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Prolonged Antigen Persistence Within Nonterminal Late Endocytic Compartments of Antigen-Specific B Lymphocytes

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Although Ag-specific B lymphocytes can process Ag and express peptide-class II complexes as little as 1 h after Ag exposure, it requires 3–5 days for the immune system to develop a population of Ag-specific effector CD4 T lymphocytes to interact with these complexes. Presently, it is unclear how B cells maintain the expression of cell surface antigenic peptide-class II complexes until effector CD4 T lymphocytes become available. Therefore, we investigated B cell receptor (BCR)-mediated Ag processing and presentation by normal B lymphocytes to determine whether these cells have a mechanism to prolong the cell surface expression of peptide-class II complexes derived from the processing of cognate Ag. Interestingly, after transit of early endocytic compartments, internalized Ag-BCR complexes are delivered to nonterminal late endosomes where they persist for a prolonged period of time. In contrast, Ags internalized via fluid phase endocytosis are rapidly delivered to terminal lysosomes and degraded. Moreover, persisting Ag-BCR complexes within nonterminal late endosomes exhibit a higher degree of colocalization with the class II chaperone HLA-DM/H2-M than with the HLA-DM/H2-M regulator HLA-DO/H2-O. Finally, B cells harboring persistent Ag-BCR complexes exhibit prolonged cell surface expression of antigenic peptide-class II complexes. These results demonstrate that B lymphocytes possess a mechanism for prolonging the intracellular persistence of Ag-BCR complexes within nonterminal late endosomes and suggest that this intracellular Ag persistence allows for the prolonged cell surface expression of peptide-class II complexes derived from the processing of specific Ag. The Journal of Immunology, 2001, 166: 6657–6664.

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mmediately upon introduction of Ag into the body, Ag-specific B lymphocytes are able to bind and internalize Ag via their cell surface B cell receptor (BCR) molecules. Moreover, Ag-specific B lymphocytes are capable of expressing complexes of Ag-derived peptides and MHC class II molecules (the result of BCR-mediated Ag processing) on their surface as little as 1 h after Ag binding. However, Ag-specific effector CD4 T lymphocytes with which these Ag-specific B lymphocytes need to interact for full development and differentiation into Ab-producing and/or memory B lymphocytes will not be present in the body until 3–5 days after the initial exposure to Ag. Presently, it is unclear how Ag-specific B lymphocytes maintain sufficiently high levels of cell surface peptide-class II complexes to allow efficient interaction with Ag-specific effector CD4 T lymphocytes when they become available.

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Abbreviations used in this paper: BCR, B cell receptor; B10, B10.Br; β-Hex, β-hexosaminidase; DAB, 3,3′-diaminobenzidine; DM, HLA-DM (human) or H2-M (murine); DO, HLA-DO (human) or H2-O (murine); DTAF, dichlorotriazin-ylino amino fluorescein; Nycodenz, 5-(N-2,3-dihydroxy-propylacetamido)-2,4,6-triiodo-N,N′-bis(2,3-dihydroxypropyl)isophthalaldehyde; EE, early endosome(s); ER, endoplasmic reticulum; F-P, fluid phase; HEL, hen egg lysozyme; IFM, immunofluorescence microscopy; LDM, low density membranes; L, lysosome; LE, late endosome; MD4, MD4.B10.Br; PC, phosphorylcholine; RE, recycling endosome(s); LAMP, lysosome-associated membrane protein.

Materials and Methods

Mice

B10.Br (B10) and MD4.B10.Br (MD4) mice (MD4 transgenic mice expressing hen egg lysozyme (HEL)-specific BCR (4)) that have been bred on the B10 background) were used from 7 to 12 wk of age. Mice were
housed under specific-pathogen free conditions and were maintained at Trudeau Institute’s Animal Breeding Facility (Saranac Lake, NY). Single-cell suspensions of splenocytes were prepared and lymphocytes isolated using Ficoll-Paque (Amersham Pharmacia Biotech, Piscataway, NJ).

**Ag (HEL) endocytosis by splenic B lymphocytes**

**BCR-mediated endocytosis.** MD4 B cells were pulsed with 100 nM HEL for 30 min on ice and then washed to remove unbound Ag. The cells were then incubated at 37°C for the indicated times to allow endocytosis of cell surface Ag-BCR complexes.

