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The development of IgE⁺ memory B cells following primary IgE immune responses

We studied whether long-lived IgE⁺ memory B cells develop following three types of primary IgE immune responses. Immunization of mice with anti-IgD antibody induced a T cell-dependent, interleukin (IL)-4-dependent primary IgE response and the formation of IgE isotype switched (IgE⁺) memory B cells. These IgE⁺ memory B cells could be stimulated *in vivo* by injection with goat anti-IgE antibodies to produce a profound IL-4-independent memory IgE response. By contrast, both infection of mice with *Nippostrongylus brasiliensis* or repeated immunization with benzylpenicilloyl-keyhole limpet hemocyanin (BPO-KLH) in alum stimulated good primary IgE responses and profound memory T cell-dependent antigen-specific IgE responses, but failed to induce the development of long lived IgE⁺ memory B cells because they could not be recalled with goat anti-IgE antibodies. Mice receiving double immunizations combining anti-IgD with either *N. brasiliensis* infection or BPO-KLH immunization mounted significant goat anti-IgE-induced secondary IgE responses, but no *N. brasiliensis* or BPO-KLH-specific IgE could be detected. This indicates that the *N. brasiliensis* and BPO-KLH induced immune responses do not suppress the development of IgE⁺ B cells, but rather, do not provide the necessary conditions for their formation. Taken together these data indicate that long-lived IgE⁺ B cells fail to develop during the primary IgE response to *N. brasiliensis* infection or BPO-KLH immunization. By contrast, significant numbers of IgE⁺ memory B cells form during the primary IgE immune response induced by anti-IgD immunization. Our observations suggest that immunization protocols involving membrane IgD cross-linking and limited duration of cognate T cell help are necessary for the formation of IgE⁺ memory B cells. It will be important to determine the relevance of membrane IgD interaction with allergens, as this would influence the design of new therapies for the treatment of allergy and asthma.

1 Introduction

In the last two decades, the molecular mechanisms which drive antibody specificity, diversity, and affinity maturation have been dissected and characterized. By contrast, much remains to be learned concerning the cellular and molecular mechanisms involved in the generation and maintenance of immunological memory [1]. One open question is to what extent immunological memory resides at either the level of antigen-specific B cells or T helper cells. This question is especially important in the case of IgE memory responses, given the strong association between allergen-specific IgE, atopy, and asthma and the current quest to discover new selective therapeutic agents for treating these diseases.

The goal of the present study was to determine which type of primary IgE immune response leads to the development of IgE⁺ memory B cells. The IgE⁺ memory B cells can be revealed by injecting immunized mice with a purified polyclonal goat anti-mouse IgE (Gt > mIgE) antibody preparation [2].

In this study three different kinds of IgE immune responses were analyzed for the formation of IgE isotype-switched memory B cells. These included infection of mice with *Nippostrongylus brasiliensis*, leading to a primary *N. brasiliensis*-specific IgE response after 14 days [3], immunization with benzylpenicilloyl-KLH (BPO-KLH) in alum adjuvant, resulting in an ongoing BPO-specific IgE response [4], and anti-mouse IgD antibody immunization, which induces a primary polyclonal IgE response after 8 days [5].

[I 15697]

Received April 23, 1996; in revised form September 13, 1996; accepted September 13, 1996.

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Abbreviations: BPO-KLH: Benzylpenicilloyl-keyhole limpet hemocyanin Gt > mIgE: Goat anti-mouse IgE M > mIgD: Mouse anti-mouse IgD *N. brasiliensis*: *Nippostrongylus brasiliensis*

Key words: Memory B cell / IgE / IgD

2 Materials and methods

2.1 Mice

Six-week-old BALB/c mice were obtained from the Ciba-Geigy Sisseln (Switzerland) breeding facility. Mice were housed under specific pathogen-free conditions, given food and water *ad libitum* and were treated according to the "Ethical principles and guide-lines for scientific experiments on animals" edited by the Swiss Academy of Medical Sciences.

2.2 Preparation of antibodies

To obtain goat anti-mouse IgE antibody, goats were immunized with a 2-mg mixture of three monoclonal IgE antibodies purified from mouse ascites fluid. After several boosting immunizations, serum was collected and goat anti-mouse IgE was purified after absorption on a series of columns containing mouse monoclonal IgM, IgG1, IgG2a, IgG3, IgA antibody, followed by subsequent binding and elution from a IgE affinity matrix column.

