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Methods for Production and Detection of Monoclonal Antibodies against Surface Components of Adhesive Implanting Mouse Blastocysts

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ABSTRACT

Monoclonal antibodies against mouse blastocysts in the adhesion stage of implantation were obtained by intrasplenic immunization with blastocysts either injected into the spleen after irradiation or transferred after drying on a piece of nitrocellulose paper. About 10 blastocysts were deposited at each of 4 immunizations before the spleen cells were used for hybridoma production. The supernatants obtained were examined for anti-blastocyst antibodies in indirect immunofluorescence labelling of native blastocysts and with ABC-immunoperoxidase staining either of blastocysts attached to nitrocellulose paper or of methanol-fixed blastocysts. Further, sections of paraffin-embedded blastocysts were used for detection of antigens.

INTRODUCTION

Methods for production of monoclonal antibodies against blastocysts of a defined functional state and techniques for antibody detection on blastocysts have previously been hampered mainly by difficulties to obtain sufficient amount of material for immunization and to a lack of easy screening techniques for checking the supernatants obtained. Since we are aiming at defining cell adhesion molecules active at blastocyst implantation, we have attempted to overcome these difficulties by using functionally homogenous blastocysts of delayed implantation (3,4) as antigen material and by exploiting the intrasplenic route for immunization (5).

This paper reports both the various techniques developed for depositing the blastocysts into the spleen and the methods used for screening the hybridoma supernatants for anti-blastocyst antibodies. Details of the monoclonal antibodies obtained will be published separately (Svalander and Nilsson, in preparation).

MATERIALS AND METHODS

Functionally defined blastocysts

Blastocysts in the adhesion stage of implantation were obtained from syngenically mated mice (C57 Bl/6 or NMRI) in an experimental delay of implantation, 18 hrs after an injection of oestrogen (1). For immunization, blastocysts were flushed from the uterine horns with Dulbecco's phosphate--buffered saline (PBS) supplemented with 1 per cent male serum from the recipient strain. For screening, blastocysts were flushed with PBS supplemented with 1 per cent bovine serum albumin (BSA) and 0.1 per cent sodium azide.

Intrasplenic immunization

Before the deposition into the spleen tissue, the blastocysts were irradiated with 5000 Rad from a cesium source to block trophoblast proliferation into the spleen. At immunization, the spleen was exteriorized through a left midscapular incision below the chest. The C57 Bl/6 blastocysts were transferred to DBA/2 male mice and the NMRI blastocysts to NMRI male mice.

Two methods for depositing the blastocysts into the spleen were used: 1) Transfer by injection of whole, irradiated blastocysts (C57 Bl/6) by a micropipette inserted through a small hole in the spleen capsule. 2) Transfer by introducing whole, dried blastocysts (NMRI) placed on a small circular (diameter 3 mm) piece of nitrocellulose paper (Schleicher & Schuell) which was inserted into the spleen. About 10 blastocysts, injected by pipette or inserted on NC paper, were deposited at each of 4 immunizations. These were performed at an interval of 3-5 weeks.

Production of hybridoma

Hybridoma cell lines were produced according to the monoclonal antibody technique (2). Briefly, three to four days after the last immunization the spleen was removed, dispersed into a single-cell suspension, and fused with the murine myeloma cell line X63-Ag8.653.

The cells were then seeded in ten 96-well plates in HAT selection medium. After 10-14 days, hybrid colonies appeared, and their supernatants were screened for antibodies reacting with adhesive blastocysts. Recloning of cell lines producing positive supernatants was made by limited dilution followed by rescreening of the obtained supernatants.

The screening was performed with an indirect immunofluorescence technique. The supernatants were initially pooled 12 in a batch. The supernatants of positive batches were then examined in groups of 3 in order

to select more rapidly a positive clone. Antibodies of positive supernatants were further examined with immunohistochemical methods of both whole and sectioned blastocysts.

Techniques for detection of anti-blastocyst antibodies

Four different techniques were used for immunohistochemical labelling of blastocysts. Conventional control labelling was run simultaneously with the experimental labelling by using an unrelated mouse monoclonal antibody supernatant.

A. Indirect immunofluorescence of native blastocysts.

The blastocysts were handled with micropipettes (new for each transfer) when transferred through a serie of watch-glasses containing the labelling reagents. PBS with 1 per cent BSA and 0.1 per cent sodium azid was used for dilution of antibodies and for washings.

Labelling Protocol:

1. Incubate in 100 µl hybridoma supernatant diluted 1:2 for 75 min.
2. Wash 5 x 5 min.
3. Incubate in 100 µl TRITC-RAM/Ig (Dakopatts) diluted 1:40 for 30 min.
4. Wash 5 x 5 min.

Similar results were obtained when labelling was made on ice.

B. ABC-immunoperoxidase staining of blastocysts attached to nitrocellulose.

After flushing, two or three washed blastocysts were transferred to circular (diameter 3 mm) pieces of nitrocellulose paper (Schleicher & Schuell) and dried at +37°C for 30 min. They were then stored at +4°C.

