Human hybridoma cells produced by electro-fusion

R. Bischoff, R.M. Eisert*, I. Schedel, J. Vienken+ and U. Zimmerman+

*Abteilung Klinische Immunologie und Transfusionsmedizin, *Abteilung Hämatologie, Zentrum Innere Medizin und Dermatologie, Medizinische Hochschule Hannover, Karl Wiechert Allee 9, D3000 Hannover 61* and *+Arbeitsgruppe Membranforschung am Institut für Medizin, Kernforschungsanlage Jülich GmbH, Postfach 1913, D5170 Jülich, FRG*

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1. INTRODUCTION

Hybridoma cells producing monoclonal antibodies can be obtained from immunoglobulin-producing B-lymphocytes hybridised with a permanent cell line of myeloma cells [1–3]. Monoclonal antibodies obtained from mouse hybridoma cells are useful for a variety of diagnostic purposes and for the preparation of natural products of biotechnological interest. The application of these antibodies to humans in immunotherapy is limited [4] due to possible adverse interspecies reactions. Since an immunisation regime would be quite unreasonable in the human system, the number of lymphocytes with a specificity against a particular determinant is relatively low. A fusion method with a particularly high yield of hybridised cells is, thus, a prerequisite for the successful preparation of hybridised cells which are capable of producing antibodies with a given specificity.

The production of hybridoma cells using human B-lymphocytes and human myeloma cells, has been done in [5,6]. As in the case of mouse hybridoma cells, fusion was achieved by the aid of PEG. The yield of hybridoma cells produced after the application of the PEG-induced fusion technique is very low, which is a considerable disadvantage. Moreover, the selection of hybridoma cells obtained by this method requires the use of HAT-sensitive myeloma cells [7], since the fusion process cannot be optically followed and the resulting hybridoma cells have to be selected via the HAT minimal medium.

Here, we report on the production of hybridoma cells with the aid of the electro-fusion technique [8–11]. This method has the special advantage that yields are high, and that the fusion process can be followed under the microscope, so that the resulting hybridoma cells can easily be identified and removed from the chamber with micromanipulators. The use of HAT-sensitive myeloma cells is thus no longer necessary. While a combined electro-hydraulic procedure was applied for the production of a high yield of mouse hybridoma cells [11], human hybridoma cells can be obtained by a relatively simple method based on the experimental observation that lymphocytes do not fuse with one another under certain field conditions. Using a larger percentage of lymphocytes to myeloma cells, myeloma–lymphocyte formation is favoured and a good yield of hybridoma cells can be achieved.

Abbreviations: AC, alternating current; DMEM, Dulbecco's modified Eagle's medium; FCS, Fetal calf-serum; F_{530} (DNA), intensity of green fluorescence emission (530 nm) corresponding to DNA content; HAT-medium, selection medium, containing hypoxanthine, aminopterin and thymidine; PEG, polyethylene glycol

2. MATERIALS AND METHODS

Mononuclear cells from healthy adults were separated by density gradient centrifugation (Ficoll, Pharmacia, Uppsala) [12]. The T-lymphocytes present in the resulting cell suspension were largely removed by gradient centrifugation after rosetting

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with sheep erythrocytes. Plastic-adhering cells were separated by incubation in a plastic Petri dish. Any T-cells remaining in the suspension were lysed by incubation with OKT 3 (Orthomune no. 96 08 11, Ortho Diagnostic Systems GmbH, Heidelberg) and rabbit serum as the complement source [13]. Using this method, OkIa-positive cells were enriched to 90–95%.

The myeloma cells (L363) were kindly supplied by Professor V. Diehl (Dept. Haematology, Medical School, Hannover). This cell line was shown to expose only immunoglobulin light chains (type λ) on its surface but does not secrete any measurable amount of immunoglobulin into the supernatant. The experimental set-up for electro-fusion is detailed in [8]. Briefly, platinum wires (diam. 0.2 mm) were mounted parallel on a perspex slide and connected to a function generator (type 7404 P, Toellner Electronic, G. Werner, Frankfurt) which was used as the voltage source for the generation of alternating electric fields (resulting in dielectrophoresis and pearl chain formation [14]. A pulse generator (type 214 B, Hewlett Packard, Boeblingen) was linked in parallel for the injection of square pulses into the cell suspension (leading to electrical breakdown of the membrane). A droplet of the suspensions containing myeloma cells (L363) and B-lymphocytes in a 1:5 ratio, suspended in 0.3 M mannitol solution with 0.5 mg/ml pronase (Serva GmbH, Heidelberg), was pipetted between the electrodes prior to field application.

After the collection of the cells by dielectrophoresis (frequency of the AC field: 2 MHz, 100 V/cm amplitude), 2 square pulses of high intensity and short duration (3.5 kV/cm, 7 μs duration) were applied, resulting in the electrical breakdown of the cell membranes followed by cell fusion. The procedure was carried out under sterile conditions in a laminar airflow cleanbench.

Three minutes after the field application, the AC field was switched off, and the cells were transferred onto microtitration plates which contained a 0.3 M mannitol solution heated to 37°C. After a further 30 min a physiological NaCl solution was added which contained 1 mM $CaCl_2$ and 1 mM $MgCl_2$. The hybrid cells were cultivated on a feeder layer of human macrophages with DMEM and 20% FCS.

