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REVIEW

Enhanced immunization techniques to obtain highly specific monoclonal antibodies

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ABSTRACT

Despite fast advances in genomics and proteomics, monoclonal antibodies (mAbs) are still a valuable tool for areas such as the evolution of basic research in stem cells and cancer, for immunophenotyping cell populations, diagnosing and prognosis of diseases, and for immunotherapy. To summarize different subtractive immunization approaches successfully used for the production of highly specific antibodies, we identified scientific articles in *NCBI PubMed* using the following search terms: *subtractive immunization, monoclonal antibody, tolerization, neonatal, high-zone tolerance, masking immunization.* Patent records were also consulted. From the list of results, we included all available reports, from 1985 to present, that used any enhanced immunization technique to produce either polyclonal or monoclonal antibodies. Our examination yielded direct evidence that these enhanced immunization techniques are efficient in obtaining specific antibodies to rare epitopes, with different applications, such as to identify food contaminants or tumor cells.

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Introduction

Despite the growing and fast advances in genomics and proteomics, monoclonal antibodies (mAbs) remain a valuable biotechnology-derived product, especially for many areas such as the evolution of basic research in stem cells and cancer, for immunophenotyping different cell populations, for diagnosing and prognosing diseases, and for immunotherapy. It is important to highlight that even for diagnosis, which one may consider an outdated theme regarding mAbs, the discovery of new biomarkers such as selective antigens from stem or tumoral cells, or different stages of diseases, still leaves an enormous gap to be explored using these tools.[1] Moreover, given that the patents for many marketed antibodies have expired or will expire soon, the development of biosimilar products further emphasize the importance of mAbs, especially for pharmaceutical companies and for emerging countries that will be able to produce their own antibodies, reducing the costs of the previously imported ones.[2] For example, it has been estimated that $378 billion could be saved during the next 20 y in the US, as a result of biosimilar competition with their reference counterparts.[2]

The production of mAbs was placed in the spotlight after the hybridoma method was described by Köhler and Milstein in 1975,[3] but the isolation of a specific antibody that recognizes rare antigens or poorly antigenic ones remained a challenge for decades. This difficulty can now be easily understood by analyzing the classic immunization protocols and techniques used after the publication of their study. Excitement around the hybridoma technology blinded researchers to the fact that a huge number of immunodominant epitopes would mask other less antigenic ones, which would have the potential of generating the desired selective and specific antibodies, especially when whole cells are used as immunogen.

After a decade of monoclonal production via hybridomas, in which researchers always adopted the classical immunization protocols, the first report of an enhanced immunization protocol came from Sharpe et al.[4] in a hypotheses paper describing that the neonatal tolerance would increase the efficacy of antibody production. In this paper, the authors wrote: "*We have shown that if mice are immunized with a human B cell line then the antisera they produce also reacts strongly with human T cells. However, if the mice are first neonatally tolerized to a human T cell line and then immunized with the B cell line the antisera becomes much more specific for the B cell line..*"[4] From the results obtained in polyclonal antibody (pAb) production,

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the authors end their paper correctly concluding that: "*In addition, this technique should theoretically make it possible to find monoclonal antibodies specific for rare cellular determinants in a much more direct fashion.*"[4]

The initial findings of Sharpe et al. lead researchers to develop other immunization techniques to obtain particularly selective antibodies. Generally known as subtractive immunizations techniques, these include subtractive immunizations using a chemotherapy agent (drug-induced subtractive immunization), the combination of neonatal tolerization with drug-induced subtractive immunization and high-zone tolerance protocols. The techniques are based on the induction of an immunosuppressive state (triggered when desired, normally during the immune response that would lead to the production of unwanted/undesired antibodies) ultimately leading to a decrease of the response toward immunodominant epitopes, favoring the response toward less antigenic epitopes, or even rare antigens.[5,6,7,8]

This review aims to summarize the uses of different subtractive immunization techniques (Table 1) in the production of antibodies with selective affinity, describing the mechanisms behind them, and detailing each different protocol used for this end. Scientific articles cited in this review were identified in *NCBI PubMed* using the following search terms: *subtractive immunization, monoclonal antibody, tolerization, neonatal, high-zone tolerance, masking immunization*. Patent records were also consulted. The documents obtained from this search included all available reports from 1985 to present describing enhanced immunization techniques to produce mAbs or pAbs.

It should be noted that mAbs can be generated using a variety of materials. In the following sections, we use the words ***tolerogen*** to refer to the agents used during the tolerization step (from which there would be no interest in obtaining mAbs) and ***immunogen*** to refer to the agents used during the immunization step (from which there is great interest in obtaining mAbs). A tolerogen is an immunogenic material/substance that carries immunodominant or commonly or highly expressed antigens/epitopes. For diagnostics, prognostics, and therapeutics, any mAb specific to such antigens/epitopes would be of no interest, given that they are shared among various cell lines/types. Such an antibody would bind to a great number of cell types, confusing and possibly misleading results. The tolerogen may be whole cells, plasma membrane lysates, purified proteins/other substances, peptides. An immunogen is an immunogenic material/substance that carries rare and/or poorly expressed antigens/epitopes. These antigens are usually directly correlated with important biologic processes, such as matrix remodeling, protein expression, apoptosis, angiogenesis, and tumor aggressiveness/metastasis. For diagnostics, prognostics, and therapeutics, any mAb specific to such antigens/epitopes would be of great interest, given that they are not shared among various cell lines/types. Such an antibody would bind to a single or, at least, to a limited number of cell types, facilitating result interpretation. As with the tolerogen, the immunogen may be whole cells, plasma membrane lysates, purified proteins/other substances, peptides.

