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Mucosal memory B cells retain the ability to produce IgM antibodies 2 years after oral immunization

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SUMMARY

In recent studies we have demonstrated that immunological B- and T-cell memory may be stimulated effectively by oral immunization, simply by admixing protein antigens with cholera toxin (CT) adjuvant. Here we extend the information by employing a hapten–carrier system allowing us to separate B- and T-cell memory and to evaluate the requirement of memory T cells for effective reactivation of mucosal memory B cells. We found that 2 weeks following oral priming immunizations with dinitrophenyl–keyhole limpet haemocyanin (DNP-KLH) plus CT adjuvant, significant serum anti-DNP antibodies of IgG, IgA and IgM immunoglobulin classes were demonstrated. However, after 2 years only IgM anti-DNP antibodies could still be detected in serum. When memory lymphocytes were isolated from these mice, from both systemic and gut-associated lymphoid tissues, and challenged with antigen *in vitro*, vigorous IgM, but no IgG or IgA, anti-DNP production was observed. By contrast, when the DNP–KLH-primed memory mice were challenged *in vivo* by an oral booster immunization with DNP–KLH plus CT adjuvant, strong systemic IgG and local mucosal IgA anti-DNP responses were recorded, while IgM anti-DNP production was poor. Moreover, the mucosal memory B cells from DNP–KLH-immunized mice were more responsive *in vivo* to an oral booster immunization with the carrier-specific antigen, DNP–KLH, compared to that provided by an unrelated carrier, DNP–human serum albumin (HSA), which gave only poor mucosal and systemic anti-DNP B-cell responses. Taken together our data suggest that mucosal memory B cells are recirculating cells that have retained their ability to produce IgM antibodies and, therefore, have not undergone switch differentiation involving gene rearrangements with constant μ-chain deletions. Furthermore, mucosal B-cell memory and $CD4^+$ T-cell memory are closely interconnected phenomena, requiring both components for effective expression and probably also for maintenance of immunological memory in the mucosal immune system.

INTRODUCTION

Immunological memory manifests itself as an accelerated and elevated response to a second challenge with the antigen.[1] The cellular basis for such memory is thought to reside in an increased precursor frequency of antigen-specific lymphocytes resulting from a primary exposure to the antigen. In addition, memory lymphocytes have been found to express antigen-specific receptors with higher-affinity and exhibit lower activation thresholds compared to naive lymphocytes.[1] Although immunological memory is a well-established phenomenon after systemic immunization, few studies have focused on memory functions of the mucosal immune system.

Vaccines delivered by the oral route are warranted. However, a fundamental basis for succesful vaccination against the vast number of pathogens that gain access to the body through the mucosal membranes is the ability to develop immunological memory.[2,3] Indeed, several clinical as well as experimental studies have indicated that immunological memory may be stimulated by mucosal immunization.[2,3] We recently reported on the effective stimulation of long-term memory following oral immunization with keyhole limpet haemocyanin (KLH) in mice using cholera toxin (CT) as the mucosal adjuvant.[4] These studies proved that (1) immunological memory in the gut mucosa may develop against a soluble protein antigen in the presence of an efficient adjuvant, and that (2) antigen-specific long-term memory B cells as well as memory T cells prevail after oral immunization.[4,5]

The existence of a common mucosal immune system in

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Abbreviations: ALPH, alkaline phosphatase; CMF-HBSS, Hank's balanced salt solution without calcium and magnesium; CT, cholera toxin; DNP, dinitrophenyl; HBSS, Hank's balanced salt solution; HSA human serum albumin; HRP, horseradish peroxidase; KLH, keyhole limpet haemocyanin; LPL, lamina propria lymphocytes; MLN, mesenteric lymph nodes; OPD, ortho-phenylenediamine; PP, Peyer's patches; SFC, spot-forming cells; SP, spleen.

