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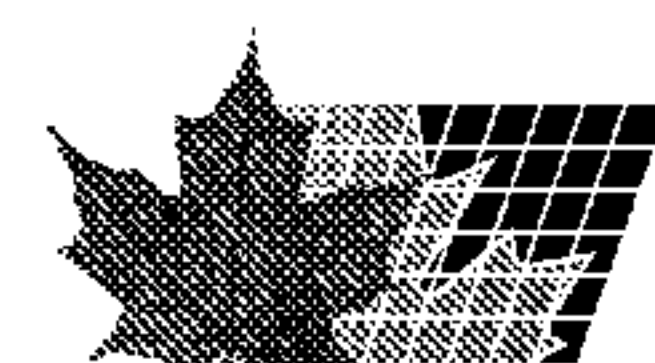
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(54) Titre : PROCEDE DE PREPARATION D'ANTICORPS AGISSANT A L'ENCONTRE D'UN POLYPEPTIDE QUI NE
CONNAIT QUE L'ACIDE NUCLEIQUE CODANT
(54) Title: METHOD FOR PRODUCING ANTIBODIES ACTING AGAINST A POLYPEPTIDE THAT ONLY RECOGNISES
THE CODING NUCLEIC ACID

(57) **Abrégé/Abstract:**

The present invention relates to a method for producing antibodies reacting specifically with a polypeptide that only recognises the coding nucleic acid, wherein said method comprises the following steps: a) the DNA coding the polypeptide is expressed in a host cell using a vector having at least one sequence coding a detection signal, and the expressed polypeptide is bound to a solid phase using the detection signal; b) regardless of step a), the DNA coding the polypeptide is introduced directly into an animal, which causes the expression of the polypeptide in the animal thus inducing the formation of antibodies acting against the polypeptide; and c), the polypeptide produced in step a) is used for reacting the antibodies formed in step b), for detecting them or for enriching them.



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Abstract

A process is disclosed for producing antibodies which react specifically with a polypeptide, the nucleic acid encoding which is known, wherein

- a) the DNA encoding the polypeptide is expressed in a host cell using a vector which possesses at least one sequence encoding a detection signal, and the expressed polypeptide is bound to a solid phase with the aid of the detection signal,
- b) independently of step a), the DNA encoding the polypeptide is introduced directly into an animal, resulting in expression of a polypeptide in the animal, which expression causes the formation of antibodies against the polypeptide, and
- c) the antibodies which are formed in step b) are reacted with the polypeptide formed in step a) and detected or enriched.

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**Process for preparing antibodies against a polypeptide,
the nucleic acid encoding which is known**

10 Because of the enormous advances which have
been made in the possibilities for sequencing nucleic
acids, the problem frequently arises in molecular
biology that, while the genetic information for a
polypeptide or protein is known, this polypeptide or
15 protein is not available in pure form. While nucleotide
sequences are continually being published as a result
of the Human Genome Project, the functions possessed by
the polypeptides or proteins encoded by these genes are
frequently completely unknown.

20 As a rule, it is very helpful for the practical
application and evaluation of these scientific findings
if these proteins can be detected using suitable
antibodies. Such antibodies can be used either to
purify the proteins or, for example, to determine the
25 location of the proteins in tissues and cells.

 An object of the present invention is therefore
to make available antibodies which are directed against
polypeptides or proteins, the nucleotide sequences for
which are known but which are not available in
30 enriched, and certainly not in purified, form.

 Conventionally, antibodies are prepared by the
proteins first of all being purified from the cells or
the tissue, or being prepared recombinantly using
bacteria, or in insect cells or mammalian cells, and
35 these proteins being used for immunizing animals. These
methods are frequently very elaborate and long-winded.
The proteins which have been prepared in bacteria are
frequently not identical to the naturally occurring

- 2 -

proteins since their secondary structure may differ from that of the native proteins and since bacteria do not possess the same post-translational modification mechanisms as those which are present in eucaryotic
5 organisms.

The present invention therefore relates to a process for producing antibodies which react specifically with a polypeptide, the nucleic acid encoding which is known, in which process

10

a) the DNA encoding the polypeptide is expressed in a host cell using a vector which possesses at least one sequence encoding a detection signal, and the expressed polypeptide is bound to a solid phase
15 with the aid of the detection signal,

b) independently of step a), the DNA encoding the polypeptide is introduced directly into an animal, resulting in expression of a polypeptide in the animal, which expression causes the formation of
20 antibodies against the polypeptide, and

c) the antibodies which are formed in step b) are reacted with the polypeptide formed in step a) and
25 detected or enriched.