Neonatal tolerization

It is now well accepted that neonatal exposure to foreign antigens results in a general failure in the immune response of mice (immunological unresponsiveness), leaving them tolerized to these foreign antigens. It is believed that, because the immune system is still under development, it recognizes the antigenic agent used in this tolerization step as a self-antigen. The mechanism behind this technique consists of maturing B cells being eliminated by the immune control natural selection during the neonatal period.[6,9,10,11] Moreover, this effect seems to be specific, as well as long lasting.[6]

As mentioned here, this immunization technique was first proposed to obtain an antibody that recognizes specific antigens, after tolerizing the newborn animals with antigens from which there would be no interest in obtaining antibodies, such as immunodominant epitopes.[4] After the tolerization step, the same animals, in adult life, need to be exposed to an antigen of interest that should contain shared epitopes with the antigen used in the tolerization step (Fig. 1). Using this approach, one can decrease the immune response toward shared epitopes between the tolerogen and immunogen, and redirect the

Table 1. Different subtractive immunization approaches that may be used during the generation of antibodies.

Subtractive Immunization Techniques in Antibody Production			
Method	Vantages	Disadvantages	Characteristics
Neonatal Tolerization	Cheaper / Possibility to combine with drug-induced tolerization / Overall satisfactory results reported	Difficulty of newborn mice manipulation / Possibility of mice death / Even longer immunization protocol	Effect is achieved via premature exposure of antigens (tolerogen) when immune system is not yet fully functional (recognized as self-antigens)
Drug Induced Subtractive Immunization	Controllable / Reliable / Overall satisfactory results reported	Expensive (due to cyclophosphamide use) / Possibility of mice death / Limited tolerization	Effect is achieved via elimination of B cell clones in proliferation (previously induced by tolerogen exposure), during exposure to cyclophosphamide
High-Zone Tolerance	Faster immunization protocol / No drugs are used	Large quantities of antigen required / Expensive / Require intense antigen production and purification	Effect is achieved via failure of helper T cell maturation during tolerance
Masking Subtractive Immunization	Faster immunization protocol / No drugs are used / Method doesn't induce helper T cell death	Needs polyclonal antibody production / pAbs used to mask cells may trigger ADCC or CDC / *Fab* fractions may be necessary	Effect is achieved via masking/cloaking of undesirable antigens using polyclonal antibodies

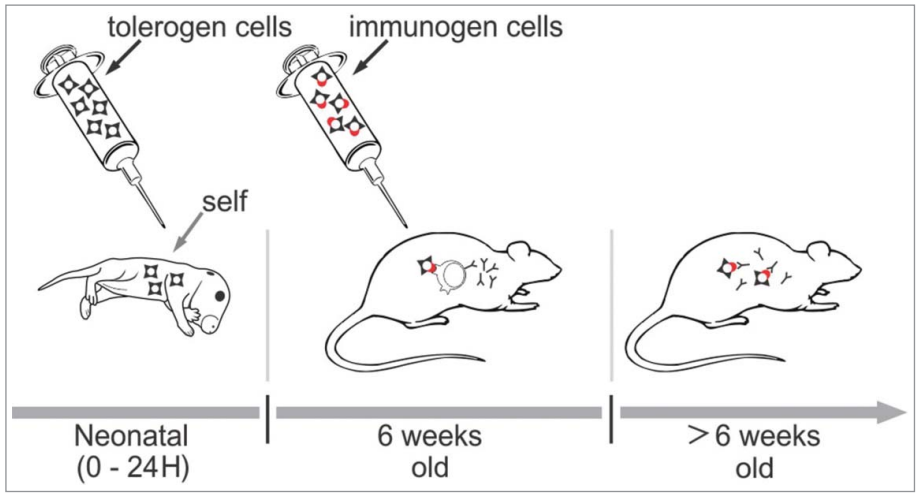

Figure 1. Neonatal subtractive immunization: Neonatal mice are administered tolerogen cells, which are recognized by the host immune system as *self*. The set of antigens anchored to cell membranes becomes tolerized (represented as black triangles). When adults (6 weeks old), the same mice are given immunogen cells, which are similar to tolerogen cells (*e.g.*, same tissue origin) but present distinct characteristics (*e.g.*, tumor associated antigens, represented as red dots). Shared antigens/epitopes (represented as black triangles) are tolerized and does not trigger a humoral reaction. B cells specific to rare/low expression antigens (red) undergo clonal selection, resulting in specific monoclonal antibody production.

