Antigen Transportation In Antibody-Mediated Immune Response *In Vivo*

Lu Zhang

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Biology Education Centre and Department of Medical Biochemistry and Microbiology, Uppsala University
Supervisor: Frida Henningson Johnson
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**SUMMARY**

Antibodies, apart from neutralizing their specific antigens, can also regulate immune response in the form of antigen-antibody complex, which is known as antibody feedback regulation. Different antibody-mediated immune responses require the help of different cells. In this study, the binding efficiency to B cells in peripheral blood, the migrating efficiency to splenic follicles, as well as the kinetic properties of antigen delivery in different antibody-mediated immune response were investigated. Facilitated by flow cytometry and confocal microscopy, we traced the *in vivo* distribution and kinetic properties of biotinylated ovalbumin-2,4,6-trinitrophenol (OVA-TNP) when administered alone or together with antigen-specific IgE/IgG2a/IgG3 to BALB/c or CD23<sup>−/−</sup> mice. It was found that IgE-antigen complexes were more efficient in binding to circulating B cells and migrating into the splenic follicles, compared to the other two immunoglobins and antigens alone. What’s more, IgE, IgG2a and IgG3 possessed totally different kinetic properties in antigen delivery, not only the time patterns but also the binding cell types, which was compatible with the believe that they used various mechanisms in regulating immune response. Finally, according to the summarized information of consecutive events in IgE-/IgG2a-/IgG3-mediated immune enhancement, including both our data and previous results, a conclusion came out that the antigen delivery from B cells to DCs probably occur during 30 minutes to 16 hours after immunization with IgE-antigen complex. The summarized information could also be a useful indication for further investigation.
ABBREVIATIONS

ACK                  ammonium chloride/potassium
APC                  allophycocyanin
B cell               B lymphocyte
BCR                  B cell receptor
BSA                  bovine serum albumin
CD                   cluster of differentiation
CD23−/−              CD23-deficient
CR                   complement receptor
DC                   dendritic cell
EDTA                 ethylenediaminetetraacetic acid
ELISA                enzyme-linked immune sorbent assay
FACS                 fluorescence-activated cell sorting
Fc                   fragment crystallizable region
FcRγ                 common gamma chain
FcRγ−/−               FcRγ-deficient
FcγR                 Fc-gamma receptor
FecR                Fc-epsilon receptor
FITC                 fluorescein isothiocyanate
FO B                 follicular B cell
HEL                  hen egg lysozyme
Ig                   immuno-globin
KLH                  keyhole limpet hemocyanin
MAb                  monoclonal antibody
MHC-II               major histocompatibility complex class II
MOMA                 metallophilic macrophages
MZ B                 marginal zone B cell
OVA                  ovalbumin
OVA-TNP              ovalbumin-2,4,6-trinitrophenol
PerCP-Cy5.5           Peridinin-chlorophyll proteins-cyanine 5.5
PBS                  phosphate buffered saline
PE                   phycoerythrin
RhD                  Rhesus antigen D
T cell               T lymphocyte
TCR                  T cell receptor
TNP                  2,4,6-trinitrophenol
INTRODUCTION

The immune system is a biological barrier which protects the organism from foreign materials such as viruses and bacteria, i.e. pathogens. It is commonly accepted that it can be divided into innate immunity and adaptive immunity. All kinds of organisms possess the innate immunity, composed of innate leucocytes, cytokines and complements, which is the first barrier and acts fast and broadly against pathogens. Only the highly evolved vertebrates possess the second barrier, a more specific and sophisticated immune system called adaptive immunity, consisting of cellular and humoral immunity. It is acquired from innate defense, thus comparatively slow and specific. Superiorly, in vertebrates, if the pathogens break through the first barrier and successfully get rid of the innate guard, the adaptive immunity will take over the protection responsibility. The pathogens will be taken through blood stream to the lymphoid organs. Take spleen as an example, they will first reach an area called marginal zone in the spleen, an interface between the non-lymphoid red-pulp and the lymphoid white-pulp, and then somehow entering into the follicles. The marginal zone is rich of marginal zone B cells (MZ B) and macrophages. The follicle can be divided into two parts - B cell zone and T cell zone. The B cell zone is rich of follicular B cells (FO B), while the T cell zone is rich of T cells and DCs. It is believed that though certain mechanisms, the pathogens can be transported from outside to the inside of the follicles, and then be endocytosed and presented out on the surface of certain antigen presenting cells. This is followed by priming, or activating the first time, the naïve T helper cells, i.e. CD4⁺ T cells, which in turn help the B cells differentiate into plasma B cells and memory B cells. Antigen-specific antibodies, when produced, will neutralize the antigens by forming immune complexes, which will accelerate the clearance of antigens.

Interestingly, apart from the function of neutralizing antigens, antibodies are also involved in regulatory immune system called antibody-mediated feedback regulation.

