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MONOCLONAL ANTIBODIES

The present invention concerns non-human primate monoclonal antibodies, hybridoma cell lines and methods for producing them, as well as the use of said cell lines for producing said antibodies.

Hybridomas are cells formed by the fusion of an immortalizing cell, such as a myeloma cell, to a what is often a non-transformed partner cell usually chosen for its ability to produce a particular, predetermined substance (e.g., a lymphocyte cell to produce antibodies). The resulting hybrid cell may be selected and cloned to obtain cell lines producing substances having a single structure and/or property. In particular, such hybridomas formed with lymphocytes can be used to produce monoclonal antibodies.

The development of hybridoma technology in recent years has been directed to obtaining cell lines which are both stable and have a high and specific production of a particular substance which it is desired to obtain. There have been various approaches to this problem which because of their historical origin have been largely concentrated in the field of immunoglobulins/antibodies.

A study of previous activities in this field provides an overview of the types of problems encountered and the various solutions so far attempted.

The original impulse for research into hybrid cell lines producing monoclonal products came from the field of immunobiology, the products being monoclonal antibodies. Conventional anti-sera usually contain a very large number of antibodies which differ structurally in the antigen binding site, but each bind to the same antigen with a greater or lesser degree of avidity and/or specificity. In addition conventional anti-sera also contain a large number of anti-

bodies directed against other antigens and reflecting previous defensive reactions of the host individual from whom the antiserum was obtained. Although for most purposes such "broad" antisera were sufficient it was felt that provision of more specificity and reproducibility would mark a significant advance and provide a scientific tool of tremendous potential, particularly in the field of diagnosis and therapy.

The first and now classical solution was that described by Kohler and Milstein [NATURE, 256, 495-497 (1975)] who succeeded in fusing pre-immunized mouse spleen cells to "drug" sensitive mouse myeloma cells. The thus immortalized fused cells could be grown in vitro and cloned from the single cell level to produce a homogenous cell population producing a homogenous antibody population (monoclonal antibodies). By selection between the many unique cells and selective recloning it was possible to obtain and grow cells which produce antibodies of the desired antigen specificity.

Mouse antibodies produced in this fashion have proved useful for research and diagnostic purposes and some have even been used therapeutically in humans. It would however involve a considerable advance in human immunoglobulin therapy if human or non-human primate antibodies of similar specificity and reproducibility could be obtained in this way. This would also reduce the danger of sensibilisation. Several approaches to the problem of producing human antibodies in vitro have been tried but have so far been largely unsuccessful. These include:

(i) Transformation of normal human lymphocytes with Epstein-Barr virus (EBV). This method has met with little success as cells of this type require a long and tedious process to become established and are extremely difficult to clone and select.

(ii) Fusion of normal (human) lymphocytes with human myeloma cells. This method represents an obvious analogy to the mouse-mouse hybridoma approach but faces the problem that in the human-system only one myeloma cell line has been made broadly available and this was found to be contaminated with mycoplasma which impedes successful fusions.

(iii) Fusion of normal human lymphocytes to an EBV-transformed human lymphoblastoid B-cell line. Whilst this is probably the most reliable and reproducible method yet described it suffers from the basic in vitro drawback encountered with lymphoblastoid cells: they are extremely difficult to clone and, as they represent an early stage in B-cell differentiation lineage, their capacity to produce and secrete antibodies is about 10 times lower than that of true myelomas.

(iv) Fusion of human lymphocytes to mouse myeloma. This method produces cells with the same excellent in vitro characteristics as the mouse-mouse hybridomas but with the great disadvantage that such hybrids have an inherent genetic instability. One particularly troublesome result of this is that they expel the human chromosome on which the genome for forming the kappa light chain of the immunoglobulin is located.

It was subsequently reported that by using a xenogenic hybridoma cell as parent for further fusion to another cell it is possible to obtain a hybridoma of greatly improved stability. (Ostberg, et al., HYBRIDOMA 2, No. 4, 361, 1983) and that these xenogeneic hybridomas present a more benign environment for the improvement of stability in terms of chromosome loss.

