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Evaluation of a time efficient immunization strategy for anti-PAH antibody development

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Abstract

The development of monoclonal antibodies (mAb) with affinity to small molecules can be a time-consuming process. To evaluate shortening the time for mAb production, we examined mouse antisera at different time points post-immunization to measure titer and to evaluate the affinity to the immunogen PBA (pyrene butyric acid). Fusions were also conducted temporally to evaluate antibody production success at various time periods. We produced anti-PBA antibodies 7 weeks post-immunization and selected for anti-PAH reactivity during the hybridoma screening process. Moreover, there were no obvious sensitivity differences relative to antibodies screened from a more traditional 18 week schedule. Our results demonstrate a more time efficient immunization strategy for anti-PAH antibody development that may be applied to other small molecules.

Keywords

PAH; mAbs; Immunization strategy; anti-sera titer; affinity; fusion

Introduction

Monoclonal antibodies (mAbs) have been used during the last decades as immuno-analytical tools for the detection of low molecular weight contaminants in food and the environment (1). The advantages of antibody-based immunoassays are that they are quick, inexpensive, simple to perform, and reasonably portable; they can also be highly sensitive and selective (2). Polycyclic aromatic hydrocarbons (PAHs) are an important class of environmental pollutants that are a concern because several of its members have been known to cause cancer (3, 4). Therefore, antibody-based immunoassays have also been widely employed in the detection of environmental PAHs, including bioelectrochemical immunoassay (5), enzyme-linked immunosorbent assays (ELISAs) (6) and biosensors (7). All of these antibody-based immunoassay methods require the use of sensitive anti-PAH mAbs.

The traditional protocol for mAb production is immunizing mice with the appropriate immunogen to induce specific B cells that are obtained from the spleen, and then fusing these with suitable myeloma cells. The resulting “hybridomas” are evaluated by immunological screening procedures, such as competitive ELISAs for the desired affinities

and specificities (8) and are able to produce a uniform population of antibodies with the preferred characteristics. This whole process is time-consuming (1) and it usually requires 3 to 6 months to produce suitable mAbs (9). Typically during the PAH mAb development procedure, half of the time is used to monitor the sera for a satisfactory titer prior to fusion. A number of mAbs against PAHs have been developed in the recent past (10-12). The immunization strategies from those reports are various. However, the immunization periods used for antiserum production were all at least 12 weeks.

Antibody persistence is thought to be determined by long-lived plasma and memory B cells (13-15). It has been reported that most Ab-secreting cells are generated during secondary (memory) immune response (16), which are present 1 month after immunization and later (17). Thus, it may be possible to use shorter immunization periods and obtain successful mAbs by optimizing the immunization strategy. To this aim, PBA-KLH (pyrene butyric acid-keyhole limpet hemocyanin) immunogen was administered to mice and the immunological response in sera and fusion success were evaluated at different week intervals post-immunization. The goal of this study was to appraise various post-immunization periods in the production of anti-PBA antibodies and to optimize a time effective immunization strategy to produce sensitive anti-PAH antibodies.

Experimental

Chemicals and reagents

Bovine serum albumin (BSA) was obtained from Fisher Scientific (Pittsburgh, PA, USA). Anhydrous N,N-dimethylformamide (DMF) was from Acros Organics (Morris Plains, NJ, USA). The Bicinchoninic acid (BCA) protein assay Kit, N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) were obtained from Pierce (Rockford, IL, USA). Keyhole limpet hemocyanin (KLH), HAT medium supplement, Freund's adjuvant complete (FAC) and Freund's adjuvant incomplete (FAI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI-1640 medium with L-glutamine and fetal bovine serum (FBS) were from Hyclone (Logan, UT, USA). Goat anti-mouse immunoglobulin G secondary antibody conjugated with horseradish peroxidase (GAM IgG-HRPO) and tissue culture plates (Costar, Corning) were purchased from Fisher Scientific (Pittsburgh, PA, USA). The 1-pyrene butyric acid (PBA) was obtained from Acros (NJ, USA). All other reagents were of analytical grade and purchased from Fisher Scientific (Pittsburgh, PA, USA).

Balb/c mice were bred at our own facility with the original mice purchased from The Jackson Laboratory, (Bar Harbor, ME, USA) All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the College of William and Mary.

