Chapter 3

Immunisation Strategies for Antibody Production

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Summary

A range of immunisation techniques can be used for the successful production of antibodies. The choice of method used is dependent on the nature of the antigen and the type of antibody required by the user.

Key words: Immunisation, Antibody, Antigen, Immunogen, Immunity, Epitope, Monoclonal, Polyclonal, Immunoglobulin.

3.1. Introduction

Vertebrate immune systems are capable of producing antibodies to a greater or lesser degree in response to the presence of a foreign protein within the tissues of the animal. The presence of the foreign protein initiates a sequence of events, mediated by the cells of the immune system that leads to the release of antibody molecules in blood and some body secretions.

Antibodies produced by vertebrate immune systems bind strongly to the protein that elicited their formation and it is this unique ability which is harnessed in all branches of immunochemistry.

Vertebrates have evolved this immunological strategy to help them to combat pathogens of viral, bacterial and fungal origin; however, almost all foreign substances regardless of source can induce antibody responses. The immunological response in mammals is particularly well developed and it is this group of vertebrates that are normally used for antibody production.

There are two main approaches for antibody production in vertebrates each having strengths and weaknesses depending upon

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intended application. Prior to discussing these two approaches it would be beneficial to describe some background to immunology.

Mammalian embryos are extremely tolerant of foreign proteins while still in utero, and all substances within the developing organism are accepted as "self". This is essential during development to ensure that immune responses are not raised to proteins and peptides produced during this time. Any immunological response to developmental proteins, hormones and growth factors would have disastrous results.

Shortly before or immediately after birth the neonatal immune system matures and learns to differentiate between "self" and "non-self" *(1)*. The immature immune system contains millions of cells within the bone marrow capable of producing antibodies (B-lymphocytes). This cell population is in effect a "starter pack" containing cells which will be capable of responding to a huge number of target proteins. These neonatal lymphocytes are produced by random re-assortment of the antibody genes and because of this many of them will recognise and respond to proteins within the developing individual. A process of clonal deletion takes place and any lymphocytes, which recognise proteins within the developing organism, are killed. As the young mammal matures it is incapable of mounting an immunological response to "self" antigens as a result of the process of clonal deletion.

The remaining B-lymphocytes in the bone marrow have the potential to respond to an enormous number of foreign substances (antigens). Once exposed to antigens the cells which have the best fit antibody to the target undergo clonal expansion to increase the cell numbers and affinity maturation to increase the specificity (fit) of the antibody molecules produced.

The ability of the mammalian immune system to respond to foreign substances is based on the molecular shape of antigen fragments produced as a result of digestion by cells called macrophages. The antigen fragments produced by this process are generally about the size that one antibody binding site can physically adhere to. These fragments are known as epitopes and although there will be many on any target substance, a single antibody will only be able to recognise and bind to one of them.

The response to any antigen will involve the recruitment of many B-lymphocytes, each making antibodies to an individual epitope on the target molecule. These lymphocytes, which have responded to the epitopes on the antigen, undergo clonal expansion so that many descendant B-lymphocytes are produced from each of the original cells. This process gives rise to a population of cells descended from single progenitors (clones) each with their own specific antibody to epitopes on the antigen. These clones of cells are resident in lymphoid tissue and are particularly concentrated in the marrow, spleen and gut associated lymphoid tissue (GALT). The resulting pool of antibody molecules produced by these cells is know

as polyclonal antibody as it is derived from multiple clones each with unique specificity to single epitopes.

Monoclonal antibodies are produced as a result of immortalising and expanding the individual antibody secreting cells artificially in tissue culture *(2)*. Cells grown in this way all have identical epitope specificity and as they are derived from single clones their product is known as monoclonal antibody. Cells that secrete monoclonal antibodies are known as hybridomas and are typically derived by the fusion of two cell types. B-lymphocytes, which have the capacity to make antibody, are obtained from a donor spleen and are physically fused to a tumour cell line, which is immortal. The resulting hybridomas are immortal and produce antibody into the synthetic medium in which they are growing.