The monoclonal anti-mouse IgD allotype (H8a/1 and FF1-4D5) [2, 5], rat anti-CD4 (GK 1.5) [6], rat anti-IL-4 (11B11) [7], rat anti-IgE (4B3-39 and 3-11) [8], rat anti-mouse IgM (b-7-6) [9], the mouse IgE anti-BPO (13-10), and mouse IgG1 anti-BPO (K1-16) [4] were purified from hybridoma ascites fluid by a combination of ammonium sulfate precipitation and DEAE anion exchange chromatography. Rat anti-mouse IgG1 antibodies (04-6100 and 04-6140) were purchased from Zymed (San Francisco, CA).

2.3 Immunizations

The mouse anti-mouse IgD immunization was performed by subcutaneous (sc) injection of BALB/c mice with 250 µg H8a/1 and 250 µg FF1-4D5 monoclonal antibodies. For BPO-KLH immunization, mice were given up to three intraperitoneal (ip) injections 4–8 weeks apart with 10 µg BPO-KLH in 0.2 ml Al(OH)₃ (aluminium hydroxide gel) (Serva, Heidelberg, Germany). *N. brasiliensis* infection involved subcutaneous injection of 700 third-stage infective larvae as previously documented [10]. The Gt > mIgE immunization involved the subcutaneous injection of 200 µg affinity-purified goat anti-mouse IgE antibodies.

2.4 Serum antibody measurement

Mice were bled from the tail vein at different times after primary or secondary immunizations. Sera were obtained after coagulation of the blood at 4°C followed by centrifugation for 3 min at 4000 rpm. The concentration of total IgG1 and IgE in the serum was determined by sandwich ELISA as described [4, 11]. For this, rat anti-mIgE antibody, 4B3-39 was used for coating and biotinylated 3-11 for detection, while the IgG1 ELISA consisted of rat anti-mouse IgG1 (04-6100, Zymed) for coating and biotinylated rat anti-mouse IgG (04-6140, Zymed) as a detection antibody. The indicated IgE and IgG1 concentrations in the serum represent the mean of groups of three to four mice. For the measurement of *N. brasiliensis* antigen-specific IgE, ELISA plates were coated with purified excretory antigen from L4 larvae, blocked, serum added and bound IgE measured with biotinylated rat anti-IgE antibody 3-11. Bound second antibody was revealed by incubation with avidin conjugated with alkaline phosphatase followed by the color reaction developed with *p*-nitrophenyl phosphate; the absorbance of *p*-nitrophenol was measured at 405 nm. BPO-specific IgE was detected by using a rat anti-mIgE antibodies 4B3-39 as a capture antibody and BPO-haptenated alkaline phosphatase for detecting the BPO-specific IgE. Bound BPO-alkaline phosphatase was detected using substrate tablets (Sigma 104, Sigma, St. Louis, MO).

3 Results

3.1 Induction of primary IgE immune responses

The serum levels of IgE were followed in mice infected with *N. brasiliensis*, immunized with BPO-KLH/alum, or injected with M > mIgD (Fig. 1). A pronounced increase in total serum IgE occurred in *N. brasiliensis*-infected mice, reaching its maximum value (200 µg/ml) at day 15 of infection. Twenty five days after infection, the serum IgE level had declined to 40 µg/ml. This serum level of IgE was maintained for at least 3 months (not shown). In mice immunized with BPO-KLH in alum, there was little detectable serum IgE after the first immunization. However, after a second immunization, the serum IgE level peaked at 33 µg/ml (Fig. 1) and was maintained at approximately 30 µg/ml for 3 months (data not shown). An ELISA detecting BPO hapten-specific IgE revealed that approximately 10% of the total IgE was BPO-specific IgE (data not shown). Mice immunized with M > mIgD monoclonal antibodies mounted a profound total serum IgE response which peaked by day 8 at 70 µg/ml (Fig. 1). The level of serum IgE then rapidly decreased to baseline levels of 5 µg/ml 12 days after immunization.

