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# Fast track selection of immunogens for novel vaccines through visualisation of the early onset of the B-cell response

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**Abstract**

A most essential step in vaccine research and development, ie vaccine studies in animals, seriously suffer from long timespans needed to arrive at effective immunogens. In this report we show how almost immediately after vaccination the antibody inducing potential of low immunogenic 'self' antigens can be accurately assessed. (We expect that this timespan can be reduced even more when 'non self' antigens are used, since such responses should be stronger.) The method takes advantage of the immediate onset after vaccination of the immune response in the spleen.

This novel method allows detection of antigen-specific B cells of the spleen as early as 7 days after immunization and at frequencies as low as 10 in 1,000,000 cells. The method depends on sequential staining with PE- and APC-conjugated tetramers, made with the same biotinylated peptide. The antigenic peptides are biotinylated and tetramerized with either PE neutravidin or APC streptavidin. We expect that this method can be generally applied to visualize B cell responses, irrespective of the way they are induced. In addition to the fast selection and development of novel immunogens, this procedure can be used to delineate the kinetics of the B cell response, to phenotypically characterize and to isolate antigen-specific B cells, and, perhaps most importantly, to count them at the clonal level before any circulating antibodies can be detected.


*Keywords:* Vaccine; Immunogen selection; Early B-cell response

## 1. Introduction

Immunization studies in animals for vaccine research and development seriously suffer from long timespans needed to arrive at effective immunogens. Vaccine efficacy studies often involves the assesment of the ability of the antibodies raised, to produce the desired biological effect: the neutralization of infection (preventive vaccines), the neutralization of signal molecules (for instance tumor associated growth factors) or the ability to bind to cell surface receptors.

Both for preventive and therapeutic vaccines the first phase always involves sets of lead antigens each of which has to be tested for its ability to induce antibodies that bind the native antigen. This initial phase may require years because the lengthy vaccination procedure to select immunogens and subsequently to improve the selected ones needs to repeated several times. This obviously forms a major stumbling block for research and development of vaccines, moreover it is a large disadvantage for the commercial potential of vaccines since it severely limits the time that vaccines can be sold before the patents run out.

However, a small but discrete population of antigen-specific B cells is present within a few days after immunization [1,2]. These antigen-specific B-cells expose on their

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surface fore runners of the antibodies that are going to be produced, i.e. the B cell receptors (BCRs) [1,2]. It is assumed that the quality of these B cell receptors and the quantity of B cells carrying them, reflect the quality and the quantity of the antibodies that will populate the circulation when the immune response has fully matured [1,2]; i.e. whether a substantial part of the expected antibodies will cross react with the native target antigen.

Because existing methods are unable to do this fast and accurately we have developed a new method which allows precise counting of specific B-cells using FACS analysis of spleens cells of mice vaccinated 7 and 14 days earlier.

This new method is based upon direct visualization of antigen-specific B-cells by flow cytometry using standardized reagents. In recent years, several flow cytometry-based strategies have been developed to improve antigen-specific B cell detection [3–7]. The field was pioneered by Hayakawa et al. [3], using the fluorescent protein phycoeryhtrin to visualize specific memory B cells at frequencies of 0.02–0.05% of splenocytes. McHeyzer-Williams and coworkers [4,5] subsequently used fluorescently labeled nitrophenol to detect specific B cells at frequencies of approximately 2% of splenocytes at day 12 after hapten immunization. Next, Townsend et al. [6] succeeded in lowering the detection threshold by staining B cells with two reagents that had been conjugated to two different labels, but which were specific for the same B cell receptor (dubbed 'single epitope multiple staining') [6]. The principle of this sequential staining procedure (in which the first reagent is used at subsaturating concentrations) is that the probability of false positive cells binding *both* reagents is much smaller than the probability of false positive cells binding only one of the reagents [2,6]. However, the authors of this study [6] only characterized adoptively transferred BCR-transgenic cells, leaving the question unanswered whether their methods could be extrapolated to the analysis of bona fide immune responses, including those induced by vaccines.

In the present study, we describe a general B cell detection method that is aimed at the visualization of low-frequency antigen-specific B cells, i.e. at frequencies $\leq$ 10/1,000,000 splenocytes. To achieve this, we have used the tetramer concept [1,7] to increase ligand avidity, and the 'single epitope multiple staining' principle [6] to reduce background due to non-specific binding. Combining these two methods was essential for the success of our method, since neither single tetramer staining, nor double staining with labeled peptides produced satisfactory results.

To demonstrate the feasibility of our approach we have used immunization with gonadotropin releasing hormone (GnRH)-like peptides [8] as a model. Vaccination against the self antigen GnRH, which can be used to achieve a shutdown of the fertility axis by anti-hormone antibodies, involves breaking of tolerance and requires modification of the antigen itself or the way it is presented. For this reason, we used three different GnRH-like peptides conjugated to ovalbumin, i.e. GnRH-monomer, GnRH-tandem and GnRH tandem dimer (TDK) [9]. Previously, we demonstrated that the enlarged GnRH molecule, GnRH tandem, consisting of twice the decapeptide is superior over the GnRH monomer. Testes development in young male pigs was fully suppressed in the GnRH tandem treated pigs whereas in the monomer treated only 60% was successfully treated [8]. Efficacy of the GnRH tandem could even be improved by dimerization of the peptide and introduction of D-amino acids [9]. This peptide (TDK) appeared to be effective in very low doses and with mild adjuvants. Herein, we show that immunization with these three GnRH vaccines induces B cell responses ranging in magnitude from 20 to 200 specific B cells per $10^6$ splenocytes (depending on the type of peptide construct used), and that these responses can be detected as early as day 7 after a single immunization. We found that all GnRH-binding cells were CD19 and B220 positive, identifying them as true B cells [10,11]. In addition, these cells were predominantly CD138 (syndecan) [12] and CD27 [13,14] negative, providing some initial clues on their phenotype.

The method that we describe allows the accurate detection and phenotypic characterization of low-frequency antigen-specific B cells as early as 7 days after vaccination. It suggested that this method is a key tool in the rapid evaluation of vaccine immunogenicity and specificity. The results for three different GnRH vaccines are in excellent agreement with their established biological efficacy. This suggests indeed that, at this very early stage after vaccination, an accurate assessment can be made of the vaccine potential of immunogens.

## 2. Results

### 2.1. Visualization and characterization of GnRH specific B cells

Several methods have been described to detect antigen-specific B cells using flow cytometry [3–7] (Fig. 1). To compare the usefulness of the different methods, we immunized OF1 mice with GnRH-like peptides conjugated to ovalbumin (OVA). We prepared three different peptides, i.e., GnRH-monomer (pEHWSYGLRPGC) GnRH-tandem (pEHWSYGLRPGQHWSYGLRPGC) and GnRH tandem dimer (TDK, the dimerized form of pEHWSYkLRPGQHWSYkLRPGC in which 'k' represents a D-lysine residue). The GnRH tandem and TDK peptide conjugated to a carrier protein have shown to be highly immunogenic, resulting in GnRH neutralizing antibodies and a full biological effect in all treated animals [8,9].

Mice were immunized once with the GnRH vaccines, or twice with an interval of 7 days. Splenocytes were harvested at day 7 (after a single immunization) and at day 14 (i.e., 7 days after the booster vaccination). To detect GnRH-specific B cells we used directly labeled native GnRH, or labeled tetramers with native GnRH, both in single and double staining protocols. GnRH tetramers were produced by incubating biotinylated GnRH with fluorescently labeled streptavidin or neutravidin. Monoclonal antibodies against the B-cell marker