Labelling Protocol:

1. Block the NC paper binding capacity by incubation in 3 per cent BSA in PBS over night.
2. Incubate in monoclonal antibody supernatant (primary antibody) diluted 1:10-1:50 with the blocking solution at +4°C over night.
3. Wash for 3x30 min in PBS.
4. Block endogeneous peroxidase activity by incubation in PBS with 0.3 per cent H_2O_2 for 30 min.
5. Block unspecific binding by incubation in PBS with 5 per cent goat serum for 15 min.
6. Incubate in biotinyl-goat-anti-mouse class specific antibody (Vector) diluted 1:200 with the goat serum blocking solution at +4°C over night.

7. Wash 3x10 min.
8. Incubate in ABC-immunoperoxidase conjugate (Vector) diluted 1:200 in PBS with 0.1 per cent BSA, 1 per cent normal goat serum, and 0.01 per cent sodium azide for 30 min.
9. Wash 3x10 min.
10. Develop with ethyl carbazole according to standard procedures.
11. Wash for 10 min.

C. ABC-immunoperoxidase staining of methanol-fixed blastocysts.

After flushing, two or three washed blastocysts in a droplet of flushing medium were placed on a glass slide and dried at +37°C for 30 min. They were then freeze-stored at -70°C.

Labelling Protocol:

1. Thaw and condition the blastocysts in cold PBS for 30 min.
2. Fix in ice-cold, absolute methanol for 30 min.
3. Wash 3x10 min in PBS.

ABC-immunohistochemistry was performed according to the section B protocol, Steps 2-11.

D. ABC-immunoperoxidase staining of sections of paraffin-embedded blastocysts.

After flushing, the blastocysts were fixed in 4 per cent neutral paraformaldehyde for 1-24 hours and embedded in a warm, fluid solution of 2 per cent agarose in H_2O applied as small drops on a slide. After gelling, small blocks, each with a blastocyst, were prepared and embedded in paraffin according to standard procedures. Since the agarose-solution was slightly stained with Pontamine Blue, the blocks of agarose were easily traced during the handling. Six µm sections were made and processed for ABC-immunohistochemistry according to the section B protocol, Steps 2-11.

COMMENTS

Deposition of the irradiated blastocysts into the spleen tissue was performed either by injecting whole, native blastocysts or by introducing small pieces of nitrocellulose paper carrying whole, dried blastocysts. An advantage of the former method is the increased chances to obtain antibodies against unchanged surface components, since only these antigens will be exposed, at least as long as the blastocysts stay undamaged in the spleen. The blastocysts on NC paper, however, have perhaps suffered a conformational change of some antigens and an exposure of intracellular

antigens due to the drying. Technically, the latter procedure is easier to apply, since the antigen material can be stored for weeks in the dried state and, being carried on NC paper, its deposition into the spleen is more simple. In the present experiments both the techniques resulted in hybridoma producing blastocyst-positive antibodies.

Immunofluorescence microscopy of native blastocysts involves transferring the blastocysts by repeated pipetting. This is a tedious work, but it probably offers the best choice for labelling the native surface antigens. We initially chose this technique for screening, since we aimed at finding only those antibodies which were directed against cell surface molecules. Both the unfixed blastocysts on a NC paper support and the methanol-fixed blastocysts on a slide support are technically easier to use and well fitted for screening procedures, but these techniques involve a risk of detecting intracellular antigens and of inducing conformational changes of surface antigens during processing. However, we did not detect any significant changes of antigen properties after drying and/or fixation since those supernatants, which were positive in immunofluorescence, also resulted in a positive staining of both unfixed and fixed blastocysts when they were processed according to the avidin-biotin-immunoperoxidase technique. Thus, it seems that at least some surface antigens will remain unchanged after both drying and/or methanol-fixation. This could imply that NC paper supported blastocysts are appropriate to use also in a screening procedure.

Intracellular antigens, as mentioned, can be exposed at immunizations both by the decomposition of the irradiated blastocysts in the spleen and by the drying of NC paper supported blastocysts before their deposition into the spleen. Neither the NC paper supported blastocysts nor the methanol--fixed ones discriminate between surface and intracellular antigens. Therefore, we developed a detection technique using sections of paraffin--embedded blastocysts. These processes involve many risks for affecting both the localization and the properties of the antigens, but due to the small size of the blastocyst this technique is the only one available for studying intracellular trophoblast antigens. Although the advantage of the technique is that it offers about 10 sections of each blastocyst, it should not be applied in search for surface antigens, since these may not be detected due to the thiness of the sections. However, for checking the labelling of intracellular antigens the technique could be useful.

The possibility to study the blastocyst surface antigens has substantially increased by the presented methods. Thus, immunization with a small number of blastocysts is now feasible by using blastocysts attached to NC paper and by applying the splenic route for the immunization. Further, the screening

of antibody-containing supernatants is rendered more simple by using NC paper supported blastocysts and the avidin-biotin-peroxidase technique instead of the indirect immunofluorescence method.

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