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Chicken Egg Yolk Antibodies, Production and Application

IgY-Technology

Springer
Lab Manual

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Preface

As early as 1893 Klemperer published his observation that there must be neutralising proteins (i.e. antibodies) in the yolk of eggs laid by immunised hens. As is often the case in the history of science, this finding was hardly acknowledged and even ignored for a long time. Only later, when animal protection came to be regarded as a serious ethical claim for the scientific community as well, did these results came back to mind. Animals are now perceived as fellow creatures in need of protection, so the extraction of specific antibodies from the eggs of immunised hens was regarded as an attractive alternative to blood-taking methods.

In 1992 – nearly a century after the original discovery – yolk immunoglobulins became the topic of a joint research project. Various research groups which already had experience with yolk antibodies started to work together and were financially supported by the German Ministry for Education and Research. The aim of this project was to find out whether these avian antibodies are as effective as the traditionally used polyclonal antibodies from mammals, especially from rabbits. It could be shown that in the case of phylogenetically highly conserved antigens, avian antibodies are even more effective than mammalian antibodies.

The project went on for six years, during which IgY-technology won increasing attention and acceptance. There are now commercially available cages for keeping hens under acceptable conditions. In addition to laboratory protocols for extracting IgY from yolk, there are also easy-to-use commercial kits for the isolation of immunoglobulins. Also, secondary antibodies coupled to detectable markers are now available. Within a few years, IgY-technology has developed to a recognized alternative which is less stressful to animals than the conventional procedures. In

other words, there is no longer any excuse for not using this well established technology.

In acknowledgement of their work on yolk antibodies, the scientists working together in the joint project received the FISEA prize in1997. FISEA – the International Foundation for the Substitution of Animal Experimentation, Luxemburg – awards this prize to researchers working on new alternative methods that eleminate or reduce the use of animals or are less stressful to animals.

Apart from the animal protection aspect, there are some advantages in using yolk antibodies compared to mammalian. For instance, a single immunised hen can yield an amazing abundance of antibodies, many of which show a different specificity than those a rabbit would produce after the same immunisation. These benefits have already led to scientific results which could not be achieved with mammalian antibodies, neither with poly- nor monoclonals.

The only obstacle was the lack of a reliable collection of protocols and laboratory instructions for this technology. Researchers or students who wished to apply this technique, were still obliged to search for information from literature in various fields of specialisation. This book now presents all relevant protocols and information needed to use IgY-technology easily and successfully.

The protocols are based on our own experience and on those of our colleagues working in the same field. All procedures have been tested and most have already reached a routine level. They are presented in an easy-to-follow way, supported by many practical hints and notes. Included are detailed instructions on how to keep and immunise hens and protocols for the extraction of antibodies from yolk and for the various applications. The protocols were selected with the aim to cover as broad a spectrum as possible. In some cases, it will of course, still be necessary to refer to special literature. Therefore, a list of the relevant references is given in the appendix.

We do hope that this laboratory manual will enable also the unexperienced scientist and everybody working in a laboratory to use this technology without initial problems.

We are aware that one can never avoid some mistakes finding their way into a text and wish to apologise for these possible errors in advance. We would welcome any suggestions which sup-

port the book's aim to disseminate IgY-technology and thus serve the protection of animals.

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Abbreviations

Ab	antibody
ABTS	2,2'-azino-bis(etylbenzthiazoline-6 – sulfonic acid)
AGIDT	agargel double immunodiffusion test ("Ouchterlony"-technique)
AI	avidity index
ALP	alkaline phosphatase
BGG	bovine gamma globuline
BSA	bovine serum albumine
CCK	cholecystokinin
CD	cell differenciation
CRP	C-reactive protein
DAB	diaminobenzidine
DMSO	dimethylsulfoxid
DNP	2,4-dinitrophenol
ECVAM	European Centre for the Validation of Alternative Methods
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence activated cell sorter
FCA	Freund's complete adjuvans
FIA	Freund's incomplete adjuvans
FITC	fluorescein isothiocynate
GABA	γ-aminobutyric acid
HPR	horseradish peroxidase
HSA	human serum albumin
i.d.	intradermal
IE	immunoelectrophoresis
IEA	immunoelectrophoretic assay
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M