Exposure to antigens can be through a variety of routes but ultimately the cellular changes leading to B lymphocyte activation are blood borne. Natural immunisation takes place as a consequence of infection through respiratory, digestive, urogenital and skin surfaces. Medical immunisation to prevent infection by pathogens is normally carried out by intramuscular injection although other routes such as oral dosing for poliomyelitis and intradermal injection for tuberculosis may be used. The main feature, which characterises immunisation, is the presentation of antigens to the cells of the immune system, which induces B lymphocyte priming. These primed B cells will undergo clonal expansion and will secrete antibodies until the antigen has been destroyed. As soon as the antigen has been removed, the B cell lineage making antibodies to it will become quiescent and will form a stable population within the tissues of the immune system (memory cells). If the antigen is encountered again by the organism these quiescent B cells can undergo rapid clonal expansion and can mount an antibody response much faster than during the primary challenge. Each time that the immune B cells are exposed to the antigen, the affinity (fit) of the antibody produced will be improved and the number of quiescent B cells after each challenge increases. Each challenge also increases the amount of antibody produced in the blood and after three or four immunisations the individual reaches a status of hyperimmunity. This is characterised by high levels of circulating specific antibodies typically in the range of 10–20 mg/ml of serum. Hyperimmunity is rarely ever seen as a result of natural immunisation but is commonly used for the in vivo production of polyclonal antibody. Risks are associated with hyperimmunity as further exposure to the antigen can lead to an overwhelming immune response known as anaphylaxis which can be rapidly fatal. Paradoxically, repeated exposure to the antigen can lead to immunological tolerance, where the B cells making the antibody are destroyed by the immune system leaving the individual unable to mount an immune response to the antigen.

As previously stated immunisation is a phenomenon mediated by the cells of the immune system and is normally the result of a blood-borne challenge by antigen. The route of introduction can be very important in determining how well the individual will respond to an antigen.

It is extremely important when immunising animals for antibody work to choose the correct approach for the type of antigen to be used. This chapter describes a number of immunisation routes for polyclonal antibody production, monoclonal antibody cell donors and also one method for inducing selective immune tolerance as a preparative method.

Polyclonal and monoclonal antibodies should be seen as complimentary in their use. Each has strengths and weaknesses and the choice of which to use should be carefully evaluated prior to embarking on antibody production. In general, polyclonal antibodies have a much broader specificity as the antiserum pool comprises many species of antibody molecule each with different target epitopes on the antigen. This lack of specificity is advantageous in situations where variation in the target substance is known and polyclonal antibodies may provide a more robust test. Monoclonal antibodies are derived from clonal cell lines and their specificity is directed to a single epitope on the antigen. The highly specific nature of the monoclonal antibody allows the development of assays where two very closely related substances can be differentiated from each other. Examples of these highly specific tests are found in virus testing for strain differentiation and in clinical assays where levels of a synthetic hormone may be detected in spite of the presence of its naturally occurring counterpart.

3.1.1. Legislation

There are strict regulations governing the welfare of laboratory animals used for antibody production in most countries. Before deciding on a particular approach for antibody production it is important that the appropriate authorities are contacted with a project proposal to ensure that the methods to be used are permissible. Local ethical review committees may also have inputs into project design to ensure that numbers of animals used are appropriate and that other diagnostic alternatives have been investigated. Legislation and ethical review typically covers animal species, numbers to be used, immunisation route, bleeding regimes, and welfare issues such as project duration.

3.2. Materials

1. Balb-c mice are the preferred laboratory strain used as cell donors in monoclonal antibody work (*see* **Note 1**).

2. New Zealand rabbits are the preferred laboratory strain used for polyclonal antiserum production (*see* **Note 2**).
3. Suitable adjuvant for addition to antigen (*see* **Note 3**).
4. Animal house facilities licensed for the specific required procedures under animal welfare legislation.
5. Parenteral anaesthetic agents as prescribed by veterinary surgeon (*see* **Note 4**) (Hypnorm + Hypnovel).
6. Diethyl ether.

3.3. Methods

3.3.1. Immunising Rabbits for Polyclonal Antiserum Production

1. Mix 0.5 ml of 1 mg/ml solution of antigen with 0.5 ml appropriate adjuvant.
2. Inject into muscle of hind leg or subcutaneously into neck scruff.
3. Repeat on days 14 and 44.
4. Test bleed (1–2 ml) on day 54 and assess for antibody activity (ELISA, etc.).
5. Bleed from marginal ear vein on day 60 and then every 28 days until the antibody titre drops.
6. Give boost dose and either commence bleeding regime 10 days later or perform terminal exsanguination under anaesthesia.