**Fluid phase (F-P) endocytosis.** B10 B cells were pulsed with 100 μM to 1 μM HEL for 30 min at 37°C to allow F-P endocytosis of detectable amounts of HEL. The cells were then washed to remove noninternalized HEL and were incubated at 37°C for the indicated times to allow intracellular trafficking of the internalized HEL.

**Immunofluorescence microscopy (IFM)**

B lymphocytes were attached to Alcian Blue-coated coverslips, fixed, permeabilized, and stained as previously reported (5). The primary Abs used were GL2A7 (antilyssosome-associated membrane protein (LAMP) 2, 1/20 dilution of tissue culture supernatant (6)), rabbit polyclonal Abs to H2-M (1/500 dilution), and H2-O (1/250 dilution), Map.DM1 (anti-HLA-DM, 1/5 dilution of tissue culture supernatant) (7), and 2D1 (anti-HEL, 1/10 dilution of tissue culture supernatant) (8). A20 cells were incubated with BODIPY-fluorescein-labeled transferrin conjugate (T-2873; Molecular Probes, Eugene, OR) at 37°C to label early endosomes (EE) and recycling endosomes (RE). Appropriate secondary Abs were used to detect primary Abs: donkey anti-mouse Ig-Texas Red (1/100 dilution); Jackson ImmunoResearch Laboratories; 712-015-153), and donkey anti-rat Ig DTAF (1/100; Jackson ImmunoResearch Laboratories; 711-016-152), donkey anti-rabbit Ig Texas Red (1/100 dilution of tissue culture supernatant (9), and anti-mouse Ig-Texas Red (1/100 dilution; Jackson ImmunoResearch Laboratories, OR) at 37°C to label late endosomes (EE) and recycling endosomes (RE). Donkey anti-mouse Ig-Texas Red (1/100 dilution; Jackson ImmunoResearch Laboratories; 711-016-152); donkey anti-rat Ig DTAF (1/100; Jackson ImmunoResearch Laboratories; 712-015-153); and donkey anti-rat Ig Texas Red (1/100 dilution; Jackson ImmunoResearch Laboratories; 712-016-153). The cells were examined with a Zeiss Axiophot 2 microscope (Carl Zeiss, Thornwood, NY) using epi-illumination.

**Cell lines**

A20 murine B cells, A20huDM (A20 cells expressing transduced human DMα- and DMβ-chains) (9), and A20 μWT (A20 cells expressing a transduced phosphorylcholine (PC)-specific human mIgM BCR) (10) were grown in MEM, 10% FCS, and 50 μg/ml of G418 for A20huDM. B10 B cells were pulsed with 100 nM HEL or B10 B cells in 100 μM HEL for 18–20 h at 37°C in complete medium, respectively). The cells were then washed to remove environmental Ag, returned to culture in complete medium for the indicated times, collected, and stained with the C4H3 mAb (13) (a 1/10 dilution of hybridoma supernatant), followed by mouse anti-rat IgG2b-FITC (1/500, no. 10054D; PharMingen, San Diego, CA) and anti-mouse CD45R/B220-PE (1/200, no. 01125A; PharMingen). After treatment with 1 μg/ml propidium iodide, the samples were analyzed with a FACScan flow cytometer (BD Biosciences, San Jose, CA). For each data point ≥10,000 live B cells (i.e., B220-positive, propidium iodide-negative lymphocytes) were analyzed, and the mean fluorescence intensity of the population was determined.

