. Cells were washed and further incubated in the same buffer at 37°C, and allowed to adhere on lamellas at 37°C for the last 10 min of incubation. Cells were then processed for immunofluorescence staining as described previously (47). The coverslips were mounted and viewed under a confocal microscope (TCS4D; Leica Lasertechnic, Heidelberg, Germany).

**Magnetic fractionation**

After washing, B cells (10⁶ cells/ml) were incubated for 1 h on ice with TeNT or C3b microbeads (final iron concentration, 1 mg/ml). Cells were washed twice with 50 ml of cold DMEM and further incubated at 37°C (5 × 10⁶ cells/ml) for the indicated time. Cells were then processed for immunofluorescence staining as described previously (47).

**Results**

**BCR and CR internalization both result in efficient Ag presentation**

The efficiency of presentation of the TeNT immunodominant epitope p30 by B lymphocytes was analyzed following three different internalization pathways: pinocytosis, and BCR- and CR-mediated endocytosis. Presentation of the epitope p30 was assessed by the stimulation of the specific T cell clone TDR11-IV2. Due to their expression of the TeNT-specific IgG, 4.2 B cells presented the immunodominant p30 epitope much more efficiently.
than did nonspecific B cells (SWEIG), which internalized TeNT by pinocytosis (Fig. 1). When TeNT was opsonized by C3b, its presentation by nonspecific B cells was almost as efficient as that mediated by 4.2 cells. In these conditions, SWEIG cells could internalize TeNT-C3b complexes by CR with an efficiency comparable with that of BCR, as previously shown using another B cell line (28). Furthermore, p30 epitope presentation is slightly enhanced when TeNT-C3b complexes are presented by TeNT-specific B cells compared with free TeNT. When 4.2 and SWEIG cells were compared, few differences in the kinetics of presentation were observed: whereas 4.2 cells induced T cell proliferation just after Ag loading (0-min chase), SWEIG cells needed >300 min to present antigenic peptide after TeNT-C3b complex internalization (Fig. 1). This could be due either to differences in the capacity of these cell lines to process Ags or to differences in the kinetics of the intracellular pathways mediated by BCR and CR. To compare the intracellular traffic induced by these two receptors, we next followed the internalization of TeNT and C3b in the same B cell line, 4.2.

Internalization by BCR and CR

To allow specific binding of C3b or TeNT ligands, 4.2 B cells were first incubated for 1 h on ice. Then, the specificity of the binding was checked: the binding of C3b was mediated by a specific receptor, because it was dose dependant, reached saturation above 20 ng (Fig. 2A), and could be inhibited by 70% by competition with 6-fold excess unlabeled C3b (B). TeNT binding was specific to BCR expression on 4.2 B cells and could be displaced by unlabeled TeNT (29, 48). Similar results were obtained with TeNT linked to microbeads (data not shown). After incubation with the cells at 4°C, ligand in excess was removed before internalization at 37°C for 0–120 min. Internalization of TeNT and C3b microbeads was analyzed by electron microscopy: after incubation at 37°C, electron-dense microbeads were localized into intracellular vesicles on ultrathin sections of cells (Fig. 3). Because B cells do not possess a strong capability of endocytosis, few microbead-positive intracellular vesicles could be observed on each section. Furthermore, TeNT and C3b microbeads were internalized with similar efficiency as shown in Table I. Intracellular endocytic compartments were separated on Percoll gradient according to their density and localized as follows: early endosomes were labeled by internalization of 125I-labeled ferri-transferrin and localized in fractions 13–15; lysosomes were in fractions 4–7, as characterized by their high β-galactosidase and cathepsin B activity (Fig. 4A). In agreement with previous results (45), microbeads did not interfere with the distribution of TeNT in light fractions (enriched in endosomes) and dense fractions (enriched in lysosomes) whether or not it was linked to the protein (Fig. 4B). Analysis by SDS-PAGE of fraction content indicated that 80–90% of 125I-labeled ligands remained complexed with microbeads (data not shown). This allowed us to follow the intracellular targeting of the ligands. Without internalization (chase, 0 min), ligands were essentially associated with the plasma membrane (fractions 18–21) (Fig. 4, B and C). Upon incubation at 37°C, radiolabeled TeNT was recovered in light endosomes and progressively accumulated in dense fractions (4–7) containing lysosomes (Fig. 4B), whereas C3b was very rapidly localized in dense fractions, and its distribution did not vary significantly during the chase time (C). These results may be explained by differences either in the targeting of the ligands or in the kinetics of their traffic in the same endocytic pathway.

