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Immune Complex-Bearing Follicular Dendritic Cells Deliver a Late Antigenic Signal That Promotes Somatic Hypermutation

Yongzhong Wu,2* Selvakumar Sukumar,2* Mohey Eldin El Shikh,* Al M. Best,† Andras K. Szakal,‡ and John G. Tew3*

We reasoned that immune complex (IC)-bearing follicular dendritic cells (FDCs) promote somatic hypermutation (SHM). This hypothesis was tested in murine germinal center reactions induced in vitro by coculturing 6-day (4-hydroxy-3-nitrophenyl) acetyl-primed but unmutated \( \lambda^+ \) B cells, chicken \( \gamma \)-globulin (CGG) memory T cells, FDCs, and ICs (anti-CGG plus NP-CGG). Mutations in primed \( \lambda^+ \) B cells were obtained only when both FDCs and immunogen were present. FDCs alone promoted B cell survival and Ab production but there were no mutations without more immunogen. Moreover, the mutation rate was enhanced when FDCs were activated. Trapped ICs ranged from 200 to 500 \( \AA \) apart on FDC membranes and this correlated with the periodicity known to optimally signal BCRs. FDCs are unique in their ability to retain ICs for months and a second signal mediated by FDC-ICs appeared to be needed a week or more after immunization by immunogen persisting on FDCs. However, the time needed to detect extensive SHM could be reduced to 7 days if ICs were injected together with memory T cells in vivo. In marked contrast, no mutations were apparent after 7 days in vivo if ICs were replaced by free Ag that would not load on FDCs until Ab was produced. The data suggest that specific Ab production leads to the following events: Ab encounters Ag and ICs are formed, ICs are trapped by FDCs, B cells are stimulated by periodically arranged Ag in ICs on FDCs, and this late antigenic signal promotes SHM. The Journal of Immunology, 2008, 180: 281–290.

Germinal centers (GCs) are a distinctive architectural feature of secondary lymphoid tissues that become apparent upon appropriate stimulation with Ag. The GC reaction is characterized by rapidly proliferating specific B cells adjacent to GC Th cells and follicular dendritic cells (FDCs) bearing immune complexes (ICs) with intact specific Ag (1, 2). Major events known to occur in GCs include Ig class switching, somatic hypermutation (SHM), and selection of somatically mutated B cells with high-affinity receptors (3–7). A variety of studies provide rationale and support for the concept that IC-bearing FDCs promote SHM (8–14). However, more direct evidence defining the role of FDCs in SHM is needed given that some studies do not support this concept. Specifically, mature FDC reticula do not develop in lymphotixin \( \alpha \) knockout mice, yet if the Ag dose is high enough, SHM is readily apparent in these FDC-reticula-defective animals (15). The authors acknowledge that FDCs may be present but in the absence of reticula they were not obvious (15). Moreover, SHM occurs in mice where Ig secretion, and thus the amount of ICs trapping on FDCs is markedly reduced (16), calling into question the need for ICs (at least at high levels) in promoting SHM. If IC-bearing FDCs participate in SHM, we reasoned that it should be possible to directly demonstrate this in vitro where T cells, B cells, ICs, and FDCs can be manipulated at will.

Hapten-protein conjugates are frequently used as immunogens because haptens provide a single defined epitope for study of SHM in a protein-specific T-dependent fashion. The hapten (4-hydroxy-3-nitrophenyl) acetyl (NP) conjugated to a carrier protein has been extensively used in SHM studies and the information available on the anti-NP response is abundant. The following are characteristic of B cells responding to NP in C57BL/6 mice: 1) they generally express the \( \lambda \) 1 chain (17, 20), 2) typically, their BCRs are encoded by the \( V_\lambda 186.2/DFL16.1/J_{H2} \) gene rearrangement (19–23), 3) Tyr\(^\text{\textsuperscript{\textcircled{Y}}} \) in the BCR appears to be involved in NP binding (20), 4) junctional diversity in CDR3 tends to be limited (21, 23), and 5) replacement of Trp (W) with a Leu (L) in position 33 in the VH region increases the affinity of BCRs expressing \( V_\lambda 186.2/DFL16.1/J_{H2} \) by \( \sim 10 \)-fold (24).

