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National Committee for Ethical Reflection on Animal Experimentation (CNREEA)

(Articles R214-134 to 136 of the Rural and Maritime Fishing Code)

Members of the Committee (Arrêtés of 02 July 2019, 03 December 2021, 24 February 2022)

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Opinion on the use of antibodies of animal and non-animal origin

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Opinion on the use of antibodies of animal and non-animal origin

Explanatory memorandum

In 2020, the European Union Laboratory for Alternatives to Animal Testing (EURL ECVAM) published a recommendation on the use of antibodies of non-animal origin (1). This approach is fully in line with the objective of replacing animals for scientific purposes. The summary conclusion of the document is that the use of animals in the creation and production of antibodies should be discontinued for any reason (creation or production) related to research, regulatory needs, diagnostics or therapeutic applications. It is added that it should no longer be possible to authorize projects using animals for scientific purposes to obtain antibodies by immunizing animals without a robust and legitimate scientific justification. As a result of this recommendation, numerous opinions have been issued by various European bodies representing both academic and private researchers (reviewed by the Deutsche Gesellschaft für Immunologie (2)).

The CNREEA wished to prepare a situation report on the creation, production and use of antibodies in France, particularly of animal origin, and to formulate an opinion adapted to the national context. This opinion will focus, but will not be limited to, situations relating to authorizations of projects using animals for scientific purposes.

Situation report

The most exhaustive assessment possible of the situation in France was carried out by means of a questionnaire which was widely distributed, focusing on both animal and non-animal antibodies. The responses received came from people working in the fields of cell or molecular biology, as well as from users of animals for scientific purposes. This questionnaire reveals a very wide variety of applications and uses of antibodies of animal and non-animal origin. The following review focuses on both antibody creation and production techniques.

Use and production of animal-derived antibodies

Therapeutic Products

While therapeutic antibodies and most antibodies used in diagnostics are produced by non-animal methods (but often initially created in animals), there are currently exceptions, and there may be special or emergency situations requiring production in animals. Some medicinal products used under marketing authorization (MA) or ATU (temporary authorization for compassionate use) have as their active ingredient polyclonal antibodies necessarily produced by immunization of animals (rabbits, horses, sheep, etc.), in the absence of a satisfactory alternative. Without giving an exhaustive list, they essentially fall into two categories: antivenom or anti-bacterial toxins (often used under ATU), and anti-thymocytes used mainly as anti-graft rejection therapy (examples: Thymoglobulin® in France, Atgam®, Grafalon®) under MA.

Diagnostics

Antibodies obtained from animal immunization have been used for years in kits for approved commercial diagnostics for human or animal infectious diseases. Even though there are new

developments in this area aiming at replacing antibodies currently in use with antibodies produced *in vitro*, this has not yet come to fruition.

It should be noted here that for regulated products for therapeutic or diagnostic purposes, any modification of the production protocol requires a modification of the corresponding regulatory authorizations, possibly in a large number of countries. In addition to the product itself, it is its production method that is subject to authorization.

Research uses

Antibodies of animal origin are also widely used in routine techniques such as ELISA, Western-blot, immunoprecipitation, flow cytometry, etc. A very large proportion of these antibodies used in research, available in catalogues, are manufactured outside the EU and mostly by immunizing animals. One of the important markets in this field is also that of secondary antibodies directed against antibodies of a given species, used in research and diagnostics, including pathological anatomy (immunostaining for example).

For various research projects, custom antibodies are essential, such as polyclonal antiserum against a new virus or monoclonal antibodies against the different epitopes of a purified protein. In this case, the antibodies can be produced in-house (laboratory itself) or externally (specialized companies, subcontractors). Regarding monoclonal antibodies used in research, when they are of animal origin, the main production technique used is intraperitoneal injection of hybridoma after conditioning, better known as the ascites production method. This method makes it possible to rapidly obtain large quantities of monoclonal antibodies. In 2017, it was already the subject of a recommendation by the CNREEA focusing on its practical implementation (3).

