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Analyses of the In Vivo Trafficking of Stoichiometric Doses of an Anti-Complement Receptor 1/2 Monoclonal Antibody Infused Intravenously in Mice

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Complement plays a critical role in the immune response by opsonizing immune complexes (IC) and thymus-independent type 2 Ags with C3 breakdown product C3dg, a CR2-specific ligand. We used a C3dg-opsonized IC model, anti-CR1/2 mAb 7G6, to investigate how such substrates are processed. We used RIA, whole body imaging, flow cytometry, and fluorescence immunohistochemistry to examine the disposition of 0.1- to 2-μg quantities of mAb 7G6 infused i.v. into BALB/c mice. The mAb is rapidly taken up by the spleen and binds preferentially to marginal zone (MZ) B cells; within 24 h, the MZ B cells relocate and transfer mAb 7G6 to follicular dendritic cells (FDC). Transfer occurs coincident with loss of the extracellular portion of MZ B cell CR2, suggesting that the process may be mediated by proteolysis of CR2. Intravenous infusion of an FDC-specific mAb does not induce comparable splenic localization or cellular reorganization, emphasizing the importance of MZ B cells in intrasplenic trafficking of bound substrates. We propose the following mechanism: binding of C3dg-opsonized IC to noncognate MZ B cells promotes migration of these cells to the white pulp, followed by CR2 proteolysis, which allows transfer of the opsonized IC to FDC, thus facilitating presentation of intact Ags to cognate B cells. The Journal of Immunology, 2004, 173: 2297–2306.

Thirty years ago, Pepys (1) first demonstrated that C can play a key role in the immune response. Although several mechanisms may underlie this phenomenon, an essential step in this process requires covalent opsonization of Ags or Ab/Ag immune complexes (IC) by C activation fragment C3b, followed by its degradation to C3dg, thus allowing binding of these substrates by immune cells via CR2 (2–10). Ags in IC, as well as thymus-independent type 2 Ags, such as bacterial-associated polysaccharides, can be opsonized by C and then bind to B cell CR2 (11–18). Studies in experimental animals indicate that the handling of these opsonized Ags in vivo can be quite complex. For example, i.v.-infused, highly cross-linked IC can be bound via FcγR on cells of the mononuclear phagocytic system, and then phagocytosed and destroyed (14, 19). However, a fraction of these complexes are rapidly opsonized with C, become associated with splenic B cells via CR2, and ultimately are transferred to follicular dendritic cells (FDC), where the Ags can act as particularly effective immunogens (9, 12, 13, 18–24). The mechanism by which C3dg-opsonized Ags are localized to FDC remains uncertain (25, 26), and in fact, several studies have demonstrated that free soluble IC and simple proteins do not gain access to the white pulp and FDC region, indicating that this delivery mechanism requires cellular transport (20, 27–29). Moreover, there is some question with respect to the identity of the cells that ferry the opsonized Ags to the FDC (25, 30, 31).

To clarify these issues we have investigated the in vivo fate and handling in mice of a rat IgG mAb specific for CR1/2. Our approach is similar to the in vitro studies of Mongini et al. (32, 33), who examined the interaction of human B cells with dextran that was conjugated with anti-IgM Abs and with an anti-human CR2 mAb. These mAb-conjugated dextrans served as surrogates for C3dg-bearing Ags. IgG mAb 7G6 binds to murine CR1/2 with high affinity and blocks its C3dg ligand binding site (34, 35). Therefore, in our analogous model we used this mAb as a surrogate for a C3dg-IC. We used RIA, dual modality imaging, flow cytometry, and fluorescence immunohistochemistry to examine the disposition of small amounts (0.1–2 μg) of mAb 7G6 infused i.v. in mice. The mAb is rapidly taken up by the spleen and binds preferentially to marginal zone (MZ) B cells. However, within 24 h the MZ B cells relocate and transfer the mAb to the FDC region; this transfer reaction occurs coincident with loss of B cell-associated CR1/2. After infusion of mAb 7G6, repopulation of the splenic MZ by CR1/2-containing B cells is not fully established until 1 wk later. Intravenous infusion of a mAb specific for FDC does not lead to comparable splenic localization or cellular reorganization, providing additional evidence for the unique role of MZ B cells in the delivery of Ags to FDC.

Our findings with the anti-CR1/2 mAb suggest that binding of C3dg-opsonized IC to noncognate MZ B cells is a key first step in the C-dependent arm of the normal immune response; this binding step is then followed by migration of the MZ B cells into the follicle to allow for transfer of the opsonized Ags to FDC, where presentation of the intact Ag to cognate B cells can then occur.