Preparation of PBA-protein conjugates

Two conjugations were prepared in this study. The immunogen PBA-KLH and the coating antigen PBA-BSA which was used in titer and affinity analyses were both carried out by the method previously described by Grabarek and Gergely (18) with some modifications.

Briefly, PBA hapten was dissolved in methanol at 1.0mg/ml. S-NHS and EDC were added to each of two aliquots of the hapten solution at a final concentration of ~500mM and ~200mM respectively, and rotated for 15min at room temperature. To quench the EDC, 2-mercaptoethanol (20mM) was added to each aliquot and rotated for 10min. Any precipitate was removed by centrifugation at 12,000rpm for 5min at room temperature; the supernatant contained the activated hapten. A 1.0mg/ml solution of both BSA and KLH were made in 0.1M sodium phosphate, pH = 7.5. The PBA was then conjugated to BSA or KLH by mixing 1ml of the activated hapten to either 10ml of BSA or 20ml of KLH to yield a molar ratio of ~20:1 (PBA:BSA) and ~50:1 (PBA:KLH). The mixtures were covered in foil and allowed to react for ~2-3hrs on a rotator at room temperature. To remove excess reactants, the two conjugates were dialyzed separately in 1XPBS with a minimum of three 1h x 1L changes, including one overnight, at 4°C. The conjugates were filter-sterilized through 0.22µm filters, and a final protein concentration was determined by bicinchoninic acid (BCA) protein assay (Pierce) and read on a microtiter plate reader (MTX Lab Systems, Vienna, VA, USA) using a 540nm filter.

Immunization and fusion

To evaluate the anti-PAH antibody production behavior of various immunization periods, the PBA-KLH conjugate was used as the immunogen. The schedule for mouse experiments is summarized in Figure 1. Eight 10-week-old female Balb/c mice were immunized with 100µl of a 1:1 FAC emulsion containing 50µg of PBA-KLH by intraperitoneal injection. Mouse whole blood was collected from the tail vein of each mouse at 0, 3, 5, 7 and 9 weeks post-immunization to obtain antisera. For the control group, whole blood was collected at 0, 3, 8 and 18 weeks post-immunization and titer and affinity were tested. The mouse whose antiserum exhibited the highest titer at each time point was selected to fuse with SP2/0 myeloma cells using the 50% polyethylene glycol method described elsewhere (19). An intravenous booster was given 3 days before the spleen was removed. To compare with traditional immunization methods, the remaining three mice served as a control group. For the control group two subsequent immunizations were administered at 6-week intervals with the same conjugate concentration emulsified 1:1 in FAI (Figure 1).

Sera titers and affinity determinations

The antisera titers of mice from different immunization time points were determined by indirect ELISA according to methods previously described by Shapiro et al. (20). The maximum rate of reaction (mOD/min) was calculated for each sample using Deltasoft JV software (BioMetallics, Princeton, NJ, USA). A unit of antibody activity is defined as the volume of sample required to produce an optical density (OD) rate of half the maximum rate. Titers were expressed as the number of antibody units per ml of serum.

The anti-PAH antibody affinities were determined by a partitioning ELISA-based analysis originally described by Nieto et al. (21) for murine sera. Briefly, 96 well microtiter plates were coated at rows B-H with increasing concentrations of PBA-BSA conjugate in carbonate coating buffer (100µl/well) reserving row A for zero concentration of PBA-BSA as controls. Plates were incubated at 4 °C overnight, unbound sites were blocked with casein blocking buffer (4% casein, 5% sucrose, 150mM NaCl, 50mM Tris base, 1mM EDTA, pH

7.6) for 1h at room temperature with slight horizontal shaking at 300rpm. Then 50µl/well serially diluted PBA inhibitor in 10% MeOH-PBS was added on columns 3-12, reserving columns 1-2 for zero concentration of PBA as controls. Meanwhile, 50µl/well of serum diluted in PBS was added yielding 1 unit to each well of the microtiter plate. Plates were incubated for 90min at room temperature and washed 3 times with Tween Tris buffered saline (TTBS, 0.1% Tween 20, 50mM Tris, 1mM EDTA, 150mM NaCl, pH8.0). Plates were then incubated with GAM IgG-HRPO (dilution 1/2000 in PBS, 100µl/well) for 1 h at room temperature. After the last washing step, substrate solution was added and absorption values at 405nm were measured with a microtiter plate reader and compared with the wells without supernatants (20). The characteristic affinity was defined by an apparent affinity constant (aK), the reciprocal concentration of free hapten (PBA) required for 50% inhibition of antibody binding to immobilized antigen (PBA-BSA). The method of aK calculation was described in detail by Ye et al. (22).