To test the hypothesis that SHM is promoted by IC-bearing FDCs, GC reactions were established in vitro by coculturing NP-primed, but unmutated, day 6 \( \lambda^+ \) B cells, chicken \( \gamma \)-globulin (CGG)-primed memory T, CCG, cells and FDCs bearing NP-CGG. After 7 days, RNA was isolated and mutations in the \( V_\lambda 186.2 \) gene switched to C\( \gamma \) were analyzed. SHM was apparent only when B cells were stimulated with both immunogen and FDCs, and ICs were more stimulatory than free immunogen. We find these data exciting, because the use of IC-bearing FDCs allowed study of SHM in GC reactions in vitro with normal B cells in an Ag-specific system for the first time. In vivo studies confirmed the importance of ICs in that SHM could be detected in just 7 days but only when FDCs were preloaded with ICs. The results suggest that the initial exposure to Ag primes T cells and stimulates specific B cells to begin making

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2 Y.W. and S.S. contributed equally to the article.

3 Address correspondence and reprint requests to Dr. John G. Tew, Department of Microbiology and Immunology, Virginia Commonwealth University, P.O. Box 980678, Richmond, VA 23298-0678. E-mail address: twt@hsc.vcu.edu

4 Abbreviations used in this paper: GC, germinal center; FDC, follicular dendritic cell; IC, immune complex; SHM, somatic hypermutation; NP, (4-hydroxy-3-nitrophenyl) acetyl; CGG, chicken \( \gamma \)-globulin; EM, electron microscopy; NIP, (4-hydroxy-3-iodo-5-nitrophenyl) acetyl.

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Ab. Specific Ab binds immunogen and forms ICs and ICs are trapped by FDCs that promote the GC reaction. The results suggest that about a week after primary immunization, a second Ag signal is needed to induce SHM and this late signal appears to be delivered to GC B cells by periodically arranged ICs persisting on FDCs.

Materials and Methods

Mice, immunization, and irradiation

Six- to 8-wk-old C57BL/6 mice were purchased from the National Cancer Institute, were housed in standard shoebox cages, and given food and water ad libitum. Mice were immunized with CGG 1 mo before the isolation of T cells that would include memory T,CCG cells as described previously (25–27).

To prime B cells, 50 μl of 200 μg/ml alum precipitated NP-CGG together with heat-killed Bordetella perriussis were injected s.c. in each front leg and hind foot to stimulate and expand NP-specific B cells, which were isolated 6 days later.

To activate FDCs, 25 μg of LPS were injected into each front leg and hind foot and mice were given 3 days for the FDCs in the draining lymph nodes to be activated. FDCs were isolated 24 h after irradiating mice with 1000 rad in a 137Cesium irradiator to kill most lymphocytes and enrich for FDCs (26). Animals were handled in compliance with Virginia Commonwealth University Institutional Animal Care and Use Committee guidelines.

Abs and reagents

Rat anti-mouse FDC (FDC-M1), biotin mouse anti-rat κ (MRK-1), anti-mouse CD21/CD35 (clone 7G6), and anti-mouse CD32/CD16 (clone 2.4-2G2) were purchased from BD Biosciences. Mouse CD45R (B220) MicroBeads, mouse CD90 (Thy1.2) MicroBeads, anti-biotin Microbeads, and MACS LS columns were purchased from Miltenyi Biotec. Biotin-labeled rat anti-mouse κ was purchased from Zymed Laboratories. Alkaline phosphatase-labeled goat anti-mouse IgG (H+L) and P-nitrophenylphosphate substrate were obtained from Kirkegaard & Perry Laboratories. OVA conjugated with (4-hydroxy-3-iодo-5-nitrophenyl) acetyl (NIP-OVA) and NP(36)-CGG were obtained from Biosearch Technologies. Anti-CGG was obtained from hyperimmunized mice with serum anti-CGG IgG levels in excess of 1 mg/ml.

Isolation of FDCs

FDCs were isolated as previously described (28). Briefly, lymph nodes from lethally irradiated mice were gently digested and the cell suspension incubated with rat anti-mouse FDC-M1, biotin mouse anti-rat κ (clone MRK-1), and anti-biotin magnetic microbeads sequentially. Finally, the FDCs were positively selected using a magnetic column (MACS). Approximately 85–95% of these cells expressed the FDC phenotype, FDC-M1+, CD32+, CD21/35+, CD40+, and ICAM+ (28).

Isolation of λ B cells and T,CCG cells

Single-cell suspensions were prepared from lymph nodes of mice primed for 6 days with NP-CGG. After depleting κ+ cells with biotin-anti κ and anti-biotin microbeads using a MACS column, the λ+ B cells were isolated with anti-B220 microbeads using MACS. To obtain memory T,CCG cells, lymphocytes from mice immunized with CGG were incubated with 40 μl of anti-CD90.2 microbeads and T cells were selected using MACS. For experiments in Fig. 5, negative selection was used to avoid tagging the B cells or T cells with Ab that might promote phagocytosis or complement-mediated lysis in vivo. The λ+ B cells from naive mice were isolated by depleting κ+ cells with biotin-anti κ and anti-biotin microbeads and T cells with anti-CD90.2 microbeads. The T,CCG cells were obtained by depleting B cells with anti-B220 microbeads.