The process according to the invention essentially consists of three steps. On the one hand, the DNA encoding the polypeptide is expressed in a
30 suitable host cell using a vector (step a)). Since the polypeptide which is expressed using the vector is as a rule only present at relatively low concentration in the host cell, the vector employed is provided, according to the invention, with a nucleotide sequence
35 which encodes a detection sequence (tag sequence). This tag sequence is linked to the sequence encoding the polypeptide, resulting in the expressed polypeptide possessing this detection peptide sequence at the C terminus, for example.

- 3 -

In step b), which is carried out independently of step a), the DNA encoding the polypeptide is introduced into a suitable animal and expressed in this animal. The genetic immunization which is employed in accordance with the invention enables antibodies to be formed directly in a host animal.

In this method of preparing antibodies, purified DNA, which contains the genetic information for the protein to be investigated and suitable control elements, is injected directly into the organism (mouse, rabbit, etc.) which is earmarked for the antibody production. The DNA is taken up by the cells of the recipient organism and the protein is expressed in native form (i.e. with correct post-translational modifications). The protein, which is foreign as far as the recipient organism is concerned, induces the immune system to produce antibodies which are directed against the foreign antigen (humoral immune response). This method has already been employed successfully for producing high-affinity monoclonal antibodies which recognise native proteins.

The expression vectors which are employed for the genetic immunization in step b), for the purpose of preparing the desired antibodies, are also to be used in vitro for producing the target protein. Transient transfection (electroporation, lipofection, etc.) is used to introduce the expression vectors into suitable target cells, in particular mammalian cells, which then synthesize the desired protein. These cells (intact or following lysis with suitable buffers) or medium supernatants (in the case of secreted proteins) are to be used for detecting the protein-recognizing antibody by means of FACScan analyses (in the case of proteins which are located in the cell) or ELISA.

When a foreign polypeptide is expressed in a host cell, the expressed polypeptide can usually be secreted to the exterior using a secretion sequence or leader sequence. In these cases, it is important that the expressed and secreted polypeptide possesses a

- 4 -

detection signal which can be used to isolate the polypeptide from the medium. If, however, the polypeptide is not secreted to the exterior but remains on the surface of the cell membrane, an additional
5 detection sequence is then not absolutely necessary. In this case, the site in the polypeptide which is responsible for the anchoring between polypeptide and cell assumes the function of the detection sequence. Since, in this case, the expressed polypeptide remains
10 linked to the cell, the antibodies which are formed can be detected by means of FACScan analyses, by binding to the polypeptide and subsequently reacting with a fluorescence-labeled antibody. As an alternative, it is also possible to carry out a cell ELISA in which the
15 bound antibodies are detected using an enzyme-coupled secondary antibody and a suitable substrate reaction. If the anchoring sequence is a signal sequence which is responsible for anchoring to a membrane by way of a glycosylphosphatidylinositol (GPI) residue, the
20 corresponding expression plasmid can then be used both for DNA immunization and for detecting the resulting antigen-specific antibodies, e.g. following transient transfection. The advantage of a GPI anchor is that it is easily cleaved enzymically from the cell surface in
25 vivo and that it is consequently possible to achieve a good antibody reaction, as is known for secreted proteins (see Example 7 for a good immune response following genetic immunization with an expression plasmid which encodes a GPI-anchored protein).

30 In the case of secreted proteins (where appropriate, also in the case of proteins which are expressed intracellularly), it is necessary to attach a detection sequence (a tag) to the antigen recombinantly. This tag sequence enables the protein to
35 be fished out of the cell supernatant or cell lysate using substances which interact with the tag sequence and which are bound to a solid matrix (e.g. antibodies which recognise the tag sequence; in the case of the His₆ tag sequence, suitable complexed Ni²⁺ ions).