IgY	immunoglobulin Y
i.m.	intramuscular
i.p.	intraperitoneal
ISCOM	immune stimulating complex
i. v.	intravenous
kDa	kilo Dalton
KLH	keyhole limpet hemocyanine
LPS	lipopolysaccharide
mAb	monoclonal antibody
MAP	multiple antigen peptide
MBP	myelin basic protein
MDP	muramyldipeptide
MHC	major histocompatibility complex
OPD	ortho-phenylenediamine
PAGE	polyacrylamide gelelectrophoresis
PBS	phosphate-buffered saline
PCSL	Pam_3Cys-Ser-$(Lys)_4$ – lipopeptide
PEG	polyethylenglycol
pNPP	p-nitrophenylphosphate
POD	peroxidase
RIA	radio immunoassay
RIE	"rocket" immunoelectrophoresis
rpm	revolutions per minute
RZ	Reinheitszahl
s.c.	subcutaneous
SD	standard deviation
SDS	sodium dodecylsulfate
SoAg	somatic antigen
SPF	specific pathogen free
SRID	single radial immunodiffusion ("Mancini"-technique)
TBS	tris-buffered saline
TMB	tetramethylbenzidin
Tris	Tris-hydroxymethyl-aminomethan
TRITC	tetramethylrhodamine isothiocynate
v/v	volume/(total)-volume
w/v	weight/ (total)-volume

Contents

Short Introduction to Hens' Humoral Immune System

MICHAEL ERHARD and RÜDIGER SCHADE

Introduction

The hens' immune system differs from mammals' in various ways. In this brief introduction we shall try to give a basic idea of the special features of the hens' humoral immune system, especially as regards the structures and functions of antibodies.

Like the immune system of mammals, that of hens is divided into two major components, one of them being non-specific and innate and the other specific and acquired. Naturally between the two there are numerous interactions of critical importance in the event of an immune response. Hens too, for instance, have antigen-presenting cells, and many functional mechanisms are regulated via interleukines. Unfortunately there are few data about hens, since there has been less research internationally into the immune system of hens than into that of rats and mice. But hens do have a feature not shared by mammals, as Klemperer (1893) pointed out: the passive immunity of the offspring, which in mammals reaches the foetus through the placenta or colostrum, has to come through the fluid parts of the egg in ovipars. While the egg is still in the ovary, hens transfer their serum immunoglobulin Y (IgY) into the yolk. As the egg passes down the oviduct, IgM and IgA from oviduct secretion are required with the albumin.

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So hens' eggs as a source of antibodies (Lösch et al. 1986) invite a wide variety of questions. We shall be brooding over them in chapters 6 - 8 of this handbook on methodology.

General basis of hen's humoral immune response

Immune competent avian cells of the specific immune system come from the thymus (T-lymphocytes) or from the bursa of Fabricius (B-lymphocytes). In the course of embryonic development, precursors of these cells reach the thymus or the bursa and there differentiate into T- and B-lymphocytes, as in mammals.

The humoral immune response, in the form of antibody-synthesis, is an important part of the specific response to foreign structures (antigens). According to the clone selection theory from Burnet (1959), an activated B-lymphocyte (plasma-cell) is genetically designed to produce an exactly defined antibody, which is firstly expressed as an antigen receptor (B-cell receptor - BCR) on its cell membrane. The BCR can bind to unprocessed antigen molecules. After proliferation induced by antigens, these B-lymphocytes differentiate into so-called effector cells, which are plasma cells producing antibodies. Antibodies are simply soluble forms of BCR secreted into the body fluids. Every antibody binds only one specific epitope of an antigen, also known as an antigen determinant. So antigens are molecules against whose surface-determinants a vertebrate is able to produce antibodies. This in turn presupposes that the respective antigen structure is recognised as foreign. Not every antigen is immunogen for every individual, so highly conserved mammal antigens from hens may set off a specific immune reaction without being immunogen to the mammal itself.