Antibody-Mediated Feedback Regulation

Antibodies, administered with their specific antigens, are able to induce complete suppression (>99%) or dramatic increase of antibody response, which is known as antibody feedback regulation [1, 2]. It has been clarified that suppressed or enhanced response is determined both by the class and isotype of antibodies and the property of antigens [3]. A well known example is the suppressive capacity of IgG when administered with large particulate antigen, like erythrocytes [4]. It has been clinically used to inhibit the immune response of rhesus D (RhD)-negative women against their RhD-positive fetuses when transplacental hemorrhage happens [5, 6]. In spite of the suppressed antibody response to large particulate antigens, IgG can also induce enhanced antibody response to soluble protein antigens [7]. However, the mechanisms behind feedback regulation still remains unknown.

Hypothetic Mechanism of IgG2a-Mediated Immune Response

There are four isotypes of murine IgGs: IgG1, IgG2a, IgG2b, and IgG3. Among the four subclasses, IgG1, IgG2a and IgG2b act in a similar way during antibody feedback regulation. The presence of activating FcγRs on bone marrow-derived cell types is required [8, 9], but not
the complement system [10]. It was observed that the antigen-specific antibody response was severely impaired in FcγR-/- mice (depletion of 80% of FcγRI and 100% of FcγRIII), in which the complement system is intact [8, 9, 10]. IgG2a has also been shown to be able to facilitate antigen presentation to T cells in vitro [11-17] and to induce an expansion of T cell population in vivo, a few days preceding the enhanced antibody response [18]. A working hypothesis depicts that IgG1-, IgG2a-, and IgG2b-antigen complexes bind to the activating FcγRs expressed on antigen presenting cells, and cause a more efficient internalization and presentation of antigens compared to non-complexed antigen. The focused antigen presentation to T helper cells will induce an expansion of T cell population, and in turn an increase of antibody response [19].

**Hypothetic Mechanism of IgG3-Mediated Immune Response**

In contrary to IgG1, IgG2a and IgG2b, IgG3-mediated enhancement requires the presence of complement system [20], but not FcγRs [8]. It was observed that the antibody response remained equally well in wild type mice, FcγR-/- mice (depletion of 80% of FcγRI and 100% of FcγRIII), FcγRIIβ-/- mice and FcγRI-/- mice [8], whereas was severely impaired in Cr2-/- mice (lack of complement receptor 1/2, CR1/CR2) and in mice depleted of complement receptor C3 by treatment with the cobra venom factor [20]. In addition, IgG3 showed a poor ability to induce the expansion of T cell proliferation both in vitro and in vivo [20]. A possible mechanism for IgG3-mediated enhancement is that the immune complex, formed by antigen, antigen-specific IgG3 and probably cooperative aggregation between IgG3s via Fc-Fc interaction [21-23], activates and induces the attachment to complement through the classical pathway. The IgG3-antigen-complement complexes subsequently co-crosslink the B cell receptors to the complement receptors (CR2/CR1 co-receptors), which in turn leads to enhanced antibody response by lowering the threshold of B cell activation [19].

**Hypothetic Mechanism of IgE-Mediated Immune Response**

Like the IgG isotypes, IgE was also recently described as an enhancing antibody which was completely dependent on the its low-affinity receptor, FcεRII/CD23 [24, 25]. IgE-antigen complexes were able to enhance the production of antigen-specific antibodies, which appeared unusually early and peaked at the sixth day post the priming administration [26, 27]. In vitro experiments showed that CD23+ B cells were required as antigen presentation cells for IgE-antigen immune complex to induce antigen-specific T-cell proliferation in both human and mouse [8-32]. In vivo experiments also showed that OVA-TNP, administered together with IgE, was able to induce OVA-specific T cell proliferation, followed by OVA-specific antibody response [33]. Furthermore, the early events leading to IgE-mediated antibody response were investigated and showed that OVA-TNP, complexed with IgE, could be captured by circulating B cells in the peripheral blood within 5 minutes, and could be traced binding to the follicular B cells in the B cell follicles within 30 minutes. Twelve hours later, OVA-specific CD4+ T cells were found localizing at the border of T and B cell zone. Three days later, an expansion of OVA-specific T cell population could be detected [34]. Recently it was found that antigen presentation to CD4+ T cells in IgE-mediated enhancement required CD11c+ DCs and not B cells, in contrary to the in vitro findings mentioned above. There was
evidence that IgE-antigen complex is delivered by CD23⁺ B cell to dendritic cell in the spleen before being presented to CD4⁺ T cells [35]. Therefore a reasonable hypothesis for IgE-mediated enhancement is that IgE-antigen complexes are first captured by circulating CD23⁺ B cells in the blood and transferred into the spleen. There they are handed over to CD11c⁺ DCs, and been processed and presented to antigen-specific CD4⁺ T cells, which subsequently induces the B cells to produce antigen-specific antibodies.