In vitro GC reactions and the anti-NP Ab response

In vitro GC reactions were initiated by culturing 1 × 10⁶ unmutated but 6-day primed B cells, 0.5 × 10⁶ T cells, and 0.5 × 10⁶ FDCs in the presence of 100 ng of NP (36)-CGG as free Ag or ICs (NP-CGG plus anti-CGG). Mutations in NP-stimulated B cells occur after day 6 (10, 23, 29) and we confirmed this in multiple experiments. The ICs were made by incubating NP-CGG with anti-CGG at a ratio of 6 ng/ml anti-CGG to 1 ng of NP-CGG for 2 h and then adding IC containing 100 ng of NP-CGG to appropriate wells in 24-well culture plates. Supernatant fluids were harvested 7 days later and were assayed for NIP-specific IgG Ab by ELISA using NIP-OVA.

Analysis of mutations in the V region

The anti-NP response predominantly uses the V,186.2 gene (20–23) and it was analyzed in this study to assess somatic mutation. Cells were collected on day 7 of culture and RNA was isolated using TRIzol (Invitrogen Life Technologies). The RNA was then reverse transcribed to cDNA with GeneAmp Gold RNA PCR Core kit (Applied Biosystems) using oligo dt. The V,186.2 gene followed by D and J regions and a proximal segment of the Cγ gene segment was amplified by two rounds of nested PCR with high-fidelity Pfu-Ultra DNA polymerase (Stratagene). The forward and reverse primers for the first-round PCR, were 5’-cagcctcctttcgacgcaa cagc-3’ and 5’-gtgcaccacgctgaggtctac-3’, for second-round PCR, 5’-caggtcactgtagccag-3’ and 5’-agttggccagcag-3’, The PCR was conducted as follows: 95°C 1 min, 56°C 1 min, 72°C 1 min for 30 cycles followed by 72°C for 10 min. The expected 400-bp PCR product was then electrophoresed on a 1.2% agarose gel, purified by gel extraction using QiAquick kit (Qiagen) and cloned using the Zero Blunt TOPO PCR Cloning kit (Invitrogen Life Technologies). The plasmids were transformed into one-shot TOP10 chemically competent cells (Invitrogen Life Technologies) and plated onto Luria-Bertani medium containing 50 μg/ml ampicillin. Multiple discrete colonies were picked the following day and grown for 24 h in Luria-Bertani broth containing 50 μg/ml ampicillin in a 37°C shaker. The plasmids were isolated using a QIAprep Spin miniprep kit (Qiagen) and the insert was sequenced using an ABI sequencer. The sequences were analyzed using the CGG Wisconsin Package (Accelrys), ClustalW (30, 31), and BioEDIT (www.mbio.ncsu.edu/BioEdit/bioedit.html) software packages.
Redundant sequences were excluded from our calculation of mutations per 1000 bases to avoid multiplying the effect of a single mutation. To avoid including N-terminal additions, changes within five bases of the V-D junction were also excluded from our calculations of mutations per 1000 bp. The background rate of mutation detected using this system was low (0.1 mutations per 1000 bases in over 100 control sequences analyzed). In addition, the C region of 24 sequences with mutated VH186.2 genes were examined and no mutations were found. In short, error attributable to random mutation or polymerase error was very low.

Adoptive transfer experiment
Recipients were irradiated with 600 rad under the maximum scatter conditions by using a 137Cs source (Mark I, Model 68-0146; J. L. Shepherd and Associates). This dose is sufficient to arrest lymphocyte populations but leave functional populations of radio-resistant FDCs (32). Twenty-four hours after irradiation, the mice were reconstituted by injecting 2.5 × 10^6 naive B cells and 2.5 × 10^6 memory T(CGG) cells in each hind foot or front leg. The B cells for these experiments were obtained by negative selection by removing T cells and B cells. These mice were divided into two groups with one group of three mice receiving 5 μg of NP-CGG in preformed ICs in the hind feet and front legs (ICs were made with 20 μg of NP-CGG plus 120 μg of anti-CGG incubated in vitro for 2 h). The control group of three mice received 5 μg of NP-CGG in each hind foot and front leg. One week later, serum and draining lymph nodes were harvested and serum Ab levels and mutations in the VH186.2 gene segment in the B cells were determined.