- 5 -

Peptide sequences which are short and/or not very immunogenic are particularly suitable tag sequences. Mouse proteins which have a stimulatory effect on antibody production (e.g. GM-CSF, IL-4, IL-10, etc.) and which at the same time are able to function as tags can be used as tag sequences which are not particularly immunogenic (i.e. for preparing antibodies in mice). Such tags have the advantage of not developing any immune response because of the tolerance of the immunized animal towards these self-proteins. If it is not possible to prevent the formation of antibodies which recognise the tag sequence of a recombinant protein, these antibodies can be identified using constructs which encode irrelevant proteins which are provided with an identical tag.

The immobilized protein, which has been prepared by transient transfection, is now used to bind the antibodies, which recognise it, from the serum or the hybridoma culture supernatant (when preparing monoclonal antibodies). The bound, specific antibodies are then detected using enzyme-coupled anti-antibodies (detection antibodies) which are quantifiable, as a rule photometrically, by way of a specific substrate reaction. When using peptide tags, the specificity and sensitivity of the detection system can be significantly increased if $F(ab)_2$ fragments of the anti-tag antibody are used as captor antibodies and an Fc region-recognising antibody is used as the detection antibody. This configuration of the ELISA rules out any cross-recognition of the captor antibody.

The transcription unit which encodes the polypeptide can have a polyadenylation sequence, which is required for stabilizing a eucaryotic mRNA, at its 3' end.

In order to ensure that the polypeptide is expressed in the host cell, the vector normally possesses a promoter, with preference being given to using strong promoters. Examples which may be mentioned

- 6 -

are the elongation factor 1 α promoter or the cytomegalovirus promoter.

In the process according to the invention, the nucleic acid encoding the polypeptide is introduced
5 directly into an animal in order to produce antibodies against the polypeptide in this animal. In a preferred form, the DNA which is employed for this purpose is present in the form of a vector which is selected such that it can be used for the two steps a) and b) at one
10 and the same time. In a particularly preferred embodiment, the polypeptide-encoding DNA is introduced using a so-called gene gun. In the gene gun method, microscopically small gold particles are coated with the DNA, preferably the vector or plasmid DNA, and shot
15 at the shaved skin of the experimental animal. The gold particles then penetrate into the skin and express the DNA which has been applied to them in the host animal. Preference is given, according to the invention, to using laboratory animals such as mice, rats or rabbits.

20 In order to achieve a more vigorous antibody formation, so-called genetic adjuvants are, in a preferred embodiment, also applied simultaneously with the polypeptide-encoding DNA. These genetic adjuvants are plasmids which express cytokines (such as GM-CSF,
25 IL-4 and IL-10) and which stimulate the humoral immune response in the laboratory animals.

Particularly when the laboratory animal employed is a mouse or a rat, there is the opportunity of forming hybridoma cells. The immunized mice are
30 sacrificed, spleen cells are isolated and fused with tumor cells, and those clones which secrete the desired monoclonal antibodies are then selected.

In a particularly advantageous embodiment, the polypeptides to be investigated are secreted from the
35 host cells in step a). Since a detection signal is linked to the polypeptides, the sought-after polypeptides can be isolated by forming a bond between the detection signal (tag sequence) and a suitable ligand. The tag sequence is preferably bound to a solid

- 7 -

phase. This solid phase can be the walls of microtiter plates, gel spheres or else magnetic beads. Magnetic beads have the advantage that the solution containing the expressed polypeptide can be readily mixed with the magnetic beads. The magnetic beads possess a ligand (for example antibody fragments) which binds to the tag sequence. The magnetic beads can then be concentrated by applying a magnetic field. By choosing suitable conditions, the sought-after polypeptide can then be eluted once again from the magnetic beads when the antibodies are to be enriched.

The present invention also relates to the antibodies which can be obtained using the process according to the invention.

Figure 1 shows the detection, by means of FACScan analysis, of anti-hp70 antibodies in serum and in the culture supernatant from hybridomas obtained from the lymph nodes of mice immunized with hp70-pcDNA3 DNA. BOSC cells which were either untransfected (gray curves) or transiently transfected with hp70-pcDNA3 DNA (white curves) were used for the FACScan analysis. GV114, mouse immunized with the hp70-pcDNA3 expression vector. The experiment is explained in more detail in example 7.

The present invention is explained in more detail with the aid of the following examples.