Alternatives to antibodies produced in animals (phage display and *in vitro* production)

Monoclonal antibodies

Manufacture of synthetic antibodies from the outset (source antibodies): phage display techniques

At present, there are several very extensive collections of synthetic antibodies in the world ((4) (5)). Screening proteins of interest against these collections can lead to antibody fragments or nanobodies targeted against parts of these proteins. These molecules differ from antibodies naturally produced by a living organism for two properties: their class (type of antibody: IgG, IgM, IgA, etc.) and their maturation (post-translational modifications). This maturation requires secondary *in vitro* work to improve their specificity and selectivity. On the other hand, the affinities are generally comparable. It should also be noted that it may be more difficult to obtain synthetic antibodies against denatured proteins as opposed to native proteins. The quality and diversity of antibodies offered in each collection also varies.

In vitro *antibody production*

Whether the source antibodies were initially synthetic (by screening phage collections) or initially obtained by immunizing animals, it is possible to subsequently produce these antibodies in cell culture systems. This is what is mostly done for therapeutic antibodies (the vast majority of which derive from source antibodies produced by immunizing animals, then humanizing by molecular cloning and *in vitro* production). However, the manufacture of fully synthetic therapeutic antibodies (from the source antibody to the authorized medicinal product) is possible, although still limited (6).

Polyclonal antibodies

Currently, polyclonal antibodies are used in research, diagnostics and therapeutics. They are all of animal origin, although it is theoretically possible to create "polyclonal" preparations by mixing monoclonal antibodies, which are themselves of synthetic origin, but these mixtures (sometimes called "multiclonal") never take up the full performance of polyclonal antibodies.

Conclusions

The survey carried out as well as a review of the literature show that the replacement of antibodies of animal origin by synthetic antibodies has been initiated for several years in various fields but that, in the current state of technology, there are still situations in which either synthetic antibodies remain less effective or even unsuitable and therefore unusable (antibodies directed against denatured proteins, anti-peptide antibodies), i.e. the move towards the use of antibodies of non-animal origin in production will be a long-term process and technical obstacles remain (antibodies for therapeutic use or diagnostics).

Recommendations

Considering:

On the one hand,

- The need to eventually phase out the use of animals in general and, in this case, for the manufacture and production of antibodies;
- The development of alternative methods to avoid the use of animals;
- The gradual reduction of technical constraints related to alternative methods.

And on the other hand,

- The current limitations and obstacles to replacement by alternative methods (access to phage-display collections, variable quality of these collections, delays in the registration of new products for regulated use);
- The still unsuitable nature of synthetic methods in terms of performance in some cases;
- The proven risk of relocation of production to third countries with lower levels of animal protection than in Europe.

The Committee issues the following opinion:

On current practices:

- The animal-free *in vitro* approach should be the first option in new research projects. The use of animals for the creation and/or production of antibodies must be demonstrated as essential in any application for project authorization (by documenting the concrete efforts made in the field, and not only by theoretical or bibliographical elements) before any possible favorable opinion from an ethics committee and then authorization. Particular attention must be paid to the methods of refinement. The project must be assessed retrospectively, in particular with an explanation of the efforts made to replace the use of animals over time.

The duration of project approval needs to be adapted to allow for regular reassessment of these efforts.

- Routine production of monoclonal antibodies by the ascites method should be strongly discouraged. It is a severe procedure that must be carried out in strict compliance with the 2017 CNREEA recommendation (3). In the event that it is unavoidable, the project should not be authorized for a period of more than two years, allowing for a rapid reassessment of whether it is still relevant thanks to retrospective assessment.

- The unavoidability of the use of initial immunization (creation of new antibodies against a protein of interest by immunization of animals, cell recovery, and creation of a hybridoma by fusion (7)) for the production of original monoclonal antibodies in research must be based on a solid and robust demonstration of the impossibility of using the selection of synthetic elements obtained by phage-display.

- The adjuvant used to obtain polyclonal antibodies in animals should be rationally selected and the use of complete Freund adjuvant should be reserved for cases where it is essential due to documented side effects (8), and in this case used at a concentration below 0,1 mg/ml.

On future developments:

- Professionals working in research or diagnostics must be aware of the origin of the antibodies they use and give preference as much as possible to synthetic antibodies, which are not produced by immunizing animals. Relocating antibody production in animals is not ethically acceptable when an alternative exists.