Electron microscopy
Purified FDCs labeled with HRP anti-HRP ICs were fixed with 1% paraformaldehyde plus 0.87–0.9% glutaraldehyde (electron microscopy grade; Electron Microscopy Sciences) in 0.1 M cacodylate buffer for 90 min, dehydrated in a graded series of ethanol and postfixed in 1% OsO₄ in 0.1 M cacodylate buffer to a thickness of 800 Å, and stained with uranyl acetate and lead citrate for better detection of the HRP labeling. Thin sections were studied with a JOEL electron microscope at 50 kV for cytochemical labeling to increase contrast on unstained sections.

Immunohistochemistry
Draining lymph nodes (popliteal, brachial, and axillary) from mice adoptively transferred with naive λ B cells and T(CGG) cells and stimulated with Ag or ICs were harvested and flash frozen in Optimum Temperature Compound (Tissue-Tec). Serial 10-μm-thin sections were cut from the frozen blocks using a Jung Frigocut 2800E cryostat and fixed in acetone. The sections were then rehydrated and incubated with IgM rat anti-GL-7 Ab (eBioscience), followed after washing by HRP-conjugated mouse anti-rat IgM (Southern Biotechnology Associates). The HRP was then developed using a diaminobenzidine substrate (Biomeda). Digital pictures were taken using an Olympus BH-2 microscope with an Optronics image acquisition device. Morphometric analysis was performed using BioQuant Nova as described previously (33).

Table I. Sequences from λ⁺ B cells stimulated with Ag or IC and normal FDCs (N-FDCs) with T(CGG) cells (data corresponds with Fig. 1)

<table>
<thead>
<tr>
<th>Sequences</th>
<th>VH</th>
<th>JH</th>
<th>IgMAb</th>
<th>Mutations</th>
</tr>
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<td>VH186.2</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
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<tr>
<td>H-FDC2</td>
<td></td>
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<tr>
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<td>H-FDC2.2</td>
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<tr>
<td>H-FDC2.14</td>
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</table>

Statistical analysis
The number of mutations per sequence was compared using a Poisson regression analysis (SAS Proc Genmod version 9.1; SAS Institute). An offset of log_{10} was used to normalize the fitted group means to the number of nucleotides. Significant χ² values were determined at p < 0.05. Analysis of GC numbers, area, and IgG in Fig. 5 were done using a two-tailed Student t test and significance was accepted at p < 0.05.
Results

SHM in vivo

The VH186.2 gene segment switched to Ig γ was analyzed using our reagents to confirm that mutations are not apparent 7 days after immunizing mice with NP-CGG but that most segments are mutated 14 days after in vivo challenge (data not shown) (10, 23, 29). A rate of 9 mutations/1000 bases was found at day 14 and this is consistent with published results (8.4–15 mutations/1000 bases) (23, 34).

SHM in the presence of FDCs and ICs in vitro

To begin testing the hypothesis that IC-bearing FDCs promote SHM, GC reactions were elicited in vitro using FDCs, CGG-primed memory T\(_{\text{CGGG}}\) cells, and B cells. The \(\lambda^+\) B cells were

![FIGURE 2](http://www.jimmunol.org/)

FIGURE 2. Clonal relatedness of sequences suggesting ongoing mutation in in vitro GC reactions. A phylogram was deduced from independent sequences derived from cultures set up with B cells, T cells, FDCs, and ICs (BTFDCIC) using the ClustalW program from the European Molecular Biology Laboratory. The first 14 sequences are listed in Table I and additional clones were sequenced yielding 7 more sequences to extend this analysis to 21.

![FIGURE 3](http://www.jimmunol.org/)