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which antigen-activated lymphocytes may traffic from one mucosal tissue to another suggests that vaccination of distant mucosal surfaces, such as the genital tract, might be achieved through oral administration of antigen.[3] However, this requires that memory lymphocytes generated after oral immunization are not anatomically restricted but may be found both systemically and in mucosal compartments, and that antigen-specific mucosal memory B and T cells interact. Indeed, a few earlier studies including our own work would suggest that mucosal memory cells may be recirculating cells.[3–6]

Following systemic immunizations, most memory B cells are thought to express surface IgG rather than IgM but little information is available about memory B cells generated by mucosal immunization.[7,8] At mucosal membranes only isotypes that associate with the poly immunoglobulin receptor, i.e IgM and IgA, may be actively transported, transcytosed, through the epithelial cells from the lamina propria (LP) to the gut lumen.[9] As the mucosal surfaces are predominantly protected by secretory IgA (sIgA) antibodies, and little if any IgG can be traced, it is assumed that mucosal memory is carried by B cells that have undergone isotype switch to IgA.[3,10] Because isotype switching to IgA involves deleting out constant heavy chain (C_H) genes upstream of the Cα gene, such memory B cells would be restricted to mucosal surfaces and have limited usefulness outside the membranes, where instead IgG is required to protect against, for example, pathogenic micro-organisms that evade the mucosal barriers.[11–13] At variance with this notion, however, we found that B cells isolated many months after oral immunization produced IgM antibodies rather than IgA upon reactivation with antigen *in vitro*.[5]

The present study was undertaken to characterize further long-term mucosal memory B cells generated after oral immunization. By using the dinitrophenyl (DNP)–KLH, hapten–carrier system, we were able to separate B-cell memory from T-cell memory and study the characteristics of the former. We focused on the isotype commitment of mucosal memory B cells 2 years after the oral priming immunizations.

MATERIALS AND METHODS

Mice

Inbred 14–15-week-old female mice of the C57Bl/6 strain, from our departmental animal breeding unit, were used for the oral priming immunizations. Age-matched control animals were included in the study when indicated.

Immunizations

The hapten DNP, conjugated to KLH (DNP–KLH; Calbiochem Corp., La Jolla, CA) or human serum albumin (DNP–HSA; Calbiochem Corp.), was used for immunizations. The number of DNP groups conjugated to each protein molecule, according to the manufacturer, was > 35 for HSA and > 400 for KLH. Mice were given 2 mg DNP-conjugate per dose, admixed with 10 μg of CT adjuvant (LIST Biological Laboratories Inc., Campbell, CA) in 0·5 ml phosphate-buffered saline (PBS) containing 3% $NaHCO_3$, intragastrically with a baby feeding tube as described elsewhere.[5] After three oral priming immunizations with 10 days between the doses the primed memory mice were rested for 2 years and kept in conventional housing at the Department of Medical Microbiology and Immunology (University of Göteborg, Sweden). When indicated, memory mice were given a single challenge immunization with antigen prior to killing. For comparison, some mice analysed for short-term immune responses were given two oral doses with antigen plus CT adjuvant prior to analysis.

Preparation of lymphoid cells

Spleen (SP) and mesenteric lymph node (MLN) cells were prepared by teasing the tissues through a nylon net.[4] Spleen cells were subject to lysis of the erythrocytes by osmotic shock using ammonium chloride, as described elsewhere.[4] Single-cell suspensions were prepared and washed three times in Hank's balanced salt solution (HBSS; Gibco, Paisley, UK) and diluted in Iscove's medium (Gibco) containing 10% fetal calf serum (FCS; Gibco). Intestinal lamina propria lymphocytes (LPL) were prepared as described elsewhere.[4] Briefly, after thorough washing in Ca^{2+}- and Mg^{2+}-free HBSS (CMF-HBSS; Gibco), the tissue pieces were incubated in CMF-HBSS containing 5 mM EDTA (Merck, Darmstadt, Germany) to remove epithelial cells and intraepithelial lymphocytes. The intestinal pieces were then incubated in RPMI-1640 (Gibco) containing 100 U/ml of collagenase type C-2139 (Sigma Chemical Co, St Louis, MO), to extract the LPL enzymatically. Finally, the single-cell suspensions of LPL were washed twice in CMF-HBSS and further purified by centrifugation on a Percoll (Pharmacia, Uppsala, Sweden) gradient prepared from 40% and 72% dilutions with PBS of a Percoll isotonic stock solution. After centrifugation at 600 *g* for 20 min at 4°, highly viable (> 95%) mononuclear cells were recovered from the Percoll interface. These cells were resuspended in Iscove's medium (Biochrom KG, Berlin, Germany) containing 10% FCS, and adjusted to an appropriate cell density.