Materials and Methods

**Monoclonal Abs**

Hybridoma cell lines producing mAbs specific for murine CR1/2 (mAb 7G6, rat IgG2b; and mAb 7E9, rat IgG2a) were kindly provided by V. M. Holers (University of Colorado, Denver, CO) and S. K. Pierce (National Institutes of Health, Bethesda, MD) and have been previously described.
mAb HB151, rat IgG2b, specific for HLA DR5, was obtained from American Type Culture Collection (Manassas, VA) and used as an isotype control. These mAbs were produced in vitro from hybridomas cultured in gas permeable bags (BioVector DCL, Oxford, CT) and purified using recombinant protein G-Sepharose (Amersham Biosciences, Piscataway, NJ). Gel filtration analysis (data not shown) revealed that mAbs produced by this procedure are >98% monomeric after purification. mAb 7B7 (IgG2a), specific for bacteriophage 8X174, has been previously described (36), and was covalently crosslinked to mAb 7G6 using published procedures (37, 38). mAbs were labeled with 125I using iodogen (39), with Alexa fluorophore (Al) dyes (Al488, Al594, or Al633; Molecular Probes, Eugene, OR) following the manufacturer’s directions, or were conjugated with NHS LC biotin (bt; Pierce, Rockford, IL) (40). The following commercial mAbs were used: PE CD19 (Caltag, Burlingame, CA); mAb FDCM1, FITC CD22.2, PE CD23, PerCP Cy5.5 CD19, and PE CD1d (BD Pharmingen, San Diego, CA); bt mAb FDCM2 (AMS Biotechnology, Abingdon, U.K.); FITC MÔMA (Serotec, Raleigh, NC); and M19, goat anti-mouse CR1/2 C terminus (Santa Cruz Biotechnology, Santa Cruz, CA). Alabeled polyclonal Abs against goat IgG (rabbit), mouse IgM (goat), and rat IgG (goat) were obtained from Molecular Probes. Affinity-purified mouse anti-rat IgG was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA; Code Number 212 005 168).

**In vivo protocols**

Female BALB/c mice (Harlan Breeders, Indianapolis, IN; or Hilltop Lab Animals, Scottsdale, PA) were housed in barrier facilities and used at 3–6 mo of age. All animal protocols were approved by the University of Virginia Animal Care and Use Committee. Mice were sacrificed and weighed, and single-cell suspensions were prepared. Mononuclear cells were separated from E by density step centrifugation on Lympholyte-M (Cedarlane Laboratories, Hornby, Ontario, Canada) according to the manufacturer’s directions. In some experiments, cells were fixed and permeabilized according to the manufacturer’s directions (Caltag). B cells were identified as PE CD19-positive (see Fig. 2) or PerCP Cy5.5 CD19-positive (see Fig. 3). M2 (CD22.2(bright), CD23(low)) and follicular (CD22.2(bright), CD23(low)) B cells were further differentiated by probing with FITC CD22.2 and PE CD23 (45). Anti-coagulated blood samples were probed with PE CD19, Al633 streptavidin (SA), and Al488 mAb 7G6, and processed as previously described (46). Calibrated fluorescence beads (Spherotech, Libertyville, IL) were used to convert fluorescence intensities to molecules of equivalent soluble fluorochrome (46).

**Fluorescence immunohistochemistry**

Spleens were snap frozen in liquid nitrogen and 5-µm sections cut. Frozen spleen sections were fixed in ice-cold acetone, blocked with dilute goat serum (Vector Laboratories, Burlingame, CA), probed with mAbs at 10 ng/ml for 1 h in the dark at room temperature, mounted with Aquamount (Lerner Laboratories, Pittsburgh, PA), and examined in an Olympus microscope, equipped with a Magnafire digital camera (Olympus, Melville, NY). Multicolor images were obtained by manually rotating the emission filter into place to coordinate with the appropriate camera filter.

**Results**

**Organ distribution**

We infused 125I-labeled anti-CR1/2 mAb 7G6 i.v. into BALB/c mice at doses ranging from 200 to 6000 ng (Fig. 1). The time course of organ distribution for a dose of 720–840 ng indicates accumulation in the spleen and liver (35 ± 3% and 13 ±1%, respectively, at 1 h), with negligible levels in the lungs, heart, and kidneys. However, the only specific organ localization is to the spleen, because at the same dose, 4% of 125I-labeled isotype control mAb HB151 was recovered in the spleen, and 18% in the liver, at 1 h (data not shown). Specific splenic localization of 125I-labeled mAb 7G6 is abrogated by i.p. pretreatment with 500 µg of unlabeled mAb 7G6, 24 h before i.v. infusion (1.5% pretreated vs 33% no pretreatment, at 1 h).