Example 1

30 Preparing murine monoclonal antibodies by means of genetic immunization without purified antigen (protein)

a) Expression construct for the genetic immunization

35 An expression construct based on the commercially available expression vector pcDNA3 (Invitrogen) was selected. In this vector, the cDNA is expressed under the control of the cytomegalovirus (CMV) promoter. However, it is also possible to use

- 8 -

other, preferably strong, usually ubiquitously active promoters (e.g. the promoter of the elongation factor 1 α [EL-1 α] gene). The human cDNA region encoding the extracellular domain of thyroid peroxidase (TPO) (2602 bp; 859 amino acids) was cloned into the BamHI/EcoRV cleavage sites in the polylinker sequence and additionally provided, at the 3' end, with a region encoding a His₆ tag and a subsequent stop codon: (TPO sol.-His-pcDNA3). The plasmid DNA was replicated in E. coli and purified using a Qiagen plasmid isolation kit (Qiagen, Hilden).

b) Genetic immunization of mice

In principle, there are two different methods for administering DNA for the genetic immunization. These methods are intramuscular injection or intracutaneous administration using gas pressure-accelerated, microscopically small gold particles coated with plasmid DNA (gene gun). We used the gene gun method for the Example. For this, 200 μ g of TPO sol.-His-pcDNA3 DNA were applied per 25 mg of gold particles in accordance with the manufacturer's instructions (gene gun optimization kit; Bio-Rad, Munich). For the genetic immunization, the abdominal fur (approx. 4 cm²) was removed, using perfume-free depilation cream (Veet), from five mice after they had been anaesthetized (intraperitoneally) with 110 μ l of ketamine/xylazine (100 mg/kg/16 mg/kg); the mice were then bombarded twice with the gene gun (Helios gene gun; Bio-Rad). 1 μ g of plasmid DNA was administered per "bombardment". The immunization was repeated after 19 days, and blood was withdrawn 14 days later for determining the quantity of specific antibodies.

35

Example 2

Expressing the protein encoded by the expression construct

- 9 -

The protein encoded by the expression plasmid has to be prepared in order to detect the specific antibodies which are formed as a result of the genetic immunization. In order to obtain the protein in native
5 form (as in the immunized animal), the expression construct was introduced by transfection into BOSC23 cells [Pear et al., (1993) PNAS, 84, 8392-8396]. BOSC23 cells are a modified adenovirus 5-transformed human embryonic kidney cell line (HEK293) which can be
10 transiently transfected very satisfactorily. The cells were plated out in 6-well cell culture dishes such that they reached 80% confluence on the following day. They were then washed three times with in each case 2 ml of serum-free and antibiotic-free *Dulbecco's modified*
15 *Eagle's medium* (DMEM) medium and treated with 2 μ g of expression plasmid/10 μ l of lipofectamine (Life Technologies, Eggenstein) in 1 ml of serum-free and antibiotic-free DMEM medium. The DNA/lipofectamine/medium mixture had previously been
20 pipetted together in a polystyrene vessel and incubated for 10 minutes at room temperature. Following a 6-hour incubation at 37°C and 10% CO₂, 2 ml of DMEM/20% fetal calf serum (FCS) were added. 24 h after transfection (corresponds to the time at which the DNA was added),
25 the medium was replaced with 5 ml of DMEM/5% FCS. After a further 48 h (72 h after transfection), the cell culture supernatant was removed and stored at -70°C.

Example 3

30

Detecting specific antibodies which are directed against the protein encoded by the expression construct

In order to bind the His₆ tag protein (TPO
35 sol.-His) prepared by transient transfection to nickel chelate microtiter plates (DUNN, Asbach), the wells were in each case incubated, overnight at 4°C, with 200 μ l of supernatant from the transient transfection mixture (see above) or of a mock-transfected BOSC23