In vitro *culture system*

SP and MLN lymphocytes were extracted from mice immunized 2 years earlier, or from unimmunized age-matched control mice, and cultured at 10^6 cells/ml in Iscove's medium containing 10% fetal calf serum, gentamicin (50 μg/ml), fungizone (0·5%, Gibco) and additional nutrients, in a volume of 1 ml/well in 24-well flat-bottomed plates (Nunc A/S, Roskilde, Denmark). The memory and naive, control, lymphocytes were cultured at 37° and 10% CO_2 for 6 days in the presence or absence of DNP–KLH antigen added to the wells at 100 μg/ml. Supernatants were harvested on day 6 and frozen at −70° until analysed. The cells were monitored for anti-DNP antibody production in various isotypes at the single-cell level using the ELISPOT (enzyme-linked immunosorbent spot assay) technique.

Enzyme-linked immunosorbent assay (ELISA)

Serum from mice bled at killing, and supernatants harvested on day 6 of culture, were studied for specific anti-DNP antibody concentrations using an ELISA. Briefly, polystyrene 96-well microtitre plates (Nunc A/S) were coated with 200 μg/ml of DNP–ovalbumin (OVA) prepared as described elsewhere,[6] and stored at 4° until used. After three washes with PBS, the plates were blocked for non-specific binding by incubation with 0·1% BSA in PBS for 30 min at 37°. The plates were subsequently washed three times with PBS–0·05% Tween-20. Supernatants and serum were added in duplicates at 1 : 2 and 1 : 100 dilutions and titrated in twofold and threefold serial dilutions, respectively, in corresponding subwells in 0·1% BSA–PBS, and incubated at 4° overnight. Following washes in PBS–Tween,

alkaline phosphatase (ALPH)-conjugated goat anti-mouse IgA, IgG or IgM-specific antibodies (Southern Biotechnology Associates Inc., Birmingham, AL), were added at 1/500 dilutions to each well. Finally, after 2 hr incubation at room temperature, the ALPH substrate [*p*-nitrophenyl phosphate (NPP) substrate tablets (Sigma), in ethanolamine buffer (pH 9·6) at 1 mg/ml] was added to the wells and the reaction was read after 45 min in a Titertek Multiscan spectrophotometer (Flow Laboratories, Irvine, UK) at 405 nm. Antigen-specific antibody titres were defined as the interpolated value giving rise to an absorbance above 0·4 over background, as described elsewhere.[4] All antibodies specific for the various mouse isotypes were tested for specificity using an ELISA with known concentrations of purified mouse IgM, IgG1 or IgA (Pharmingen, San Diego, CA) added to each well. The antisera used were highly isotype specific and did not give rise to detectable cross-reactivity.

ELISPOT assay

Determinations of antigen-specific antibody production at the single-cell level were performed using the ELISPOT technique.[4] Antibody production was detected as spot-forming cells (SFC). Polystyrene Petri dishes (Nunc A/S) were coated with DNP–OVA at 200 μg/ml. Following three washes in PBS, the plates were blocked for unspecific binding with 0·1% BSA/PBS at 37° for 30 min. After three subsequent washes with PBS–Tween, freshly isolated SP, MLN or LP cells were added to the wells at 400 000 cells per Petri dish in Iscove's medium containing 5% FCS, and incubated for 4 hr at 37° in 10% CO_2. Thereafter the plates were washed three times in PBS–Tween, and incubated overnight at 4° with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse immunoglobulin at 1/200 dilution (Dako, Glostrup, Denmark), followed by HRP-conjugated swine anti-rabbit immunoglobulin (Dako) at 1/200 dilution, and allowed to incubate at room temperature for 2 hr. Following three washes in PBS–Tween, the 1,4-*p*-phenylene diamine free base (PPD) substrate (Sigma) in PBS–1% agarose was added to the Petri dishes as described previously[4] and the SFC were enumerated under low magnification and expressed as means ± SD of duplicate wells.