A substantial fraction of infused 125I-labeled mAb 7G6 is still specifically localized to the spleen after 24 h. We find that 9%, corresponding to ~70 ng mAb 7G6, is found in the spleen at this time (Fig. 1A); based on our fluorescence immunohistochemistry analyses we believe that much of the material in the spleen at 24 h is intact mAb (see Fig. 5, E and F, below). Dose-response experiments for the 1-h time point comparing the liver and spleen localization suggest that the specific binding capacity of the spleen for mAb 7G6 is near saturation at i.v. doses of 2 µg or more, as demonstrated by the relative increase in uptake by the liver at higher doses (Fig. 1B). Further evidence for saturation is the finding that the 720- to 840-ng (Fig. 1A) and 2000-ng doses (data not shown) lead to localization of 70 and 100 ng, respectively, of mAb 7G6 in the spleen at 24 h. A smaller number of experiments conducted with 125I-labeled anti-CR1/2 mAb 7E9 demonstrated the same preferential targeting to the spleen as observed with mAb 7G6 (data not shown). Finally, in a separate series of experiments that made use of a different batch of 125I-labeled mAb 7G6, infusion of 650–800 ng into BALB/c mice led to splenic localization of 27 ± 2% of the counts at 1 h (n = 5), and 9.5 ± 0.1% of the counts at 24 h (n = 2), in agreement with the results in Fig. 1A.

We used dual modality whole body imaging to examine the kinetics of the organ distribution of several doses of infused 125I-labeled mAb 7G6 and isotype control mAb HB151 (Fig. 1, C–E). Specific localization of the anti-CR1/2 mAb 7G6 to the spleen is rapid and reaches a steady state in ~15 min when 250 ng of mAb 7G6 is infused (Fig. 1C). In agreement with the whole organ gamma-counting experiments, isotype control mAb HB151 does not localize to the spleen. However, the time to equilibration of this mAb is also rapid (Fig. 1D). The kinetics of splenic localization of anti-CR1/2 mAb 7E9 show a similar pattern (data not shown).
When the dose of mAb 7G6 is increased to 2000 ng, spleen saturation is achieved even more rapidly (Fig. 1E), but the spleen to liver density ratio decreases relative to the 250-ng dose. Our findings of splenic localization are not biased due to generation of abnormal weights for the liver and spleen induced by the infused mAbs. For example, the respective liver and spleen weights for the 1 h infusions of 0.5 to 6/260 ng of 125I-labeled mAb 7G6 were 0.98 ± 0.12 g and 0.15 ± 0.05 g, and for infusion of mAb HB151 they were 0.90 ± 0.05 g and 0.13 ± 0.02 g. Alternatively, after normalization for organ weights, the specific organ localization, in percentage of infused counts per milligram of tissue, were 0.24 (spleen) and 0.014 (liver) for the 720–840 ng infusions of 125I-labeled mAb 7G6 at 1 h (n = 9).

Analysis of mAb 7G6 in the circulation

In view of the rapid localization of 125I-labeled mAb 7G6 to the spleen, we also examined counts in the plasma for either cellular binding or IC formation, as IC would also be expected to localize, at least in part, to the spleen. Multiple blood samples were obtained within the first hour after i.v. infusion of 125I-labeled mAb 7G6 (650–800 ng), and aliquots were washed and pelleted. Less than 2% of the circulating counts were found in the cell pellet (Table I), presumably due to the small number of circulating B cells. Alternatively, larger amounts of plasma were isolated, and then tested for the presence of IC based on precipitation with 3.5% PEG (42). The results in Table I indicate 125I-labeled mAb 7G6 did not form IC when it was infused into the naive BALB/c mice. However, when small amounts of mouse anti-rat IgG were added to the isolated plasmas as a positive control, 125I-labeled mAb 7G6 did precipitate in 3.5% PEG. Finally, ELISAs conducted as part of an immunization procedure confirmed that the naive BALB/c mice did not have endogenous Abs specific for rat IgG (data not shown).

Dose-response analyses of mAb 7G6 binding to circulating and splenic B cells

Because i.v. infusion of microgram quantities of the anti-CR1/2 mAbs appeared to saturate the binding capacity of the spleen, it is reasonable to postulate that the targeted cells, presumably B cells, should take up the infused mAbs and demonstrate saturation at the same low doses. Therefore, we infused i.v. varying amounts of bt mAb 7G6, and then used flow cytometry to examine its binding to circulating and splenic B cells. At the 2-h point, bt mAb 7G6, revealed by probing with Al633 SA or Al488 SA, was bound to both circulating, as well as to splenic, B cells (Fig. 2, A and C). Ex vivo probing with Al488 mAb 7G6 at the 2-h point revealed that available CR1/2 on both circulating and splenic B cells decreased as the dose increased.