Evaluation of the antibody specificity and sensitivity

The sensitivity of antibodies against PAHs was evaluated from every successful fusion with screening by indirect competitive ELISA. The original PAH derivative, PBA, and its homologous unsubstituted PAH, pyrene, were selected as inhibitors. Briefly, 96-well plates were coated with 100µl of PBA-BSA conjugate (5µg/ml) in carbonate coating buffer at 4 °C overnight and then blocked with TTBS 200µl/well at room temperature for 1h. Subsequently, 50µl of high to zero concentrations of inhibitor working solutions were added mixed with the same volume of supernatants from hybridoma wells exhibiting cell growth. The following steps were in the same format as described above. The inhibition curves were obtained through the calculation of the difference between the concentrations of inhibitor working solutions and inhibition values. The inhibition values were expressed as % B/B₀, where B was the absorbance at each concentration of inhibitor and B₀ was the absorbance in the absence of inhibitor (23). The individual PAH compounds; benzo(a)pyrene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene and chrysene were also used as inhibitors to evaluate the specificity of the antibodies.

Results and discussion

Antibody titers and affinities induced to PBA-KLH

Mouse whole blood from both the experiment and control group were harvested just prior to immunization (week 0) and weeks 3, 5, 7 and 9 post-immunization for experiment group and weeks 3, 8 and 18 post-immunization for the control group. The comparison of antisera's titer and affinity response is shown in Figure 2. Although different immunization strategies were used between the experimental and control group, it can be seen that the kinetics of titer behaved in a comparable pattern with a dramatic increase between weeks 0 and 5, and then a more gradual increase over time. The maximal titer for the experiment group was 100units/µl at week 9 and the maximal titer for the control group was 125units/µl at week 18. Meanwhile, the antisera affinity showed a trend of slowly increasing over time with the average affinity post-immunization of approximately 10⁵.

The analysis according to Nieto (21), not only provides a weighted average affinity for an antiserum sample, but can also provide an antibody affinity distribution profile, which was compared at each time point. The affinity distribution was converted into the percent response for the entire serum. The discrete individual aK values for different time points were summed within quarter-log aK increments (3.75-4.00, 4.01-4.25, 4.26-4.50, 4.51-4.75, 4.76-5.00, 5.01-5.25, 5.26-5.50 and 5.51-5.75) (Figure 3). Although the average affinity showed only a slight increase throughout the whole immunization period, there is a clear subpopulation affinity shift over time. The proportion of each affinity subpopulations appeared hypodispersed prior to week 5 post-immunization. Then, at 7 weeks post-immunization, the higher affinity subpopulations ($\log aK > 4.76$) were beginning to assume greater dominance within the population. The trend keeps going, with the affinity subpopulations all becoming higher than 5.01 at week 18 for the control group. Moreover, a new set of antibodies with a quarter-log greater affinity ($\log aK$ 5.51-5.75) began to emerge.

As a previous study showed, cells producing antibodies with the highest aK for a particular antigen will bind the greatest amount of that antigen and be selectively triggered to produce those specific, high-affinity antibodies (24). The control group's data from week 18 show an emergence of a quarter-log greater affinity ($\log aK$ 5.51-5.75) indicating the possibility of getting antibodies with higher affinity (Figure 3). Yet, the antisera titer results at 5 weeks post-immunization show that a relatively high titer has already appeared and an even higher titer at week 7. Thus, a shorter immunization period of 5-7 weeks should be sufficient to produce anti-PBA antibodies. Our results indicate that it will be more time efficient to obtain anti-PBA antibodies after 7 weeks post-immunization.