**Results**

**Prolonged Ag persistence within Ag-specific B cells**

To study the endocytosis and intracellular trafficking of BCR-internalized Ag in normal B lymphocytes, we took advantage of the MD4 BCR transgenic mouse (4) (referred to hereafter as MD4) in which all of the B lymphocytes express an HEL-specific BCR. Incubation of MD4 splenic B cells with 10–100 nM HEL allows for the BCR-mediated binding, endocytosis, and presentation of HEL in the absence of detectable F-P endocytosis or presentation of HEL (14). To allow for comparison of the intracellular trafficking of BCR-internalized Ag to the intracellular trafficking of the same Ag internalized via F-P endocytosis, we also used splenic B cells from non-BCR transgenic B10 mice. Incubation of B10 B cells with 100 μM HEL allows for the F-P endocytosis, processing, and presentation of HEL by these B cells. In addition, we took advantage of two distinct anti-HEL mAbs (i.e., 2D1, Ref. 8; and HyHEL10, Ref. 15) to follow the intracellular movements of HEL internalized by either BCR-mediated or F-P endocytosis. Importantly, the 2D1 mAb recognizes an epitope on HEL that is on the opposite face of HEL from the epitope recognized by the MD4 BCR (8). Therefore, the 2D1 mAb can recognize both free HEL as well as HEL bound to the MD4 BCR. Contrarily, the HyHEL10 mAb recognizes precisely the same epitope as the MD4 BCR (4). Therefore, the HyHEL10 mAb can only recognize free HEL and will not recognize HEL bound to the MD4 BCR.

To follow BCR-mediated and F-P endocytosis of HEL, MD4 and B10 B cells were pulsed with Ag (i.e., 100 nM and 100 μM HEL, respectively), and the intracellular distribution of the internalized HEL was analyzed by double-label IFM. The results presented in Fig. 1 (A and B) demonstrate that under these conditions, sufficient HEL is internalized by both BCR-mediated as well as F-P endocytosis to allow detection of internalized Ag. Interestingly, when B cells that had internalized Ag via BCR-mediated endocytosis were washed (to remove environmental Ag) and then returned to culture for 24 h before analysis, they still contained detectable HEL (Fig. 1C). Contrarily, B cells that had internalized HEL via F-P endocytosis contained no detectable intracellular...
Ag 24 h after removal of environmental Ag (Fig. 1D). Quantitation of these results is presented in Fig. 2, A and B. The results in Fig. 2A demonstrate that even 24 h after removal of environmental Ag, essentially every Ag-specific B cells still contained detectable levels of BCR-internalized intracellular Ag. Contrastingly, as little as 8 h after removal of environmental Ag, very few if any B cells that had internalized HEL by F-P endocytosis contained detectable levels of Ag (Fig. 2B). These results demonstrate that BCR-internalized Ags can persist for a prolonged period of time within B lymphocytes. Furthermore, because the 2D1 mAb recognizes a conformational epitope on the HEL molecule (8), these results also suggest that similar levels of HEL protein were internalized via F-P- and BCR-mediated endocytosis.

When a similar analysis was performed using the HyHEL10 mAb (which is able to recognize free HEL, but not HEL associated with the MD4 BCR) an interesting result was obtained. The results of HyHEL10 staining of B cells that had internalized HEL via F-P endocytosis (Fig. 2D) closely mirrored the results obtained with the 2D1 mAb. This was expected, because both mAbs should be able to recognize the non-BCR-associated HEL in these cells. However, the results obtained with the MD4 B cells were significantly different. As shown in Fig. 2C, HyHEL10 initially did not detect any free HEL on or within MD4 B cells. However, after 1–8 h of incubation at 37°C, non-BCR-associated HEL could be detected within intracellular compartments of the vast majority of MD4 B cells, demonstrating that some of the HEL had dissociated from the BCR (notably, no HyHEL10 staining of HEL-BCR complexes present at the plasma membrane of HEL-pulsed MD4 B cells was observed at any time point). Furthermore, after 24 h of incubation, the HEL that had been released from the BCR was, like the HEL internalized via F-P endocytosis, degraded, as indicated by the decrease in HyHEL10 at this time. However, these B cells still contain HEL-BCR complexes, as indicated by the high level of staining with the 2D1 mAb (Fig. 2A). These results are in line with our observation that BCR-mediated processing and presentation of HEL by MD4 B cells exhibits a lag of 2–4 h before expression of detectable peptide-class II complexes at the cell surface.
(J. R. Drake, unpublished observation) and demonstrate that Ag-specific B lymphocytes possess a mechanism to allow the prolonged intracellular persistence of cognate Ag-BCR complexes (compared with Ag internalized via Fc-receptor endocytosis).