This invention concerns a hybridoma cell line comprising a parent immortalizing cell fused to a xenogeneic partner cell, the resulting immortalizing cell being then fused to a cell capable of producing a non-human primate monoclonal antibody. This latter cell is to be genetically compatible with the said partner cell in the xenogeneic hybridoma. By genetically compatible is meant the characteristic of resembling the related cell as to species and genera of origin such as to avoid significant loss of chromosomes and therefore retain a desirable degree of stability after fusion.

It now appears that preparation and use of non-human primate monoclonal antibodies prepared by such xenogeneic hybridoma fusion technique may prove to be a useful approach to the treatment of humans using monoclonal antibodies and in diagnostic applications. No prior report of preparation or isolation of non-human primate monoclonal antibodies is known.

It is accordingly an object of this invention to provide a method for producing stable cell lines which generate non-human primate monoclonal antibodies useful in the treatment of humans and in diagnostics. It is a further object of this invention to provide immunoglobulins derived from non-human primate lymphocytes which are capable of effective use in humans. The non-human primates included in this aspect of the invention include in essence the apes, e.g., organgutans, gorillas,

or chimpanzees, and the monkeys, such as new world monkeys, e.g. cebus monkeys, and old world monkeys, e.g. rhesus monkeys. As between the apes and monkeys, it is clear that for practical purposes, the use of animals small enough to be readily handled in laboratory conditions is preferred but this aspect is intended to cover all non-human primates.

In a particular aspect the xenogeneic hybridoma chosen as an immortalizing cell is a myeloma hybrid which has lost its own ability to produce immunoglobulin. An example of such a xenogeneic hybridoma cell would be that between a myeloma cell and a lymphocyte cell. Examples of suitable myeloma cells are those obtained from mice and rats and will preferably produce no immunoglobulin. These myelomas can themselves be hybrids (e.g. mouse myeloma x mouse lymphocyte) which are in effect myelomas. Such a cell is, e.g., the mouse SP-2 myeloma cell line. This may then be fused to a monkey lymphocyte, for instance, to produce monkey monoclonal antibody.

As described in the HYBRIDOMA 2 paper previously referred to (as well as in corresponding patent applications, e.g. UK Patent Application 2,113,715A, published August 10, 1983), the xenogeneic hybridoma used for immortalization is made drug resistant prior to further fusing, and the lymphocyte to which it is fused is obtained preferably after pre-sensitization for improved production of the desired substance. The methods used in drug resistance and pre-sensitization treatments are conventional, as is the process of fusing these cells. Selection of the desired hybrids and cloning are also performed in a conventional manner, as earlier described, and published, e.g.

in the references quoted above.

In a particular embodiment according to the present invention, the SP-2 cell line is used. It was originally itself a hybridoma between P3-X63-Ag8 line and mouse spleen cells which produce antibodies to sheep red blood cells and has lost its ability to produce antibodies (C.F. M. Shulmann et al., NATURE 276, 269, 1978).

It is obtainable e.g. from the NIGMS Human Genetic Mutant Cell Repository Ref. GM 35669 A (see US DHHS 1982 Catalog of Cell Lines). This cell line is made drug resistant and then fused with normal human peripheral lymphocytes by conventional techniques [C.F. G. Galfre et al., NATURE 266, 550 (1977) and R. Nowinski et al., SCIENCE 210, 537 (1980)].

A large number of hybrids is obtained and, after approximately five weeks, five clones are selected which show fast growth and no antibody production. These cells are selected for resistance to 8-azaguanine and with three of these lines it is possible to obtain mutants which are resistant to 20 ug/ml of 8-azaguanine. These cells are at the same time sensitive to Hypoxanthine-Aminopterin-Thymidine (HAT) medium which showed that they had lost their ability to produce hypoxanthine phosphoribosyl transferase. One of these lines is SPAZ 4 which may be used for further fusion to obtain the antibody producing hybridoma.