FIGURE 3. Activation of FDCs enhanced SHM. FDCs express TLR-4 and 3 days after injection of LPS, the levels of molecules known to promote FDC accessory activity including ICAM-1, VCAM-1, and Fc\(\gamma\)RIIB were significantly elevated (37). GC reactions were set up as described in Fig. 1 except the FDCs were activated. The contents in each culture are indicated at the bottom of the figure and LPS was used as a control in the second set of cultures at 25 \(\mu\)g/ml. The rate of mutations per 1000 bases in each of the five conditions was calculated after analyzing: 10, 7, 18, 20, and 14 clones, respectively. The data from these clones were used to look for significant \(\chi^2\) values in statistical analysis. The analysis indicated that both Ag and IC stimulated more mutations with LPS-activated FDCs than normal FDCs (\(p < 0.01\)). It was also clear that ICs, which would load on FDCs immediately, stimulated better than Ag that would be converted into ICs over time as Ab was produced (\(p < 0.01\)).
obtained from mice immunized 6 days earlier with NP-CGG. The 6 days allowed NP-specific B cells to clonally expand, class switch, and to begin producing IgG anti-NIP under physiological conditions in vivo. However, as shown by others (10, 23, 29) and confirmed here, these B cells taken 6 days after primary immunization are not mutated. However, somatic mutation would be one of the next major events in a GC reaction. These 6-day B cells were cultured for 7 additional days in vitro and the production of specific anti-NIP Ab and mutations in the VH186.2 gene segment were determined (Fig. 1). In the absence of FDCs, the anti-NIP IgG levels were low (20–25 ng/ml; Fig. 1A, columns 1–3) and sequences were germline after the 6 days in vivo and 7 days in vitro (Fig. 1B, columns 1–3). Addition of FDCs to activated B cells and memory T(CGG) cells dramatically increased anti-NIP production (Fig. 1A, column 4), but did not promote SHM as indicated by a lack of mutations (Fig. 1B, column 4). In vivo, specific Ab production leads to IC formation followed by IC trapping and retention on FDCs in GC light zones. To model this in vitro, Ag (NP (36)-CGG) or ICs were added to the T and B cells as a source of immunogen and this addition correlated with a reduction in free anti-NIP levels persisting in cultures with FDCs (Fig. 1A, columns 5 and 6) suggesting the hapten was interacting with the newly produced anti-NIP. Moreover, in marked contrast with all previous conditions, numerous mutations were obtained upon second exposure to immunogen (Fig. 1B, columns 5 and 6). All sequences sampled were germline until immunogen was added with the FDCs and the 3–5 mutations per 1000 bases with immunogen was significantly different from total lack of mutations in all previous conditions (p < 0.01; by χ²). The numbers of clones sequenced for each culture condition and negative controls including FDCs with irrelevant ICs and irrelevant T cells are described in the legend to Fig. 1.

B cells do not survive well in the absence of FDCs and FDCs produce molecules including B cell activating factor belonging to the TNF family (BAFF) that promote B cell survival (35). Approximately 80% of our B cells survive for a week in the presence of FDCs compared with only 20% in the absence of FDCs (36). We reason the low levels of Ab in cultures lacking FDCs is, at least in part, a reflection of a loss in B cells. Nevertheless, the critical comparison is with cultures containing FDCs in the absence of specific immunogen and cultures with FDCs plus immunogen. The addition of FDCs gave a major boost in Ab production that correlated with increased B cell survival but no mutations. In marked contrast,
the addition of FDCs plus immunogen resulted in SHM. We examined B cell survival in the presence of FDCs with specific and non-specific ICs and without immunogen and were unable to detect any difference in B cell number or viability as long as FDCs were present.

The specific nucleotide changes in the mutated \textit{VH186.2} gene segment presented in Fig. 1 are indicated in Table I and the resulting changes in amino acid sequences are indicated in Table II. In cultures stimulated with FDC plus IC, there were 14 independent clones analyzed and these clones contained 21 mutations. Among the 21 mutations, 17 were unique mutations distributed over 16 different positions in the V region and all mutations were substitutions and not the consequence of insertion or deletion. As expected the mutations show a bias for transition (76%) over transversion (24%), and AGT/C (this usually represented as RGYW) intrinsic hot spots for somatic mutations were found 10 times. Moreover, these point mutations were concentrated in CDRs (62%) rather than framework regions (38%). As expected, changes in amino acid sequence were apparent in framework regions in Table II but changes were most frequent in CDRs 1 and 2. Clone N-FDCIC 8 has tryptophan replaced with leucine in position 33 and that shift is known to be associated with higher affinity anti-NP (24). In addition, a phylogram was deduced from sequences obtained from the cultures with FDCs and ICs (Fig. 2). Note the clonal relatedness of some sequences suggesting ongoing mutation in the cultures.