3.2 Injection of Gt > mIgE antibody stimulates memory IgE responses in anti-IgD primed mice

The effect of injecting Gt > mIgE on the serum IgE level of mice previously immunized with M > mIgD is shown in Fig. 2. The IgE response peaked around day 5–7 at almost 1000 µg/ml, which corresponded to a 1000-fold increase in serum IgE concentration. The IgE response was selective in that no induction of secondary IgM or IgG1 responses occurred in these mice (data not shown) and control non-antigen-specific goat IgG failed to induce IgE responses in the anti-IgD-immunized mice. Furthermore nonimmunized mice failed to mount a significant secondary IgE response (6 µg/ml) to Gt > mIgE (Fig. 2). These data indicate that Gt > mIgE selectively stimulates IgE-switched B cells in mice which have previously mounted a

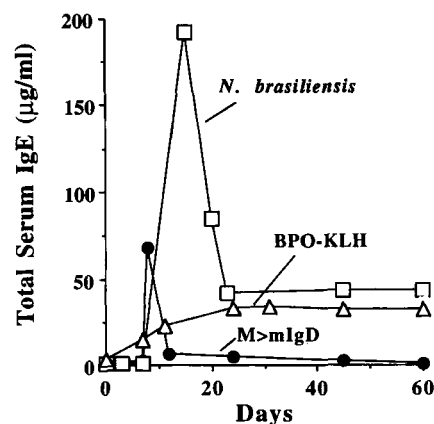


Figure 1. Primary IgE response of mice following primary infection with 700 *N. brasiliensis* larvae (open squares), two immunizations with 10 µg BPO-KLH in alum (open triangles), and primary immunization with 500 µg M > mIgD (closed circles). Serum was collected from groups of five mice each and analyzed for levels of total IgE by ELISA.

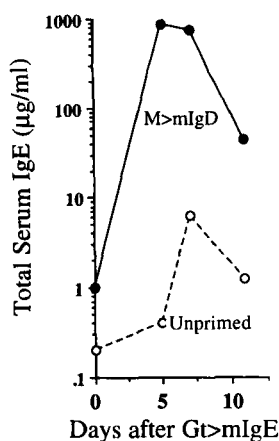


Figure 2. IgE response of mice injected s.c. with Gt > mIgE antibodies. Mice immunized 3 months previously with M > mIgD (closed circles) or saline injected controls (open circles) were injected s.c. with 200 µg Gt > mIgE antibody. Serum was taken at days 5, 7, and 11 and analyzed for the levels of total IgE by ELISA.

primary immune response. This conclusion is further supported by previous studies which demonstrated that the secondary Gt > IgE-induced IgE response is IL-4-independent, while the primary M > mIgD-induced IgE responses is inhibited by neutralizing anti-IL-4 antibodies. These results indicate that the IgE-producing B cell which responds to Gt > mIgE has previously undergone an IL-4-dependent isotype switch to IgE [2]. Taken together, these data show that the IgE response induced with Gt > mIgE is derived from a previously IgE isotype-switched B cell.

3.3 Anti-IgD-induced IgE⁺ memory B cells are long-lived cells

It was important to establish the life span of the IgE⁺ memory B cell population following primary immunization with M > mIgD antibody. Mice were immunized with M > mIgD antibody 1, 3, 5, and 8 months before Gt > mIgE challenge. The effect on IgE production at 7 days after Gt > mIgE administration is shown in Fig. 3. Strikingly, in all groups of mice, the magnitude of the Gt > mIgE-induced IgE response consistently peaked at 800 µg/ml IgE. Control, unprimed littermates, which were also boosted with Gt > mIgE, failed to mount any IgE response greater than 10 µg/ml. This result dramatically indicates that once formed, the switched IgE⁺ memory B cells can persist for 8 months in the absence of any further antigen challenge.

3.4 Gt > mIgE does not trigger an IgE response in *N. brasiliensis* or BPO-KLH/alum-primed mice

The Gt > mIgE treatment of *N. brasiliensis*-infected mice or BPO-KLH/alum-primed mice failed to induce any increase in serum IgE (Fig. 4), indicating that no IgE⁺ memory B cells were present in the tissues. This result suggests that no IgE⁺ memory B cells were induced during the course of the primary immunization or infections. However, the *N. brasiliensis*-infected mice were capable of

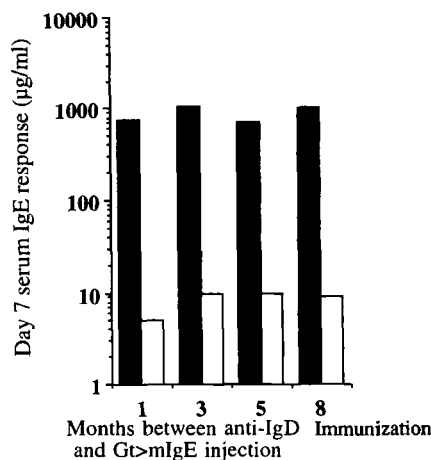


Figure 3. Life span of Gt > mIgE-induced IgE response in mice previously immunized with M > mIgD. Mice were injected with Gt > mIgE either 1, 3, 5, or 8 months following primary immunization with M > mIgD antibody (solid bars). Age-matched control mice were also injected with Gt > mIgE antibody. Serum was taken at days 3, 5, 7, and 11 following Gt > mIgE immunization and total serum IgE levels determined by ELISA. Only the peak day 7 total IgE serum level is shown in the figure.