response to epitopes of interest present only in the immunogen, thereby generating a specific immune response.[9,10,11]

Generally, the neonatal tolerization technique is performed through serial inoculations of foreign agents (as a tolerogen), starting 15–40 hours after mouse birth, as shown by different authors.[9,11] There are small variations in the details of the protocols described so far, but all of them follow the same critical steps. In the first work[4] reported on the use of neonatal tolerization technique, human T cells were used as the tolerogen and human B cells as the immunogen. The authors of this first report injected mice following 15 hours of birth with the tolerogen and then after 4 weeks, this tolerization step was repeated. Finally, they measured antibody titers on the tolerized mice, and the mice bearing the lowest levels of anti-T cell titers were selected for the following immunization step, which comprised of a single inoculation of B cells as immunogen. Control mice were also immunized with the regular immunization technique.[4] As a result, the authors show that all the mice that underwent neonatal exposure to T cells had negative titer of anti-T cell antibodies, and positive titers of anti-B cell antibodies. Control mice had positive titers of both antibodies. At this point, the authors did not present any explanation on how the neonatal tolerization would work.

Following Sharpe and colleagues findings, Golumbeski and Dimond[6] made use of the neonatal tolerization for the generation of antibodies specific for a lysosomal enzyme of *Dictyostelium discoideum*. These authors aimed at the production of mAbs against the *N*-acetylglucosaminidase and acid phosphatase lysosomal enzymes produced by this organism; however, the mAbs produced were specific to the same antigenic determinant, an *N*-linked sulfated oligosaccharide, which was then classified as an immunodominant epitope present on the lysosomal enzymes of this species. Over 30 hybridoma cell lines secreting the mAbs with similar specificity were produced, and no cell lines secreting mAbs specific to the enzymes were generated. After getting similar results when trying to generate mAbs against a different lysosomal enzyme, a β-glucosidase, the authors devised a neonatal tolerization protocol.[6] They initially tolerized mice 30–40 hours after birth with a single intraperitoneal (ip) injection of 10 μg of purified *N*-acetylglucosaminidase, which highly express the immunodominant *N*-linked sulfated oligosaccharide epitope. Five days later, the tolerization step was repeated. After 18 weeks, the surviving tolerized mice were immunized 5 times with purified β-glucosidase. These authors also used control mice, immunized with the regular schemes. Classical hybridoma technology was used for the generation of the cell lines, and a final screening step was used to determine if the antibodies were specific to the common immunodominant epitope or to unique epitopes expressed in β-glucosidase. Nine different hybridoma cell lines were obtained from the tolerized mice, and all produced mAbs specific for the antigen of interest, and with zero cross-reactivity. The authors concluded that the technique not only yields more specific antibodies, but also a wider variety of antibody specificity compared with non-tolerized mice, suggesting that, by decreasing the immune response to immunodominant epitopes during the neonatal stage, one can obtain an increase on the immune response to other less antigenic ones during the immunization in adults. These authors both corroborated the previous cited research and predicted something that would be achieved decades later, which will also be reviewed in this paper: *"Clinically, tolerization could be used to selectively produce monoclonal antibodies against antigens specific to tumor cells.."*

Hockfield[12] also identified the difficulty of obtaining antibodies against antigens of interest due to the presence of a few immunodominant ones and, in this sense, used neonatal tolerization to generate antibodies that specifically recognize antigens from gray matter spinal cord. This author devised an immunization schedule that involved tolerizing newborn mice through 10 d after birth with rat spinal cord white matter (1 mg/50 μL of 0.1 M phosphate buffer, ip). Littermate controls were not subjected to tolerization. From 3 to 15 weeks of age, all the animals were immunized with rat gray matter tissue emulsified with Freund's complete adjuvant (in the hind footpads, ip).

The next step in the protocol was additional immunization with gray matter every 4 days; the dose used varied between 0.05 to 2.5 mg (when injected in the hind footpads) and from 0.1 to 5.0 mg (ip). It is notable that, although the major cerebellar cell classes were already described, this new immunization technique enabled the isolation of a mAb that recognized a new epitope, which identified a new cerebellar cortical neuron cell. This procedure also generated a high frequency of IgG antibodies of the desired specificity.

Drug-induced subtractive immunization

The subtractive immunization technique, induced by drug, has proved its efficiency in the production of antibodies capable of recognizing desired cell types that have high similarity with other cell types, and reducing the production of undesirable antibodies (e.g., antibodies that cross-react with other cells). Matthew and Sandrock[13] first proposed the use of cyclophosphamide (Cy) as a modulator of the immune response, also envisioning that this drug could be used as a key aspect in a subtractive immunization approach, specifically for the production of mAbs. Cy is an immunosuppressive drug that, given its classical anti-proliferative action, eliminates the B and T lymphocytes that initiate clonal proliferation after exposure to a set of antigens. Cy is not a reactive compound when delivered to the mice; initially is must be activated in the body, via cytochrome P450 oxidation at the liver, which results in the formation of 4-hydroxycyclophosphamide and its aldophosphamide tautomer. Next, the 4-hydroxycyclophosphamide/aldophosphamide enters target cells by diffusion, releasing aldophosphamide inside the cell. This compound then spontaneously decomposes to produce phosphoramide mustard, the first alkylating agent produced in the metabolism of cyclophosphamide, which will ultimately eliminate the targeted cells.[14]