**Activated FDCs are potent inducers of SHM**

We recently reported that FDCs express TLR4 and that LPS upregulates many FDC molecules. Moreover, FDC activation correlates with increased accessory activity as indicated by a 2- and 10-fold increase in specific IgG production (37). If FDC molecules promote SHM, then activated FDCs should have enhanced ability to promote SHM. To test this prediction, LPS-injected mice were given 3 days for FDCs in the draining lymph nodes to be activated and then FDCs were isolated, which involves multiple washes which should remove any persisting LPS. Nevertheless, to control for carryover, we added LPS to selected cultures to determine whether we could find an LPS effect (Fig. 3, column 2). LPS has not been found to promote SHM (38) and that was confirmed in these studies. Like normal FDCs (Fig. 1), activated FDCs failed to promote SHM in the absence of second exposure of B cells to immunogen (Fig. 3, column 3). However, addition of Ag doubled the frequency of mutations found with normal FDCs (\(p < 0.01\)) and preformed ICs yielded \(12\) mutations/1000 bases at day 13 (6 days in vivo and 7 days in vitro) which compared favorably with the \(9\) mutations/1000 bases obtained in vivo at day 14. Tables III and Table IV illustrate the alterations in the \(V_{\text{H}186.2}\) gene segment and alterations in amino acids, which are similar to those shown in Tables I and II except changes are more frequent.

**ICs on FDCs are periodically arranged**

Immunogens in ICs on FDCs induce more potent Ab responses than free Ag plus FDCs (39, 40) and ICs with FDCs were more potent stimulators of SHM than free Ag as illustrated in Figs. 1 and 3. Repeating epitopes are known to simultaneously engage multiple BCRs and promote B cell activation (41). Moreover, T cell-independent GCs that include low levels of hypermutation can be obtained when multiple NP epitopes are conjugated to Ficoll (42). Studies of DNP on polymers indicate that epitopes spaced

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**Table IV. Amino acid sequences from \(\lambda^+\) B cells stimulated with Ag or IC and activated FDCs (A-FDCs) with \(T_{\text{CGG}}\) cells (data corresponds with Fig. 3)**

<table>
<thead>
<tr>
<th>Clones</th>
<th>Sequence</th>
<th>CDR1</th>
<th>CDR2</th>
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<td>Vh.186.2</td>
<td>SQVQLQLQPGRGELVKFASWKLSCASGTTTSTGMDWVQRFPGERLEW1GRHPSSGTTKBKFSKATLTVKDFSTAYMQLSSLTSEDSAVYCAR</td>
<td></td>
<td></td>
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</table>
As expected on the basis of previous work (39), IC-injected mice were divided into two groups with one receiving 5 µg of NP-CGG in preformed ICs in the hind feet and front legs. The control group received 5 µg of NP-CGG in each hind foot or front leg. After 7 days, λ⁺ B cells were isolated from lymph nodes and analyzed for V_{H}186.2 mutations. A, NIP-specific IgG measured by ELISA in three mice per group and the error bar represents SD and the differences are statistically significant (p < 0.01) and the data are representative of two experiments. B, The number of mutations per 1000 bases of V_{H}186.2 clones sequenced. The λ⁺ B cells from the draining lymph nodes of the three mice were pooled and RNA extracted. We analyzed 10 clones in each condition and the difference in mutation frequency was statistically significant (p < 0.01). C and D, Representative illustrations of 10-µm-thin sections of draining lymph nodes from the two groups of mice labeled with anti-GL7 to identify GC B cells. E and F, Cumulative data representing total number of GCs and area of GCs per midsagittal section of all six mice challenged with Ag or IC.

**Discussion**

The concept that IC-bearing FDCs promote GC reactions and play an important role in promoting SHM is supported by a number of studies (8–14) but not all (15, 16). Demonstrating SHM with normal B cells in vitro, where cell types can be readily manipulated,
FIGURE 6. Kinetics of events that promote SHM in GC reactions. Events in the first panel occur in the first 4 days after Ag challenge. Dendritic cells (DCs) trap Ag and transport it into the deep cortex, where they complete Ag processing and prime naive T cells emerging from the high endothelial venules (HEVs). Specific B cells in afferent lymph and emerging from HEVs also interact with Ag. As soon as specific T cells are primed, they provide help for specific B cells to begin making Ab (AbY; see arrows) detectable 3–4 days after Ag challenge. This specific Ab begins to form ICs which fix complement and these ICs are readily cleared by macrophages (Mc) particularly in the medullary sinuses (MS) and subcapsular sinuses (SS). In addition, some ICs are trapped by FDCs that attract B and T cells into the follicles. In the 4- to 7-day period (second panel), IgG production expands, illustrated by plasma cells migrating toward medullary cords, and clearance of ICs or “immune elimination” continues. FDCs trap more ICs including IgG-ICs and the FDC associated ICs promote GC reactions with dark and light zones (light green in panel 2). The GC reactions lead to enhanced Ig class switching and more IgG production. By day 7, soluble Ag has flowed through the node or has been cleared by phagocytes except for Ag persisting in ICs trapped on FDCs in GC light zones. In the 7- to 14-day period (panel 3), a strong Ag signal is delivered to GC B cells by periodically arranged ICs on FDCs that promotes SHM leading to cells with higher affinity BCRs that compete for limited Ag persisting on FDCs. These mutated cells are induced to produce high-affinity IgG important in affinity maturation.