Table I. Internalization of TeNT and C3b in 4.2 B cells

<table>
<thead>
<tr>
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<th>TeNT</th>
<th>C3b</th>
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<tr>
<td>Binding (ng/10⁶ cells)</td>
<td>11.1</td>
<td>11.3</td>
</tr>
<tr>
<td>Internalization (%)</td>
<td>72</td>
<td>89</td>
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*4.2 B lymphocytes were incubated with 125I-labeled C3b or 125I-labeled TeNT microbeads (3.6 μg of protein for 10⁷ cells) for 1 h at 4°C, washed, and incubated at 37°C for 30 min to allow internalization. Internalization is expressed as percentage of bound ligand. Results from one representative experiment are shown.
BCR and CR intracellular targeting of Ags

The intracellular pathways of CR and BCR ligands were analyzed by confocal microscopy. TeNT and C3b were labeled with Alexa 488 and fixed on microbeads. After 10-min internalization, TeNT microbeads were observed in peripheral and pericentriolar vesicles, positive for TIR (Fig. 5A). After 60 min of chase, TeNT partly colocalized with MHC-II and Lamp-2. BCR intracellular targeting has been extensively studied, and in agreement with previous results from different laboratories (1, 2, 49, 50), we found that, in 4.2 B cells, BCR is first delivered to TIR-positive endosomes before reaching late endocytic compartments containing MHC-II and Lamp-2 that have been characterized as MIIC.

The intracellular traffic of C3b is quite different. C3b microbeads remained mostly in peripheral vesicles and never colocalized with TIR (Fig. 5B). After 60 min of chase, C3b partly colocalized with MHC-II but not with the lysosomal Lamp-2 (Fig. 5B). Observations in immunoelectron microscopy, confirmed that, after 60 min of chase, C3b microbeads were not found in Lamp-2-positive compartments (data not shown). Furthermore, when TeNT (labeled with Alexa 488) and C3b (labeled with Cy3) were simultaneously internalized, the proteins were localized in distinct compartments after 10 or 60 min incubation at 37°C (Fig. 5C and data not shown). After 5 h of chase, although the remaining fluorescent signals were very weak, some colocalization of TeNT and C3b could be observed, suggesting that at least part of the ligands may finally converge.

These results demonstrate that BCR and CR ligands are targeted to different early intracellular compartments. C3b do not access the MIIC presentation compartment at least during the first hour after internalization.

Comparison of vesicular content

To characterize each endocytic pathway, compartments targeted by BCR and CR were isolated by magnetic fractionation, after internalization of TeNT or C3b microbeads, respectively. Their content in specific proteins, involved in vesicular transport (Rab) or in Ag presentation, was analyzed by Western blot. As shown in Fig. 6A, after 120 min of chase, MHC-II, Ii, and HLA-DM, as well as the late endosome marker Rab7, were detected in isolated compartments whether microbeads were internalized by BCR or CR.

Nevertheless, the kinetic evolution of the protein content in both pathways was very different. When internalization was mediated by BCR, compartments containing microbeads progressively acquired Rab7, with a maximum between 60 and 120 min of chase (Fig. 6B). However, in compartments reached after internalization by CR, a constant but low level of Rab7 was observed, which corresponded to the level found after 10-min internalization of BCR. In both cases, the early endosome and plasma membrane marker Rab5, was found at the very beginning of the chase and decreased later on (Fig. 6, C to E). In conclusion, after 60 min of chase, BCR ligand is found in late endocytic compartments, equipped to load antigenic peptides on class II molecules. Vesicles purified after CR internalization also contained MHC-II, Ii, and HLA-DM, but in constant amounts during the first 120 min of

FIGURE 4. Subcellular distribution of TeNT and C3b microbeads. A, 125I-labeled ferri-transferrin was internalized by 4.2 B cells for 20 min at 37°C. Cells were homogenized and fractionated on Percoll gradient. Fractions were analyzed for their content in 125I-labeled ferri-transferrin, β-galactosaminidase, and cathepsin B activity. B—D, 4.2 B cells were incubated at 4°C (1 h) with 125I-labeled TeNT (B), 125I-labeled TeNT microbeads (B and C), or 125I-labeled C3b microbeads (D) and chased at 37°C for 0, 10, 30, 60, or 120 min. Cells were homogenized and fractionated on Percoll gradient. Fractions were collected and the amount of iodinated ligand in each fraction was counted. B, Comparison of the percentage of 125I-labeled TeNT found in light fractions enriched in endosomes (fractions 13–15) and dense fractions enriched in lysosomes (fractions 4–7) after 30 min of chase, when TeNT is linked or not to microbeads. C and D, 125I distribution on Percoll gradients. Data are expressed as percentage of total radioactivity recovered in the gradient.
chase and increased only after 240 min of chase (Fig. 6, C–E). Therefore, Ag processing in these distinct compartments may be quite different.