- 10 -

culture supernatant and then washed 4 times with buffer A (50 mM tris/HCl, pH 7.5, 1 M NaCl) and twice with buffer B (phosphate-buffered saline (PBS), 0.1% BSA, 0.05% Tween 20). Nonspecific binding sites were then
5 blocked by incubating with 300 μ l of 3% bovine serum albumin (BSA)/PBS at room temperature for 1 h, after which the washes with buffer A and buffer B were repeated. The pre-immune sera and the immune sera from the immunized mice were diluted 1:100 with buffer B. In
10 each case 100 μ l of the diluted mouse sera were added to the wells of the nickel chelate microtiter plates. After incubating at room temperature for 1 hour, the wells were in each case washed four times with buffer C (50 mM tris/HCl, pH 7.5, 0.5 M NaCl, 0.1% BSA, 0.05%
15 Tween 20) and twice with buffer B and then treated with 100 μ l of rabbit anti-mouse Ig peroxidase conjugate (DAKO, Hamburg) diluted 1:2000 with buffer B. After a one-hour incubation, the wells were washed four times with buffer C and twice with buffer B and in each case
20 treated with 100 μ l of 3,3',5,5'-tetramethylbenzidine substrate solution (Fluka, Buchs, Switzerland). After sufficient development, the color reaction was stopped by adding 50 μ l of 0.5 M H₂SO₄ and measured in an ELISA reader at a wavelength of 450 nm.

25 In order to check the serviceability of the invention which is presented here, the specific antibodies directed against TPO were detected "classically" by means of a commercially available TPO antibody ELISA (Varelista TPO antibody; Pharmacia-
30 Upjohn, Freiburg). In this test system, anti-TPO antibodies are detected using purified recombinant TPO. The content of anti-TPO antibodies in the pre-immune and immune sera from the immunized mice was determined at a dilution of 1:100 in accordance with the
35 manufacturer's instructions.

- 11 -

Results:

It was possible to detect anti-TPO antibodies unambiguously, as compared with the pre-immune sera, at a dilution of 1:100, in the serum obtained from all the five mice which were immunized with TPO sol.-His-pcDNA3 DNA. The results are presented in Table 1.

Table 1: Detection of anti-TPO antibodies in the serum of TPO sol.-His-pcDNA3 DNA-immunized mice using purified TPO protein (*Varelixa TPO Antibodies* detection system).

Mouse	Optical density _{450 nm}	
	Pre-immune serum	Immune serum
GV1	0.09	2.53
GV2	0.06	1.97
GV3	0.07	1.13
GV4	0.08	1.63
GV5	0.08	0.60

The detection system according to the invention was used to investigate the pre-immune serum and immune serum from a mouse (GV1 in Table 1) as an example. As can be seen from Table 2, it is possible to detect anti-TPO antibodies unambiguously, at a serum dilution of 1:100, in the immune serum whereas the pre-immune serum did not exhibit any reaction.

Table 2: Detection of anti-TPO antibodies in the serum of a TPO sol.-His-pcDNA3 DNA-immunized mouse using TPO sol.-His protein which was produced by transient expression.

Serum or buffer	Dilution with buffer A	Optical density	
		TPO sol.-His	Medium
pre-immune	1:100	0.17	0.15
immune	1:100	0.55	0.19

- 12 -

buffer A	--	0.03	0.01
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Example 4

5 **Preparing polyclonal antibodies by means of genetic immunization without purified antigen (protein) in rabbits**

a) Expression construct for the genetic immunization

10 For the second case example, the ubiquitously active promoter of the elongation factor 1 α (EF-1 α) gene was used for controlling the expression. The expression vector employed is based on the pBluescript vector (Stratagene, Heidelberg), into which a 1.2 kb
15 fragment of the human EF-1 α gene promoter, an 0.7 kb EcoRI fragment containing the polyadenylation signal from the cDNA for human G-CFS (Mizushima and Nagata, 1990), and also, between the BamHI and NotI cleavage sites, the oligonucleotide sequence encoding the
20 influenza virus hemagglutinin (HA) tag were incorporated. The human cDNA region encoding the extracellular domain of the activin receptor IIA (431 bp; 135 amino acids) was cloned into the ClaI/BamHI cleavage sites of the polylinker sequence such that the
25 HA tag-encoding region and a subsequent stop codon came to lie at the 3' end (pEF-1 α -ActRII-HA).

b) Genetic immunization of rabbits

30 For the genetic immunization, 100 μ g of pEF-1 α -ActRII-HA DNA were applied per 25 mg of gold particles in accordance with the manufacturer's instructions (*gene gun optimization kit*; Bio-Rad, Munich). After having been anaesthetized with 15 mg of
35 pentobarbital/kg and having 200 cm² of the abdominal fur depilated with depilation cream, two rabbits (Chinchilla Bastard; Charles River, Sulzfeld) were

- 13 -

bombarded 30 times with the gene gun. 1 μ g of plasmid DNA mixture was administered per "bombardment". The immunization was repeated after 21 days and blood was removed 21 days later for determining the quantity of specific antibodies.