3.3.2. Immunising Mice for Monoclonal Antibody Production

1. Mix 0.15 ml of 0.5 mg/ml solution of antigen with 0.15 of appropriate adjuvant and mix (volumes based on group of three mice).
2. Inject 0.1 ml of adjuvant/antigen mixture per dose intraperitoneally (IP).
3. Repeat on days 14 and 44.
4. Obtain test bleeds by tail tip amputation under light anaesthesia on day 54 (*see* **Note 5**) and assess for antibody titre. Mice should be marked by ear punching or tattooing to allow subsequent identification.
5. Rest the mice for a period of 60 days or more to allow the B cells to become quiescent.
6. Inject (boost) the best responding mouse with an IP injection of 0.05 ml antigen (0.5 mg/ml solution) without adjuvant.
7. Kill the mouse 3 days later by cervical dislocation and remove its spleen aseptically for cell harvesting.

3.3.3. Modified Immunisation Protocol for Non-Anamnestic Antigens

When it is known from other work that the antigen is highly glycosylated or comes from a source known to be rich in polysaccharides (bacterial cell walls, etc.) it is highly unlikely that the animal will ever produce a classic full immune response. Usually these antigens do not produce quiescent B cells following the rest period and each immunisation is seen as a primary challenge. Substances which invoke this incomplete response are known as non-anamnestic antigens and immunoglobulins produced to them are always class M. There is no point in carrying out a full immunisation protocol over several months and a shortened one is recommended.

1. Mix 0.15 ml of 0.5 mg/ml solution of antigen with 0.15 of appropriate adjuvant and mix (volumes based on group of three mice).
2. Inject 0.1 ml of adjuvant/antigen mixture per dose intraperitoneally (IP). Repeat on day 14.
3. Take a test bleed by tail tip amputation under light anaesthesia on day 21 and assess for circulating antibodies. All antibodies produced will be IgM and so the assessment method (ELISA, etc.) must take this into account. Mark the mice by ear punch or tattoo for subsequent identification.
4. Give the mouse exhibiting the highest titre of circulating antibody an IP injection of 0.1 ml (0.5 mg/ml solution) of antigen on day 23.
5. Harvest the spleen and perform the cell fusion on day 30.

3.3.4. Induction of Immune Tolerance in Neonatal Mice (3, 4)

As previously mentioned foetal and new-born mammals have immature immune systems which do not yet have the capacity to differentiate self and non-self. This lack of maturity can be harnessed to our advantage when working with antigens, which are naturally found along with closely related (cross-reacting) substances. This technique does not guarantee success but can swing the odds in favour of the researcher producing hybridomas with the desired specificity. Situations where this methodology is used include work on viruses, bacteria and fungi where there may be many shared epitopes between the organism of interest and closely related species. The cross-reacting antigen used for the technique is a whole preparation of the closely related species. This technique causes the suppression of an immune response to *shared* epitopes which may swamp the immune response and favours specific immunity to epitopes found only on the species of interest.

1. Obtain a "time mated" female Balb-c mouse 10 days into the pregnancy and maintain in standard cage used for mouse breeding.
2. Observe until litter are born.

3. Inject the neonates daily on days 1–5 after birth with 0.025 mg of *cross-reacting* antigen into the neck scruff. (The neonatal immune systems are maturing at this time and because the cross-reacting antigen is present they will adopt these proteins as "self" and lose the ability to mount an immune response to them.)
4. Immunise three of the animals with specific antigen of interest when 6 weeks old using the standard monoclonal immunisation protocol given above.

3.3.5. Intrasplenic Immunisation

Intrasplenic immunisation is used for the production of hybridomas in situations where only very small quantities of the antigen are available. Typically it lends itself extremely well to producing antibodies to proteins that have been purified by electrophoresis and subsequent blotting onto nitrocellulose. Antibodies produced by this route are always immunoglobulin class M as only one immunisation is used. This method is covered by Animal Procedures legislation in most countries as it is an invasive surgical procedure and welfare issues must be addressed.