The simple hybridoma cell lines of this invention which produce non-human primate monoclonal antibodies may also, if desired or required, be fused further, such as with a further lymphocyte cell from a monkey, to provide cells of either still greater stability or somewhat different desired characteristics.

The antibodies according to the invention may then be used in humans to fight infectious diseases, malignancies, allergies, and may be used to curb transplantation rejections and for other purposes known in the art, such as in toxicity caused by pharmaceuticals, e.g. digoxin. They may also be used in diagonostic applications such as in kits and the like.

Examples of the types of cell lines to which this invention relates are cell lines comprising mouse myeloma cells and human and non-human primate cells, such as from a mouse-human xenogeneic cell to produce a (mouse x human) x non-human primate cell line, e.g. a (mouse x human) x monkey hybridoma cell line, or a (mouse x non-human primate) x non-human primate cell line, such as a (mouse x monkey) x monkey cell line. Further cell lines provided according to this invention include, e.g. a [(mouse x human) x non-human primate] x non-human primate cell line, for instance a [(mouse x human) x cebus monkey] x cebus monkey cell line, a [(mouse x human) x rhesus monkey] x rhesus monkey cell line, a [(mouse x human) x chimpanzee] x chimpanzee cell line, and a [(mouse x human) x rhesus monkey] x chimpanzee cell line.

The monoclonal antibodies produced according to the invention may be confectioned and administered in conventional manner for therapeutic application and also got up in conventional kits for diagnostic use.

The following examples illustrate the invention.

Example 1

Digoxin is dissolved in 2 ml of absolute ethanol, and 2 ml of 0.1 molar sodium periodate is added dropwise and the mixture incubated for 45 minutes. Then 60 microliters of ethylene glycol is added. After 5 minutes incubation 50 mg of keyhole limpet hemocyanin (KLH) in 10 ml of water at pH 9.5 is added. When the pH stabilizes at 9.5, 0.3 grams of $NaBH_4$ in 2 ml of water is added. The resulting preparation is dialyzed overnight against saline.

About 4 mg of KLH-digoxin in a volume of 0.5 ml is emulsified together with 0.5 ml of Freund's complete adjuvant. The emulsion is injected intramuscularly in both thighs of a cebus monkey. Following this first injection, the animal is boosted four times with a similar amount of KLH-digoxin but this time emulsified in 0.5 ml of Freund's incomplete adjuvant. After this immunization the test bleeding shows that the animal has produced antibodies reacting with digoxin that were able to inactivate the toxicity of digoxin in in vitro studies. This is shown by noting that dilutions of monkey serum neutralize the toxic (lethal) effect of digoxin (or its analog oubain) on sensitive human lymphoblastoid cells in cell culture. After resting the animal for two months, another injection of 0.5 ml of KLH-digoxin in saline is given intravenously.

On the third day after the last intravenous injection, cells are removed from the animal for fusion.

a. Blood is removed from a peripheral vein into a syringe containing 50 µl heparin solution (10,000 USP units/ml). The blood lymphocytes are purified by gradient centrifugation on a cushion of PERCOLL® (Pharmacia, Inc., Piscataway, N.J.). After centrifugation the cells are

washed in Hank's balanced salt solution (Gibco Laboratories, Grand Island, N.Y., catalog no. 310-4020, widely available commercially).

b. By using aseptic surgical techniques, lymph nodes are removed from the inguinal area. The lymph nodes are mashed through a fine mesh steel net in order to prepare single cell suspensions. The isolated cells are washed in Hank's balanced salt solutions.

c. Using aseptic surgical techniques, a partial splenectomy is performed on the animal. A single cell suspension is prepared from the spleen fragment by mashing the organ through the fine mesh steel net. This isolated single cell suspension is washed in Hank's balanced salt solution.