### Table I. Analysis of 125I-labeled mAb 7G6 in the bloodstreama

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<tr>
<th>Percent of Plasma cpm Precipitated with 3.5% PEG</th>
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<tr>
<td>Percent of cpm Cell Bound</td>
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<tr>
<td>No addition 1.65 ± 0.35 10.6 ± 1.4 81.0 ± 5.1</td>
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<tr>
<td>Plus mouse anti-rat IgG 3 mice, 6 bleeds each 4 mice, 3 bleeds each</td>
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A total of 650–800 ng of 125I-labeled mAb 7G6 was infused i.v., and blood samples were drawn at 2, 10, and 12 min (three bleeds), or 2, 5, 10, 15, 30, and 60 min (six bleeds) after infusion.
CD22.2, and CD23. After 1 h, both the MZ B cells (CD22.2 high, CD23 low), and the follicular B cells (CD22.2 high, CD23 high), in the spleen were prepared and probed with mAbs specific for CD19, CD22, and CD23. Varying amounts of bt mAb 7G6 were infused into BALB/c mice and blood samples were taken 2 and 18 h after infusion (A and B), immediately before euthanization. Single-cell suspensions were prepared from the spleen (C and D). Samples were probed with Al633 SA (A) or Al488 SA (C) to identify cell-bound bt mAb 7G6, and with Al488 mAb 7G6 to measure available unliganded CR1/2 (B and D) by flow cytometry. Note that although most bt mAb 7G6 is removed from both circulating and splenic B cells at 18 h, the amount of available CR1/2 is not restored to levels seen in the naive (0-μg dose) mice. E. In a similar infusion experiment (2 μg of bt mAb 7G6 infused), anti-CR1/2 mAb 7E9, which does not compete with mAb 7G6, was used to assay for available CR1/2 on both circulating cells and on intact, as well as on permeabilized, spleen (spl.) cells. n = 2 for all points.

**mAb 7G6 binding to MZ vs follicular B cells in the spleen**

To further characterize the localization of mAb 7G6 within the spleen, we used a flow cytometry gating scheme that differentiates between splenic MZ and follicular B cells (45). Following i.v. injection of 2 μg of bt mAb 7G6, single-cell suspensions from the spleen were prepared and probed with mAbs specific for CD19, CD22,2, and CD23. After 1 h, both the MZ B cells (CD22,2high, CD23low), and the follicular B cells (CD22,2high, CD23high), bound bt mAb 7G6 as revealed by the increase in binding of the Al633 SA probe (Fig. 3A), and the decrease in binding of Al633 mAb 7G6 (Fig. 3B). However, while 72 ± 3% of the MZ B cells had bound bt mAb 7G6, only 39 ± 2% of the follicular B cells contained bound bt mAb 7G6 (Fig. 3D). At 24 h, the Al633 SA signal is markedly reduced compared with the signal at 1 h for both MZ and follicular B cells, and there is no corresponding increase in binding of Al633 mAb 7G6. B cell CR1/2 is known to be very susceptible to proteolysis (47–49). To determine whether only the extracellular portion of B cell-associated CR1/2 is removed, we used a polyclonal Ab specific for the cytoplasmic region of CR1/2 to assay the B cells before and after permeabilization. Fig. 3C shows that at 24 h, substantial levels (>50%) of the epitope recognized by M19 remain associated with both types of B cells, after most available extracellular CR1/2, defined by binding of Al633 mAb 7G6, is removed.

**Visualization of splenic localization**

In view of the observations that a substantial fraction of the 125I-labeled mAb 7G6 is found in the spleen at 1 and 24 h after infusion, and that available CR1/2 on B cells is reduced considerably 24 h after bt mAb 7G6 infusion, we next investigated the site(s) of localization of mAb 7G6 within the spleen. We examined sections from spleens taken at several times after infusion of 2 μg of bt mAb 7G6 (Fig. 4, A–C). Sections were probed with green Al488 SA to identify infused bt mAb 7G6, and residual available CR1/2 was identified by probing with red Al594 anti-CR1/2 mAb 7E9 that does not compete with mAb 7G6. Thirty minutes after infusion the bt mAb 7G6 (Fig. 4C, 30 min) is localized to the green rim of a region rich in CR1/2 (identified as the MZ, see Fig. 5 below). Based on the yellow color in the rim (Fig. 4A, 30 min), it is clear that the region in which bt mAb 7G6 is found still has available CR1/2 recognized by mAb 7E9; moreover, large areas of available CR1/2, apparently not yet chelated by the infused bt mAb 7G6, are identified by the red fluorescence of the Al594 anti-CR1/2 mAb 7E9 probe. However, at 24 h after infusion, available CR1/2 is reduced considerably. Moreover, at this time the infused bt mAb 7G6, identified by the green fluorescence of the Al488 SA, is colocalized in the region containing the residual available CR1/2 (compare Fig.
with Al633 SA. Percent of total CD19-positive cells is indicated. follicular B cells that bound infused bt mAb 7G6 were revealed by probing for the intracellular C terminus of CR1/2 (recognized by the ex vivo Al594 mAb 7G6 probe) and infused bt mAb 7G6. The points shown at 2 and 4 h indicate some rearrangement does occur, and this rearrangement and condensation appear to be complete by 24 h.