RESULTS

Long-term persistence of serum IgM production after oral immunization

Using the hapten DNP conjugated to the T-cell dependent carrier protein KLH, we observed the development and maintenance of long-term immunological memory in B lymphocytes following oral immunizations. Mice were given oral priming immunizations with DNP–KLH plus CT adjuvant and monitored for hapten-specific serum antibodies of various immunoglobulin classes. After 2 weeks the serum contained anti-DNP antibodies of IgM as well as IgG and IgA classes. By contrast, after 2 years the serum anti-DNP IgG and IgA levels were undetectable, whereas significant anti-DNP IgM antibody titres were still demonstrable (Fig. 1). Naive age-matched control mice had no detectable serum antibodies directed against DNP. Thus, while both IgG and IgA anti-DNP production was stimulated by oral priming immunization, only IgM anti-DNP production persisted for more than 2 years following immunization with DNP–KLH plus CT adjuvant,

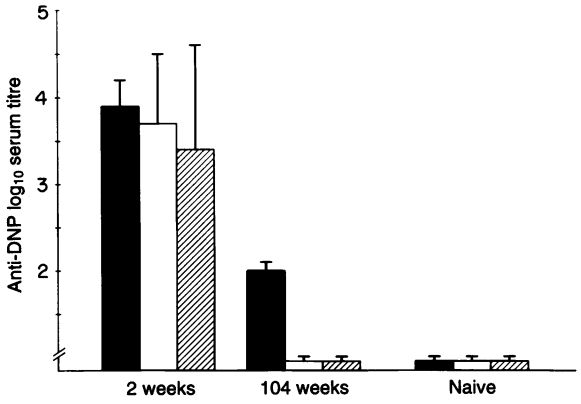

Figure 1. Long-term persistence of antigen-specific serum IgM after oral immunizations. Mice were primed by two or three oral immunizations with DNP–KLH plus CT adjuvant and rested for 2 or 104 weeks, respectively. Serum samples from six immunized mice from each group were analysed for IgM (filled bars), IgG (open bars) or IgA (hatched bars) anti-DNP-specific antibodies by ELISA. For comparison, age-matched, old (> 104 weeks) naive control mice were analysed in parallel for the presence of anti-DNP antibodies. Anti-DNP serum antibodies of the various isotypes were determined and expressed as $\log_{10}$ titres ± SD. The detection limit for significant titres above background was set to 1·0. This is one representative experiment of three.

suggesting that maintenance of long-term immunological memory after mucosal immunization may involve B cells that have not switched to IgA or IgG isotypes but rather have retained the ability to produce IgM antibodies.

Memory B cells responding to recall antigen *in vitro* are found both in local and systemic lymphoid tissues following oral priming immunizations

As after 2 years the hapten-specific serum IgM production in previously immunized mice may have been derived from newly formed B cells encountering persistent antigen, rather than reflecting the production by long-lived memory B cell clones, we isolated lymphocytes from naive and memory mice and cultured these cells in the presence of DNP–KLH antigen. Only lymphocytes from mice previously immunized responded with detectable anti-DNP production (Table 1). Lymphocytes from naive mice and lymphocyte cultures without antigen demonstrated no anti-DNP SFC (Table 1). Irrespective of whether the memory lymphocytes were isolated from gut-associated lymphoid tissues (GALT), MLN, or systemic lymphoid tissues, SP, following oral immunization, strong anti-DNP SFC responses were found to recall antigen *in vitro* (Table 1). Serum IgM anti-DNP production persisted in memory mice and IgM anti-DNP antibodies were predominantly produced by the isolated lymphocytes after stimulation *in vitro* with recall antigen (Table 2), indicating that long-term memory after oral immunization may be carried by secondary B cells capable of producing IgM antibodies upon stimulation. No specific anti-DNP antibodies of any isotype were detected in cultures containing recall antigen and naive lymphocytes (Table 2).

Antigen restimulation of mucosal memory B cells *in vivo* results in IgG- and IgA-dominated responses

Memory mice that had been primed orally with DNP–KLH

Table 1. Anti-DNP SFC responses by memory B cells restimulated with antigen *in vitro* 2 years after oral priming immunizations with DNP–KLH plus CT adjuvant

	MLN*		SP	
Stimulent	Memory	Naive	Memory	Naive
DNP–KLH	3125 ± 1000†	< 200	2275 ± 550	< 200
None	< 200	< 200	< 200	< 200

* Mice were primed by three oral immunizations with DNP–KLH plus CT adjuvant and rested for 2 years prior to analysis. Cells from gut-associated MLN or systemic SP tissues were freshly isolated from perorally primed memory mice or naive, unimmunized, age-matched control mice.