In general, a distinction has to be drawn between antigens and antigen determinants. All kinds of macro-molecules such as proteins and polysaccharides and hence glycoproteins, nucleoproteins, lipoproteins but also lipids, steroids or nucleic acids may act as antigens. The best antigens are proteins and polysaccharides, whereas lipids and nucleic acids are much less clearly recognised by the immune system. The antigen determinant epitop of an antigen is actually the site where the antibody binds the antigen and makes up only a small part of the whole

molecule. Antigen determinants of proteins may, for instance, be made up of 3 to 10 amino acids.

On an antigen, the number of epitopes able to bond depends on the antigen's structure and size. Generally there is a difference between sequential determinants, such as one defined by a sequence of amino acids, and determinants defined by a configuration. Thus antigens can be typified immunologically by antibodies. Small molecules such as haptens, which are not themselves immunogen, can stimulate lymphocytes to synthesise antibodies, if these haptens are coupled to larger molecules serving as carriers. So in the case of hens too, antigens range from proteins, viruses, bacteria and parasites to the cells of organisms. The potency of an antigen depends in turn on the size of the molecule, and few molecules with a molecular weight below 10,000 Daltons can induce an immune response without a carrier. The structure of the antigen is always the crucial factor. Moreover, non-specific stimulating substances, known as adjuvants, can positively influence the immunogenicity.

Characterisation of antibody-antigen binding

Antibodies are typified according to their specific responses and related affinities. The human immune system seems to have antibodies with about 10^7 to 10^8 specific responses. The scope of the hens' immune system is not assessed in the literature, but in hens too, each plasma cell synthesises precisely defined molecules, which are identical antibodies with their specific biological function. Raised to the level of immunological dogma, this axiom has been the prerequisite for the production of monoclonal antibodies. Every hybridoma cell clone comes from one B-lymphocyte stimulated by an antigen and accordingly produces a precisely defined antibody, the monoclonal one. Unfortunately, no suitable myeloma cell lines are presently available as fusion-partners in the hens' system. Polyclonal serum-antibodies on the other hand consist of a mixture of antibodies of various classes with various specificities and affinities, since they have come from various B- cell clones. Antigen-antibody binding is reversible in accordance with the law of mass action. The binding force between an antibody and antigen is taken as a measure of the antibody's affinity with the respective antigen.

There are no known differences between antibodies from mammals and hens in this respect.

Immunoglobulins in hens

Antibody molecules are glycoproteins called immunoglobulins. Hens' immunoglobulins are like mammals' in having light (L) and heavy (H) chains, bridged by disulphide bonds. The molecule is made up of a variable part with an antigen binding site and a constant part. In the case of hens, a distinction is made between the immunoglobulins M (IgM), Y (IgY) and A (IgA). Hens' IgM has the same function as mammals' IgM. Present in all vertebrates, IgM delivers the first response with its high molecular weight. Likewise avian IgA is not essentially different to mammals' IgA, as it too is found in the fluids of the gall bladder and in secretions active in the body. Already in 1969, Leslie and Clem suggested that hens' main serum-immunoglobulin should be called IgY instead of IgG, and the results of more recent genetic research suggest that the IgY molecule is phylogenetically a progenitor of mammals' IgG and IgE. According to Warr et al. (1995) this conclusion is justified by the antibody's function and structure and the corresponding expression of the H-chain genes. IgY, like mammals' IgG, is the immunoglobulin delivering the second response with its high serum-concentration and low molecular weight. But in the case of hens, IgY can also take part in anaphylactic reactions unlike mammalian IgG.

Structurally there is a clear difference between IgY and mammals' IgG, as the heavy chain of hens' IgY has an additional constant domain instead of the hinge region of mammals' IgG. So the molecular weight of IgY (about 190 kDa) is rather more than IgG's (Figure 1). In the literature, the terms IgG and IgY are sometimes used as synonyms as regards hens, so on the basis of the newest findings it has been decided within the framework of an international ECVAM workshop that the term IgY should be used throughout (Schade et al. 1996).