To confirm that infused bt mAb 7G6 was bound to B cells, we probed spleen sections from treated mice with red Al594 goat antimouse IgM and green Al488 SA (Fig. 5A). Only 15 min after infusion, the bt mAb 7G6 (yellow-orange) is located on the surrounding edge of the red B cell follicles, but at 24 h, the infused anti-CR1/2 mAb (yellow) is located near the center of the follicle, in a region highly enriched with B cells. Alternatively, consecutive sections were probed with red Al594 SA and green FITC MOMA, specific for metallophilic macrophages (50), to identify the boundary between the MZ and the follicle (Fig. 5B). The infused bt mAb 7G6 (now revealed as red) clearly moved from the MZ at 15 min to the inside of the follicle at 24 h.

To further characterize the cells that the infused mAb 7G6 was ultimately associated with, spleen sections from mice infused with green Al488 mAb 7G6 were probed with markers specific for FDC (Fig. 5C and D). At 1 h after infusion, the Al488 mAb 7G6 is contiguous with, but not overlapping, the red FDC-positive region. At 24 h, the infused Al488 7G6 is found in the FDC region. These observations, taken along with Figs. 4 and 5 (A and B), indicate that the ultimate (24-h) destination of the infused anti-CR1/2 mAb is in an FDC-rich region closely associated with B cells; moreover, the only residual CR1/2 available for chelation ex vivo also is colocalized with the FDC-positive region. Slides from Fig. 5D at the 24-h mark were also examined under ×100 objective magnification. We observed substantial numbers of single cells that stained with both the red probe, specific for FDC, and with the green probe, specific for deposited bt mAb 7G6. Finally, we found no evidence for localization of mAb 7G6 to splenic T cells (data not shown).

Because one of the goals of this work is to develop a general approach for delivery of Ags to FDC for purposes of immunization, we investigated whether infusion of a bispecific mAb construct, anti-CR1/2 × anti-ΦX174, would lead to a similar localization pattern in the spleen. To analyze spleen sections, we used the Ag, green Al488 ΦX174, to detect the anti-ΦX174 mAb, and red Al594 anti-rat IgG, to detect the anti-CR1/2 mAb 7G6 (Fig. 5E). Alternatively, we determined the location of the green Al488 ΦX174 with respect to the FDC (Fig. 5F, red). The results indicate that the intact and immunologically active bispecific reagent is identified by an orange color (Fig. 5E), based on probing for both components of the construct. Moreover, the staining patterns suggest that the reagent is bound to MZ B cells at 1 h, but after 24 h, the infused material (Fig. 5F, orange) again appears to be condensed and localized to a region enriched in FDC.

We next performed pulse-chase experiments to determine the kinetics of CR1/2 depletion and recovery on MZ B cells. Mice were first pulsed with a 2-μg infusion of Al488 mAb 7G6, then chased at varying times after pulse (1–7 day) with a 2-μg infusion of bt mAb 7G6, and finally euthanized 1 h after chase. In control mice that were only chased, the bt mAb 7G6 shows a characteristic rimlike appearance, indicating localization to CR1/2 on MZ B cells (Fig. 6A, chase only). We find, based on the weak Al594 SA signal for the 1 day chase (Fig. 6A, 1 d), that there is very little splenic localization of bt mAb 7G6 when it is infused 1 day after the initial 2-μg pulse of mAb 7G6. However, after 2–3 days, the Al594 SA signal is weakly visible (data not shown), and at day 7, the staining patterns closely resemble the patterns observed in the untreated.

FIGURE 3. Infused bt mAb 7G6 binds to MZ and follicular B cells in the spleen and induces loss of CR1/2 after 1 day. A, Infused bt mAb 7G6 (2 μg) is bound to B cells at 1 h (n = 3), but is removed by day 1 (n = 1). B, At day 1, available CR1/2 is reduced considerably, relative to naive (Na, n = 2) levels. C, Splenic B cells were probed before and after permeabilization with a polyclonal Ab specific for the intracellular C terminus of CR1/2 (n = 1). Molecules of equivalent soluble fluorochrome values were calculated for the entire population of either MZ or follicular B cells. D, Representative histograms from experiments illustrated in A. MZ and follicular B cells that bound infused bt mAb 7G6 were revealed by probing with Al633 SA. Percent of total CD19-positive cells is indicated.

4, A–C, at 24 h). That is, the only available CR1/2 detectable by mAb 7E9 is located in the same region as the infused bt mAb 7G6. When consecutive spleen sections were instead probed with Al488 SA and Al594 mAb 7G6 (Fig. 4D), a somewhat different pattern is evident at the 30-min mark. There is no overlap between available CR1/2 (red) and infused bt7G6 (green), presumably due to simple competition. However, by 24 h, we find colocalization of available CR1/2 (recognized by the ex vivo Al594 mAb 7G6 probe) and infused bt mAb 7G6. The points shown at 2 and 4 h indicate some rearrangement does occur, and this rearrangement and condensation appear to be complete by 24 h.
mouse at 1 h. These observations suggest that functional MZ B cell CR1/2 is restored within 7 days after infusion of 2 μg of mAb 7G6.