† Anti-DNP total immunoglobulin SFC/10^7 cells were determined in memory or naive cells after 6 days in culture in the presence or absence of DNP–KLH antigen. SFC activity is expressed as means ± SD of duplicate determinations. The threshold for detection of specific SFC over background activity was set to 200 SFC/10^7 cells. This is one representative experiment of three giving similar results.

plus CT adjuvant 2 years earlier were compared with naive age-matched control mice for responsiveness to an oral challenge immunization with the same antigen given together with CT adjuvant. We found significant anti-DNP SFC responses in both gut mucosal and systemic lymphoid tissues following oral immunization in memory mice, whereas naive mice gave no or poor anti-DNP responses (Fig. 2). Antigen challenge of previously immunized mice thus elicited strong serum IgG and IgA anti-DNP antibody responses, while serum IgM anti-DNP titres were increased to a much lesser extent (Fig. 3). A plausible interpretation of these results would be that mucosal long-term memory B cells upon antigen re-exposure *in vivo* may undergo isotype switching from IgM to IgG and IgA production.

Table 2. Memory B cells generated by oral immunization with DNP–KLH plus CT adjuvant produce predominantly IgM anti-DNP *in vitro*

	Distribution of anti-DNP isotypes in supernatants from restimulated memory B cells (log_{10} titres)*			
	MLN		SP	
Isotype	Memory	Naive	Memory	Naive
IgM	3·9 ± 0·4†	< 1·0	< 3·4 ± 0·3	< 1·0
IgG	< 1·0	< 1·0	< 1·0	< 1·0
IgA	< 1·0	< 1·0	< 1·0	< 1·0

* Mice were primed by three oral doses of DNP–KLH plus CT adjuvant and rested for 2 years before killing. Single-cell suspensions from MLN and SP of memory and naive, unimmunized, mice were prepared and cultured for 6 days in the presence or absence of DNP–KLH. Supernatants were then harvested and the anti-DNP-specific antibody concentration was determined by ELISA.

† The anti-DNP titre was expressed in log_{10} titres ± SD of duplicate cultures analysed in triplicate ELISA wells, respectively. Antibody concentrations below the limit for detection are shown as < 1·0. The experiment is representative of three others giving similar results.

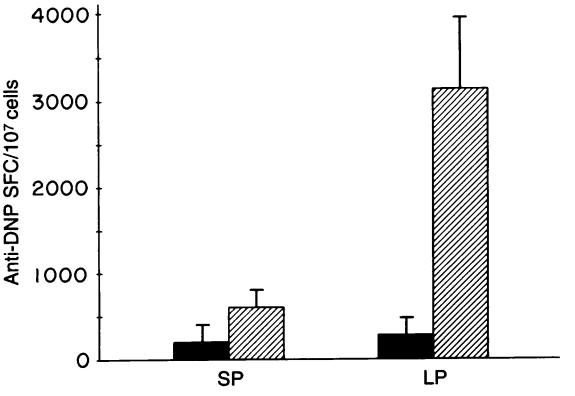

Figure 2. Strong memory B-cell responses may be triggered *in vivo* 2 years after oral priming immunizations. Memory mice, primed by oral immunizations with DNP–KLH plus CT adjuvant 2 years earlier (striped columns), and unimmunized age-matched control mice (filled columns) received a single oral challenge immunization with DNP–KLH plus CT adjuvant. The mice were killed 7 days after the challenge immunization and single-cell suspensions from LP and SP were prepared and analysed for anti-DNP SFC activity. Anti-DNP SFC numbers were expressed as mean SFC/10^7 cells ± SD of six mice per group. This is one representative experiment of three giving similar results.

Elicitation of a specific mucosal memory B-cell response *in vivo* requires participation of memory T cells

Next we investigated whether secondary and naive T cells differ in their ability to promote efficient help for elicitation of a memory B-cell response *in vivo*. Using the B-cell specific hapten, DNP, conjugated to two different carrier molecules, KLH or HSA, we analysed which of these conjugates would stimulate anti-DNP memory B-cell responses most efficiently. At 2 years post-priming immunization, DNP–KLH mice were given a single oral challenge immunization with DNP–KLH or DNP–HSA plus CT adjuvant and the local and systemic anti-DNP