mounting profound secondary IgE immune responses when reinfected with *N. brasiliensis* (Fig. 5A), with the serum IgE level increasing from 40 to 1300 µg/ml 7 days after infection. When *N. brasiliensis* antigen-specific IgE was measured by ELISA, these sera had titers in the range of 1/500–1/1000. Injection of these double-infected mice with Gt > mIgE antibodies also failed to induce an IgE response (data not shown). Therefore, despite the fact that *N. brasiliensis* infection can stimulate mice to produce profound primary and secondary IgE responses, it appears that no IgE-switched memory B cells are formed. Therefore, together with the data of Katona et al. [2], it would appear the cellular basis of *N. brasiliensis*-induced IgE

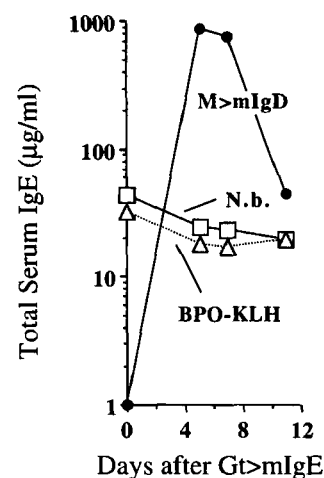


Figure 4. Gt > mIgE-induced serum IgE response of immunized mice. Mice previously immunized with either *N. brasiliensis* (N. b.; squares), BPO-KLH in alum (triangles) or M > mIgD (closed circles) were compared in their ability to mount secondary IgE responses following immunization with Gt > IgE antibody. Serum was collected at days 5, 7, and 11 and the levels of total IgE determined by ELISA.

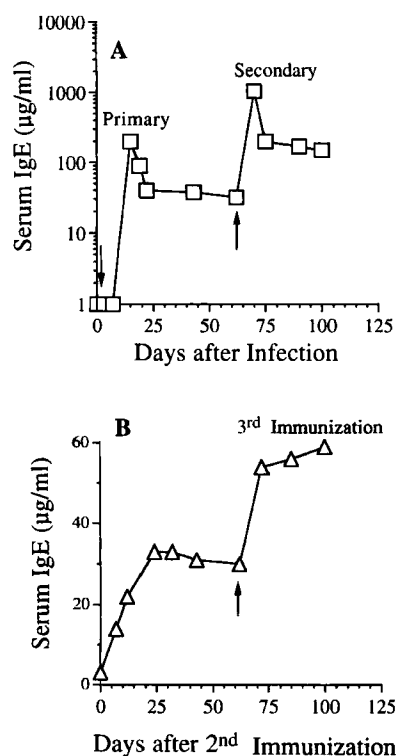


Figure 5. Memory serum IgE responses in mice infected twice with *N. brasiliensis* and three times with BPO-KLH. (A) Mice previously infected with *N. brasiliensis* were infected at day 62 a second time (arrow) and the production of total serum IgE determined. (B) Mice twice previously immunized with BPO-KLH in alum were immunized a third time (arrow) at day 62 and the production of total serum IgE followed. Serum was collected at the indicated time points and the levels of total IgE determined by ELISA.

memory responses is entirely at the level of IL-4 producing T cells.

Mice receiving a third BPO-KLH/alum immunization also mounted large and rapid IgE responses, increasing after 12 days from 32 to 52 µg/ml (Fig. 5B). However, injection of these triple-immunized mice with Gt > mIgE also failed to induce an IgE⁺ memory B cell response (data not shown). These data are in agreement with observations made in an earlier study by Finkelman and colleagues [12], who demonstrated that a major proportion of the secondary DNP-KLH/alum-specific IgE response was inhibited by anti-IL-4 neutralizing antibodies.

A possible explanation for the inability of *N. brasiliensis*-infected or BPO-KLH/alum-immunized mice to mount IgE⁺ memory B cell responses to Gt > mIgE is that the high concentration of IgE (10–30 µg/ml) in the serum could neutralize the injected Gt > mIgE antibodies. We found that increasing the dose of Gt > mIgE to 1 mg/mouse was able to clear all circulating IgE, but was still not able to induce IgE responses in *N. brasiliensis*-infected or BPO-KLH/alum-immunized mice (data not shown).