Subcellular localization of persisting Ag-BCR complexes

Having demonstrated that Ag-BCR complexes can persist for a prolonged period within Ag-specific B lymphocytes, we next sought to determine the identity of the intracellular compartment(s) in which these complexes were residing. To accomplish this, the distribution of persisting Ag-BCR complexes was compared with the intracellular distribution of markers for the late aspects of the endocytic pathway (i.e., LAMP-2, a marker of both late endosome LE and lysosome L, and FITC-dextran chased as a marker of the early endosome EE). These results demonstrate that persisting Ag-BCR complexes reside within a subpopulation of LAMP-2-positive vesicles. However, the persisting Ag-BCR complexes exhibited no colocalization with FITC-dextran, which was introduced into the cells at the same time as the Ag, but then subsequently chased into terminal L (Fig. 1F). These results demonstrate that persisting Ag-BCR complexes reside within nonterminal L. Additionally, the lack of colocalization between persisting Ag-BCR complexes and FITC-dextran (which were initially internalized into the cell at the same time) suggests that B cells possess a mechanism to prevent the delivery of Ag-BCR complexes to terminal L.

Trafficking of Ag-BCR complexes through DM- and DO-containing endocytic compartments

B lymphocytes express two intracellular proteins that are known to be intimately involved in Ag-mediated Ag processing and class II peptide loading, DM and DO (16). Therefore, we sought to compare the distribution of persisting Ag-BCR complexes to the subcellular distribution of DM and DO in normal B lymphocytes. As illustrated in Fig. 3 (A and B), upon initial Ag binding, Ag-BCR complexes are present exclusively at the surface of the B cell, whereas the DM and DO proteins are restricted to intracellular compartments. However, after 1–4 h of incubation at 37°C, a large fraction of internalized Ag-BCR complexes is localized to endocytic vesicles that contain detectable levels of both DM and DO (yellow vesicles in Fig. 3, E–H). Nevertheless, at these time points internalized Ag was also detected in endocytic vesicles that did not contain detectable levels of DM or DO protein (e.g., red vesicles indicated by arrows in Fig. 3, F and H). In addition, at these time points some B cells contained DM- and DO-positive vesicles that were not accessed by detectable amounts of internalized Ag-BCR complexes (green vesicles in Fig. 3, E–H). Unexpectedly, when the distribution of long term (i.e., 24-h) persisting Ag-BCR complexes was compared with the distribution of DM and DO (Fig. 3, I and J), it was observed that while long-term persisting Ag-BCR complexes exhibit a high degree of colocalization with DM, they exhibit a notably lower level of colocalization with DO. These results demonstrate that some of the endocytic vesicles that contain persisting Ag-BCR complexes are more highly enriched for the MHC class II chaperone DM than for the negative regulator of DM activity, DO. Moreover, these results suggest that DM and DO have somewhat distinct steady-state distributions within the endocytic pathway of normal B lymphocytes.

Because many laboratories have demonstrated that DM and DO are tightly associated within B lymphocytes (17–20), we were surprised by this finding. To further investigate the distribution of DM and DO within the endocytic pathway of the B lymphocyte, we analyzed the distribution of DM and DO within the A20 murine B cell line. We chose the A20 model system for three reasons: 1) the availability of a human DM transfected A20 cell line (i.e., A20huDM) (9), in which we could use available reagents to analyze the intracellular distribution of DM and DO by double-label IFM; 2) our extensive experience analyzing the cell biology of Ag processing within these cells using the techniques of double-label IFM and subcellular fractionation (5, 9, 11, 12, 21, 22); and 3) the availability of a transfected A20 cell line (i.e., A20 μWT) (10) that expresses an Ag-specific BCR that can mediate the endocytosis, processing, and presentation of BCR-bound Ag.