has been challenging. In the present study, we sought to set up GC reactions in vitro where the hypothesis that FDCs promote SHM could be tested under defined conditions. We recently refined methods for FDC isolation (28) and found that FDCs bearing the appropriate ICs promote activation-induced cytidine deaminase production, Ig class switching, and affinity maturation by NP-specific B cells (25). These results prompted this study to determine whether FDCs promote SHM in GC reactions in vitro. The results reported here indicate that, over a 13-day period, B cells primed in vivo can be stimulated with activated IC-bearing FDCs (37) in vitro and exhibit SHM at rates comparable with those seen in vivo. In brief, the data suggest that the initial Ag exposure primes T cells and stimulates specific B cells to begin making specific Ab that interacts with Ag to produce ICs. Most ICs will be efficiently trapped and eliminated by phagocytic cells but some are trapped by FDCs in GC light zones and SHM is promoted in GC B cells restimulated by Ag in FDC-ICs after day 6. The sequence of these events is illustrated and summarized in Fig 6.

Passive transfer studies indicate that it is important for ICs to be made using IgG to promote SHM (13). SHM is typically not apparent in the first week after primary immunization (10, 23, 29) and we reason that IgG Ab production, IgG-IC formation leading to trapping by FDCs, takes time and helps explain the delay. As illustrated in Fig 5, SHM can be shifted into the first week if specific ICs and specific T cells are injected into normal mice but not by specific T cells alone. Thus, the rate-limiting step appears to be IgG production. We think it is important to note that, in the absence of a second exposure to Ag, no mutations were apparent in B cells (after 6 days in vivo and 7 days in vitro) even though Ab production was abundant in the presence of FDCs. Taken together, the data suggest that class switch recombination leads to IgG production and IC formation and that IgG-ICs trapped by FDCs deliver a follow-up antigenic signal that promotes SHM in conventional immune responses. Although macrophages and dendritic cells internalize, degrade, and clear ICs with a T1/2 in macrophages of ~30 min (45), ICs trapped by FDCs are retained for months to years (45). This unique ability to retain intact surface-bound Ag (in the form of ICs) in GC light zones makes FDCs an attractive candidate to deliver an antigenic signal to GC B cells a week or more after the initial immunization. Moreover, ICs on FDCs were arranged periodically between ~200 and 500 Å apart and were more potent than free Ag in their ability to stimulate SHM (Figs. 1 and 3).

Mutations in the in vitro GC reactions had numerous similarities with mutations observed in vivo. Specifically, sequences from cultures containing IC-bearing FDCs accumulated numerous mutations in the V\textsubscript{\textmu}186.2 region while the IgG C region remained unchanged (data not shown). An increased rate of mutation in CDR 1 and 2 is typical of in vivo data (46) and suggests a selective pressure and the same trend is apparent in the in vitro data reported here. Evidence for selection in the in vitro GC reactions is also suggested by the observation that changes at the VD junction in control sequences were never found (B cells alone, B and T cells plus Ag or ICs, B and T cells plus FDCs without Ag). However, changes at the VD junction in cultures with IC-bearing FDCs were found, especially with activated FDCs (see Tables I and III). Changes at the VD junction included multiple bases in sequence and these alterations likely reflect rare B cells with end-region changes that were selected and expanded by Ag on FDCs. Tyr\textsuperscript{29} appears to be important for NP binding (20); tyrosine was maintained or replaced by phenylalanine which is a conservative change that has been found in anti-NP responses (29). We also reasoned that leucine at position 33 would be selected in the in vitro GC reaction but it was rarely observed and this is not understood. However, a lack of leucine at position 33 was obtained in vivo when SCID mice were injected with T and B cells and given ICs (47) and the same result was obtained when we injected ICs in our in vivo study (data not shown). Remarkably, the tryptophan to
leucine replacement at position 33 is not found during the late stages of immune responses when the selection process should be virtually complete and Ab affinity is highest (29). ICs given at the beginning of an experiment may result in levels of ICs on FDCs that are comparable with FDC-ICs at later stages of a normal immune response. This raises the possibility that sequences with tryptophan at 33 may be favored by mature levels of FDC-ICs resulting in a rapid transition through the leucine stage. The observation that leucine at position 33 was only occasionally found in the present study when ICs developed rapidly in culture or preformed ICs were given would be consistent with this interpretation.