**Aims**

The objective of the present study is to further investigating the early events leading IgE-/IgG2a-/IgG3-mediated antibody response and/or T cell proliferation. Specific targets include:

1. To obtain potent antigens either by conjugating 2,4,6-trinitrophenol (TNP) with ovalbumin (OVA) or bovine serum albumin (BSA), and further label them with biotin for *in vivo* investigation.
2. To trace the distribution of IgE/IgG2a/IgG3-antigen complexes over time in vivo.
3. To figure out when, where and how the antigen delivery taken place by detecting the co-localization of CD11c⁺ DCs and antigen positive B cells, especially for IgE-mediated immune enhancement.

**MATERIALS and METHODS**

**Ethics Statement**

Mice were bred and raised in the animal facilities at the National Veterinary Institute (Uppsala, Sweden). All animal experiments were carried out with the approval of Uppsala Animal Research Ethics Committee.

**Mice**

BALB/c mice were obtained from Bomnice (Ry, Denmark). CD23-deficient (CD23⁻/⁻) mice [25], previously backcrossed to CBA/J mice for 12 generations [36], were further backcrossed to BALB/c mice for 10 generations [33]. DO11.10 mice were obtained from Dr. Westerberg (Karolinska Institute, Stockholm, Sweden) with the permission of Prof. K. Murphy (Washington University School of Medicine, St Louis, MO). DO11.10 mice carry rearranged transgenic T cell receptor (TCR)α and TCRβ gens which encode a TCR recognizing OVA peptide 323-339 bound to I-A⁺ Class II molecules [37]. Animals were matched for ages and sexes within each experiment.

**Antigens**

OVA, BSA and TNP were obtained from Sigma-Aldrich. TNP was conjugated to OVA as described [38]. After 170 minutes of incubation at 22.5°C water bath in the dark, the reaction were stopped by adding excess of glycyl-glycine (9-10 mg/ml; Merck). Proteins were dialyzed against PBS, sterile filtered, and stored at 4°C for later use. The coupling ratio of
TNP to OVA was determined by Nanodrop 1000 (Spectrophotometer) and the TNP over OVA ratio around 2.5 was used. OVA-TNP was further biotinylated using 4.8 mg of sulfo-NHS-LC-Biotin (Pierce) per 10mg of OVA-TNP, 40-fold molar excess of biotin were used. The reaction took place at room temperature for 2 hours and then 4°C in the dark over night. The biotinylated proteins were dialyzed against PBS, sterile filtered, and stored at 4°C in the dark for later use.

Antibodies

Monoclonal antibodies (mAb) used for immunization were derived from B cell hybridoma producing murine IgE anti-TNP (IGElb4) [39], IgG2a anti-TNP (C4007B4, 7B4) [7], IgG3 anti-TNP IM-H11) [20, 40] and were purified, stored, and measured as described [18, 20, 33, 40]. For flow cytometry, the following mAbs were used: phycoerythrin (PE)-labeled anti-CD4 (GK1.5; BD Bioscience), fluorescein isothiocyanate (FITC)-labeled anti-CD45R/B220 (RA3-6B2; eBioscience), allophycocyanin (APC)-labeled streptavidin (SA) (eBioscience), FITC-labeled anti-CD21/CD35/C2R2/Cr1 (eBio8D9; eBioscience), PE-labeled anti-CD23/FcRII (B3B4; eBioscience), Peridinin-chlorophyll proteins-cyanine 5.5 (PerCP-Cy5.5)-labeled anti-CD45R/B220 (eBio8D9; eBioscience) and Fc-block (2.4G2, BD Bioscience). The DO11.10-transgenic TCR was detected with a FITC-labeled KJ1-26 mAb (Caltag Laboratories) which is specific for this particular TCR heterodimer. For immunostainings, the following mAbs were used: Pacific Blue-labeled anti-CD45R/B220 (RA3-6B2; BD Bioscience), PE-labeled anti-CD11c (HL3; BD Bioscience), FITC-labeled anti-Metallophilic Macrophage (MOMA) (MCA947F; SeroTec), and APC-labeled SA (eBioscience). For enzyme-linked immune sorbent assay (ELISA), the following mAbs were used: alkaline phosphatase-conjugated sheep anti-mouse IgG (Jackson ImmunoResearch Laboratories).

Immunizations

OVA-TNP or biotin-OVA-TNP was mixed with IgE/IgG2a/IgG3 anti-TNP at room temperature and was given to mice in PBS through the tail vain within 1 hour in a total volume of 200μl each. The amount of antigens and antibodies are indicated in each figure legend respectively. This experimental setup applied to all experiments except for the optimization test shown in Figure 1A.

Adoptive transfers

Spleens of DO11.10 mice were removed and smashed in 10 ml PBS each to single cell suspensions. Cells from every spleen were centrifuged at 400 rpm for 5 minutes and resuspended in 600 μl PBS. BALB/c recipient mice were immunized through the tail vain with a total volume of 200 μl each, i.e. one third of DO11.10 donor mouse spleen cells were transferred into each BALB/c recipient.