Using A20huDM cells in which we have previously demonstrated a similar intracellular distribution of the endogenous murine DM and transfected human DM molecules (9, 21), we examined the relative distributions of DM and DO by double-label IFM. As shown in Fig. 4A, the human DM and endogenous murine DO proteins have notably distinct subcellular distributions within A20huDM cells. As previously reported (9), the DM protein is most highly enriched within relatively large intracellular vesicles located near the periphery of the cell. This pattern of staining is reminiscent of the distribution of the LE/L marker LAMP-2 (Fig. 4C) (9), which we have previously reported to extensively colocalize with DM in A20 cells (9). Contrastingly, the DO protein is most highly enriched within a population of intracellular vesicles whose distribution is reminiscent of the distribution of the Golgi apparatus within these cells (9, 11, 22). To further characterize the subcellular distribution of DO in A20 cells, we compared the subcellular distribution of DO to markers of the early and late aspects of the endocytic pathway (i.e., EE/RE and LE/L, respectively). As illustrated in Fig. 4B, DO exhibits a moderate degree of overlap with internalized transferrin present in EE and RE. Although most of the transferrin-containing vesicles contain DO, there is a population of DO-positive vesicles that does not contain detectable levels of transferrin. Contrastingly, and as expected from the low level of colocalization between DM and DO (Fig. 4A), DO exhibits little if any colocalization with the LE/L marker LAMP-2 (Fig. 4C). These results suggest that in A20 cells, DO is most highly enriched within the biosynthetic pathway as well as early aspects of the endocytic pathway. Contrastingly, DM is most highly enriched within LE and terminal L.

To confirm the results obtained by IFM, the steady-state intracellular distribution of DM and DO in A20 μWT cells was also analyzed by subcellular fractionation. Because we have previously used the technique of Nycodein density gradient centrifugation to localize DM to LAMP-2-positive LE and L in A20 cells (9), we used the same approach to compare the intracellular distribution of the endogenous murine DM and DO proteins within A20 μWT cells. As previously reported (9) and again illustrated in Fig. 4D, the endogenous murine DM protein of A20 cells is most highly enriched in gradient fractions that contain LAMP-2-positive, β-Hex-negative LE as well as LAMP-2-positive, β-Hex-negative L. Contrastingly, the endogenous murine DO protein bands to two distinct regions of the gradient. The population of low buoyant density DO-containing vesicles near the top of the gradient has been tentatively labeled EE/RE (see brackets above Western blots) because of the previously published localization of the EE/RE marker Rab4 to this region of the Nycodein gradient (9) as well as the high level of DO colocalization with internalized transferrin illustrated in Fig. 4B. The high buoyant density DO-containing vesicle near the bottom of the gradient comigrates with vesicles derived from the plasma membrane as well as comigrating vesicles derived from the early aspects of the biosynthetic pathway (i.e., the endoplasmic reticulum ER and Golgi apparatus). Because DO is an intracellular protein, the DO molecules within these gradient fractions must reside in either ER- or Golgi-derived vesicles. Importantly, although the precise identities of the two populations of DO-containing vesicles remain to be conclusively determined, the
subcellular fractionation results are completely consistent with the IFM analysis of the distribution of DM and DO shown in Fig. 4,
A–C, and conclusively demonstrate that in A20 B cells, the majority of the DM and DO molecules have distinct steady-state intracellular distributions.

Moreover, the IFM and subcellular fraction studies suggest that DM and DO have distinct distributions within the endocytic pathway of murine B cells. Therefore, we analyzed the trafficking of BCR-internalized Ag through DM- and DO-containing endocytic compartments of A20 WT B cells. For this analysis, we used rabbit anti-DO followed by donkey anti-rabbit IgG-DTAF. Shown are representative results from one of four independent experiments. In all four experiments the majority of the observed B cells displayed a staining pattern for HEL and DM or DO similar to that presented in the figure. For example, in one representative experiment only 22 of 100 randomly scored B cells containing 24-h persisting Ag contained one or more intracellular vesicles that were Ag positive but DM negative. Contrastingly, when the distribution of persisting Ag and DO was analyzed in the same sample, 76 of 100 randomly scored B cells contained one or more intracellular vesicles that were positive for persisting Ag, but DO negative.

subcellular fractionation results are completely consistent with the IFM analysis of the distribution of DM and DO shown in Fig. 4, A–C, and conclusively demonstrate that in A20 B cells, the majority of the DM and DO molecules have distinct steady-state intracellular distributions.