To determine whether the transient loss of binding capacity for bt mAb 7G6 at the 1-day point in the pulse-chase experiment was associated with movement of MZ B cells, we treated mice with bt mAb 7G6 and euthanized them at times varying from 1 h to 7 days after infusion. Then, we probed the spleen sections with a mixture of Al350 goat anti-mouse IgM (to identify MZ and follicular B cells), FITC MOMA (to identify the macrophages at the boundary between the MZ and the follicle), and PE CD1d (to distinguish MZ cells from follicular B cells) (51). As shown in Fig. 6B, 1 day after infusion of 2 μg of bt mAb 7G6, the B cells in the MZ are depleted, as defined by loss (noted by an asterisk) of the IgM high, CD1d high phenotype had returned to the MZ along with the ability to capture infused bt mAb 7G6 (Fig. 6, A and B, 7 d). Similar results demonstrating loss followed by repopulation of the MZ by B cells were obtained when the MZ B cells were defined as IgM high, IgD low (data not shown). Thus, taken together, these results support the hypothesis that engagement of CR1/2 on MZ B cells by mAb 7G6 leads to movement of the ligated MZ B cells to the splenic follicles, ultimately allowing transfer of CR1/2 and bound mAb 7G6 to the FDC-rich area.

To gain further insight regarding the accessibility of FDC to proteins in the bloodstream, we infused either 2 μg of mAb FDCM1, specific for FDC, or 2 μg of anti-CR1/2 mAb 7G6, or simply vehicle (0.4 mg/ml mouse IgG). After 24 h, the mice were euthanized and sections of the spleens were blindly analyzed for the presence of these rat mAbs by development with Al594 anti-rat IgG (Fig. 7A). All mice infused with mAb 7G6 had demonstrable rat IgG in the spleens at 24 h, but there was no evidence for splenic localization of the infused mAb FDCM1 in the mice that received this reagent; the spleens of these mice had the same appearance as spleens of mice treated with vehicle alone. However, ex vivo probing of adjoining sections with mAb FDCM1 and Al594 anti-rat IgG confirmed the specificity of the mAb FDCM1 (Fig. 7B). Finally, we infused 125I-labeled mAb FDCM1 into mice, and observed no localization to the spleen. mAb FDCM1 showed the same pattern of organ localization as seen for isotype control mAb HB151 (data not shown).

Discussion

Transport of substrates by MZ B cells

The key finding in this mouse model is the observation that i.v. infusion of a prototype C3dg-opsonized IC, anti-CR1/2 mAb 7G6, leads to its processing by a pathway that was postulated 30 years ago to be used in IC processing (19). MZ B cells are uniquely located to interact with blood-borne pathogens (16–18, 24, 28, 29, 52–55), and our results indicate mAb 7G6 infused i.v. is localized to the spleen (Fig. 1) and binds to MZ B cells. Over a 24-h period, the opsonized cells leave the MZ and transport the mAb to the FDC region (Figs. 4–7). It is likely that by the 24-h mark, the MZ B cell-bound mAb 7G6 is in fact transferred to FDC.

Infusion of 2 μg of mAb 7G6 is sufficient to induce substantial rearrangement of MZ B cells. Although flow cytometry analyses indicate these cells are demonstrable in the spleen 24 h after infusion (Figs. 2 and 3), fluorescence immunohistochemistry experiments reveal the cells are not in the MZ at this time (Fig. 6). In addition, restoration of MZ B cells able to bind infused anti-CR1/2 mAb 7G6 requires ~1 wk. After infusion and clearance of 2 μg of bt mAb 7G6, a saturating dose with respect to B cell CR2, ex vivo binding of additional mAb 7G6, as well as binding of noncompetitively bound anti-CR1/2 mAb 7E9, is abrogated, strongly suggesting that CR1/2 was removed from the B cell during the transfer reaction (Fig. 2). Based on an estimate of 1 × 10^8 B cells per mouse (53), infusion of 2 μg of mAb 7G6 corresponds to an input of ~75,000 mAb molecules per B cell. Because there are ~10,000 CR1/2 molecules per B cell and mAb 7G6 binds to CR1/2 with high affinity, it is reasonable that the 2-μg dose achieves saturation of B cell CR1/2.