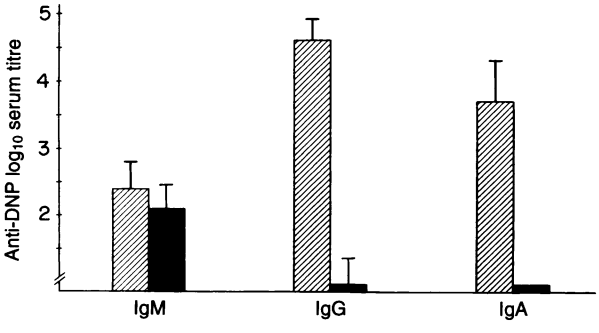

Figure 3. Isotype distribution of long-term memory B-cell responses *in vivo* after oral challenge with antigen. Memory mice, primed by oral immunizations with DNP–KLH plus CT adjuvant 2 years earlier, were either untreated (filled columns) or recieved a single challenge immunization (striped columns) with DNP–KLH plus CT adjuvant. The mice were bled when killed 7 days after the challenge immunization, and individual serum samples were analysed for anti-DNP antibodies of the various isotypes. The anti-DNP serum activity was expressed as means ± SD of anti-DNP log_{10} titres of six mice in each group. This is one representative experiment of three giving similar results.

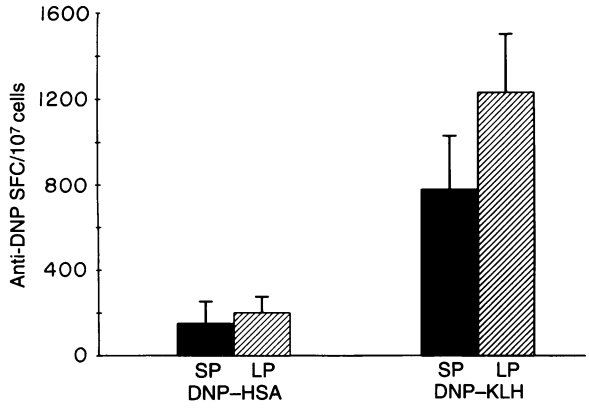

Figure 4. Carrier-specific memory T cells are required for efficient elicitation of memory B-cell responses *in vivo*. Memory mice, primed by oral immunizations with DNP–KLH plus CT adjuvant 2 years earlier, recieved a single challenge immunization with DNP–KLH plus CT adjuvant or DNP–HSA plus CT-adjuvant. The mice were killed 7 days after the challenge immunization and single-cell suspensions from LP and SP were prepared and analysed for anti-DNP SFC activity. Anti-DNP SFC numbers were expressed as mean SFC/10^7 cells ± SD of six mice per group. This experiment represents two identical experiments giving similar results.

responses were recorded. As illustrated in Fig. 4, only DNP–KLH was effective at stimulating a memory B-cell response. Strong anti-DNP SFC responses were demonstrated in both gut mucosal (LP) and systemic (SP) lymphoid tissues to DNP–KLH, whereas, in contrast, DNP–HSA seemed not to stimulate sufficient T-cell helper activity in the HSA naive mice (Fig. 4). In previously unimmunized mice, the DNP conjugates were equally potent as priming antigens for stimulation of mucosal immune responses. Thus naive mice given DNP–KLH or DNP–HSA orally together with the CT adjuvant displayed similar increases in serum anti-DNP titres (Table 3). By contrast, the DNP–KLH memory mice responded with similar increases in IgM anti-DNP titres to DNP–KLH and DNP–HSA, but serum anti-DNP IgG and IgA titres were greatly augmented only after challenge with DNP–KLH (Table 3). This latter antigen effectively stimulated KLH-specific secondary/memory T-cell help whereas the T-cell help generated by DNP–HSA was poor (Table 3). Our results suggest that memory B cells require secondary/memory T-cell help for an efficient stimulation of antibody production and isotype-switch differentiation.

DISCUSSION

A prerequisite for effective vaccination of mucosal tissues is the ability to develop immunological memory. Several studies have documented that immunological memory is indeed a property of the mucosal immune system.[2–4,6,14] The present study confirms and further extends our information on immunological memory after oral vaccination, using CT as the adjuvant. Most important, we demonstrate that mucosal memory may be carried for several years by recirculating B lymphocytes that have retained the ability to produce IgM antibodies upon restimulation.

A shortcoming of our earlier studies on immunological memory after mucosal immunizations was the inability to separate T- and B-cell memory.[4,5] However, by employing a hapten–carrier system, DNP conjugated to KLH or HSA, we could separate these two entities from each other. This gave us a better and more controlled experimental system to work with. We could then study DNP-specific memory and ask to what extent mucosal B-cell memory required T-cell help. From our results it is suggested that for effective elicitation of the DNP-specific B-cell memory 2 years after the priming immunizations, carrier-specific memory T cells are required. This was evident by the failure of DNP–HSA to elicit IgA or IgG responses in DNP–KLH-primed memory mice, whereas DNP–KLH given as an oral challenge stimulated strong anti-DNP IgA and IgG responses.