Origin of MHC-II in both endocytic pathways

Because MHC-II found in endocytic compartments may come either from the plasma membrane after reinternalization or from the Golgi apparatus after neosynthesis, we have analyzed the origin of these molecules in compartments reached by BCR and CR ligands. Neosynthesized molecules were metabolically labeled, and cell surface molecules were biotinylated before incubation in the presence of TeNT or C3b microbeads. After receptor internalization and 120 min of chase, compartments containing TeNT or C3b microbeads were magnetically isolated. Mature MHC-II were immunoprecipitated using L243 Ab. The proportion of neosynthesized and molecules reinternalized from plasma membrane was determined on the same protein blot, by radioactivity and biotin detection, respectively. Controls performed on the PNS showed that the overall amount of MHC-II immunoprecipitated was equivalent whether internalization was mediated by BCR or CR ligands. Neosynthesized molecules were metabolically labeled, and cell surface molecules were biotinylated before incubation in the presence of TeNT or C3b microbeads. After receptor internalization and 120 min of chase, compartments containing TeNT or C3b microbeads were magnetically isolated. Mature MHC-II were immunoprecipitated using L243 Ab. The proportion of neosynthesized and molecules reinternalized from plasma membrane was determined on the same protein blot, by radioactivity and biotin detection, respectively. Controls performed on the PNS showed that the overall amount of MHC-II immunoprecipitated was equivalent whether internalization was mediated by BCR or CR ligands (data not shown). In isolated vesicles, the amount of biotinylated MHC-II did not significantly differ in BCR and CR compartments, whereas the amount of neosynthesized MHC-II in CR compartments was significantly reduced (−28.5 ± 2.5%) compared with BCR compartments (Fig. 7).

Discussion

Using biochemical analysis and microscopic observations, we have shown that the intracellular trafficking induced by the internalization of BCR and CR are independent. BCR endocytosis is mediated by clathrin-coated pits (51), leading to colocalization with TR in early peripheral sorting endosomes and pericentriolar recycling endosomes. After 1 h of chase, the Ag concentrates in pericentriolar compartments, accumulating MHC-II, HLA-DM, and Ii, labeled by late endosomal markers Rab7 and Lamp-2. These compartments have the characteristics of MIIC. In contrast, endocytosed C3b is retained in peripheral vesicles devoid of TR and Lamp-2. These compartments contain MHC-II, Ii, and HLA-DM at a constant level for 2 h. They are rapidly accessed by endocytosed C3b, but they are distinct from BCR-targeted early endosomes by the lack of TR and their apparent high density on Percoll gradient. The absence of intracellular colocalization of BCR and CR ligands at early stage of the endocytosis process indicates that the receptors follow distinct internalization pathways. Their ligands are found in compartments differentiated by their composition and their intracellular evolution, C3b remaining confined in specific compartments for at least 2 h. BCR and CR ligands may converge later on. Therefore, C3b opsonization of Ags not only enhances their capture by nonspecific cells, using CR that are widely expressed, but also modifies their targeting within B lymphocytes.

These results raise the question of the targeting of C3b-Ag complexes in specific B cells. As shown previously, cross-linking of BCR and CR by such complexes induces intracellular signaling leading to B cell activation (39), and compared with resting cells, these activated cells may also have modified intracellular traffic. In conclusion, more investigation will be necessary to analyze the effect of C3b-Ag complexes on the intracellular traffic in specific B cells to discriminate between consequences of cell activation and modification of intracellular pathway due to receptor internalization, because both may modify Ag processing and presentation.

Although internalization via BCR and CR results in very different trafficking within the cell, we have shown, in agreement with previous results (28), that both pathways result in efficient Ag presentation of an immunodominant epitope, although with different kinetics. C3b compartments are equipped to load antigenic
peptides, because they contain neosynthesized and membrane MHC-II, HLA-DM, and HLA-DO (data not shown). Furthermore, we found comparable amounts of MHC-II in C3b compartments isolated after 1 h as in TeNT-containing compartments reached after 1 h of BCR internalization (Fig. 6C) that were characterized as MIIC. The presence of HLA-DM in C3b compartments after 10 min of internalization is in agreement with the finding of HLA-DM at the cell surface and in endosomes of B lymphocytes (52), and not only in lysosomes as described initially. Moreover, we have also detected this molecule by Western blot in Percoll gradient light fractions enriched in endosomes prepared from our B lymphocytes (data not shown). The generation of antigenic peptides by limited proteolysis of Ags is supported by the presence of active proteases throughout the endocytic pathway even in early endosomes, where peptide loading on recycling MHC-II takes place (18) and may be influenced by HLA-DM (53, 54). Therefore, Ag processing and the resulting loading of antigenic peptides onto MHC-II could occur in C3b compartments. The delay in Ag presentation observed when TeNT is linked to C3b (Fig. 1) may result either from differences in the capacity of 4.2 and SWEIG cells to present Ags or from the retention of C3b-TeNT complexes in the compartments that we describe, either because these compartments do not allow Ag presentation despite their content in class II and HLA-DM molecules or because the processing in these compartments is slower than that occurring in MIIC. In all cases, the particular trafficking induced by CR modify the conditions of processing of the opsonized Ag.