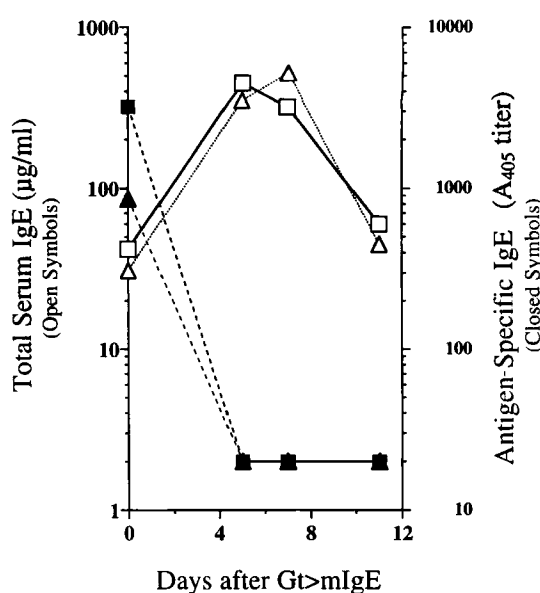


Figure 6. Serum IgE responses in mice double-primed with M > mIgD and *N. brasiliensis* (squares) or M > mIgD and BPO-KLH/alum (triangles). Mice were initially primed with M > mIgD and 2 months later were either infected with *N. brasiliensis* (squares) or immunized twice with BPO-KLH and left for one month (triangles). All groups of mice were then injected with Gt > mIgE antibodies and the level of total IgE (solid lines, open symbols) and *N. brasiliensis*-specific (dotted line, closed squares) and BPO-specific IgE (dotted line, closed triangles) evaluated by ELISA.

3.5 Double-primed mice mount secondary IgE responses following Gt > mIgE antibody injection

To demonstrate that IgE⁺ memory B cells could be activated by Gt > mIgE in both *N. brasiliensis*-infected or BPO-KLH/alum-immunized mice, double immunizations were performed. Mice were primed first with anti-IgD and 2 months later were either infected with *N. brasiliensis* or immunized twice with BPO-KLH. One month after the second treatment, mice were injected with Gt > mIgE antibodies and the total serum IgE response and *N. brasiliensis*-specific and BPO-specific IgE responses evaluated (Fig. 6).

Despite the high total serum IgE level (30–40 µg/ml) at the time of Gt > mIgE treatment, a profound polyclonal IgE response was produced in both groups of mice. It became clear that this IgE response was derived from the anti-mIgD immunization, as there was no detectable *N. brasiliensis*-specific or BPO-specific IgE antibodies produced (Fig. 6). Presumably, the injected Gt > mIgE antibody cleared all existing antigen-specific IgE from the serum while at the same time activating the IgE⁺ memory B cells to IgE production.

Therefore, it appears that IgE switched memory B cells are not induced by *N. brasiliensis* infection or immunization with BPO-KLH and that this lack of induction is not the result of a suppressor mechanism preventing such cells from being revealed. Furthermore, once IgE⁺ memory B cells are induced by anti-IgD treatment, they may persist and be activated independently of other IgE immune responses.

4 Discussion

The goal of these studies was to determine what contribution IgE⁺ memory B cells make to a memory IgE response and in particular, to investigate what types of primary IgE immune responses generate long-lived IgE⁺ memory B cells in mice. The formation of IgE⁺ switched memory B cells was specifically revealed by injecting Gt > mIgE antibodies. The secondary response induced by this treatment is IgE isotype-specific, IL-4-independent, but CD4 T cell-dependent [2, 13]. Three distinct types of IgE immune responses were compared. These included infection of mice with *N. brasiliensis*, repeated immunization with BPO-KLH in alum adjuvant and anti-mouse IgD monoclonal antibody immunization. The only IgE response which led to the formation of IgE-switched B cells was anti-mouse IgD immunization. In fact, IgE⁺ B cells in these mice could be reactivated up to 8 months after the initial priming, indicating that the IgE⁺ B cells were long-lived.