Approximately $2x10^7$ isolated lymphocytes are mixed with a similar amount of SPAZ-4 myeloma cells (available according to methods described in the art, such as the HYBRIDOMA 2 reference aforementioned) in serum free medium and pelleted together in a centrifuge at low speed. The cell pellet is treated for one minute with 1.0 ml of 50% PEG-1500 in Hank's balanced salt solution at a pH of 8.0. After one minute incubation at 37°C., one ml. of Hank's balanced salt solution is slowly added. In the following one minute, ten more ml of Hank's solution is added. The cells are recovered by centrifugation and resuspended in 20 ml of Dulbecco's Modified Eagle Medium (No. 430-2100, Gibco Laboratories) VIROLOGY 8, 396 (1959); VIROLOGY 12, 185 (1960); Tissue Culture Standards Committee, IN VITRO, Vol. 6, No. 2, P. 93, containing 20% fetal bovine serum and using HAT (hypoxanthine-aminopterin-thymidine) as a selection system.

After about three weeks, when good growth is seen in the culture wells, supernatant is removed from the cultures for testing. The testing is performed by an ELISA technique using digoxin coupled to bovine serum albumin (BSA) as the antigen (the BSA-digoxin is produced in a way analogous to the KLH-digoxin). Positive clones are detected using a rabbit anti-human or anti-monkey immunoglobulin - peroxidase system. Positive clones are picked for scaling up and at the same time cloned by limiting dilution in microtiter wells using mouse thymocytes as filler cells. As soon as a large number of cells are available the clones are frozen in liquid nitrogen at -196°C.

Antibodies are produced by growing the hybridoma cell lines either in stationary culture or in roller culture using Dulbecco's MEM with a maximum of 10% fetal bovine serum. Antibodies, typically at a concentration of 20 to 50 micrograms per milliliter, are purified by affinity chromatography on Sepharose Protein A columns (Pharmacia, Inc.), by loading the material to be chromatographed onto the column, saturating the column's binding capacity and washing the column with phosphate buffered saline. After removal of the unbound material from the column, the column is eluted with 0.05 M sodium citrate containing 0.5 M sodium chloride (pH 3.0). The eluate is rapidly neutralized by addition of 1 M Tris-HCl, pH 8.0).

Example 2

Cells produced according to the cell production and selection processes of Example 1, which cells have over time lost their ability to generate immunoglobulin, are made drug resistant by treatment in the presence of 20µg/ml of 8-azaguanine making it possible to use HAT as a selection system in conventional fashion. The resulting cells are then fused to immunized cebus monkey lymphocytes and after treatment again with HAT-medium, positive cell cultures are selected using the same procedures as described in Example 1 above.

According to this procedure, three antibodies have been obtained from fusions using cells from the cebus monkey. The hybridomas are designated 7-1, 11-1 and 11-3 respectively. All three antibodies have been tested for their ability to inhibit the toxicity of digoxin on a human lymphoblastoid cell line, IM-9. When the antibodies were used in a final concentration of 100 µg/ml the following result was obtained:

Addition	Digoxin dose causing no damage
phosphate buffered saline	2.9×10^{-8}M
7-1	4.7×10^{-7}M
11-1	9.4×10^{-7}M
11-3	2.3×10^{-7}M

The 7-1 antibody has also been tested for its specificity against other types of digitalis alkaloids. The result of this specificity testing is summarized in the following table:

Drug	Drug dose causing no damage	
	phosphate buffered saline	7-1
oubain	2×10^{-8}M	2×10^{-8}M
digitoxin	5.9×10^{-9}M	2.3×10^{-8}M
deslanosid	2.3×10^{-8}M	1.9×10^{-7}M

These data taken together show that antibodies produced by this methodology can be protective to the toxic effect of drugs, and that in the case of digitalis alkaloids one antibody can cover two of the drugs used on an out-clinic patient basis (digoxin and digitoxin) and against one parenterally used drug (deslanosid). It has no activity against another parenteral drug (oubain).

