Eur J Cancer Clin Oncol, Vol. 20, No. 1, pp. 123–128, 1984.
Printed in Great Britain.

0277-5379/84$3.00+0.00

Immunoperoxidase Staining on Frozen Tissue Sections as a First Screening Assay in the Preparation of Monoclonal Antibodies Directed Against Small Cell Carcinoma of the Lung

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Abstract—*To prepare monoclonal antibodies against small cell carcinoma of the lung (SCCL), we have used an SCCL-derived cell line as immunogen. A first screening of hybridoma supernatants was performed on frozen tissue sections of a biopsy with histologically proven SCCL involvement. Screening on tissue sections is a valuable technique, especially for the isolation of monoclonal antibodies directed against tumor antigens. A drawback of this procedure is that it is laborious. To circumvent this, we have reduced the number of supernatants to be screened by increasing the number of seeded hybridomas per well. Although this resulted in the growth of 5–20 hybridomas per well, among which there was always at least one that secreted antibodies against 'common antigens', clones that secreted specific antibodies could still be revealed by testing supernatants which were preabsorbed with thrombocytes. This has resulted in the isolation of 7 monoclonal antibodies directed against SCCL associated antigens.*

INTRODUCTION

SMALL cell carcinoma of the lung (SCCL) is a clinicopathologic distinct form of lung cancer [1, 2]. The development of monoclonal antibodies against SCCL associated antigens may add to our knowledge of the biology of SCCL and also improve diagnostic and therapeutic possibilities.

The type of screening assay used in the preparation of monoclonal antibodies is of utmost importance. This is true especially in the case of preparing monoclonal antibodies against tumor associated antigens, because a major portion of the immune response evoked by the injection of tumor cells can be expected to be directed against 'common antigens', i.e. antigens which tumor cells share with normal cells. In this paper we show the feasibility of an immunoperoxidase staining technique on frozen tissue sections as a first screening assay in the preparation of monoclonal antibodies directed against SCCL.

Accepted 4 July 1983.
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MATERIALS AND METHODS

Neuraminidase treated cells (0.1 U/10^6 cells/ml for 30 min at 37°C) of a low passage (p3–6) cell line derived from pleural effusion of a patient with SCCL were used as immunogen. Pretreatment with neuraminidase is carried out to enhance the immunogenicity of the tumor cells [3, 4]. The cell line is designated OC Rol and will be described in detail elsewhere. Briefly, the cell line can be characterized as a 'converter' SCCL line [5] on the basis of its growth behaviour (growth in selective, serum-free HITES medium), ultrastructural morphology (sparse occurrence of dense core vesicles), enzyme content (moderate levels of Dopa-decarboxylase), hormone production (ACTH) and karyotype (presence of the 'SCCL-marker' del 3p). Immunization was performed in BALB-c mice by i.p. injection in complete Freund's adjuvant, followed after 1 month by a first booster given also i.p. in incomplete Freund's. After an additional 3 weeks the last booster was given i.v. in phosphate

buffered saline (PBS) 3 days before removal of the spleen.

Fusion protocol

The HGPRT negative myeloma cell line X63 was used as the fusion partner. The fusion protocol was according to the procedure of Kennett [6] as adapted by Astaldi [7], except that the fusion was carried out at a pH of 8.0 [8]. The fusion mixture was divided over six 96-well cluster trays, which yielded 10–20 hybridomas per well. After 10–14 days incubation the hybridomas were cryopreserved after programmed freezing of complete microtitre plates according to a freezing protocol which has been described elsewhere [8]. Following appropriate screening of previously collected supernatants, positive wells were thawed, recultured and cloned. During this procedure the specific clones, which were selected, stayed viable and could be isolated.