Flow Cytometry

Blood samples (around 200 μl blood in 100 μl heparin for anticoagulation) were depleted of
erythrocytes by treated with ACK lysis buffer (0.15 M NH₄Cl, 1.0 M KHCO₃, 0.1 M Na₂EDTA, pH 7.3) for 3 to 5 minutes at room temperature. Cells were then washed and resuspended in FACS buffer (PBS with 2% fetal bovine serum). Total blood cells were suspended in a total volume of 50 μl. For spleen samples, erythrocytes in the spleen cell suspensions were depleted by treated with ACK lysis buffer (0.15 M NH₄Cl, 1.0 M KHCO₃, 0.1 M Na₂EDTA, pH 7.3) for 3 to 5 minutes at room temperature. The same amount of PBS were added to terminate the lysis process. Spleen cells were then washed and resuspended in FACS buffer (PBS with 2% fetal bovine serum). Appropriate amount of spleen cells were suspended in a total volume of 50 μl. The fluorescence staining were performed at 4°C in the dark for 30 minutes by addition of 50 μl predetermined optimal amounts of labeled antibodies and Fc-block. After 30 minutes, cells were washed twice using FACS buffer. No less than 200 000 events were acquired by a LSRII cytometer (BD Bioscience) through gating on all cells with the forward- and side- scatter properties of lymphocytes. Data were analyzed using FlowJo software (Tree Star Inc). Follicular B cells were gated as CD23hiCD21lo and marginal zone B cells were gated as CD23loCD21hi [41, 42].

**ELISA Analysis**

Mice were bled from the tail and sera were tested in ELISA for OVA-specific IgG. Microtitre plates (Immunolon 2B; Dynex Technologies) were coated with 100 μl of 5% OVA (50 μl/ml OVA in PBS with 0.05% NaN₃) at 4°C overnight and blocked with 200 μl of 5% dry milk (50 μg/ml OVA in PBS with 0.02% NaN₃). Sera samples and polyclonal OVA-specific standard IgG, serially diluted in PBS containing 0.05% Tween-20, 0.25% dry milk, and 0.02% NaN₃, were added and incubated for 3 hours at room temperature. After washing, 100 μl of substrate (P-nitro-phenylphosphate; Sigma-Aldrich) diluted in diethanolamine buffer was added and the absorbance at 405 nm was measured after 30 minutes of incubation at room temperature. For OVA-specific ELISA, a polyclonal OVA-specific standard serum with a starting concentration of 0.295 μl/ml was used.

**Immuo-Fluorescence Staining for Confocal Microscopy**

Spleens were flash frozen in O.C.T. compound (Sakura Finetek). Eight microliter-thick sections were harvested using a cryostat (Leica CM 1800), and air dried onto microscope slides (Menzel-Gläser). The slides were rehydrated and washed in PBS for fifteen minutes at room temperature, and blocked with 5% horse sera (Sigma) for 1 hour at room temperature. Excessive blocking solution was tapped off. B cells were detected using 2 μg/ml Pacific Blue-labeled anti-CD45R/B220. DCs were detected by 2 μg/ml PE-labeled anti-CD11c. Biotinylated-antigens were detected by 3 μg/ml APC-labeled SA. The border of follicle was lined out by detecting marginal zone macrophages using 1 μg/ml FITC-labeled anti-Metallo Macro. The slides were stained at room temperature for 1 hour in the dark and then washed twice in PBS for 5 minutes each and finally mounted in Fluoromount G (Southern Biotech). Immuno-fluorescence was detected by a LSM 700 confocal microscope (Carl Zeiss). Photos of three non-consecutive sections from each spleen were taken by ZEN 2009 software (Carl Zeiss). Data were analyzed using ImageJ software (NIH).
Statistical Analysis

Statistical differences between groups were determined by Student’s t-test. Values for P>0.05 (no significance), P<0.05, P<0.01, or P<0.001 are indicated in figure legends.

RESULTS

Immune Complex Induces the Expansion of OVA-Specific CD4+ T Cell Population and the Enhancement of IgG Anti-OVA Response

To test the antibody-mediated feedback regulation, the first thing is to get a potent antigen which, when administered with its specific antibody, has the ability to induce enhanced antibody response and augmented T cell proliferation. OVA-TNP is a potent candidate which worked well previously in our lab [34]. To determine whether our newly conjugated OVA-TNP is potent or not, it was given to BALB/c mice with or without IgE at different dosages and incubation conditions. It showed that 20 μg of OVA-TNP given to mice had better results compared to 50 μg dose, and incubation at room temperature or at 37°C both gave the same result [Figure 1A]. Therefore we decided to immunize mice with 20 μg of OVA-TNP alone or mixed with 50 μg of antibodies at room temperature 1 hour before injection. The ELISA also showed that OVA-TNP, administered with IgE/IgG2a/IgG3, induced significant enhancement of anti-OVA IgG production [Figure 1B]. These results demonstrate that the newly conjugated OVA-TNP performed potently for our designed experiment system.