A surprising result from this study was that mice which had mounted IgE responses following *N. brasiliensis* infection or repeated BPO-KLH/alum immunization did not generate such IgE⁺ memory B cells as revealed by Gt > mIgE. However, these same *N. brasiliensis*- or BPO-KLH-immunized mice could mount profound secondary IgE responses if either reinfected with *N. brasiliensis* or reimmunized. Although it has been previously demonstrated that secondary IgE responses are largely IL-4-dependent in both *N. brasiliensis*- and KLH/alum-primed mice, it has been shown *in vitro* that an IL-4-independent IgE response does occur using B cells from BPO-KLH-primed mice [4, 14]. This made it all the more surprising that no IgE⁺ switched B cells developed or could be revealed during these immunizations.

Several trivial explanations for the absence of an IgE response to Gt > mIgE in *N. brasiliensis*-primed or BPO-KLH/alum-primed mice were considered. The potential for the high serum levels of IgE in the *N. brasiliensis*- or BPO-KLH/alum-primed mice to interfere with the Gt > mIgE binding of IgE⁺ B cells was excluded by injecting saturating amounts (>1 mg/mouse) of the Gt > IgE antibody (200 µg/mouse is sufficient for complete clearance of the serum IgE; data not shown).

The possibility that the *N. brasiliensis*- or BPO-KLH/alum-primed lymphoid environment was somehow suppressive for Gt > mIgE-induced memory B cell responses was excluded by the observation that Gt > mIgE could reveal IgE⁺ memory B cells in double-primed (*N. brasiliensis* + M > mIgD or BPO-KLH/alum + M > mIgD) mice.

The suggestion that the IgE response induced by the Gt > mIgE antibodies was dependent on the T cells being previously primed to possible cross-reactive determinants shared between goat IgG and the M > mIgD monoclonal antibody was excluded in the experiments involving double immunization with the anti-IgD antibodies and either *N. brasiliensis* infection or BPO-KLH immunization. In these situations, any *N. brasiliensis*-specific or BPO-KLH-specific IgE⁺ B cells should also be revealed by

Gt > mIgE, as they would have also been able to receive T cell help from the mouse IgD (b) allotype-primed T cells. However no BPO-KLH- or *N. brasiliensis*-specific IgE could be detected in the double-primed mice following Gt > mIgE injection. This indicated that no IgE⁺ memory B cells were present in *N. brasiliensis*- or BPO-KLH/alum-immunized mice.

In the context of IgE-mediated diseases, it is important to understand the mechanism of IgE memory B cell formation and thus the difference between the anti-IgD immunization and the *N. brasiliensis* and BPO-KLH/alum immunizations in generating IgE⁺ memory B cells. All three IgE response models studied here are T cell dependent and IL-4 dependent. However, the relative proportions of B and T cells which might be involved during primary immunization differ greatly. The anti-IgD immunization characteristically provides a strong polyclonal activation of all the B cells, but only a limited amount of Ig allotype-specific cognate T cell help which is of short duration [15, 16]. This is largely because the relatively high levels of injected anti-IgD antibodies are quickly cleared (within 7 days) from the body. In fact, there may be enough IL-4 produced to switch the B cells to IgE, but not enough cognate T cell help to drive B cell differentiation towards IgE synthesis. By contrast, BPO-KLH and *N. brasiliensis* immunization are both characterized by the chronic (>14 days) supply of potent T cell antigenic epitopes, leading to a longer duration of T cell help to B cells which are activated. Taken together, we conclude that IgE⁺ memory B cells develop under conditions where cognate T cell help is of limited duration.

There are several other features of the anti-IgD immunization which could explain its unique ability to generate IgE⁺ memory B cells. One obvious feature is that anti-IgD immunization leads to the activation of IgM⁺/IgD⁺ B cells exclusively via surface IgD, providing the B cell with a restricted set of signals compared to those provided by surface Ig-mediated antigen uptake and antigen-specific cognate T cell help. Such restricted signaling may be important in differentiating the activated B cell into a memory B cell. For instance, cross-linking of membrane IgM may inhibit the generation of IgE⁺ memory B cells in the *N. brasiliensis*- and BPO/KLH-primed mice, which would obviously not occur in the anti-IgD-immunized animals. If true, it may be that only low concentrations of antigens with a low degree of epitope multivalency could trigger IgE⁺ memory B cell formation via IgD. Indeed, it has been reported that large memory IgE immune responses are preferentially induced by low concentrations of antigen during the primary immunization [14].

These results demonstrate that not all IgE responses lead to the formation of IgE⁺ memory B cells. Given the interest in seeking to understand the mechanisms which drive IgE-mediated allergic and asthmatic reactions, it is important to determine whether the allergen-specific IgE immune response in humans resides at the level of the T cell or the IgE⁺ B cell.

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