Transfer to FDCs appears to be mediated by extracellular proteolysis of CR1/2

B cell CR2 can be released by proteolysis at an extracellular site close to the cell membrane (47–49) and our previous studies, both
in vitro and in vivo in a monkey model (56), demonstrate that after ligation by IC, the IC- and B cell-associated CR2 are transferred to macrophages in a process in which CR2 is removed from B cells. Our results presented in this study are consistent with cleavage and removal of mAb 7G6-ligated CR1/2 from MZ B cells, thus allowing uptake of these substrates by the FDC. Indeed, after CR1/2 loss, permeabilized B cells were found to still have substantial levels of epitopes associated with the cytoplasmic portion of CR1/2 (Fig. 3), implying that it was the extracellular portion of CR1/2 that was removed from the B cell.

Although splenic B cell CR1/2 is substantially reduced 24 h after infusion of mAb 7G6, the mAb is retained in the spleen in an area enriched in available nonligated CR1/2. This CR1/2 may represent FDC-associated CR1/2 that is less labile and less susceptible to proteolysis than B cell CR1/2 (57, 58). Whether the localized mAb 7G6 is held on FDC by FcR (30, 31), or by FDC-associated CR1/2, or by CD23, which binds CR2 in primates (59, 60), is not revealed presently, but this question may be addressed in appropriate knockout animals. Support for proteolytic release of extracellular B cell CR1/2 is also found in studies that indicated cleavage of a structurally related protein, primate E CR1, is required for the transfer of CR1-bound IC from E to acceptor cells (61–63). In fact, in diseases associated with C activation and IC processing, levels of both E CR1 and B cell CR2 are reduced substantially (47, 64–66).

**On the fate of CR1/2 on follicular B cells**

Fig. 4 indicates that infused bt mAb 7G6 is strongly bound to MZ B cells between 30 min and 2 h, but we observe little binding to follicular B cells. However, CR1/2 is clearly available when these cells are probed ex vivo with either anti-CR1/2 mAb. Thus, although we detect moderate binding of bt mAb 7G6 to follicular B cells 1 h after infusion as determined by flow cytometry (Fig. 3), there is almost no discernible binding as revealed in the fluorescence immunohistochemistry studies on spleen sections. These observations may be reconciled if there is re-equilibration of infused mAb 7G6 in the single-cell suspension after the three-dimensional integrity of the spleen is destroyed, leading to some binding to follicular B cells in the flow cytometry experiments. At later times, available CR1/2 on follicular B cells clearly decreases, as indicated in both fluorescence immunohistochemical measurements and flow cytometry experiments (Figs. 2–4).

A complex steady state may characterize the interaction of follicular B cells with infused mAb 7G6. Because follicular B cells recirculate, they can encounter mAb 7G6 in the bloodstream or in the splenic follicle. In addition, bound mAb 7G6 and CR1/2 can be recirculate, they can encounter mAb 7G6 in the bloodstream or in the splenic follicle. In addition, bound mAb 7G6 and CR1/2 can be removed from follicular B cells as they traverse the mononuclear phagocytic system (56). The net result is that by 24 h, follicular B cells have lost almost all CR1/2, but at any given time a smaller fraction of these cells appeared to have the mAb bound compared with the MZ B cells, which remain fixed in the spleen and also have a larger number of CR1/2 per cell.

**The white pulp and FDC region are not readily accessed**

The immune system can prevent inappropriate responses by physical isolation. For example, soluble Ags in the bloodstream may be restricted from entry into certain sites, thus preventing untoward stimulation or immunization. In fact, in the spleen, the white pulp, including the FDC region, constitute such relatively inaccessible regions (12, 19, 26–29). Although mAbs specific for FDC can stain these cells ex vivo, i. v. infusion of the anti-FDC mAb did not lead to its localization to the FDC in the spleen, while infused mAb 7G6 does indeed localize to the FDC after 24 h (Fig. 7). Based on these observations with the anti-CR1/2 mAb model, we suggest that the ferrying of C3dg-opsonized substrates, initially captured by CR1/2 on MZ B cells, to FDC provides an important and reasonable delivery mechanism. Recognition and opsonization by C

![FIGURE 5](image-url). Infused mAb 7G6 binds initially to MZ B cells and is then transferred to the follicle, where it colocalizes with FDC. A and B. Relative location, at 15 min and 24 h, of infused bt mAb 7G6 (2 $\mu$g) compared with B cells and metachromatic macrophages. Spleen sections were probed with Al488 SA and Al594 anti-mouse IgM (A) or Al594 SA and FITC MOMA (B). Representative of similar results in at least two mice. C and D. Infused Al488 mAb 7G6 is colocalized with FDC after 24 h. Spleen sections were probed with bt mAb FDCM1 or bt mAb FDCM2, followed by Al594 SA. Representative of similar results in three mice. E and F. Infused bispecific mAb complex is colocalized with FDC after 24 h. Bispecific mAb complex (5 $\mu$g) mAb 7G6 × mAb 7B7 was infused i.v. Spleen sections were stained with Al488 FITC followed by Al594 anti-rat IgG to locate mAb 7G6 (E), or with Al488 FITC and bt mAb FDCM2 followed by Al594 SA (F) to identify FDC. Representative of similar results with multiple sections from one mouse. All sections were viewed with sequential TR, then FITC filters. Objective magnification, ×10.
of either IC or thymus-independent type 2 Ags provides the necessary ligand that promotes binding to MZ B cell CR1/2. This binding reaction can provide a signal that promotes movement of the MZ B cells to the FDC, resulting in localization of the Ags to a site that is particularly effective at presenting intact Ags to cognate B cells, thus promoting a vigorous immune response (3, 9, 15, 23, 25, 30, 31, 67). Indeed, binding of several ligands to different receptors on MZ B cells promotes movement of MZ B cells to the follicles and to FDC (12, 15, 18, 28, 52, 54, 55, 68, 69).