The DNP–HSA antigen promoted stronger serum IgM anti-DNP responses compared to DNP–KLH, probably indicating that whereas the memory B cells recognized the DNP antigen the absence of adequate T-cell help hampered terminal differentiation and isotype switching of the DNP-specific memory B cells. Therefore, our finding underscores that for the elicitation of a secondary-type mucosal B-cell response against a given antigen, B- and T-cell specific memory may be closely interconnected phenomena. In addition, in recent reports on the requirements of T cells for the induction of

Table 3. Optimal stimulation of mucosal anti-DNP memory B-cell responses requires activation of carrier-specific memory T-helper cells

	Primary response			Memory response		
Challenge	IgM	IgG	IgA	IgM	IgG	IgA
DNP–KLH	2·9 ± 0·1	2·3 ± 0·3	2·7 ± 0·1	2·4 ± 0·4	4·6 ± 0·3	3·7 ± 0·6
DNP–HSA	2·9 ± 0·1	2·1 ± 0·1	2·6 ± 0·1	2·8 ± 0·2	1·6 ± 0·8	2·1 ± 0·2

Naive 2-year-old mice were primed by two (primary response) oral immunizations with DNP–KLH or DNP–HSA plus CT adjuvant and rested for 14 days, or naive 8-week-old mice were given three (memory response) oral immunizations with DNP–KLH plus CT adjuvant and rested for 2 years before analysis. After 2 years the memory mice were challenged by oral administration with DNP–KLH or DNP–HSA plus CT adjuvant 8 days prior to killing. Serum anti-DNP concentrations in various isotypes were determined by ELISA and expressed as mean titres ± SD of duplicate determinations. The anti-DNP titer was expressed in $\log_{10}$ titres ± SD of duplicate determinations. This is one representative experiment of two.

immunological B-cell memory, elegant studies have shown that activated T cells are necessary to interact via the surface molecule CD40L (gp39) with the B-cell CD40 molecule,[15–17] again supporting the notion that antigen-specific $CD4^+$ T-cell and B-cell memory are closely interconnected. Whether it is the CD40–CD40 ligand (gp39) interaction that is required for the elicitation of mucosal memory B-cell responses is currently not known. Of note, both supporting and conflicting findings regarding the notion that a CD40 ligand interaction is required for effective elicitation of systemic memory B-cell responses have been reported.[15,17]

An important aspect of the close relationship between memory B and T cells concerns the construction of new vaccines using isolated B-cell epitopes. As immunological memory is a T-cell dependent phenomenon, T-cell independent antigens most often do not stimulate immunological memory.[1,18,19] The finding that memory B and T cells are interconnected also alludes to current attempts to vaccinate against bacterial infections using T-cell independent polysaccharide antigens conjugated to protein carrier molecules, such as tetanus toxoid or cholera B subunit.[19–21] In this way memory B cells are generated and high titres of polysaccharide-specific antibodies may be obtained following immunization.[20]

The long-term maintenance of such a memory and the elicitation of the specific memory B-cell response would require helper activity by memory T cells. Whereas the B-cell epitope is present at exposure to the live bacterial infection, the helper T-cell epitopes are missing. Consistent with this prediction we found poor responses after DNP–HSA challenge of the orally DNP–KLH primed memory mice , while the oral DNP–KLH challenge elicited strong anti-DNP B-cell memory responses. The same consequences and limitations would also apply to peptide vaccines in which B-cell epitopes are linked to carrier protein molecules, providing T-cell epitopes that may not be involved in infections with live microbial agents against which the vaccine is directed.[22]

The maintenance of immunological memory has been ascribed to the persistence of antigen in the form of antigen–antibody complexes trapped on the interdigitating follicular dendritic cells (FDC) in the organized lymphoid tissues.[23,24] This hypothesis places the recirculating memory B cell in a central position as the most effective cell to sample antigen from the FDC via its high-affinity receptor, and because of its antigen-presenting ability the memory B cell is, perhaps, also the key element in propagating antigen-specific memory $CD4^+$ T cells.[24] One may speculate that the low but persistent serum concentrations of IgM anti-DNP antibodies that we observed after 2 years post-oral immunizations represent recirculating memory B-cell clones being restimulated in lymph nodes with antigen bound to FDC.[25] As anti-DNP IgG and IgA antibodies were absent at this time, it may be that memory B-cell clones undergo partial activation with only few cells differentiating into antibody production. Consistent with such a theory of low-level continuous restimulation of mucosal memory B-cell clones, IgM antibodies, requiring the least T-cell help, dominated the response.