A number of FDC molecules are thought to be involved in GC reactions, including CxCL-12 and CxCL-13 that attract GC T and B cells, IL-6 that promotes terminal B cell differentiation, CD21L and CD320 that promote Ab production, FcγRIIB that minimizes ITIM signaling in B cells, and ICAM and VCAM that promote adhesion between FDCs and B cells (26, 39, 48–50). How significant these molecules are in promoting SHM remains to be determined. However, the in vitro model where GC reactions are easily manipulated should be useful for such studies. Given that blocking FDC-CD21L-B cell CD21 interactions inhibit Ab and activation-induced cytidine deaminase production (25), we thought that FDC-CD21L would likely be important for SHM, and that was tested by blocking FDC-CD21L-CD21 interactions but blocking did not have a significant effect (data not shown). Nevertheless, lack of inhibition in this study corresponds with reports of SHM occurring normally in CD21 knockout mice that also have reduced Ab responses (51, 52). It is also known that FDCs are activated as a consequence of interacting with ICs (53) and the need for ICs could reflect the need for FDC-FcγRII occupancy to produce factors needed to sustain mutating B cells that have been adequately signaled in vivo. However, if SHM has initiated in vivo and the need for ICs is to properly activate FDCs via FcR engagement, then irrelevant ICs should work as well as relevant ICs. However, cultures with FDCs stimulated with either irrelevant ICs (ova anti-OVA) or containing irrelevant T cells did not induce the SHM that is so readily apparent with relevant ICs and relevant T cells. The data reported here are most compatible with the interpretation that the B cell population needs stimulation by specific Ag and specific T cells late in the primary response for optimal SHM. The need for T cells in the SHM process is well-known (54) and these data suggest that activated T cells are needed for over a week. A kinetic study to establish exactly when SHM first occurs in our cultures has not been done, but Gray and colleagues (38) found that B cells isolated at day 10 after Ag challenge and stimulated in vitro gave good SHM but before that point SHM was difficult to detect. Accordingly, we reason that the first mutations will appear 3–4 days after culture initiation. We hope to test this postulate in future studies.

Addition of FDCs promoted a dramatic anti-NIP response, but there were no mutations unless immunogen was also present to provide a late antigenic stimulus. This need for a second late exposure to Ag is consistent with the observations of Klinman and colleagues (10) in cultures of splenic fragments that likely contained FDCs. These authors found that a second exposure to Ag 6–8 days after initiation of the response was necessary to get SHM (10). In addition, we found that preformed ICs, which would be rapidly trapped by FDCs, stimulated SHM more effective than free immunogen. Furthermore, it should be appreciated that the day 6 B cells were producing Ab; free immunogen would likely have been converted into ICs and thus ICs may have promoted SHM in these cultures. Activating FDCs via TLR4 engagement (37) nearly doubled the mutation rate, further supporting a regulatory role for FDCs in SHM. Electron microscopy revealed that ICs are arranged periodically between ~200 and 500 Å apart on FDC membranes that have trapped ICs in vitro. Repeating epitopes are known to simultaneously engage multiple BCRs and promote B cell activation (41). Studies of DNP on polymers indicated that epitopes spaced 120–670 Å apart are optimal (43) and the range of 200–500 Å found with ICs trapped by FDCs in this in vitro study fits that periodicity. Moreover, T cell-independent GCs can be obtained when NP is conjugated to Ficoll which should allow simultaneous engagement of multiple BCRs and that arrangement includes low levels of hypermutation (42). Similarly, SHM could be demonstrated in an in vitro culture system by cross-linking BCRs and providing T cell help (38). The periodic organization of ICs on FDCs should also allow immunogenic epitopes to be arranged in a repeating pattern much like a T-independent Ag and facilitate cross-linking of BCRs. In short, extensive interaction between the B cell membrane and long FDC dendrites bearing epitopes that simultaneously engage numerous BCRs likely provides a potent signal that would not be delivered by free Ag. This periodic arrangement of epitopes on FDCs may explain why ICs were more potent immunogens than free Ag. Adoptive transfer experiments confirmed that IC-bearing FDCs were important for optimal GC reactions and SHM was obtained in just a week. In short, use of FDCs to model GC reactions in vitro made it possible to demonstrate SHM in Ag-stimulated B cells in vitro. The results suggest that induction of specific Ab leads to IC formation, ICs being trapped by FDCs, GC B cells being stimulated by Ag in these FDC-ICs after about a week in vivo, and this late second Ag signal leads to the promotion of SHM.

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Disclosures

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References