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HRP-labeled Ag and took advantage of the observation that delivery of HRP-labeled ligands to various endocytic compartments allows the HRP-catalyzed DAB-mediated cross-linking of the constituent proteins of those vesicles (23–25). The results presented in Fig. 5A demonstrate that HRP-labeled Ag is rapidly and efficiently internalized after binding to the BCR of A20 μWT cells. After 15 min of incubation at 37°C, the Ag has attained a steady-state distribution in which approximately half the total amount of cell-associated Ag remains at the cell surface while the other half resides within early endocytic compartments. Importantly, after an additional 45 min of incubation at 37°C, the same relative distribution is maintained. However, after this time BCR-internalized Ag would have gained access to latter aspects of the endocytic pathway.

To assess the kinetics of delivery of BCR-internalized Ag to DM- and DO-containing endocytic compartments, we analyzed the extent of HRP-catalyzed DAB-mediated cross-linking of various intracellular proteins at different times after initiation of BCR-mediated endocytosis of PC-BSA-HRP. As illustrated in Fig. 5B, as soon as 15 min after initiation of endocytosis, Ag-HRP-mediated depletion of DO attained a maximal level, consistent with the hypothesis that the DO protein in A20 μWT cells is most highly enriched within the early aspects of the endocytic pathway. The fact that Ag-HRP-mediated DAB cross-linking of DO never exceeded ~30% of the total cellular pool of DO suggests that a significant portion of the DO protein within the cells resides within the biosynthetic pathway of the cell (i.e., ER and Golgi apparatus) and is therefore, like the ER-resident protein calnexin, not accessible to BCR-internalized Ag. This would be consistent with the partial colocalization between DO and internalized transferrin in A20 cells as presented in Fig. 4B as well as the partial overlap between DO and BCR-internalized Ag observed in normal splenic B cells (Fig. 3E). Contrastingly, Ag-HRP-mediated depletion of both DM and the terminal L marker β-Hex was greatest after 60 min of incubation at 37°C, consistent with the hypothesis that the majority of the DM protein within the endocytic pathway of A20 B cells is restricted to LE and L. The incomplete depletion of DM and β-Hex suggests that either BCR-internalized Ag is unable to access every LE and L of the cell or that insufficient time was allowed to attain maximal DM or β-Hex depletion.

Taken together, the results presented in Figs. 3–5 demonstrate that DM and DO have different steady-state distributions within the endocytic pathway of both normal murine B cells as well as the A20 B cell line. In A20 μWT B cells, DO appears to be most highly enriched within the biosynthetic pathway and early aspects of the endocytic pathway, whereas DM is present at highest levels within LE and L. Moreover, internalized Ag-BCR complexes are first delivered to EE, which have a low DM to DO ratio. Subsequently, Ag-BCR complexes are transferred to nonterminal LE, which have a high DM to DO ratio, where these Ag-BCR complexes may persist for a prolonged period of time.

Prolonged expression of antigenic peptide-class II complexes by B cells harboring persisting Ag-BCR complexes

Although the results presented above demonstrate the prolonged intracellular persistence of native Ag-BCR complexes within nonterminal late endocytic compartments of Ag-specific B lymphocytes, the immunological impact of this finding remains to be determined. Therefore, because BCR-mediated Ag internalization is the first step in the pathway of Ag processing and presentation, we analyzed the level of Ag presentation in B cells harboring persistent Ag-BCR complexes. Specifically, we used a flow cytometric assay for Ag processing (based on the binding of the peptide-class II-specific mAb C4H3 that specifically recognizes HEL46–61-I-Ak peptide-class II complexes) (13) to analyze the duration of expression of antigenic peptide-class II complexes by Ag-specific B cells after removal of environmental Ag.