Example 5

Expressing the protein encoded by the expression construct

The protein encoded by the expression plasmid pEF-1 α -ActRII-HA was prepared, as described in Example 2, by transiently transfecting BOSC23 cells.

Example 6

Detecting specific antibodies which are directed against the protein encoded by the expression construct

In order to bind the HA tag protein (EF-1 α -ActRII-HA), prepared by transient transfection, to microtiter plates, the wells were first of all coated with the F(ab)₂ fragment of the anti-HA tag antibody. For this, 150 μ l of the antibody fragment were added to each well of the microtiter plate, after which the plate was washed with PBS at room temperature and free protein-binding sites were blocked by incubating with 200 μ l of 0.2% BSA/PBS/well.

The supernatant from the transient transfection mixture (see Example 5), or from a mock transfected BOSC23 culture supernatant[sic], was then incubated at room temperature for 2 h, after which the plates were washed three times with phosphate-buffered saline (PBS). The pre-immune sera and immune sera from the immunized rabbits were diluted 1:100 and 1:500, respectively, with 0.2% BSA/PBS. 100 μ l of the diluted rabbit sera were in each case added to the wells of the coated microtiter plates. After the plates had been

- 14 -

incubated at room temperature for one hour, the wells were in each case washed three times with PBS, after which 100 μ l of goat anti-rabbit Ig peroxidase conjugate (DAKO, Hamburg), diluted 1:2000 with PBS/0.2% BSA) were added to each well. After the plate had been incubated for one hour, the wells were washed three times with PBS, after which 100 μ l of 3,3',5,5'-tetramethylbenzidine substrate solution (Fluka, Buchs, Switzerland) were added to each well. After it had developed sufficiently, the color reaction was stopped by adding 50 μ l of 0.5 M H₂SO₄ to each well and measured in an ELISA reader. The results showed that it is also possible to use the process according to the invention to produce specific polyclonal antibodies against an unknown gene product in rabbits.

Example 7

Using genetic immunization to prepare murine monoclonal antibodies against a human GPI-anchored surface protein

a) Expression construct for the genetic immunization

For the genetic immunization, the complete hp70 cDNA, encoding a 70 kDa GPI-anchored human surface protein, was cloned into pcDNA3 (hp70-pcDNA3) and replicated (see Example 1). Approx. 70% of the residues of the human hp70 amino acid sequence tally with those of the murine hp70 sequence.

b) Genetic immunization of mice

The mice were immunized with the gene gun (see Example 1b) using a short protocol (6 immunizations within 13 days), as described by Kilpatrick et al. (1998), Hybridoma 17: 569-576.

c) Preparing hybridomas for producing monoclonal antibodies

- 15 -

In order to prepare hybridomas, lymphocytes were isolated from the regional (axillary, brachial, inguinal and popliteal) lymph nodes from three mice and fused, in accordance with a standard protocol, with exponentially growing SP2/0 mouse myeloma cells (American Tissue Type Culture Collection) using polyethylene glycol (Sigma) (Campbell A M (1986). Monoclonal antibody technology: The production and characterization of rodent and human monoclonal antibodies. Book series: Laboratory Techniques in Biochemistry and Molecular Biology (R H Burdon and P H van Knippenberg, eds.), Elsevier Science Publishers, Amsterdam). 2×10^5 fused lymph node lymphocytes were plated out in each well of a 96-well microtiter plate and in each case cultured in $100 \mu\text{l}$ of hypoxanthine/aminopterin/thymidine (HAT)-containing DMEM medium (Sigma) containing 20% FCS and 5% *Hybridoma Enhancing Factor* (Sigma).

20

d) Detecting specific antibodies using cells in which the expression construct used for the genetic immunization is expressed following transient transfection

25

Candidate hybridoma clones were identified by means of a cell ELISA. For this, BOSC cells, as described in Example 2, were transiently transfected with the hp70-pcDNA3 expression construct, resuspended in 4% formaldehyde in PBS and fixed for 10 min. The cells were then diluted 1:10 with PBS and stored at 4°C for up to four weeks.