Figure 1. Immune Complexes Induce the Expansion of OVA-Specific CD4+ T Cell Population and the Enhancement of IgG Anti-OVA Response. (A) BALB/c mice were adoptively transferred i.v. with one third of spleen cells from DO11.10 mice. 24 hours later, the recipients were immunized with 20 μg of OVA-TNP alone, 20 μg of OVA-TNP mixed with 50 μg of IgE at room temperature, 20 μg of OVA-TNP pre-incubated with 50 μg of IgE at 37°C for 1 hour, 50 μg of IgE alone, 50 μg of OVA-TNP alone, or 50 μg of OVA-TNP mixed with 50 μg of IgE at room temperature. The antigens or immune complexes were given in PBS in a total volume of 200 μl through the tail vain within 1 hour after
mixing. Three days after immunization, the spleens were removed and prepared for FACS. The percentage of OVA-specific KJ1-26+ cells among CD4+ T cells were determined by flow cytometry. (B) BALB/c mice were immunized with 20 μg of OVA-TNP alone, or together with 50 μg of IgE/IgG2a/IgG3. Fourteen days later, mice were bled and the sera was tested by ELISA. Statistical significance were determined by student’s t-test. *, p<0.05; **, p<0.01; ***, p<0.001; ns, no significant difference. SI, stimulation index. n = two mice per group.

Enhancement of Immune Response In Vivo Can Be Detected and Visualized In Flow Cytometry and Confocal Microscopy

To trace the antigen distribution in vivo, we labeled OVA-TNP with biotin. Biotinylated OVA-TNP with or without IgE, were administered to BALB/c and CD23−/− mice. The antigen-carrying B cells, both in the peripheral blood and in the spleen, were traced by detecting the biotinylated antigen using APC-labeled SA, which has an high affinity for biotin. In BALB/c mice, nearly 80% of the IgE-complexed OVA-TNP were captured by peripheral B cells, which showed a huge increase of antigen-positive B cells compared to antigen given alone [Figure 2A]. In CD23−/− mice, there was no such difference, which confirms that CD23 is important for IgE-mediated antigen transportation [24, 25, 34]. The same trend was showed in the spleen of BALB/c mice, while there was a decrease in antigen-carrying B cells in CD23−/− mice, when antigen were administered together with IgE [Figure 2B]. We could also visualize the antigen location by staining the spleen sections 30 minutes post administration in both BALB/c and CD23−/− mice [Figure 2C]. The percentage of antigen-carrying B cells in each B cell follicle was determined using Manders’ Coefficient in the ImageJ software. The average percentage of antigen-carrying B cells per follicle increased in BALB/c mice but not in CD23−/− mice, comparing antigen given with or without IgE [Figure 2D and 2E]. In summary, biotinylation is a useful labeling method for in vivo antigen tracing using flow cytometry and confocal microscopy, at least within 30 minutes.
Figure 2. Enhancement of Immune Response In Vivo Can Be Detected and Visualized In Flow Cytometry and Confocal Microscopy. (A-B) BALB/c mice and CD23−/− mice were immunized with 150 μg of biotin-OVA-TNP alone, or together with 50 μg of IgE. The percentage of antigen-carrying B cells among B220+ B cells in peripheral blood 5 minutes after immunization or in the spleen 30 minutes after immunization was determined by flow cytometry. (C) The spleen FACS used half of the spleens, while the other halves were frozen, sectioned, and stained for B cells using Pacific Blue-labeled anti-CD45R/B220 (blue) and biotinylated antigen using APC-labeled SA (red). (D) The percentage of antigen-carrying B cells per cell follicle and (E) the average percentage of antigen-carrying B cells per mouse were determined by ImageJ using Manders’ Coefficient. Statistical significance were determined by student’s t-test. *, p<0.05; ns, no significant difference. IC, IgE-antigen immune complex. n= two mice per group.

IgE-Antigen Immune Complex Binds to Circulating B cells and Migrates to B Cell Follicles Most Efficiently