Accordingly, normal splenic B lymphocytes were allowed to generate HEL46–61-I-Ak peptide-class II complexes via either BCR-mediated or F-P Ag processing (i.e., MD4 B cells plus 10 nM HEL or B10 B cells plus 100 μM HEL, respectively). The B cells were then washed to remove environmental Ag and were returned to culture for various periods of time before quantitation of cell surface antigenic peptide-class II complexes by flow cytometry. As illustrated in Fig. 6A, removal of environmental Ag from B cells that are generating peptide-class II complexes via F-P Ag processing results in a rapid decrease in the level of cell surface peptide-class II complexes such that 24 h after removal of Ag the level of cell surface peptide-class II complexes has dropped by 40–60%. Contrastingly, removal of environmental Ag from B cells that are generating cell surface peptide-class II complexes via BCR-mediated Ag processing (and possess persisting intracellular antigenic peptide-class II complexes) fails to significantly effect the level of cell surface peptide-class II complexes such that 24 h after removal of Ag there is essentially no decrease in the level of peptide-class II complexes expressed by the cells (Fig. 6B). Importantly, the prolonged peptide-class II expression by the Ag-specific B cells is not simply a consequence of physiological changes elicited by Ag binding to the BCR, because Ab-mediated ligation of the BCR on B10 B cells that are generating peptide-class II complexes via F-P Ag processing does not change the observed level of peptide-class II cell surface persistence on these cells (Fig. 6A). Therefore, these results demonstrate that Ag-specific B lymphocytes that are harboring persistent intracellular Ag-BCR complexes are capable of prolonged cell surface expression of antigenic peptide-class II complexes that are the direct result of the processing and presentation of BCR-internalized Ag.
moved onto ice, and the level of cell surface HEL\textsubscript{46-65}-I\textsubscript{A\*} complexes Ag, returned to culture at 37°C for the indicated chase periods, and re-

moved onto ice, and the level of cell surface HEL\textsubscript{46-65}-I\textsubscript{A\*} complexes was determined by staining with the C4H3 mAb, followed by analysis via

flow cytometry. Shown is the level of detectable cell surface peptide-class II complexes, where the initial level of expression has been normalized to a value of 1 to allow direct comparison between experiments. Each line represents the results obtained from a single independent experiment. B, MD4 B cells were incubated in 10 nM HEL overnight to allow for the F-P endocytosis of HEL and cell surface expression of HEL peptide-class II complexes. The cells were then washed to remove environmental Ag, returned to culture at 37°C for the indicated chase periods, and re-

moved onto ice, and the level of cell surface HEL\textsubscript{46-65}-I\textsubscript{A\*} complexes was determined by staining with the C4H3 mAb, followed by analysis via flow cytometry. Shown is the level of detectable cell surface peptide-class II complexes, where the initial level of expression has been normalized to a value of 1 to allow direct comparison between experiments. Each line represents the results obtained from a single independent experiment. Results from concurrent experiments are denoted by the same icon.

FIGURE 6. Prolonged cell surface expression of antigenic peptide-class II complexes on Ag-specific B cells harboring persistent Ag-BCR complexes. A. B10 B cells were incubated in 100 µM HEL (either without (open icons) or with (filled icons) 10 nM anti-\mu Ab) overnight to allow for the F-P endocytosis of HEL and cell surface expression of HEL peptide-class II complexes. The cells were then washed to remove environmental Ag, returned to culture at 37°C for the indicated chase periods, and removed onto ice, and the level of cell surface HEL\textsubscript{46-65}-I\textsubscript{A\*} complexes was determined by staining with the C4H3 mAb, followed by analysis via flow cytometry. Shown is the level of detectable cell surface peptide-class II complexes, where the initial level of expression has been normalized to a value of 1 to allow direct comparison between experiments. Each line represents the results obtained from a single independent experiment. B. MD4 B cells were incubated in 10 nM HEL overnight to allow for the BCR-mediated endocytosis of HEL and cell surface expression of HEL peptide-class II complexes. The cells were then washed to remove environmental Ag, returned to culture at 37°C for the indicated chase periods, and removed onto ice, and the level of cell surface HEL\textsubscript{46-65}-I\textsubscript{A\*} complexes was determined by staining with the C4H3 mAb, followed by analysis via flow cytometry. Shown is the level of detectable cell surface peptide-class II complexes, where the initial level of expression has been normalized to a value of 1 to allow direct comparison between experiments. Each line represents the results obtained from a single independent experiment. Results from concurrent experiments are denoted by the same icon.