Example 3

In a manner similar to that described in Example 1, KLH-digoxin is used as immunogen in an adult male chimpanzee. After a total of five injections of this antigen, given essentially two weeks apart, a final challenge is administered intravenously with 0.5 ml of KLH-digoxin (approximately 4 mg of protein). Blood sample is taken from the animal on the sixth day after the last i.v. injection and, one day after the bleeding, is used for cell fusion with the SPAZ-4 myeloma cell line using the procedure previously outlined. From the sample obtained on day 6, IgG antibody producing hybridomas designated CH 4-14 and CH 4-25 are obtained, having respectively a lambda and a kappa light chain. Characterization of the light chains is obtained by using the reagents against human immunoglobulins in conventional manner. Such reagents give unambiguous results and the reactivity is stronger to anti-human reagents than to anti-monkey reagents prepared against the cynomolgus monkey. Both of these (mouse x human) x chimpanzee antibodies have affinities for digoxin that are distinctly higher than cebus antibody 11-1 mentioned in Example 2 above.

Claims:

1. A hybridoma cell line producing a non-human primate monoclonal antibody, comprising an immortalizing cell fused to a cell producing a non-human primate antibody, the immortalizing cell comprising a xenogeneic hybridoma cell fused from a parent immortalizing cell and a partner cell, said antibody producing cell being genetically compatible with said partner cell.

2. A hybridoma cell line as claimed in claim 1 wherein said antibody producing cell is of the same genera as the partner cell in the xenogeneic hybridoma cell parent.

3. A hybridoma cell line as claimed in claim 1 wherein said antibody producing cell is of the same species as the partner cell in the xenogeneic hybridoma cell parent.

4. A hybridoma cell line as claimed in claim 2 wherein the cell producing the non-human primate antibody is a lymphocyte.

5. A hybridoma cell line as claimed in claim 1 wherein the non-human primate monoclonal antibody is a monkey monoclonal antibody.

6. A hybridoma cell line acording to claim 1 wherein the parent immortalizing cell is of the SP-2 cell line.

7. A method of producing the hybridoma cell line of claim 1 which comprises making a xenogeneic hybridoma cell drug resistant, and fusing this latter cell to a antibody producing cell which is genetically compatible with the partner cell in the xenogeneic hybridoma, and selecting a desired hybrid.

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8. A method as claimed in claim 7 wherein selection of a desired hybrid takes place on the basis of lack of sensitivity to HAT and assay for the ability to produce a non-human primate monoclonal antibody.

9. A cell fusion system which comprises a non-human primate monoclonal antibody producing cell and a xenogeneic hybridoma cell fused from an immortalizing cell and a partner cell genetically compatible with said antibody producing cell, in a nutrient culture medium together with an agent which promotes fusion of said cells.

10. Non-human primate monoclonal antibody.

11. An antibody according to claim 10 which is monkey monoclonal antibody.

12. A (mouse x human) x non-human primate hybridoma cell line.

13. A (mouse x non-human primate) x non-human primate hybridoma cell line.

14. A cell line according to claim 12 which is a (mouse x human) x monkey hybridoma cell line.

15. A cell line according to claim 13 which is a (mouse x monkey) x monkey hybridoma cell line.

16. A hybridoma cell line comprising a trioma cell fused to a cell producing a non-human primate monoclonal antibody, the trioma cell comprising an immortalizing cell fused to a non-human primate lymphocyte, the immortalizing cell comprising a xenogeneic hybridoma cell fused from a parent immortalizing cell and a partner cell, said lymphocyte being genetically compatible with both said partner cell and said antibody producing cell.

17. A hybridoma cell line as claimed in claim 16 wherein said antibody producing cell is of the same genera as the lymphocyte.

18. A hybridoma cell line as claimed in claim 17 wherein the lymophocyte is of the same genera as both the partner cell and said antibody producing cell.

19. A cell line according to claim 16 which is a [(mouse x human) x non-human primate] x non-human primate cell line.

20. A cell line according to claim 19 which is a [(mouse x human) x chimpanzee] x chimpanzee cell line.

21. A cell line according to claim 14 which is a (mouse x human) x chimpanzee cell line.

22. An antibody according to claim 10 which is chimpanzee monoclonal antibody.