The antigen must be in a highly aggregated or immobilised form. This technique is frequently used to produce antibodies to proteins separated by electrophoresis. The gel is stained in the normal manner and the proteins blotted over onto nitrocellulose. The band containing the protein of interest is then excised from the blot and used as the antigen. The presence of protein stains and other reagents makes little difference and as the "in vivo" part of the protocol is very short there are no long term animal welfare issues.

1. Induce and maintain anaesthesia using a mixture of fentanyl/fluanisone with midazolam (Hypnorm/Hypnovel) (*see* **Note 4**). The dosage for these agents is 0.25 ml of each active ingredient plus 0.5 ml water given IP at a rate of 0.1 ml per mouse.
2. Shave the hair along the mid-scapular line above the position of the spleen and make a 1 cm incision made through the skin. Cut through the muscle wall to expose the spleen, and then deliver it through the opening complete with its pedicle.
3. Introduce the antigen into a pocket made in the spleen through the capsule and return the organ to the body cavity.
4. Close the muscle layer with three or four sutures and then close the skin likewise.
5. Keep the mice warm and close to a source of drinking water until they recover from the anaesthetic which takes about 1–2 h. Generally the mice suffer no side effects and will be feeding, grooming and showing no signs of discomfort soon after recovery from anaesthesia.

6. Use the mice as donors for cell fusion 7 days after the intrasplenic immunisation.

3.3.6. In Vitro Immunisation

This technique is performed in tissue culture and works well when only small quantities of antigen (1–2 µg) are available. It also allows the production of antibodies to substances that are toxic in whole animals. There is no immunological processing of antigens so only soluble, simple antigens such as peptides can be used for this approach.

A source of interleukins 4 and 5 is required for the method to work and the easiest way of obtaining them is from thymocyte conditioned medium.

3.3.6.1. Thymocyte Conditioned Medium

1. Kill two 6-week-old Balb-c mice and remove their thymus glands aseptically.
2. Homogenise the tissue to produce single cells and resuspend in 10 ml RPMI 1640 medium containing 15% FBS.
3. Incubate the medium at 37°C/ 5% CO_2 for 24 h (*see* **Note 6**) and then harvest the supernatant by centrifugation (700 × *g*). Store the conditioned medium at –20°C until required.

3.3.6.2. Immunisation

1. Kill a non-immunised Balb-c mouse and remove its spleen aseptically.
2. Homogenise the spleen to produce single cells, and then resuspend them in 10 ml of the thymocyte-conditioned medium.
3. Add 1–2 µg of antigen to the cell suspensions and incubate at 37°C/ 5% CO_2 for 72 h (*see* **Note 7**).
4. Harvest the spleen cell by centrifugation (500 × *g*) and use immediately for a cell fusion.

3.4. Notes

1. Balb-c mice are an inbred strain ideally suited to monoclonal antibody work. Females are usually used, as they do not fight when housed together in project groups of 3–5 individuals. This mouse is also known as the "barber" strain, as the dominant female will remove the whiskers from the others in the group.
2. New Zealand rabbits are normally used for serum production; they are easily handled and adapt well to individual cages or group floor pens. This strain has half-lop ears, which make blood collection from marginal veins a fairly straightforward procedure.

3. Most antigens require an adjuvant to increase their immunogenicity and a number of formulations can be used. Regulations on their use should be consulted prior to embarking on a course of immunisations. For many years Freund's complete and incomplete adjuvants were the formulation of choice for all immunisation work. In recent years welfare issues have been raised over the use of these adjuvants and a number of alternatives based on water soluble bacterial cell wall components have become available.
4. Parenteral anaesthetic agents are preferable to gaseous ones as the size of the mouse creates problems when using standard anaesthetic machines.
5. Tail tip amputation is normally used to obtain test bleeds from mice and this is normally carried out under light anaesthesia induced with diethyl ether.
6. It is extremely important that the medium is harvested after 24 h of incubation. Longer incubation periods may induce the formation of suppressing cytokines, which will block the desired cell stimulation.
7. It is important that the thymocytes are not disturbed for the full 72-h incubation and that they are rapidly harvested and fused after this period of time.

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