To compare the binding and migrating efficiency of IgE/IgG2α/IgG3-mediated antigen transportation, BALB/c mice were immunized with antigen alone, or together with IgE/IgG2α/IgG3 and analyzed. IgE-mediated transportation seemed to be the most efficient, since about 80% of IgE-antigen complexes were quickly taken up by circulating B cells in the peripheral blood within 5 minutes and could also be detected in the spleen within 30 minutes, whereas IgG2a- or IgG3-mediated antigen transportation are less efficient to be detected [Figure 3A and 3B]. The confocal-microscopy results of antigen distribution mediated by IgE/IgG2α/IgG3 are shown in Figure 3C. The border of follicle is lined out by marginal zone macrophages detected by FITC-labeled anti-MOMA (green). The percentage of
antigen-carrying B cells in each B cell follicle was determined using Manders’ Coefficient in the ImageJ software. The average percentage of antigen-carrying B cells per follicle increased dramatically in IgE-antigen complex immunized mice and increased a little bit in IgG3-antigen complex immunized mice, comparing to mice given antigen alone or together with IgG2a [Figure 3D and 3E]. Therefore, generally, IgE-antigen immune complex possess the highest efficiency in both binding to peripheral B cells and migrating into B cell follicles.
Figure 3. IgE-Antigen Immune Complexes Bind to Circulating B Cells and Migrate to B cell Follicles Most Efficiently. (A-B) BALB/c mice were immunized with 150 μg of biotin-OVA-TNP alone, or together with 50 μg of IgE/IgG2a/IgG3. The percentage of antigen-carrying B cells among B220+ B cells in peripheral blood 5 minutes after immunization or in the spleen 30 minutes after immunization was determined by flow cytometry. (C) The spleen FACS used half of the spleens, while the other halves were frozen, sectioned, and stained for B cells using Pacific Blue-labeled anti-CD45R/B200 (blue), marginal zone cells using FITC-labeled anti-MOMA (green), and biotinylated antigens using APC-labeled SA (red). The pictures in the left part of each group are original ones, while the right part are pictures with enhanced signals processed by ImageJ using Find Edge. (D) The number of antigen-carrying B cells per follicle and (E) the average number of antigen-carrying B cells per mouse were determined by ImageJ using Manders’ Coefficient. Statistical significance were determined by student’s t-test. *, p<0.05. n= two mice per group.

Kinetics of IgE-/IgG2a-/IgG3-Antigen Distribution in the Spleen

To determine the sub-locations of the different immune complexes, several cell populations were checked for antigen binding. Spleen cells were analyzed and two common populations were first gated out: CD23highCD21lo as follicular B cells, CD23loCD21high as marginal zone B cells [Figure 4A and 4B]. 10 minutes post administration, IgE-antigen complexes localized mostly inside the B cell follicles, while IgG3-antigen complexes localized at the marginal zone of B cell follicle [Figure 4E and 4F]. A new cell population was later discovered which showed a very strong signal of IgG2a-antigen [Figure 4G]. The cell marker profile of this population was B220interCD21interCD23lo, as indicated by Figure 4C and 4D.

To further study the kinetic distribution of different antibody-mediated immune complexes, mice were immunized with antigens alone or complexed with IgE/IgG2a/IgG3. Spleen cells were analyzed at six different time points post administration: 10 minutes, 30 minutes, 1 hour, 4 hours, 16 hours, and 24 hours. Most of the IgE-antigen complexes reached the marginal zone and started entering into the B cell follicles within 10 minutes. They accumulated in the B cell follicles within 30 minutes, with a peaking percentage around 18%, i.e. 18% of the follicular B cells were detected as antigen positive. Later they were decreased and subsequently diminished within 16 hours [Figure 4H]. IgG3-antigen complexes reached and accumulated in the marginal zone of B cell follicles within 10 minutes, and then were somehow partly transported into the B cell follicles within 30 minutes [Figure 4H and 4I], followed by a reverse accumulation and peaked within 1 hour after immunization, with a peaking percentage around 5%. Later they decreased and completely diminished within 4 hours [Figure 4I]. IgG2a-antigen complexes accumulated into the new population extremely quickly and massively within 10 minutes after immunization, with a peaking percentage about 61%, followed by a sharp decrease and a total diminish at 4 hours after immunization [Figure 4J]. In summary, different antibody-mediated immune complexes accumulate into different kinds of cells and possess a totally different kinetic pattern in antigen delivery.
Figure 4. Kinetics of IgE-/IgG2a-/IgG3- Mediated Antigen Distribution in the Spleen. BALB/c mice were immunized with 150 μg of biotin-OVA-TNP alone, or together with 50 μg of IgE/IgG2a/IgG3. 10 minutes later, spleens were removed and prepared for FACS. (A-B) Follicular B cells (FO B) and marginal zone B cells (MZ B) were gated as described in Methods and Materials. (C-D) The new population was gated as B220<sup>int</sup>CD21<sup>int</sup>CD23<sup>lo</sup>. (E-G) 10 minutes post immunization, IgE-antigen immune complex were found mostly bound to follicular B cells, IgG2a-antigen immune complex bound to marginal zone B cells, and IgG3-antigen immune complex bound to a new population gated as B220<sup>int</sup>CD21<sup>int</sup>CD23<sup>lo</sup>. (H-J) The kinetic distribution of antigens in different cell types.
populations in mice immunized with antigen alone, or together with IgE/IgG2a/IgG3. n= two mice per group.

DISCUSSIONS

Soluble protein antigens, when administered together with their specific IgE/IgG2a/IgG3, could induce dramatic increase of T cell proliferation (except for IgG3) and antibody responses [18, 33, 40]. There are several kinds of soluble carrier proteins which have the potency to induce increased immune response, such as OVA, BSA, KLH (keyhole limpet hemocyanin). In this study, we have tested the immunogenicity of both OVA and BSA. Unfortunately, BSA didn’t show stable performance in antibody response (data not shown). Instead, we concentrated on OVA test. After obtaining the newly conjugated OVA-TNP, we optimized the dosage and incubation time of OVA-TNP and TNP-specific IgE in T cell proliferation test [Figure 1A]. Then we followed the optimized experimental set up and found that OVA-TNP , when administered together with TNP-specific IgE/IgG2a/IgG3, could induce OVA-specific CD4+ T cell expansion (except for IgG3) and enhanced OVA-specific IgG response [Figure 1, 40]. The potent OVA antigens were then labeled with biotin and given to mice alone or together with IgE. The enhancement of antibody response and T cell proliferation in vivo can be detected and visualized in flow cytometry and confocal microscopy [Figure 2].

Different antibody-mediated immune response require the aid of different cells which are summarized in Table 1. IgE-mediated enhancement of antibody response and T cell proliferation needs the aid of CD23+ B cells, as well as CD11c+ cells as antigen presenting cells [33, 34, 35, 36, Table 1]. IgG2a-mediated immune enhancement is dependent on activating FcγR expressing cells [8, 9, 18, Table 1]. IgG3, in analogy to IgM, requires the complement system, typically CD21/CD35 (CR1/2) expressing cells, to induce augmented feedback regulation [20, Table 1].

Table 1. Comparison of Different Antibody-Mediated Immune Responses to Soluble Antigens

<table>
<thead>
<tr>
<th>Immune Complex</th>
<th>Transporting / Binding Cells</th>
<th>Cells Needed for Inducing Immune Responses</th>
<th>Enhancement of T Cell Proliferation/ Antibody Response</th>
<th>Immune Complexes Localization in the Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgE-Ag</td>
<td>CD23+ B cells [33, 34, 36]</td>
<td>CD11c+ cells [35]</td>
<td>Yes/Yes [33]</td>
<td>FO B [Figure 4A and 4E]</td>
</tr>
<tr>
<td>IgG2a-Ag</td>
<td>?</td>
<td>Activating-FcγR expressing cells [8, 9, 18]</td>
<td>Yes/Yes [18]</td>
<td>B220intCD21intCD23lo [Figure 4C and 4G]</td>
</tr>
</tbody>
</table>
Therefore the antigen-delivery efficiency mediated by different antibodies might vary. Our data showed that IgE-antigen immune complexes have the highest efficiency in binding to circulating B cells and migrating to B cell follicles, compared to antigen alone and IgG2a-/IgG3-antigen complexes [Figure 3]. Impressively, IgE-antigen complexes migrated so rapidly and massively into the B cell follicles within 30 minutes. While during the same time period, there were only a small amount of IgG3-antigen immune complexes in the follicles, and almost all IgG2a-antigen complexes were still remained in the marginal zone [Figure 3C].

For IgE-mediated immune response, Henningson et al. showed the evidence that CD11c⁺ cells are required for antigen presentation [35]. Given to the good performance of biotinylated antigens, it might be the high time to find evidence for the antigen delivery from B cells to DCs. Spleen sections of 30 minutes after immunization were stained for both B220⁺ B cells and DC cells, disappointedly we could not find any signals indicating for DCs in the sections (data not shown). It might be that the biotinylated antigens had already been over-processed 30 minutes after immunization, which made it difficult for us to detect the antigens if only using APC-labeled SA. And actually we've also tried another labeling kit called PKH26, but still nothing happened. And it is also possible that we checked at a wrong time point. We thought we had better narrow down the time period first. Study of the consecutive events during antibody-mediated immune enhancement had already been carried out by Hjelm et al.[34]. However, they only tested three time points during the very early phase, which was far from enough for our purpose. Therefore we decided to check more time points between 10 minutes to 24 hours after immunization. It showed that the accumulation of IgE in B cell follicles peaked at 30 minutes after immunization and totally diminished within 16 hours after immunization. Hjelm et al.[34] had shown that OVA-specific CD4⁺ T cells migrated and localized close to the T-B border during 6 to 12 hours after immunization, and proliferated massively 3 days after immunization [Table 2]. Take all these information into consideration, one conclusion is that antigen delivery from B cells to DCs probably occur between 30 minutes to 16 hours after immunization.

**Table 2. Consecutive Events in IgE/IgG2a/IgG3-Mediated Immune Enhancement**

<table>
<thead>
<tr>
<th>Time after Primary Immunization</th>
<th>Effect of IgE-Mediated Immune Response</th>
<th>Effect of IgG2a-Mediated Immune Response</th>
<th>Effect of IgG3-Mediated Immune Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 minutes</td>
<td>Antigens captured by circulating CD23⁺ B cells in the blood [34, Figure 2A]</td>
<td>Antigens NOT captured by circulating B cells in the blood [Figure 2A]</td>
<td>Antigens NOT captured by circulating B cells in the blood [Figure 2A]</td>
</tr>
<tr>
<td>10 minutes</td>
<td>Antigens accumulating on</td>
<td>Antigens peaked on</td>
<td>Antigens accumulating on</td>
</tr>
</tbody>
</table>
FO B in the spleen [Figure 4E]  
B220<sup>high</sup>CD21<sup>high</sup>CD23<sup>low</sup> cells [Figure 4G and 4J]  
MZ B cells [Figure 4F and 4I]  