30

Cell ELISA

35

96-well round-bottom microtiter plates were blocked at room temperature by adding $300 \mu\text{l}$ of 1% BSA in PBS per well for 1 h. After the solution had been removed by inverting the plates, $75 \mu\text{l}$ of the hybridoma

- 16 -

cell supernatant and 10 μ l of transiently transfected BOSC cell suspension (6×10^6 cells/ml of 1% BSA in PBS) were added per well and the plates were incubated at 4°C for 1 h. After 100 μ l of 1% BSA in PBS had been added to the well, the plates were centrifuged at 300 x g for 4 minutes and the supernatants were tipped out as above. The cells were washed once again with 200 μ l of 1% BSA/PBS/well, resuspended in each case in 75 μ l of peroxidase-coupled goat anti-mouse immunoglobulin antibody (DAKO), diluted 1:2000 in 1% BSA/PBS, and incubated at 4°C for 1 h. 100 μ l of 0.1% Tween 20/PBS were then added per well and the plates were centrifuged as above and the supernatants discarded. The cells were then washed three times with in each case 200 μ l of 0.1% Tween 20/PBS and twice with in each case 200 μ l of PBS. Peroxidase-coupled goat anti-mouse IgG antibody (diluted 1:2000) or goat anti-mouse IgM antibody (diluted 1:2000) (Southern Biotechnologies Associates) was used to determine the immunoglobulin class (IgG or IgM) of the monoclonal antibodies in the hybridoma supernatants. The peroxidase bound to the cells by way of the antibodies was quantified by adding 3,3',5,5'-tetramethylbenzidine substrate solution as described in Example 3.

25

Results:

In all, 176 hybridoma-covered microtiter wells were obtained using the above-described fusion. 64 supernatants from these wells proved to be positive for anti-hp70 antibodies when an OD₄₅₀ value which was twice as high as the blank value obtained with medium (blank value: 0.035) was used as the threshold value. Table 3 lists the values which were measured for a negative (N1B10) hybridoma supernatant and for a positive (N1F4) hybridoma supernatant. The OD values obtained in the same test for the immune serum and pre-immune serum from a mouse (GV114) used for preparing the hp70 hybridoma are shown for comparison. The same N1B10 and

- 17 -

N1F4 hybridoma supernatants were also tested for the presence of specific anti-hp70 antibodies by means of a FACScan (*fluorescence-activated cell scanning*) analysis (see below).

5

Table 3: Use of a cell ELISA to detect anti-hp70 antibodies in serum and in culture supernatants from hybridomas obtained from the lymph nodes of mice immunized with hp70-pcDNA3 DNA. BOSC cells which were transiently transfected with hp70-pdDNA3 DNA were used for the cell ELISA.

10

Serum or hybridoma supernatant	Dilution	Optical density _{450 nm}
Pre-immune serum GV114	1:100	0.08
Immune serum GV114	1:100	1.21
Hybridoma supernatant N1B10	undiluted	0.05
Hybridoma supernatant N1F4	undiluted	1.07

FACScan analysis

15

In each case 10 μ l of the suspension of fixed, transiently transfected BOSC cells (20×10^6 in 3% FCS/PBS), as described under cell ELISA, were pipetted into the wells of a 96-well round-bottomed microtiter plate, after which 75 μ l of the given hybridoma supernatant were added. As controls, cells were treated either with 25 μ l of pre-immune or immune sera diluted 1:100 with 3% FCS/PBS or with 25 μ l of a control monoclonal antibody (50 μ g/ml of 3% FCS/PBS). After the plate had been incubated at 4°C for 30 min, 200 μ l of 3% FCS/PBS were added to each well and the cells were centrifuged down, as described above, and the supernatants discarded. After the plate had been washed once with 200 μ l of 3% FCS/PBS per well, 25 μ l of a phycoerythrin-coupled goat anti-mouse immunoglobulin antibody (Southern Biotechnologies Associates), diluted 1:50 with 3% FCS/PBS (final concentration: 10 μ g/ml),

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- 18 -

were added per well and the plate was incubated at 4°C for 30 min. The cells were subsequently washed twice as above and the fluorescence was measured in a FACScan appliance (Becton Dickinson).