Cy is generally inoculated in mice shortly after the tolerogen, thus suppressing their immune response so that all lymphocytes in clonal proliferation, after exposure to the tolerogen, are eliminated. Therefore, no or low amounts of antibodies are generated and no memory cells are allowed to arise. Following Cy inoculation, the immunogen is introduced into the mice. The immunogen should consist of a similar cell type, which carries specific antigens/epitopes, usually important for biologic processes, such as angiogenesis and tumor metastasis/aggressiveness, or rare or poorly expressed antigens. This technique allows production of discriminatory antibodies that recognize a specific protein that shares up to 90% amino acid sequence identity to another protein.[15] The rationale behind this technique is that the immunogen probably contains shared antigens/epitopes with the tolerogen (given that both have a common origin, e.g., same tissue origin). Due to the previously tolerized nature of the animals, any shared antigens/epitopes carried by the immunogen cells should be immune-suppressed. In this sense, only the specific/rare antigens/epitopes are left (that are desired for mAb production) as the immunogenic material to trigger selection of B cell clones and antibody production[5,7,8,16,17] (Fig. 2).

The subtractive immunization with Cy immunosuppression technique has been widely used in research, especially for cancer, as showed by Brooks *et al.*[5]who made use of subtractive immunization to obtain mAbs by using a highly metastatic human epidermoid carcinoma cell line (M^+ HEp3) and a non-metastatic variant (M^- HEp3) as the immunogen and tolerogen, respectively. The generated hybridomas were screened by ELISA with tolerogen and immunogen cells. Brooks *et al.* selected 2 mAbs, DM12–4 and 1A5, for further testing, and, following purification, their effects were investigated in a chick embryo metastasis model. The authors reported that both mAbs inhibited metastasis of HEp3 cells, ranging from 86% to 90% inhibition. The authors also performed regular immunizations (without Cy treatment) as controls, and compared the final results to the proposed technique, using ELISA assays. As a conclusion, the authors claimed that the subtractive immunization increased the number of cell lines that secreted mAbs specific to the immunogen by 8-fold compared with immunization control. This study demonstrated the efficacy of the subtractive immunization to find epitopes that are expressed in tumor cells, stimulating new studies about metastasis control strategies.

A considerable number of papers corroborating Brooks' findings were subsequently published. In one of these, Dorrell *et al.*[22] developed a panel of biomarkers for the study of the major cell constituents of the human pancreas. The authors needed a more complex understanding of the different cell types present in this organ for the proper identification, isolation, and manipulation of cells suitable for transplantation. In addition, the identification of a novel biomarker could be a breakthrough in the elucidation of pathological conditions, but they can only be found using highly specific antibodies, which are difficult to obtain due to the presence of immunodominant epitopes. Having realized this, these authors used a subtractive immunization protocol to produce different antibodies that could recognize different cell types of the human pancreas. Following BALB/C mice tolerization with unwanted antigens, which included 1×10^6 human peripheral blood cells, fetal bovine serum, and small amounts of trypsin, the authors administered 2 injections of Cy, at 200 mg/Kg, and concluded the protocol using islet inoculations as the immunogen. The authors ultimately generated a panel of cell-surface-binding mAbs that allowed the isolation of different pancreatic cell types, including endocrine, exocrine, and duct cells.

It is interesting to note that the authors correctly chose to include, in the tolerizing material, components widely used during the expansion of cells *in vitro*, such as fetal bovine serum and trypsin. Trace amounts of these reagents should always be expected to be present if an *in vitro* step was performed to obtain the immunogen, and, therefore, should always be presented to the mice during the tolerization step to minimize the clones of B cells secreting undesirable antibodies.

Almost 20 y after this technique was described, Hamabashiri *et al.*[8] produced 4 mAbs (2P-1–2–1, 2P-1–17–1, 6P-3–2–4 and 7P-9–11–6) specific to pancreatic juice from cancer patients, which have a potential application for differential diagnosis. Their immunization protocol was performed using female BALB/c mice, 5–6 weeks old, tolerized with 25 μg of inflammatory pancreatic juice diluted in phosphate-buffered saline (PBS) and adjuvant. Mice received, after 24 and 48 hours, 200 mg/Kg of Cy. At the day 52, 25 μg of cancerous pancreatic juice was injected as the immunogen, diluted in PBS and adjuvant. The

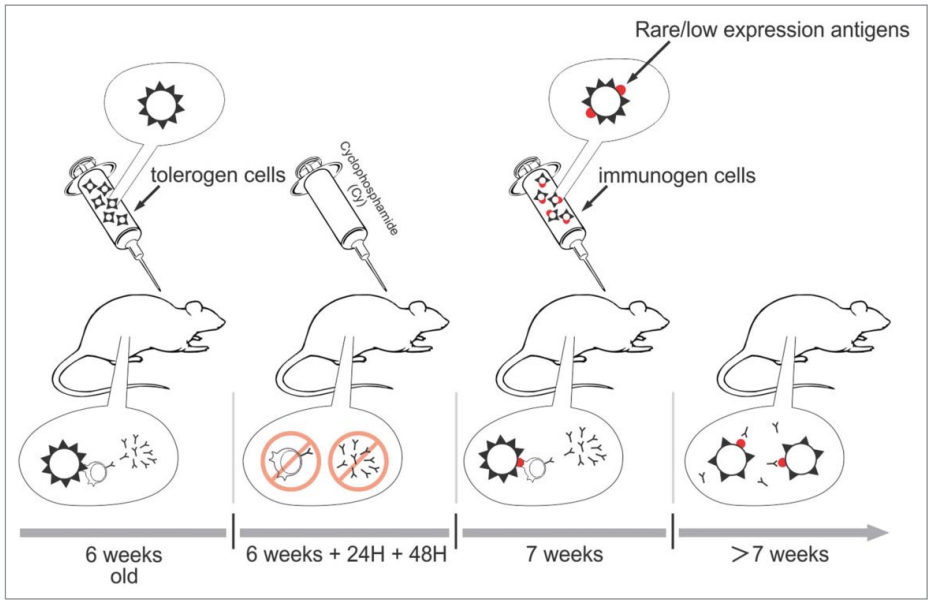

Figure 2. Drug-induced subtractive immunization: Adult mice (6 weeks old) are administered tolerogen cells, which are recognized by the host immune system as *non-self*. The set of antigens anchored to cell membranes (represented as black triangles) would trigger a humoral reaction; however, after 24 h and 48 h, the same mice are given cyclophosphamide (Cy), typically at 200 mg/kg. This immunosuppressive drug eliminates B cells that underwent clonal selection and expansion, triggered by the previous exposure to the tolerogen antigens. It is important to note that neither B cells nor memory cells are produced. Finally, the same animals are given immunogen cells, which are similar to tolerogen cells (*e.g.*, same tissue origin); however, they present distinct characteristics (*e.g.*, tumor associated antigens, represented as red dots). Shared antigens/epitopes (represented as black triangles) are tolerized (by the elimination of B cells, induced by Cy) and does not trigger a humoral reaction. B cells specific to rare/low expression antigens (red) undergo clonal selection, resulting in specific monoclonal antibody production.

animals received 2 more immunogen injections, and sera was collected to investigate the reactivity of pAbs against the tolerogen and immunogen. The obtained mAbs were screened by western blotting. The authors of this report claimed that the pancreatic juice from diseased patients carries specific antigens that would be efficient biomarkers for pancreatic cancer and, in this sense, could provide highly specific mAbs. However, this same antigenic material also carries immunodominant antigens/epitopes, which should be first tolerized. This is, according to Hamabashiri et al., the rationale to use drug-induced subtractive immunization. Hamabashiri *et al.*[8] showed that their approach is an effective technique to obtain specific mAbs to rare or poor immunogenic antigens, and that it can be used to provide tools for new strategies in the premature diagnosis of cancer.

Another interesting report came from Villavedra *et al.*, in which the authors produced mAbs that are effective in the identification of amoebic gill disease, which is of substantial concern to the salmon industry.[18] They reported a subtractive immunization protocol using non-infective *Neopapamoeba* spp. as the tolerogen, followed by 2 Cy inoculations and then exposure to different antigen preparations (whole parasites or cell membrane extracts) from infective counterparts. The authors showed that the nature of antigen preparation may influence the final outcome of the subtractive immunization technique, as they found a 3-fold increase of immunogen-specific mAbs when using whole parasites and a 2-fold increase when using cell membrane extracts from the same parasites. These authors were the first to demonstrate a concern regarding only one cycle of tolerization, as they cited that some of the experiments were performed with more tolerization cycles. However, they did not present relevant conclusions about the efficacy of additional tolerization cycles.[18]

Subtractive immunization has not provided specific antibodies for the oncology area only. The identification of food contamination may be possible using mAbs capable of recognizing specific species of bacteria, as shown by Jin *et al.*,[7] who produced 3 mAbs that have high affinity for *E. coli* O157:H7 and do not cross-react with 80 other strains of bacteria. In this study, the authors used *E. coli O157:H19* and *E. coli O157:H7* as the tolerogen and immunogen strains, respectively. On the first day of immunization, 1×10^8 CFU of the H19 strain were injected i.p. in mice. The authors gave Cy injections after 10 minutes, 24 hours and 48 hours later. This complete tolerization cycle was repeated 3 more times at 14-day intervals. Finally, on days 55, 65, 72, and 86, the mice were immunized with 1×10^8 CFU of the H7 strain, i.p.. At day 89 the mice were killed, and the spleen was removed to obtain B cells. After cell fusion (Sp2/0 myeloma cells were used as partners), the hybridomas were screened using ELISA assays. Positive hybridomas to *E. coli* O157:H7 were cloned by limiting dilution twice. The scale-up concentration of mAbs was done using 2 different techniques, *in vivo* ascites fluid and *in vitro* using batches from RPMI-1640 medium with 10% of fetal bovine serum. They produced 19 hybridoma clones that secreted mAbs reactive against the immunogen strain, H7. They also tested these mAbs, using indirect ELISA, against other 84 strains of bacteria, reporting 3 mAbs with no affinity to these other strains that might considered promising tools for the detection of food contamination by *E. coli* O157:H7.

The only report that compared neonatal tolerization to drug-induced Cy subtractive immunization dates to 1991, and was performed by Ou, McDonald and Patterson.[9] These authors used different tissue extracts to compare both the techniques. In the chemical immunosuppression experiment, Cy

was used to suppress the mouse's immune reaction against immunodominant antigens of the tolerogen extract before injection of the immunogen extract, which contained the rare/target antigens. In another experiment, mice were neonatally tolerized against immunodominant antigens using the tolerogen extract before the introduction of the immunogen extract, which carried rare/target antigens. The authors concluded that even though it was a small scale experiment (a limited number of animals were used), results obtained from immunodot assays clearly indicated that Cy subtractive immunization is superior to neonatal tolerization.[9]

High zone tolerance

Immunologic tolerance is a natural phenomenon defined as *a non-responsivity state to an antigen which is triggered by a previous exposition to it*. The same antigen material can behave as a tolerogen or an immunogen, depending on how this antigen is presented to lymphocytes. Currently, it is well known that the injection of large quantities of soluble non-aggregated antigens, in the absence of adjuvant, generates what is known as *High Zone Tolerance*, a failure in obtaining a specific T-cell response. This phenomenon culminates an anergy of the immune system, in which tolerogen-specific B cells are eliminated.[19, 20] This tolerance may be used as a subtractive immunization technique since the mice becomes tolerized, and the desired immunogen can then be inoculated, resulting in an immunological response directed to the desired epitopes (Fig. 3).

Lebrón *et al.*[20] generated *in vitro* pAbs against one polypeptide chain of 2 heterodimeric proteins (HFE/β_2m; CD94/NKG2A). Tolerization was performed using the immunodominant heterodimeric protein (β_2m; also known as CD94). 250 μg of this soluble immunodominant protein was inoculated, without adjuvant, into 5-week-old female BALB/c mice at days 1 and 5. At day 9, the tolerized mice were immunized with HFE/β_2m or CD94/NKG2A. The hybridomas were generated using classical methods, and before cloning, the secreted pAbs were assayed by ELISA. The authors reported that 7–39% of the hybridomas from tolerized mice produced antibodies that reacted against the β_2m portion alone and 59–93% reacted with the HFE portion, representing an increase of up to 90-fold of hybridomas producing the desired antibodies. The authors showed that only 18% of hybridomas reacted to CD4/NKG2A heterodimers, but not to CD94 homodimers. As a conclusion of this study, Lebrón *et al.*[20] wrote: "*This method should be of general utility for the production of mAbs against weakly antigenic proteins in mixtures of antigens.*"

The high zone tolerance technique may also be applied in oncology studies, such as the one reported by Krueger *et al.*[19] In this study, the authors generated mAbs capable of recognizing distinctive epitopes expressed by human myeloma cells using 750 μg of cell membrane lysate from the K562 cell line (which is a chronic myelogenic leukemia cell line) as the tolerogen, adjuvant absent, injected in 6–8-week-old female mice, followed by a pool of irradiated multiple myeloma cell lines (also as cell membrane lysates) one week and 2 weeks later. The popliteal lymph node B cells were fused with a mouse myeloma cell line (Sp2/0). The supernatants from hybridomas were screened by ELISA and the positive hybridomas were screened again in a secondary ELISA and in a flow cytometry-based assay. The authors obtained a panel of 240 hybridomas, from which the mAb VAC69 was selected and further explored using flow cytometry, western blotting and additional ELISA assays. The authors showed that the mAb VAC69 reacted exclusively with the multiple myeloma cells used as immunogens.

High zone tolerance may be an efficient manner to generate mAbs against poorly immunogenic antigens, using a mixture of immunodominant epitopes after the tolerization step. We also highlight that the tolerization can be performed in a week, and that the time between the tolerization and immunization is shorter when compared with other subtractive immunization approaches. However this technique has several limitations because the tolerization must be performed using a pure and soluble presentation of the tolerogen, which may be a limiting factor when the tolerogen cannot be purified, and large amounts of tolerogen are needed, which can also be a limiting factor.

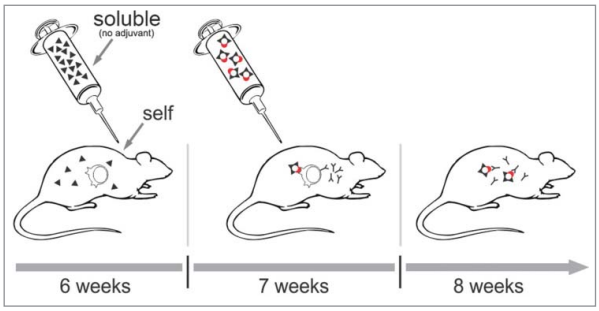

Figure 3. High-zone tolerance immunization: Adult mice (6 weeks old) are administered tolerogen purified soluble antigens (represented as black triangles), at a very high quantity, and in the absence of adjuvant. These antigens are recognized by the host's immune system as *self*, and becomes tolerized. One week later, the same mice are given immunogen cells, which presents a degree of shared antigens to the tolerogen antigen solution (*e.g.*, same tissue origin); however, they present distinct characteristics (*e.g.*, tumor associated antigens, represented as red dots). Shared antigens/epitopes (represented as black triangles) are tolerized and do not trigger a humoral reaction. B cells specific to rare/low expression antigens (red) undergo clonal selection, resulting in specific monoclonal antibody production.

Masking subtractive immunization

All the subtractive immunization methods discussed above, which are the most commonly used, are based in tolerizing a host animal to undesirable or immunodominant antigens, followed by immunization with the desirable antigen to generate the specific antibodies. All these methods have shortcomings, such as those demonstrated in some studies regarding neonatal immunization[21] in which the authors showed that the tolerization was not really induced; in fact what happened was the contrary: a peptide administered in neonatal life triggered T-cell activation and antibody production. Even the cyclophosphamide-induced tolerization, which so far has been the most effective method, may have its flaws because cyclophosphamide not only kills B-cell clones in proliferation, but it also kills helper T-cells, which are necessary for B cells to maturate and differentiate (collateral damage effect). Should this happen, when the animals receive the immunogen, only low affinity IgM antibodies could be produced.[23] To avoid these potential

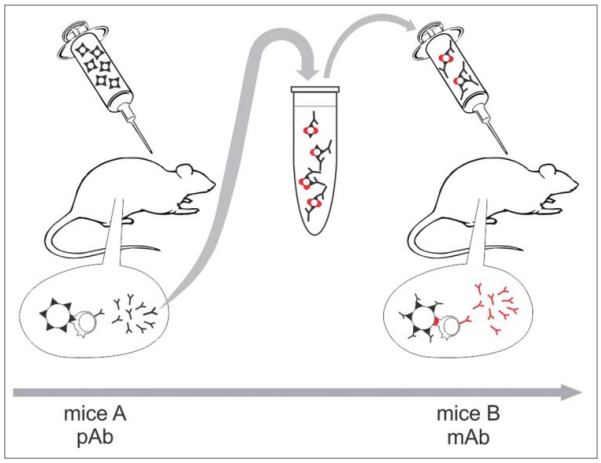

Figure 4. Masking subtractive immunization: Adult mice (6 weeks old) are administered tolerogen cells, which are recognized by the host immune system as *non-self*. The set of antigens anchored to cell membranes (represented as black triangles) triggers a humoral reaction, resulting in normal polyclonal antibodies (pAb) production. This mouse is used solely to produce pAbs reactive to tolerogen antigens, which are then harvested from peripheral blood, purified, and used to mask shared antigens from immunogen cells (represented as black triangles with an antibody - Y). Finally, another mouse is given immunogen cells coupled with pAbs. These cells are similar to tolerogen cells (*e.g.*, same tissue origin) but they present distinct characteristics (*e.g.*, tumor associated antigens, represented as red dots). Shared antigens/epitopes (represented as black triangles) are masked by pAbs (Y) and do not trigger a humoral reaction. B cells specific to rare/low expression antigens (red) undergo clonal selection, resulting in specific monoclonal antibody production.

problems, some authors have designed a different approach in immunizing the mice. For example, the masking immunization is a patented method[23] that promises little or no setbacks as those described previously.

The cells used as immunogens are injected together with mAbs or with antisera from mice previously immunized with immunodominant epitopes, which aren't of interest for mAb generation. In this scenario, these epitopes are masked/cloaked to the host's immune system, and therefore do not trigger B-cell clonal proliferation. The unmasked epitopes should be more visible/evident, and therefore trigger a selective clonal selection of specific B cells (Fig. 4). In this approach, it may be necessary to modify unmasked epitopes using a hapten so these unmasked epitopes are more easily recognized by the host's immune system. Moreover, successful immunization requires repeating the process several times, allowing the amplification of the immune response to less immunogenic but specific epitopes of target immunogen cells. The antisera used to mask the immunogen cells' undesirable/common epitopes can be produced using non-cancer cells, red blood cells, white blood cells, total blood cells or a pool with different normal cell lines, as tolerogens.[23]

The only shortcoming of this approach is that the antibodies used in the masking process may bind to the immunogen cells, resulting in cell death through antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC), which may decrease the efficacy of the whole process. To avoid this collateral damage, a pool of antigen-binding fragments (Fabs) can be used as an alternative to full-length antibodies to mask the target cells. By doing so, the *Fc* portion of the antibodies, required for ADCC and CDC, will not be present.

Combined subtractive immunization strategies

The secret to obtaining the best mAbs may be to combine different approaches, as a way to correct or avoid the problems peculiar to each method, and also to combine the desirable characteristics from each one. Ensrud and Hamilton[24] must have foreseen this, when they combined the neonatal and chemical immunosuppression with Cy in a successful attempt to produce mAbs against maturation-specific sperm surface antigens. They made use of neonatal tolerization with 50 μg from caput epididymal sperm plasma membranes, prepared without adjuvant, in which each animal was tolerized 24 hours after birth. Seven weeks later, these neonatally tolerized mice

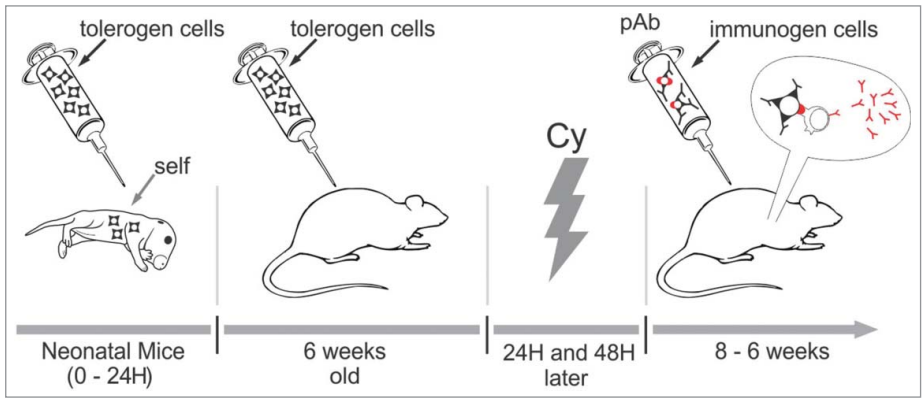

Figure 5. Combined subtractive immunization: Neonatal mice are administered tolerogen cells, which are recognized by the host immune system as *self*. The set of antigens anchored to cell membranes becomes tolerized (represented as black triangles). When adults (6 weeks old), the same mice are given more tolerogen cells, whose antigens/epitopes (represented as black triangles) were partially tolerized neonatally. To enhance tolerization, following 24 h and 48 h, the same mice are given cyclophosphamide (Cy), typically at 200 mg/Kg. This immunosuppressive drug eliminates remaining B cells that escaped from neonatal tolerization, and underwent clonal selection and expansion, triggered by the previous exposure to the tolerogen antigens. It is important to note that neither B cells nor memory cells are produced. Finally, the same animals are given immunogen cells, coupled with pAbs previously produced in other mice. These cells are similar to tolerogen cells (*e.g.*, same tissue origin), but present distinct characteristics (*e.g.*, tumor associated antigens, represented as red dots). Shared antigens/epitopes (represented as black triangles) were tolerized neonatally, by drug-induction (by the elimination of B cells, induced by Cy), and are also masked with pAbs (Y). B cells specific to rare/low expression antigens (red) undergo clonal selection, resulting in specific monoclonal antibody production. This approach combines all the others.

were again injected with 15 μg of the same tolerogen preparation, and after 48 hours they were immunosuppressed with Cy. At the end of the tolerization process (neonatal + drug-induced), after 4 weeks, the mice were immunized twice with cauda epididymal sperm plasma membranes, which carries the maturation-specific sperm surface antigens (immunogen).The authors then proceeded to cell fusion and screening, and 5 hybridoma cell lines that produced mAbs with higher specificity to target antigens were selected. These cell lines were cloned and subjected to further characterization. In their conclusion, Ensrud and Hamilton[24] affirmed: "*By combining the 2 techniques, we may have enhanced the specificity of the immune response by altering more aspects of the immune system than either technique alone.*" As previously shown by these authors, and in theory, the combination of different subtractive immunization techniques may enhance the specificity of the mAbs; however, more studies involving different combinations are required to prove this.

We propose that combining 3 of the techniques detailed in this paper may be a very promising approach, as follows: 1) at birth, mice may be tolerized with tolerogen cells; 2) at mature age, the same animals that were neonatally tolerized should receive more tolerogen cells, followed by 200 mg/Kg of cyclophosphamide (drug-induced subtractive immunization); and 3) finally, these tolerized animals should receive more immunogen cells, masked with polyclonal sera from other mice that were previously tolerized (masking immunization approach) (Fig. 5).

Conclusions

The production of mAbs reinvented research worldwide, especially in the biomedical area, but this revolution also brought the need for a greater specificity of these molecules. Over the years, different approaches in immunizing mice were designed, and the subtractive immunization was developed as an attempt to solve the lack of desired specificity by controlling the manipulation of the immune system. The available studies revealed that the subtractive immunization techniques have greatly increased effectiveness in obtaining more specific antibodies. They also showed that all the techniques have peculiar characteristics that may be hurdles in obtaining the best mAbs, and that combining different approaches, may provide the best results. Our review of the literature thus suggests that a 40 year-old classical technology, i.e., Köhler and Milstein's hybridomas, still can provide insights in biomedical research.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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