The anatomical localization for the persistent antigen driving mucosal memory B-cell clones after oral immunizations is at present unknown.[2] A possible candidate might be the FDC network in Peyer's patches (PP). However, in our previous work as well as in the present study, we have demonstrated that memory cells may be isolated from both systemic and GALT, other than PP, at any time and reactivated to antibody production *in vitro*.[5] This observation agrees with the findings in a recent study by Bachmann *et al.*[26] who demonstrated that memory B cells were localized both to draining and non-draining lymph nodes after a local injection of antigen.[26] Moreover, we have preliminary findings to suggest that memory B cells, after adoptive transfer to major histocompatibility complex (MHC) syngeneic nude mice lacking functional PP,[27] may be reactivated to antibody production by oral challenge immunization with specific antigen (N. Lycke, unpublished data). Therefore, the proposed model may be overemphasizing the role of PP for the maintenance of mucosal memory, as it would require the recirculating memory B cells to express surface homing receptors for the high endothelial venules in the PP rather than for peripheral lymph nodes or lamina propria.[28] To our knowledge, no information in support of a distinct homing pattern for long-term mucosal memory B cells has as yet been reported. Nor has any documentation on long-term antigen deposition following oral immunization with soluble antigen been presented.

In our present study we have demonstrated that even 2 years after oral immunization we were able to elicit IgM anti-DNP antibody production by isolated memory B cells. This finding suggests that mucosal memory B cells may retain the ability to produce all isotypes, including IgM, which is otherwise normally associated with primary responses to T-cell-dependent antigens.[1] An indirect implication of the data at the gene level is that mucosal memory B cells have not undergone isotype-switch differentiation involving deletion of C_H chain gene segments.[11] Our finding is at some variance with earlier studies on intestinal memory B cells isolated from PP, of which a majority were membrane IgA^+ and produced IgA antibody.[10] However, it is possible that the complex system of analysis employed in the latter studies, e.g. the splenic fragment assay, may account for some of the differences between the two results.[10] Indicative of such an assumption is that these investigators also observed antigen-specific IgM production by memory PP B-cell cultures, albeit at a lower frequency and magnitude compared to IgA.[10]

Nevertheless, we isolated memory B cells from both systemic and GALT, and irrespective of localization we obtained predominantly IgM anti-DNP production. Theoretically, it would be advantageous for mucosal memory B cells not to rearrange and delete C_H chain genes and to retain the whole repertoire of isotypes. In this way the mucosal memory B cell is not restricted to IgA production and effector function only at mucosal sites, but rather may be influenced by the requirements dictated by microenvironmental factors, such as T-cell activities, in any given tissue. Thus, secondary lamina propria IgA and systemic IgG responses may be elicited from the same recirculating precursor population. Analysis of surface expression of immunoglobulin isotypes on memory B cells has given disparate information. In fact, the litterature is confusing with reports on memory B cells expressing almost all different isotypes including studies demonstrating double isotype-expressing memory B cells, i.e IgG^+/IgM^+, IgA^+/IgM^+, etc.[1,29,30] Probably, the heterogeneity in membrane isotype expression reflects a significant proportion of long-term memory B cells that do not undergo switch differentiation involving C_H chain gene deletion, but rather use long mRNA

transcripts to produce both up- and downstream-located C_H chain genes.[31,32] This way the mucosal memory B cell may give rise to any isotype-committed progeny of IgA- as well as IgG-producing effector cells. In support of such a notion, we found predominantly IgM production after *in vitro* restimulation of mucosal memory B cells with antigen, whereas oral challenge immunization of mice 2 years after the priming immunization resulted in concomitant strong mucosal IgA and serum IgG anti-DNP responses. Future studies will attempt to enrich for antigen-specific mucosal memory B cells to allow for more detailed analysis of the isotype differentiation patterns and activation requirements.

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