Immunoperoxidase staining on frozen tissue sections

The procedure is essentially as previously described [9]. Snap frozen tissue blocks, stored at −70°C, are cut into sections of 6 μm thickness in a cryostat and put on glass slides. These preparations can be stored for some weeks at −20°C. Before use, they are dried at room temperature for 10 min with the aid of a hairdryer, fixed in acetone for 10 min at room temperature and washed in PBS. Subsequently the area around the tissue is dried with tissue paper and 25 μl of undiluted culture supernatant is pipetted on top of the wet sections. After incubation for 30 min in a humidified atmosphere at room temperature, the sections are washed in three changes of PBS for 5 min. The second step reagent, horse radish peroxidase conjugated rabbit anti-mouse immunoglobulin (Dakopatts, Copenhagen, Denmark), is applied for 15 min. This antibody is diluted 1:20 in a 1% solution of human AB serum in PBS to prevent reactivity with human immunoglobulin which may be present in the tissue. After another washing in PBS, the staining reaction is performed. For this step, 10 mg 3-amino-9-ethylcarbazole is dissolved in 2.5 ml dimethylformamide and added to 50 ml acetate buffer, 0.05 M, pH 4.9. After adding 0.05 ml 30% H_2O_2, the sections are incubated in this mixture for 5–10 min. A positive reaction is indicated by a red deposit on the section. The reaction is stopped by washing in acetate buffer. Nuclei are counterstained with Mayer's haematoxilin and the sections are mounted with Aquamount. The results are evaluated with a light microscope. A maximum of about 200 supernatants can be processed and evaluated by two experienced persons in one day.

Removal of monoclonal antibodies against 'common antigens' from hybridoma supernatants

Before screening, a major part of the antibodies reactive with 'common human antigens' are removed by preabsorption with thrombocytes. To this end the hybridoma supernatants are sampled in 100 μl quantities in round-bottomed 96-well microtiter plates and about 25 μl of a 1:4 diluted suspension of packed thrombocytes are added. After 20 min incubation at room temperature with occasional shaking, the plates are centrifuged for 20 min at 800 *g*. The clear supernatant is subsequently pipetted off and used in the screening assay.

Miscellaneous

Common HLA-ABC and HLA-Dr directed monoclonal antibodies were obtained from BRL and Ortho, respectively.

RESULTS

A newly established SCCL derived cell line, OC Rol, which will be described in detail elsewhere (manuscript in preparation, see also Materials and Methods) was used for immunization. Screening of hybridoma supernatants was performed on 'composed' SCCL preparations, made by combining on one glass-slide a cytospin of the cell line used for immunization and a frozen section of a histologically proven SCCL metastasis in a lymph node. All supernatants reacted heavily with both the cytospin and the SCCL cells in the sections (Figs 1a and 2a). However, in all cases the normal cells in the lymph node (fibroblasts, lymphoid cells) were also stained (Fig. 1a). This finding suggested that in addition to some possible specific clones all wells contained at least one clone secreting antibodies reacting with 'common human antigens'. These 'common antigens' were not HLA, since neither the cell line which was used for immunization nor the tumor cells in the section expressed HLA, as shown by the fact that they did not react with commercially available anti-HLA-ABC or HLA-Dr directed monoclonal antibodies (not shown). To remove a substantial portion of antibodies directed against 'common antigens' we subsequently pre-incubated all supernatants with thrombocytes, after which a number of supernatants were obtained that stained tumor cells selectively. For instance, Fig. 1b shows a monoclonal antibody specifically reacting with SCCL cells in the biopsy. This reaction pattern was not seen before the incubation with thrombocytes (Fig. 1a). Out of 192 supernatants (the equivalent of 2000–3000

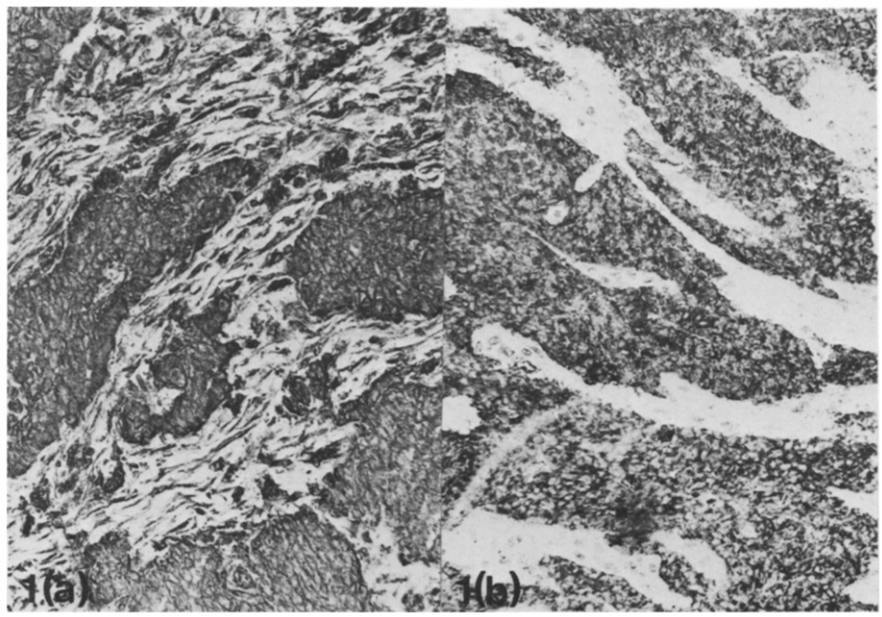

Fig. 1. *Immunoperoxidase staining of a cryostat section made from a SCCL tumor metastasis present in a lymph node. in (a) unabsorbed primary hybridoma culture supernatant has been applied to the section. Tumor cells are strongly positive, but in addition fibroblasts present in the stroma are heavily stained. In (b) the primary hybridoma culture supernatant has been absorbed with thrombocytes to remove monoclonal antibodies recognizing 'common antigens'. Only tumor cells react positively, whereas the fibroblasts in the stroma are negative* (×140).

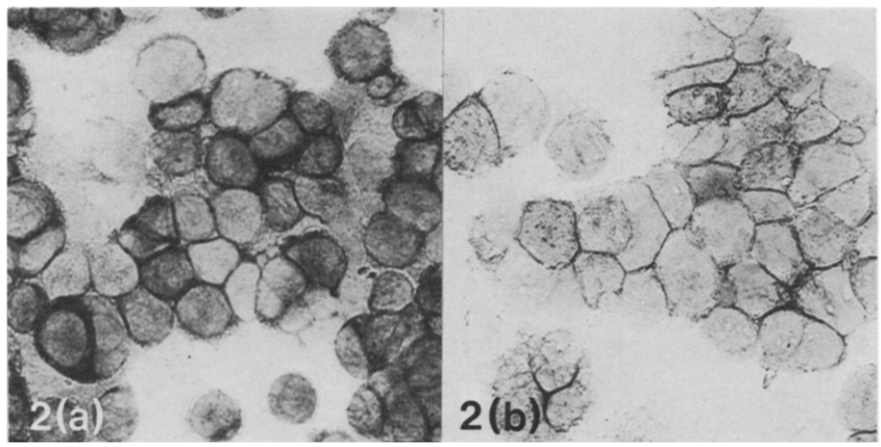

Fig. 2. *Cytospin preparations made from the SCCL cell line OC Rol, which was used for immunization. Immunoperoxidase staining before (a) and after (b) removal of monoclonal antibodies directed against 'common antigens'. The same hybridoma culture supernatants as in Fig.* 1 *have been used. From this culture well MOC* 1 *has been isolated* (×350).

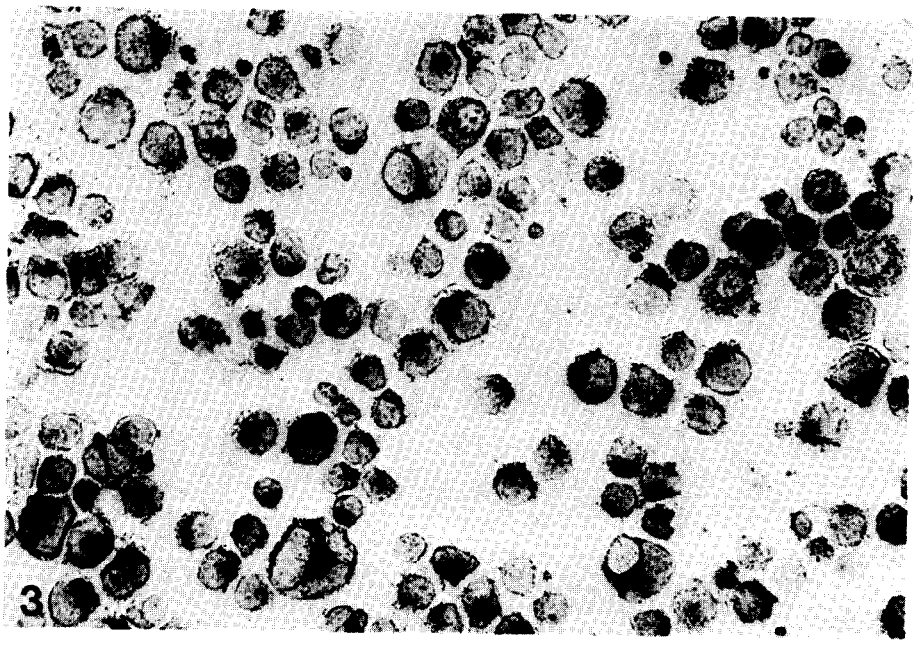

Fig. 3. Cytospin preparation made from the SCCL cell line OC Rol. Immunoperoxidase screening with MOC 4 (×350).