5

Results:

20 supernatants giving OD₄₅₀ values of >0.2 were selected from the hybridoma supernatants judged to be positive in the cell ELISA (see above) for the determination of anti-hp70 antibodies by means of FACScan analysis. Figure 1B shows the histograms which were obtained, using BOSC cells which were transiently transfected with the hp70-pcDNA3 expression vector or which were not transfected, for an irrelevant antibody (26/3/13), used as negative control, and for the positive hybridoma supernatant N1F4. The histograms which were obtained in the same test for the immune and pre-immune sera from a mouse used for the hybridoma preparation are shown for comparison (Figure 1A). All the 20 hybridoma supernatants selected proved to be positive in the FACScan analysis. The immunoglobulin class of the hp70-specific antibodies was determined in 19 out of the total of 20 supernatants. Two of the tested supernatants contained hp70-specific IgM antibodies and 17 supernatants contained hp70-specific IgG antibodies.

- 19 -

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Patent claims

1. Process for producing antibodies which react
5 specifically with a polypeptide, the nucleic acid
encoding which is known, wherein
 - a) the DNA encoding the polypeptide is expressed in a
host cell which is derived from a mammal using a
10 vector which possesses at least one sequence
encoding a detection signal, and the expressed
polypeptide is bound to a solid phase with the aid
of the detection signal,
 - 15 b) independently of step a), the DNA encoding the
polypeptide is introduced directly into an animal,
resulting in expression of a polypeptide in the
animal, which expression causes the formation of
antibodies against the polypeptide and the
20 expression vector employed for the genetic
immunization in step b), for the purpose of
preparing the desired antibodies, is also used in
vitro for producing the target protein, and
 - 25 c) the antibodies which are formed in step b) are
reacted with the polypeptide formed in step a) and
detected or enriched.
2. Process according to claim 1, characterized in
that the vector used in step a) possesses, at the C-
30 terminus of the DNA encoding the polypeptide, a
sequence which encodes the detection signal.
3. Process according to claim 2, characterized in
that the detection sequence is selected from the His₆
tag sequence, the hemagglutinin sequence of an influenza
35 virus or the myc tag sequence.

AMENDED SHEET

- 20 -

4. Process according to one of the preceding claims, characterized in that the vector encoding the polypeptide possesses a polyadenylation sequence at the C-terminal end of the detection sequence.
- 5 5. Process according to one of the preceding claims, characterized in that the vector encoding the polypeptide possesses a strong promoter at the 5' end of the DNA sequence encoding the polypeptide.
6. Process according to claim 5, characterized in
10 that the strong promoter is selected from the group consisting of strong eucaryotic promoters, in particular the elongation factor 1 α promoter or the cytomegalovirus promoter.
7. Process according to one of the preceding
15 claims, characterized in that the polypeptide-encoding DNA, which is introduced directly into an animal in accordance with step b), is present in a vector.
8. Process according to one of the preceding claims, characterized in that the polypeptide-encoding
20 DNA is introduced into the animal in step b) using a gene gun.
9. Process according to one of the preceding claims, characterized in that the animal employed in step b) is a mouse, a rat or a rabbit.
- 25 10. Process according to one of the preceding claims, characterized in that, in step b), a genetic adjuvant is administered in addition to the polypeptide-encoding DNA.
11. Process according to claim 10, characterized in
30 that the genetic adjuvant is selected from a group comprising cytokine expression vectors which increase antibody production.
12. Process according to one of the preceding claims, characterized in that suitable cells from an
35 animal which has been immunized in accordance with step b) are used for preparing hybridoma cells for forming monoclonal antibodies.

AMENDED SHEET

- 21 -

13. Process according to one of the preceding claims, characterized in that polypeptide formed in step a) is bound to a solid phase by means of the detection signal being bound to an antibody or an antibody fragment which is directed against it.

14. Process according to claim 13, characterized in that the solid phase is microtiter plates, gel spheres or magnetic beads.

15. Process according to one of the preceding claims, characterized in that the antibody formed in step b) is detected, after having been bound to the polypeptide formed in step a), using an anti-antibody which is directed against the antibody.

16. Process according to one of the preceding claims, characterized in that the antibody which is bound to the expressed polypeptide in step c) is released by elution.

17. Process according to one of the preceding claims, characterized in that the detection signal is a sequence which is responsible for membrane anchoring using a GPI residue.

18. Antibody, characterized in that it can be obtained using one of the processes according to claims 1-17.

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Fig. 1