C and the immune response
An increasing literature now documents multiple links between C, a component of the innate immune system, and the adaptive immune system (8–10, 15, 17). C is thought to influence the immune response due to the costimulatory effect on B cell activation that occurs when C3dg-opsonized Ags bind to cognate B cells, thus allowing signaling through both the BCR and CR1/2 (2, 6, 8–10). In fact, C3dg opsonization can occur after Ags bind to their

FIGURE 6. A, Infusion of mAb 7G6 induces temporary loss of CR1/2 on MZ B cells. Naive, Naive mouse spleen was probed ex vivo; Chase only, mice were infused with 2 μg of bt mAb 7G6 and then euthanized 1 h later (no pulse); 1 d and 7 d, mice were infused with 2 μg of Al488 mAb 7G6 (pulse), chased with an infusion of 2 μg of bt mAb 7G6 1 or 7 days later, and euthanized 1 h after chase. Spleen sections were probed with Al594 SA to reveal the capacity of the splenic B cells to capture the infused bt mAb 7G6. B–E, Infusion of mAb 7G6 induces temporary loss of MZ B cells. Mice were infused with 2 μg of bt mAb 7G6 and euthanized at the indicated times after infusion. Spleen sections were probed with the three indicated reagents and viewed with DAPI, FITC, and TR filters either sequentially (B) or singly (C–E) to identify MZ B cells, denoted by the arrows. At day 1, the asterisk (*) indicates the lack of detectable MZ B cells. Representative of similar experiments in a minimum of three mice for each time point. Objective magnification, ×10.

FIGURE 7. Infusion of 2 μg of mAb FDCM1 does not lead to its localization to the spleen at 24 h, although anti-CR1/2 mAb 7G6 is localized. A, Spleen sections were stained with Al594 anti-rat IgG to reveal infused mAb FDCM1 or mAb 7G6. B, Ex vivo probing of spleen sections from mice previously infused with mAb FDCM1 verified the specificity of the anti-FDC mAb. Representative of similar results for three mice treated with each of the three paradigms. Objective magnification, ×4.
cognate BCR, with subsequent engagement of CR1/2 and enhanced signaling (70). The transfer of C-opsonized ligands, initially bound to noncognate MZ B cells, to FDC is likely to represent another independent mechanism by which C enhances the immune response. After opsonization with C, Ags in IC might bind to noncognate B cell CR1/2 and stimulate the cell inappropriately. However, additional ligation of FcγRIIb by IgG on the IC would provide a negative or down-regulatory signal to prevent inappropriate activation of MZ B cells (8, 9, 31). Thus, the cell can act simply as an inert transporter of the C3dg-IC.

During natural infections, C3dg opsonization of infectious agents, mediated in part through the alternative pathway of C, insures that they will be directed to MZ B cells and then delivered to FDC, as has been demonstrated by several investigators (15, 17, 18, 24). However, immunization protocols based on use of large quantities of Ags incorporated into IgG-containing IC have generated substantial immune responses in mice depleted of C3 or lacking CR1/2 (71–73). Moreover, quite recently, Haas et al. (74) have reported that C3d opsonization of Ags can enhance the immune response in CR1/2 knockout mice in a pathway that must be independent of CR1/2 expression. It is likely that these paradigms promote Ag processing by pathways that completely circumvent requirements for C activity.

Summary

We have demonstrated in a mouse model that i.v. infusion of small amounts of a model C3dg-opsonized IC, anti-CR1/2 mAb 7G6, leads to its binding to MZ B cells, which subsequently transport the mAb to the FDC region, in a reaction first postulated by Brown (19). This transport reaction appears to play a key role in the normal immune response, and we are now investigating whether this paradigm will allow the generation of a robust immune response against Ags coupled to intact IgG anti-CR1/2 mAbs.

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References


69. Appelquist, S. E., J. Dahlstrom, N. Jiang, H. Molina, and B. Heyman. 2000. Antibody production in mice deficient for complement receptors 1 and 2 can be induced by IgU/Ag and IgE/Ag, but not IgM/Ag complexes. J. Immunol. 165:2198.