<table>
<thead>
<tr>
<th>Time</th>
<th>FO B cells</th>
<th>B220&lt;sup&gt;high&lt;/sup&gt;CD21&lt;sup&gt;high&lt;/sup&gt;CD23&lt;sup&gt;low&lt;/sup&gt; cells</th>
<th>MZ B cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 minutes</td>
<td>Antigens peaked on FO B cells [Figure 4H]</td>
<td>Antigens decreasing from B220&lt;sup&gt;high&lt;/sup&gt;CD21&lt;sup&gt;high&lt;/sup&gt;CD23&lt;sup&gt;low&lt;/sup&gt; cells [Figure 4J]</td>
<td>A partial of Antigens delivered from MZ B cells to follicular B cells [Figure 3B, 4H and 4I]</td>
</tr>
<tr>
<td>1 hour</td>
<td>Antigens decreasing from FO B cells [Figure 4H]</td>
<td>Antigens decreasing from B220&lt;sup&gt;high&lt;/sup&gt;CD21&lt;sup&gt;high&lt;/sup&gt;CD23&lt;sup&gt;low&lt;/sup&gt; cells [Figure 4J]</td>
<td>Antigens peaked on MZ B cells [Figure 4I]</td>
</tr>
<tr>
<td>4 hours</td>
<td>Antigens decreasing from FO B cells [Figure 4H]</td>
<td>Antigen diminished from B220&lt;sup&gt;high&lt;/sup&gt;CD21&lt;sup&gt;high&lt;/sup&gt;CD23&lt;sup&gt;low&lt;/sup&gt; cells [Figure 4I]</td>
<td>Antigens diminished from MZ B cells [Figure 4I]</td>
</tr>
<tr>
<td>6-12 hours</td>
<td>Antigen-specific T cells localized to the T-B border in spleen [34]</td>
<td>Antigen-specific T cells localized to the T-B border in spleen [34]</td>
<td>-</td>
</tr>
<tr>
<td>16 hours</td>
<td>Antigens diminished from FO B cells [Figure 4H]</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3 days</td>
<td>Massive proliferation of antigen-specific T cells [33, 34]</td>
<td>Massive proliferation of antigen-specific T cells [34]</td>
<td>No proliferation of antigen-specific T cells [40]</td>
</tr>
<tr>
<td>7-14 days</td>
<td>Increased number of germinal centers in the B cell zone [34]</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>14 days</td>
<td>Enhanced primary antibody response [Figure 1B]</td>
<td>Enhanced primary antibody response [Figure 1B]</td>
<td>Enhanced primary antibody response [Figure 1B]</td>
</tr>
<tr>
<td>138 days</td>
<td>Enhanced recall response when boosted with antigen alone [26]</td>
<td>-</td>
<td>-</td>
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</table>

Incidentally, we noticed a new cell population bound by IgG2a-complexed antigens, since about 61% of IgG2a-antigen complexes localized and peaked in the new population within only 10 minutes, which is even faster and more massive than antigen complexed with IgE to some extent. However, few IgG2a-antigen complexes were detected in follicular B cells during the whole testing period. We’ve further checked the cell marker profile of the new population, and found that they were low in CD23 expression but high in major histocompatibility complex class II (MHC-II) expression. What’s more, they had autofluorescence in both B220 and CD21 detecting channels, which explained the intermediate expression of B220 and CD21 in Figure 4C. Our preliminary conclusion is that they might be neutrophils, and macrophages as well. As to the IgG3-mediated immune response, the preferred binding cells seemed to be marginal zone B cells, although the results were not significant compared to IgE and IgG2a.
In summary, IgE, IgG2a and IgG3 use totally different mechanisms to regulate immune response and thus possess various kinetic properties in antigen distribution, for example various time pattern and binding cells types. The summarized consecutive events in different antibody-mediated immune response in Table 2 will help us to subdivide the whole process more accurately. And for this specific project, we can tell that antigen delivery from B cells to DCs in IgE-mediated immune enhancement is probably taken place between 30 minutes to 16 hours after immunization.

Therefore the next step is to find a more sensitive method to trace the biotinylated antigens, or to use other advantageous labels instead of biotin. Hen egg lysozyme (HEL) is now under test which has the advantage of being processed and presented on MHC-II molecules of APCs in the form of HEL peptides. We have a monoclonal antibody which can recognize the MHC-II molecule together with the HEL peptide presented on its surface. If the HEL peptide can be detected as presented on the MHC-II molecules of DCs, we can be sure that DCs somehow take over the antigens from CD23+ B cells. After that we can use confocal microscopy to look for the direct evidence of antigen delivery between B cells and DCs.
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REFERENCES