Comparison of fusions at each time point

The mouse whose antiserum exhibited the highest titer at each time point (3, 5, 7, 9 and 18 weeks) after injection with PBA-KLH was selected for fusion with SP2/0 myeloma cells. Fusions were successful, with greater than 60% of fused cells visible 10-15 days after fusion. Indirect ELISAs and indirect competitive ELISAs were performed to screen antibodies against the target hapten PBA, usually testing 300-600 supernatant wells for each fusion. The hybridoma cell supernatants showing reactivity with PBA were considered to be positive colonies. The total number of colonies versus positive colonies from each fusion was summarized (Figure 4). No positive colonies against PBA were found from the fusions at week 3 and week 5. To evaluate the reason for the lack of PBA affinity, Ig(M+G) antibody class was also determined in the colony supernatants from both of these fusions. ELISA plates were coated with goat-anti-mouse Ig(M+G), only 1 out of 36 anti-Ig(M+G) positive colonies were found in the fusion from week 3, and 2 out of 45 colonies were positive from week 5. These results indicated that there was very few IgM or IgG class antibodies produced during the first 5 weeks after immunization. These results were consistent with the previous study by Černohorská et al. (25), which showed that after the fifth week of immunization with pyrene-KLH, the presence of IgG class antibodies was detected in mouse serum. With regard to the production of monoclonal antibodies, it is generally preferred to obtain the IgG class of antibodies (25). This is supported by the low amount of IgG class antibodies found from the third and fifth week fusion supernatants and may explain the lack of anti-PBA positive colonies during screening of the first two fusions.

Positive anti-PBA colonies from weeks 7, 9 and 18's harvests were evaluated and the positive rates were 8.28%, 5.66% and 5.12%, respectively (Figure 4). The results in this study revealed that anti-PBA antibodies can be obtained successfully as early as 7 weeks post-immunization. Based on the results that higher affinity antibody subpopulations ($\log aK > 4.76$) were beginning to assume greater dominance within the population at 7 weeks post-immunization (Figure 3) the fusion results were in agreement with this previous finding. Fusions conducted with cells harvested at 7 weeks post-immunization resulted in successful anti-PBA antibody production. Additionally, there is not a large difference in the number of positive colonies from fusions after 7 weeks, even though mice sera from week 18 exhibited the highest titer against PBA-BSA.

Antibody affinities of different fusion times

Based on the results above, we found that fusions 7 weeks after PBA-KLH immunization were sufficient to produce antibodies that recognize free PBA. However, was there any difference in the sensitivity between the antibodies produced from different time points? The hapten PBA and its homologous free PAH, pyrene, were used as inhibitors to evaluate the sensitivity of the antibodies over the time series. The comparison between the IC_{50} 's of the experimental group (7 weeks and 9 weeks) and control group (18 weeks) are listed in Table 1. Although the IC_{50} values for the different supernatants vary, there is a typical pattern with all antibodies more sensitive against the original hapten than pyrene. However, there is no distinct increase in sensitivity to PBA for the supernatants screened from fusion produced from the 18 week harvest. There were no obvious sensitivity differences observed between antibodies produced from fusions at 7 weeks, 9 weeks and 18 weeks post-immunization.

The antibody 2G8, which was produced by fusion 7 weeks post-immunization, was selected to evaluate reactivity to other specific PAH compounds. A comparison of the reactivity of our antibody 2G8 and the commercial antibody [BAP-13] against benzo(a)pyrene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene and chrysene is presented in Table 2. Benzo(a)pyrene was defined as the reference analyte in this study. The antibodies 2G8 and [BAP-13] show a similar pattern of PAH recognition with higher reactivity to 4-ring PAHs such as pyrene and chrysene than the 3-ring PAHs, phenanthrene and anthracene.

Antibodies are a key component of adaptive immunity and the response to immunization. In the presence of a particular antigen (i.e. injection), naïve B cells with B cell receptors specific for the antigen bind the antigen. During the course of an initial infection, plasma cells or effector B cells produce and secrete large quantities of antibodies (9). Short-lived plasma cells are generally disseminated into blood and tissues, where they secrete millions of copies of the clonal antibody. The antibodies secreted from the short-lived plasma cells are typically the IgM isotype, which have a relatively lower affinity (26).

As a result of somatic hyper-mutation and affinity maturation, antigen-specific B cells emerge from germinal centers as either high-affinity memory B cells or long-lived plasma cells (27, 28). Along with affinity maturation, simultaneous antibody class switching occurs, generally from an IgM to an IgG isotype. The presence of specific IgG antibodies generally corresponds to the maturation of the antibody response, with usually high affinity for the

antigen and is detectable 5 weeks after immunization (25). The failure of high affinity antigen specific mAb production for our 3 and 5 weeks post-immunization fusions could be a reflection of the absence of the IgG isotype.