Discussion

The results presented in this report demonstrate that after internalization, Ag-BCR complexes persist for a prolonged period within nonterminal late endocytic compartments of B lymphocytes. Moreover, the ability of a conformation-specific mAb to recognize the Ag within these Ag BCR complexes demonstrates that at least a portion of the Ag within these complexes is in a native conformation. In addition, B cells that harbor persisting Ag-BCR complexes are also capable of prolonged cell surface expression of antigenic peptide-class II complexes, which are the direct result of the processing and presentation of BCR-internalized Ag. These results suggest that the reason for the prolonged peptide-class II expression by these B cells is the continued processing of the Ag within the persisting Ag-BCR complexes and transport of newly formed peptide-class II complexes to the cell surface. This possibility is consistent with our preliminary observation that LEs containing persisting Ag-BCR complexes also contain peptide-class II complexes derived from the processing of this Ag (as detected with the HEL\textsubscript{46-65}-I\textsubscript{A\*} complex-specific mAb C4H3) (13) (T. A. Gondré-Lewis and J. R. Drake, unpublished observation). Importantly, it is unlikely that the reason for the prolonged expression of specific peptide-class II complexes on the surface of the Ag-specific B cells is due to a change in the physiology of the B cell because of Ag-induced BCR signaling, because signaling of B cells undergoing F-P Ag processing (with anti-BCR Abs) fails to extend the duration of peptide-class II complex expression by these B cells.

An additional interesting property of the persisting intracellular Ag-BCR complexes is that they exhibit a higher degree of colocalization with the MHC class II peptide exchange catalyst DM than with the DM modulator DO. DO is widely thought to be an inhibitor of the peptide exchange activity of DM (18, 20), and it has been proposed that the function of DO is to focus Ag processing in B cell onto BCR-internalized Ag (16, 26). The colocalization results presented in this report suggest that DO may achieve this focusing by restricting DM-mediated class II peptide loading to intracellular compartments where Ag-BCR complexes persist for a prolonged period of time, allowing a greater time for the processing of BCR-internalized Ag and increasing the efficiency by which antigenic peptides are loaded onto MHC class II molecules. However, this observation raises the question of how DM and DO (two proteins that are known to associate within the ER of the B cell) (19) establish different steady-state intracellular distributions within the endocytic pathway of these cells.

Although the limited number of published reports on the intracellular distribution of DM and DO in nontransfected B lymphocytes suggests that DM and DO have similar intracellular distributions (19, 26), the precise distributions of these two proteins within the endocytic pathway of normal B cells and the ability of the BCR to deliver Ag to these subcellular compartments remain to be extensively studied. Although double-label IFM analysis of murine splenic B cells suggests that DM and DO have an overlapping intracellular distribution (Fig. 1 in Ref. 19), the relative intensity of staining of individual vesicles for DM and DO was variable, suggesting that in some cases there may be differences in the intracellular distributions of these two proteins. Moreover, it is known that B cells produce DM in excess over DO (leading to the presence of both DM-DO complexes as well as free DM molecules) (17, 18). Therefore, it is possible that (as already suggested by Alfonso and Karlsson; Ref. 16) “free DM and DM-DO complexes may be sorted to different compartments of the endosomal/lysosomal system.” Furthermore, DM and DO molecules may access the endocytic pathway as DM-DO complexes, which subsequently dissociate, allowing the active intracellular targeting motifs in the cytoplasmic tails of DM (27) and DO (28) to establish distinct steady-state distributions of the two dissociated proteins. Therefore, although the results presented in this report do not distinguish between these or other possibilities, they do demonstrate that BCR-internalized Ags access DO- and DM-enriched endocytic compartments with distinct kinetics and show that persistent intracellular Ag-BCR complexes exhibit a higher degree of colocalization with DM than with DO.

Finally, the prolonged intracellular persistence of Ag-BCR complexes within nonterminal LE raises the question of the molecular mechanism of intracellular trafficking of this receptor-ligand complex. Unlike Ag internalized by F-P endocytosis (which is rapidly delivered to terminal L for degradation), internalized Ag-BCR complexes are delivered to a nonterminal LE and form a hybrid organelle followed by hybrid organelle fission to regenerate LE and L (29), it is possible that the actin-based cytoskeleton is necessary for the sorting of Ag-BCR
complexes into reforming LE and/or exclusion of the Ag-BCR complexes from reforming L. Presently, we are working to further examine the molecular mechanism of Ag-BCR persistence within nonterminal LE.

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