- Public authorities, academic and private research centers and learned societies have an important role to play in supporting – each in their own fields of competence – a resolute move towards reducing the use of antibodies of animal origin:

    o by providing all antibody users with extensive information about the possibilities of non-animal *in vitro* approaches and organizing training on this topic;

    o by encouraging the development of consortia to identify existing collections of synthetic antibodies, including those with the most complete annotations possible, making it possible to have a quality index of each antibody identified and to know its possible or documented uses;

    o by also strongly encouraging the development of platforms or service providers in the fields of synthetic antibody selection and *in vitro* antibody production;

    o by encouraging research on the performance of multiclonal antibodies (mixtures of synthetic monoclonals) compared to polyclonal antibodies obtained by immunizing animals.

- In all cases, since the use of animals is the subject of mandatory applications for project authorizations, it would be useful for the Ministry of Research to put in place a procedure to identify these projects and the experimental approaches and techniques on which they are based. This would allow progress to be assessed over time and to revise, if necessary, this Recommendation, which should be considered as scalable.

These approaches are fully in line with the aim of reducing the use of animals for experimental purposes by replacing them with alternative approaches wherever possible. This also opens up new opportunities for research and development in the biomedical field. The CNREEA will monitor progress in this area and will regularly revise this recommendation if necessary to reflect developments in alternative approaches.

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Glossary:

Antibodies: An antibody is a molecule produced by the adaptive immune system in a living organism to specifically detect and neutralize foreign agents (antigens) to the body. It is an immunoglobulin secreted by plasma cells, the final stage of differentiation of B cells. Immunization of an organism consists of administering an antigen in order to induce the production of antibodies (which is the product being sought).

Monoclonal antibodies: Monoclonal antibodies are antibodies produced naturally by the same line of activated B cells or plasma cells, recognizing the same epitope of an antigen.

Polyclonal serum: This is the result of the immune response to stimulation by a protein containing several epitopes. The immune response will naturally induce the production of antibodies against several epitopes, resulting from the expansion of several plasma cell clones. This is the normal response to an organism's exposure to a foreign antigen.

Nanobody: This is a single-chain antibody (they are much smaller than common antibodies, which have two light chains and two heavy chains). They were discovered in camelids, but are now synthesized *in vitro* and can be humanized and recombined to form common antibodies.

Epitope: An epitope is the singular part of a protein that can be recognized by the variable part of an antibody or membrane receptor of B cells (BCRs) and T cells (TCRs), to determine whether it belongs to the realm of the self or the realm of the non-self (i.e., foreign to the organism).

Antibody affinity: Affinity is the specific attraction between an antibody and an antigen. The affinity is variable according to the antibody/antigen pairs, the post-translational maturation of the antibodies and the environmental conditions (pH, ionic strength, temperature).

Class of an antibody: There are 5 classes of immunoglobulins (A, D, E, G, and M). The constant regions of the heavy chains determine the category of immunoglobulin to which the antibody belongs. The immunoglobulin category is also called an isotype.

Antibody maturation: Affinity maturation is a specific process of the adaptive immune response leading to the production by B cells of immunoglobulins of increasing affinity for the antigen. These processes can be implemented *in vitro* in some cases.

Phage-display: Phage exposure is used for high-throughput screening involving protein interactions. A very large collection of proteins is made up (e.g. synthetic nanobodies), to mimic the immune system's repertoire of recognition of non-self proteins. The exposure of this collection to a protein of interest makes it possible to determine the nanobodies that interact with the protein to be studied (thus having the protein sequence of these nanobodies). Subsequently, it is possible to reconstruct complete humanized antibodies by *in vitro* synthesis.

Hybridoma: A hybridoma is a hybrid cell that results from the artificial fusion of normal mammalian lymphoid cells and myeloma cells from malignant tumors of the immune system. Hybridoma combines the properties of the two starting cells: specific production of antibodies for the lymphocyte (monoclonal) and immortality for the cancer cell.

Ascites: Ascites is a buildup of fluid in the abdomen, specifically in the peritoneal cavity. In this case, it is an inflammatory reaction triggered by inflammatory molecules when implanting an immortalized monoclonal antibody-secreting hybridoma in the peritoneal cavity of a mouse. The ascites fluid is then collected by puncture, and it usually contains large amounts of monoclonal antibodies.

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