It can be seen from our results that the traditional immunization strategy (a long immunization period) could result in a higher titer (Figure 2) and a high affinity subpopulation shift in mice anti-sera (Figure 3). However, the highest anti-sera titer does not necessarily result in the production of higher affinity mAbs to the target antigen. Based on our results, antibodies showing inhibition to the free PAH can be successfully screened from fusions as early as 7 weeks post-immunization. In addition, it is important to note that no obvious sensitivity differences were observed in antibodies produced from fusions at later time points. Moreover, the reactivity of 2G8 (produced at 7 weeks) and the commercial PAH antibody [BAP-13] show a similar PAH recognition pattern. An immunization strategy with cell harvest and fusion at 7 weeks post-immunization is sufficient for successful anti-PAH antibody production.

Conclusions

The four-ring PAH-pyrene derivative, PBA, was successfully coupled to keyhole limpet hemocyanin and used for the immunization of mice. A time efficient immunization strategy was compared with conventional immunization methods by examining in detail anti-serum titer, affinity distribution profiles and antibody production. It was demonstrated that anti-PBA antibodies could be successfully produced as early as 7 weeks post-immunization. The sensitivity differences between antibodies produced by fusions at various times post-immunization were minimal although sera obtained from conventional long immunization times exhibited higher titer and a higher affinity subpopulation. Our results strongly suggest that the time efficient immunization strategy for anti-PAH antibody production is to conduct a one-time injection of immunogen emulsified with FAC and wait 7 weeks until harvest and fusion. These results may also provide useful insight for saving time for the production of other antibodies against low molecular weight contaminants.

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Figure 1.

Experimental schedule for the PBA-KLH immunized mice. Experiment group, female Balb/c mice were immunized by intraperitoneal injection with 50 μ g PBA-KLH emulsified 1:1 in FAC. Serum samples were collected at 0, 3, 5, 7 and 9 weeks post-immunization. The mouse whose antiserum exhibited the highest titer was selected for fusion at each time point. Control group, two subsequent immunizations were administered at 6-week intervals with the same conjugate concentration emulsified 1:1 in FAI. Whole blood was collected at 0, 3, 8 and 18 weeks post-immunization. The mouse whose antiserum exhibited the highest titer was selected for fusion at week 18.

Figure 2.

Antisera response for mice immunized with PBA-KLH and bled at different weeks. The bars indicate the average of antisera affinities and the lines show the average of antisera titers. Error bars represent the standard deviation of different mice samples.

Figure 3.

Change in antisera affinity profiles over time. The subpopulation responses are summarized at each time point. The affinity distribution was converted into the percent of entire serum response. The average affinities for weeks 3, 5, 7 and 9 were calculated using individuals from experimental groups (black bar). The average affinity for week 18 was calculated from the control group (gray bar).

Figure 4. Summary of colonies from each fusion. The blue bars show the number of total colonies while the red bars show the number of anti-PBA positive colonies from harvests and fusions at weeks 3, 5, 7, 9 (Experiment Group) and 18 (Control Group).

Table 1

Comparison of antibody sensitivity from each fusion time.

supernatants	Fusion weeks	IC ₅₀ µg/L ^a	
		PBA	Pyrene
10E5	7	0.81	34.37
8A7	7	2.31	60.66
2G8	7	1.29	24.26
10C3	9	2.88	13.75
5H6	9	7.21	16.18
8G3	9	3.75	0.81
1F4	18	0.86	52.57
4C2	18	0.29	64.70

^aThe IC₅₀ values for selected cell fusion supernatants against PBA and Pyrene were estimated by indirect competitive

Table 2

Comparison of the reactivity of antibody 2G8 and commercial antibody [BAP-13].

PAHs	Reactivity $\pm$ SD [%]	
	2G8	[BAP-13] ^I
Benzo[a]pyrene	100	100
Phenanthrene	12 $\pm$ 1	23 $\pm$ 4
Anthracene	20 $\pm$ 5	25 $\pm$ 4
Fluoranthene	49 $\pm$ 7	60 $\pm$ 9
Pyrene	35 $\pm$ 7	47 $\pm$ 7
Benzo[a]anthracene	65 $\pm$ 4	44 $\pm$ 7
Chrysene	42 $\pm$ 9	42 $\pm$ 6

^I Reactivity data of commercial antibody [BAP-13] is cited from abcam website: