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Monoclonal Anti-cytokeratin Antibody from a Hybridoma Clone Generated by Electrofusion

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Abstract—*Hybridomas producing mouse monoclonal antibodies to antigens of the human mammary carcinoma cell line, MCF-7, have been generated by electric field-mediated fusion at a frequency ten times higher than by polyethylene glycol. One of the monoclonal antibodies obtained recognizes a cytoskeletal structure restricted to epithelial cells and carcinomas with a distribution pattern resembling cytokeratin 19.*

INTRODUCTION

IT IS now widely accepted that intermediate filaments represent reliable tissue-specific markers [1, 2]. Monoclonal antibodies (mabs) against the different filament proteins promise to become extremely helpful new tools in histology and pathology, tumour diagnosis included [3–5]. In previous experiments we obtained a monoclonal antibody (A45-B/B3) which recognizes a cytokeratin epitope widely distributed in epithelia and carcinomas [6]. We now report on another antibody (A53-B/A2) with a more restricted cytokeratin specificity. The hybridoma clone secreting this antibody has been produced by electic field-mediated fusion [7] instead of polyethylene glycol (PEG)-induced fusion.

MATERIALS AND METHODS

Cell culture

The parental mouse plasmacytoma cell line X63-Ag8.653 [8] was held in logarithmic growth in RPMI 1640 medium supplemented with L-glutamine (2 mM), 2-mercaptoethanol (50 μM) and 10% heat-inactivated selected horse serum (HS), without antibiotics. The cells were regularly checked for the absence of mycoplasmas. Hybridoma cells were grown in the same medium except that the serum content was increased (15% HS) and 200 μg/ml gentamicin added. Target cell lines were cultured in different media as listed in Table 1.

Immunization

The spleen cell donor was a female BALB/c mouse injected 5 times with about 10^7 live MCF-7 cells i.p. without adjuvant at large intervals, the last injection being done 4 days prior to fusion. The MCF-7 cells were harvested mechanically in Dulbecco's PBS and washed once before injection.

Electric field-mediated fusion

Spleen cells were exposed to osmotic shock in order to remove erythrocytes, washed and resuspended in tissue culture medium. One million X63-Ag8.653 cells were treated with pronase (0.5 mg/ml) at 37°C for 30 sec, spun down, suspended in 300 μl sucrose solution (9%), and 2×10^6 spleen cells in about 50 μl added. After three washes in sucrose solution the cell pellet was resuspended in 50 μl sucrose solution and pipetted into the fusion chamber. The latter consisted of two parallel electrode plates made of stainless steel separated by an insulating ring, providing a chamber 0.5 mm in height and 28 mm in diameter. The cell drop was given into the centre of this chamber and exposed to an alternating electric field (f = 2 MHz, E_{max} = 1.2 kV/cm) for about 20 sec, followed by a single unipolar square pulse (3 μsec, E_{max} = 4.2 kV/cm). During the single pulse the dielectrophoresis was interrupted and was continued thereafter for about 10 sec. Finally the cells were carefully

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Table 1. Antigen A53-B/A2 distribution among cell lines as compared to the epithelium specific mab A45-B/B3 [6]

Cell line	Species	Tissue of origin/ cell type	Ref.	Source	Medium + serum	Reactivity in immunofluorescence A53-B/A2	A45-B/B3
MCF-7	human	mammary carcinoma/ epithelial	9	Salk Inst., La Jolla	RPMI + FCS	+	+
T47D	human	mammary carcinoma/ epithelial	10	ICRF, London	RPMI + FCS	+	+
BT-20	human	mammary carcinoma/	11	ICRF, London	MEM* + FCS	+	+
CaMa 1	human	mammary carcinoma/	12	ICRF, London	RPMI + FCS	+	+
MaTu	human	mammary carcinoma/ epithelial	13	Dept Virology, CICR	RPMI + NCS	-	+
Hs578T	human	carcinosarcoma of breast	14	Univ.-Frauenklinik, Innsbruck	RPMI + NCS	-	-
H184D1	human	normal breast/ epithelial	15	Lawr. Berk, Lab., Berkeley	MCDB 170* + FCS	-	+
H5/83	human	skin of adult breast/ fibroblastoid	—	Dept Virology, CICR	MEM + NCS	-	-
T-24	human	bladder carcinoma	16	Inst. Molec. Genet., Prague	RPMI + FCS	+	+
HT-29	human	colon carcinoma	17	Ludwig Inst., Cambridge	RPMI + NCS	+	+
LS-174T	human	colon carcinoma	18	MRC Lab. Molec. Biol., Cambridge	RPMI + FCS	+	+
HeLa-S	human	epidermoid carcinoma of cervix	19	Salk Inst., La Jolla	DMEM/F12 + BS	-	+
A-431	human	epidermoid carcinoma of vulva	20	Salk Inst., La Jolla	RPMI + FCS	-	+
A-204	human	rhabdomyosarcoma	21	J. L. Smith Mem., Maywood	RPMI + NCS	-	-
Tu 131	human	Ewing sarcoma	22	Dept Virology, CICR	MEM + BS	-	-
Reh	human	null cell ALL	23	ICRF, London	RPMI + FCS	-	-
SKW-3	human	T cell leukemia	24	Univ.-Kinderklin., Jena	RPMI + FCS	-	-
HMy 2	human	plasmacytoma	25	Ludwig Inst., Cambridge	RPMI + FCS	-	-
RL-19	rat	liver/epithelial	26	CICR	RPMI + NCS	-	+

ICRF = Imperial Cancer Research Fund Laboratories: CICR = Central Institute of Cancer Research, Berlin-Buch. Sera: FCS = foetal calf, NCS = newborn calf, BS = bovine; *indicates additional medium components.

transferred into 1 ml culture medium. The whole procedure (enzymatic plus electric treatment) took about 12 min.

PEG-induced fusion

For comparison, 2×10^7 cells from the same spleen without prior removal of erythrocytes were fused with 2×10^6 X63-Ag8.653 cells using polyethylene glycol, mol. wt 1500 (Ferak, Westberlin), essentially according to standard procedures as described earlier [6].

Treatment after fusion

After fusion cells were incubated in culture medium at 37°C for 4 hr and then distributed to the wells of 2 microtitre plates onto 2×10^4 mouse peritoneal cells per well.

The average number of spleen cells pipetted into individual wells were around 10^4 in the case of electrofusion and 10^5 in the PEG fusion. Beginning 24 hr after fusion, the medium was replaced by azaserine selection medium [culture medium as above but with 20% HS, 5.8 μM azaserine (Calbiochem) and 0.1 mM hypoxanthine (Reanal, Budapest) in successive half-medium changes, thus ensuring effective azaserine concentrations for at least 7 days]. Thereafter, medium lacking azaserine was used for another week and, finally, hypoxanthine was also omitted and HS reduced to 15%. Hybridoma growth was evaluated on days 7 and 14 after fusion.

Antibody tests

Antibody tests were done by indirect immunofluorescence as described [6]. For optimal

demonstration of cytoskeletal antigens cells were made permeable by either methanol or digitonin.

Antibody subclasses were determined by double immunodiffusion using commercial antisera (Bionetics, Kensington).

Cloning and further processing

Cloning was effected by a rational and optimized variant of the limiting dilution technique using mouse peritoneal feeder cells. Selected hybridoma clones were expanded and stored in liquid nitrogen. Some of them were thawed, recloned and again expanded and frozen, and also grown as ascites in BALB/c mice.

Flow cytometry

Vital hybridoma cells were stained with a mixture of ethidium bromide and olivomycin in the presence of 0.01% Triton X-100 and measured by means of a Phywe ICP 11 flow cytometer.

RESULTS

After preliminary studies aimed at optimizing individual steps of the electric field-mediated fusion we directly compared both types of fusion in one experiment using spleen cells from a single hyperimmune mouse. The PEG-induced fusion was done with 2×10^7 spleen cells as in earlier work, whereas the electrofusion was performed three times on a smaller scale using about $1\text{-}2 \times 10^5$ spleen cells each. The results evaluated 2 weeks after fusion are summarized in Table 2. All three electrofusions were successful, yielding viable hybrids at high frequencies (around 1 in 5000 spleen cells). This was ten times that of the PEG-induced fusion (about 1 in 50,000 spleen cells), which in itself is a reasonable result when compared with other PEG-induced fusions from this laboratory, as well as from the literature. Although the spleen cell:plasmacytoma cell ratio was different in the two types of fusions, it seems obvious that the fusion frequency of the electrofusion is higher. The proportion of cultures producing specific antibodies was similar in both groups. This means that the electric field technique could lead to an increased hybridoma yield in real terms. Three hybridomas from the electrofusion and four from the PEG-induced fusion were selected for further processing. One from each group lost antibody production; the others were cloned, tested, expanded and stored in liquid nitrogen. There was no obvious difference between the two groups in terms of viability, growth behaviour *in vitro* or as ascites, or antibody production. The clones obtained by electrofusion could be re-established and recloned easily after freezing and showed cloning efficiencies fully comparable to other hybridomas. Both were found free from mycoplasma contamination. To further confirm the hybrid status of the clones B/A2 and B/A8 which had been selected from the electrofusion, we analyzed the DNA distribution patterns by flow cytometry. Both hybridomas showed significantly enhanced DNA contents compared to the parental cell line (Fig. 1). The $G_{1/0}$ peaks were at channel No. 34 for B/A2 and 51 for B/A8, compared to channel 22 for the parental cell line. Considering the well-known segregation phenomenon, the DNA distribution patterns thus suggest that A53-B/A2 is probably a two-cell fusion product, whereas A53-B/A8 may have arisen from at least three cells. The small peak at channel 22 in the A53-B/A2 suspension could be interpreted as reflecting some degree of instability, although upon recloning no non-producing clones were found.

The A53-B/A8 clone consists of very large, rapidly growing cells producing an IgM antibody which detects a common membrane antigen.

A53-B/A2 cells are comparable to hybridomas of conventional origin in size and appearance (Fig. 2b). After an adaptation phase they also grew well. The antibody found in the supernatant (3.1 μg/ml) is an IgG 2a. It recognizes a cytoskeletal structure (Fig. 2c). Table 1 presents the distribution of the corresponding antigen among cell lines of normal and malignant origin. For comparison the reactivity of the epithelium-

Table 2. Comparison of results obtained by electrofusion vs PEG-induced fusion

Type of fusion*	Spleen cells per well	Wells with growth/total No. of wells	%	Mean No. of colonies per well	Fusion frequency†	Antibody production (%)‡
PEG	10^5	144/156	92	1.9	2	47
E_1	10^4	12/12	100	3.1	31	40
E_2	10^4	12/12	100	3.8	38 (27)	30
E_3	10^4	10/12	83	1.3	13	50

*PEG = polyethylene glycol induced fusion; E_1, E_2, E_3 = electrofusions 1, 2 and 3.

†No. of hybrid colonies per 10^5 spleen cells. Mean of the electrofusions in parentheses.

‡Wells with supernatants reacting in immunofluorescence with MCF-7 cells expressed as per cent of wells with hybridoma growth.

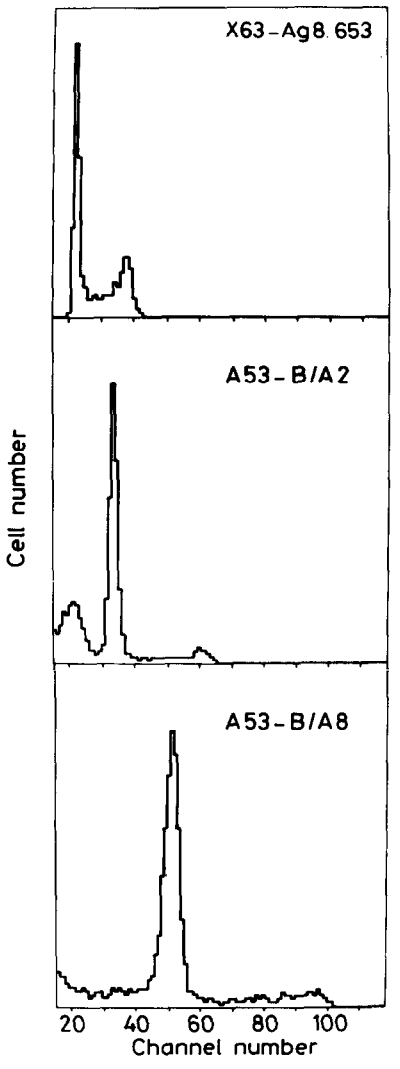

Fig. 1. DNA distribution patterns of parental plasmacytoma cells (above) and two hybridoma clones generated by electric field-mediated fusion. DNA content per cell is roughly proportional to channel number.

specific mab A45-B/B3 [6] is also shown. Whereas A45-B/B3 stains all cells of epithelial origin, the A53-B/A2 antigen is more restricted but is in no case found in non-epithelial cells. At present no cytoskeletal proteins other than cytokeratin(s) are known which would fit into the reaction pattern of A53-B/A2. In cryostat sections of mammary carcinomas this mab clearly stains tumour cells but not stroma cells (Fig. 2d).

DISCUSSION

Although plant protoplasts and mammalian cells have already been fused [7] and viable hybridomas have been produced by electrofusion [27, 28], only one very recent paper reported on monoclonal antibodies generated by this technique [29]. These authors used a very efficient coupling method to construct pairs of antigen-specific B cells and myeloma cells prior to fusion which resulted in a high yield of antigen-specific hybridomas. This method is, however, not applicable to experiments aimed at producing mabs against unknown cellular antigens.

In the experiments described here we were able to demonstrate the feasibility of the electric field-mediated fusion technique to generate hybridomas which produce specific monoclonal antibodies against cellular antigens. The hybridoma clones obtained in this way were in almost every respect similar to those fused by means of PEG. However, the yield of hybridomas per unit number of spleen cells was higher by an order of magnitude in the electrofusion irrespective of whether the total number of colonies or the number of wells producing specific antibodies is considered. If confirmed in further experiments, this would mean that by employing electric field-mediated fusions the antibody repertoire will be more completely reflected in the spectrum of hybridomas obtained. Methods to enhance the hybridoma yield are of special interest in human-human fusions where fusion frequencies are rather low. Even more advantages of the electrofusion can be envisaged with devices aimed at fusing defined cell pairs instead of mixed populations [28].

One of the antibody producing clones, A53-B/A8, has probably arisen from a three-cell fusion as concluded from its DNA content. Although not proved, we assume from the very rapid proliferation of the fusion product that two X63-Ag8.653 cells and one lymphocyte were involved in the fusion. The possibility of intentionally constructing three-cell products to improve certain properties (e.g. growth characteristics) would be another advantage of the single-cell electrofusion. The antigen specificity of the mab A53-B/A8 remains to be identified. It is directed against a common membrane constituent.

The second clone derived from the electrofusion, A53-B/A2, produces a mab recognizing a cytoskeletal protein restricted to epithelial and carcinoma cells. According to all available evidence, this strongly suggests cytokeratin(s) to be the target antigen.

Cytokeratins are a family of around 20 proteins characteristic of epithelial [2] and mesothelial cells [30] but absent in mesenchymal cells. They are major cytoplasmic proteins organized in a cytoskeletal network (intermediate filaments), the functions of which are not yet fully understood. Different epithelia contain lineage-specific sets of individual cytokeratins. These sets are reasonably stable even under conditions where other markers are lost, especially in tissue culture or after malignant transformation. The analysis of cytokeratins is considered to be valuable in

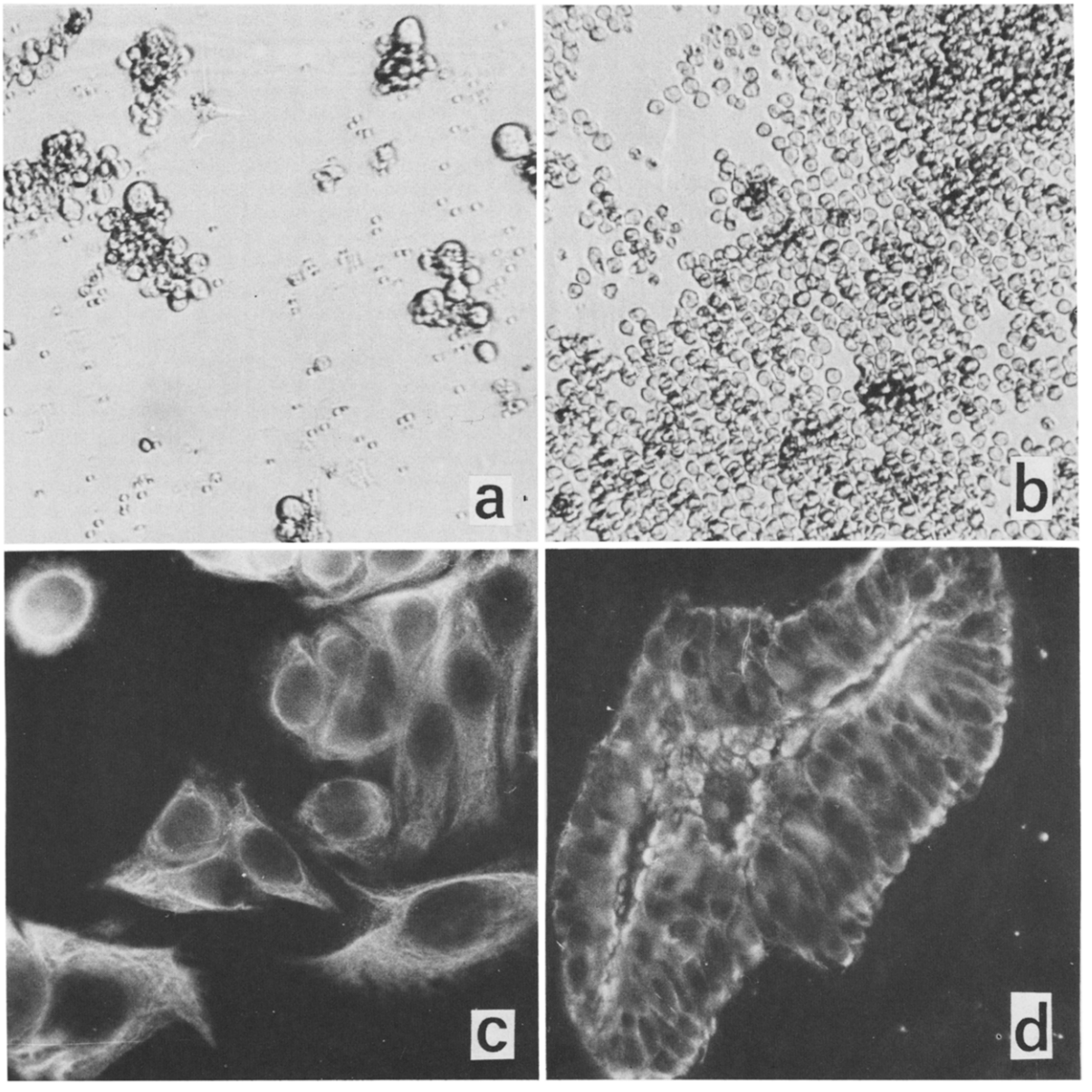

Fig. 2.(a) Spleen cell-plasmacytoma cell mixture in azaserine-hypoxanthine selection medium 3 days *after electrofusion. Unfused cells are dying. Some peritoneal feeder cells can be seen.* ×150. *(b) Suspension culture of hybridoma clone A53-B/A2* 6 weeks *after electrofusion.* ×150. *(c) MCF-7 cells stained with A53-B/A2 supernatant in indirect immunofluorescence test.* ×590. *(d) Human mammary adenocarcinoma (cryostat section) stained with A53-B/A2 supernatant in indirect immunofluorescence test. Carcinoma cells are stained, while stroma cells do not react.* × 590.

tumour diagnosis [5, 31]. For obvious reasons mabs specific for the individual cytokeratin proteins would be extremely helpful in this respect. Interestingly, some cytokeratin epitopes recognized by mabs are shared with many, if not all, members of the family [32, 33] or even with cytoskeletal proteins other than cytokeratins [34]. The mab A53-B/A2 described here seems to be specific for an individual cytokeratin protein, probably No. 19 of the catalogue of Moll *et al.* [2]. Although direct proof is lacking, our conclusion is based on the following facts: (1) MCF-7 cells have been shown to contain only three cytokeratins, 8, 18 and 19 [2]; (ii) the occurrence of the A53-B/A2 antigen is clearly different from that recognized by A45-B/B3 (also raised against MCF-7 cells and probably specific for cytokeratins 8 and/or 18), and (iii) mab A53-B/A2 does not stain cell lines A-431 and HeLa, which are lacking cytokeratin 19 [2].

Cytokeratin 19 is a small acidic cytokeratin present as a major component in most epithelia and carcinomas but absent in epidermis, basal cell epitheliomas and hepatocytes [2]. In any case, our monoclonal antibody A53-B/A2 seems to provide a histological reagent able to discriminate between different epithelial lineages and their malignant derivatives.

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