Two types of peptide-loading compartments have been described and named MIIC and class II-containing vesicles (CIIV). MIIC are late endocytic compartments, containing Lamp-2, where neosynthesized MHC-II and HLA-DM molecules accumulate (3, 55). CIIV were first isolated from B lymphocytes by subcellular fractionation (4). The absence of lysosomal markers such as Lamp-2 distinguish them from MIIC. They are connected to endocytic compartments, because internalized material can reach them, and they contain stable MHC-II-peptide complexes and few HLA-DM molecules. They are a site of terminal degradation of Ii and probably of peptide loading (56). More recently, Turley et al. (57) have described, in dendritic cells, nonlysosomal peripheral vesicles involved in the transport of these complexes from MIIC to the plasma membrane that corresponded to CIIV. Ags were first targeted to MIIC where processing and peptide loading took place, and then MHC-II-peptide complexes were delivered to CIIV to be transported to the membrane. Compartments reached by C3b share some features with CIIV: they are nonlysosomal peripheral vesicles enriched in HLA-DM compartments, containing HLA-DM molecules, and devoid of Lamp-2 and TfR. Therefore, either C3b is retained in specialized atypical endosomal compartments devoid of TfR, or C3b is directly targeted to CIIV export vesicles where peptide loading can occur as shown previously (56). In conclusion, C3b is internalized independently of BCR and remains for several hours in compartments that differ from MIIC by their physical properties and their protein content. This will have some repercussions on Ag processing according to proteases and other enzymatic activities located in these compartments. The repertoire of peptides that will be selected for presentation may also be affected by the difference in the ratio between reinternalized and neosynthesized MHC-II.
Indeed, C3 induces some differences in antigenic peptides that can be eluted from MHC-II (V. A. Serra, unpublished observations). Similarly, as in early endosomes (18), peptides of high affinity may be loaded on recycling MHC-II and could form highly stable MHC-II-peptide complexes.

There are several candidates for the receptors inducing the targeting of C3b to CHV-like compartments; among them are the following: decay-accelerating factor (DAF; CD55), membrane cofactor protein (MCP; CD46), and CR1 (CD35). DAF is a GPI-anchored receptor mainly localized at the plasma membrane in lipid rafts (58). Like other GPI receptors, it may be internalized into caveolae vesicles devoid of TIR. MCP is a transmembrane receptor that can induce efficient Ag presentation by MHC-II (59, 60), indicating that this receptor gains access to some peptide-loading compartments, but its intracellular traffic has not been characterized. Furthermore, DAF and MCP are strongly expressed on B lymphocytes. CR1 binds C3b and its internalization is not mediated by clathrin-coated pits (61), but this receptor is very weakly expressed on our cells and may be excluded as a single candidate for intracellular targeting of C3b. However, it has been established that CR1 could cooperate with another receptor: CR2 (CD21). CR2 has high affinity for C3dg and to a lesser degree for iC3b, two fragments generated by cleavage of C3b, and it was shown to induce very efficient presentation of C3d-HEL complexes (62). CR2/CR1 complexes induce intracellular signaling and up-regulation of costimulatory molecules (63); these two receptors could also cooperate to enhance endocytosis of C3b and its fragments (64), but their endocytic pathway has not been explored. In conclusion, CR1 and DAF could account for the peculiar intracellular pathway of C3b, and MCP and CR2 could account for efficient Ag presentation. Blocking agents specific to each receptor could not individually inhibit C3b internalization, whereas their use in combination is much more efficient (data not shown), strongly suggesting that these receptors are not mutually exclusive.

The formation of complexes between Ags and C3b is a physiological process occurring naturally in vivo when complement system is activated, by immune complexes, for example. In vivo experiments on immunization have shown that Ag opsonization by C3b improves the immune response, inducing a long-lasting Ab response to low doses of Ag, indicating that this complement protein has an adjuvant effect (25, 26). In this study, we show that CR and BCR are first targeted to distinct compartments, likely able to induce modifications of the peptides generated, and then presented to T cells. In contrast, a smaller fragment of C3, C3d rapidly targets Ags toward MHC, via internalization by CD21/CD19 complexes (62, 65), illustrating the multiple roles of C3 that can be achieved by several receptors. Taken together, these results indicate that binding of C3b to Ags is an intrinsic and important step of the immune response. The modification of Ag targeting induced by C3b provides new explanation for the understanding of the adjuvant effect of C3b on humoral immunity.

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