Table 1. *Reaction pattern of SCCL directed monoclonal antibodies*

Monoclonal antibody	Lab. designation	Reaction with OC Rol	Specific reaction with SCCL in biopsy	Membrane reaction	Intracellular reaction
MOC 1	1-g-12	+	+	+	-
MOC 2	1-c-6	+	+	+	+
MOC 3	1-f-1	+	+	+	+
MOC 4	1-a-11	+ (50%)	+	-	+
MOC 5	1-b-1	+	+	-	+
MOC 6	1-e-1	+	+	-	+
MOC 7	6-c-12	+	+ (also with granulocytes)	-	+

clones) that have been screened up to now, 7 hybridomas reacting specifically with SCCL cells in tissue sections of a lymph node metastasis have been detected in this way. These results are summarized in Table 1. In all cases both cultured cells in the cytospin and the tumor cells in the tissue sections were positive, whereas fibroblasts, endothelial cells and lymphocytes also present in these sections were negative. MOC 7 reacted, in addition, with granulocytes in the section. From these 7 monoclonal antibodies one reacted with membrane determinants only (MOC 1, Fig. 2b), two reacted with both membrane and intracellular determinants, whereas four reacted with intracellular antigens only (e.g. Fig. 3).

DISCUSSION

To obtain specific monoclonal antibodies against SCCL associated antigens, we developed the following strategy. As immunogen an early passage SCCL cell line (p3–6) was used. Immunization with a cell line is advantageous, since only tumor cells are injected and contamination with normal tissue components is avoided. A drawback of the use of established cell lines may be that tissue culturing could induce changes in the tumor cells. Since screening is performed, however, on both the cell line and cryostat sections of SCCL biopsies, it is certain that only those antibodies are selected which react with tumor cells present in their '*in vivo*' environment, i.e. in the patient. Screening on cryostat sections with the immunoperoxidase technique offers two additional advantages.

Firstly, it is a highly informative test with respect to specificity of the antibody reaction pattern. This stems from the fact that in addition to the recognition of desired reactions against histologically identifiable tumor cells, a number of 'control cells' also present in this section can be evaluated at the same time. This advantage can be expanded by the use of so-called 'composed' sections, i.e. by mounting two sections of different tissues on the same object glass or alternatively by combining a cryostat section with a cytospin preparation (Figs 1 and 2).

Secondly, the staining reaction can provide information on a subcellular level. Due to the fact that tissue sections are used as a substrate for the screening test, intracellular structures are also accessible to the monoclonal antibodies. Figures 2b and 3 represent examples of the detection of antibody reactions against a membrane and an intracellular antigen, respectively. A drawback of the immunoperoxidase screening on frozen tissue sections is, however, that this method can only become fully exploited when sufficient time is available. To this end we used a freezing protocol which permitted the early cryopreservation of newly formed hybridomas in complete microtitre plates [8]. As a result the screening can be subdivided into a number of smaller screening protocols, each consisting of a minimum of 96 samples (one thawed microtiter plate). In addition, it is also possible to increase the number of hybrids seeded per well. When an average of 15 clones is present in each of the seeded wells, this results, however, in the growth of clones producing antibodies against common antigens in all wells (Fig. 1). In the first tests these antibodies obscured all possible specific reactions. Only after removal of these antibodies directed against 'common antigens' by absorption with thrombocytes could the antibodies directed against the desired antigens be detected (compare Figs 1a and b). The advantage of this set-up is that by using pre-absorbed hybridoma supernatants, about 9000 clones can be screened in six 96-well plates for the production of specific monoclonal antibodies.

In conclusion, the results presented in this paper show that immunoperoxidase screening of a large number of primary hybridomas on frozen tissue sections is technically feasible, thereby enabling the use of this highly informative screening method as a first screening step for the isolation of SCCL directed monoclonal antibodies. With this protocol 7 anti-SCCL mono-

clonal antibodies have been isolated to date and both membrane and intracellular antigens have been detected.

Acknowledgements—We thank Pieter Klok for performing the animal experiments, Biddy Schilizzi for critical reading and Kiki Bugter for typing the manuscript.

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