To ascertain the DNA-content of the cells, the individual cell populations, the cell mixtures, as well as the hybrid populations were examined with a cytofluorograph (System 30L, Ortho Instruments Inc. Raritan NY) around 10 h after field application. For this purpose the cells were stained with acridine orange.

The identity of hybridoma cells was demonstrated after 3 weeks of cultivation by the indirect immunofluorescence technique using monoclonal anti-Ia sera (no. 96 08 61, Ortho Diagnostic Systems GmbH, Heidelberg) and fluorescein-conjugated anti-mouse Ig (code F 232, Dako Diagnostika, Boehringer Ingelheim).

3. RESULTS AND DISCUSSION

Pearl chain formation of myeloma cells and lymphocytes was induced by application of the AC field in the fusion chamber. These chains consist of different combinations of myeloma cells and lymphocytes. An electrical breakdown pulse (3.5 kV/cm, 7 μs duration) does not lead to fusion of the lymphocytes with one another under these experimental conditions. In contrast, myeloma cells easily fuse with one another and with lymphocytes. The lack of lymphocyte/lymphocyte fusion may be due to the smaller radius of these cells [9] and/or to the different properties of the cell membrane or of the cytoskeleton. Formation of myeloma/myeloma combinations could be largely avoided by mixing the 2 cell types in a 5:1 ratio. This ratio ensures that almost every myeloma cell has contact with a lymphocyte.

The electro-fusion of human B-lymphocytes with human myeloma cells (L363) gave a high yield of hybridoma cells (fig.1). These cells became round within 10 min of application of the breakdown pulse, and this process was accelerated by the addition of mannitol solution warmed to 37°C. The hybridised cells could thus be transferred to culture media after a further 30 min. Under the specified cultural conditions, hybridoma cells went through 3 asynchronous phases of division (fig.2) within 3 weeks, and the first division was observed after 4 days.

In order to identify the hybridoma cells, the cells were stained with acridine orange [15] after 10 h cultivation, and their DNA content was examined with a cytofluorograph. As a control, the individual populations of peripheral blood lymphocytes and myeloma cells as well as mixtures of the two

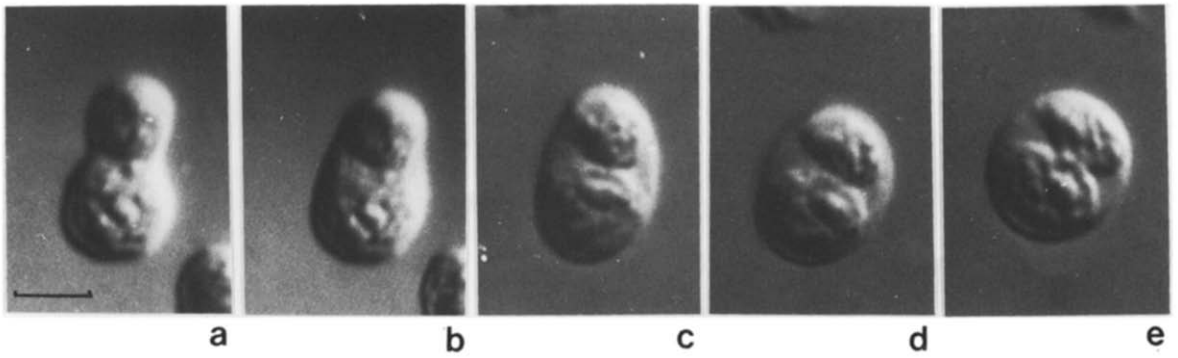

Fig.1.(a–e) Electric field induced fusion between a human B-lymphocyte and a human myeloma cell (L363). Lymphocyte and myeloma cell are brought in close membrane contact by dielectrophoresis. Pronase (0.5 mg/ml) was added to the suspension prior to the experiment. Photographs show the time course of fusion: (a) 10 s; (b) 20 s; (c) 40 s; (d) 60 s; (e) 120 s after the application of 2 square pulses (3.5 kV/cm, 7 μs). Bar: 10 μm.

cell types not subjected to the electrical field were examined at the same time and under the same conditions.

The results are shown in fig.3a–d. The DNA content is plotted as a function of the number of cells for B-lymphocytes in fig.3a, for myeloma cells in fig.3b, for the mixture in fig.3c and for the hybridoma cells in fig.3d.

The B-cell enriched polyclonal lymphocyte fraction is characterised by a peak with a narrow base which is attributable to diploid cells in the G1 and G0-phases. In the case of lymphocytes, the proportion of cells in the S-phase is small, as no significant numbers appear in this region of the histogram (fig.3a). In contrast, the myeloma cell line L363 demonstrates a peak with a broad base which corresponds to the G0/G1-phase. Due to the S-phase in cultured cells, an elevated plateau is observed compared with the B-lymphocyte graph, this distribution being typical of fast-growing cells [16]. The mixture of lymphocytes and myeloma cells exhibits clearly a two-peaked distribution, as expected from the linear superposition of the fluorescence pattern of the two cell types. Fig.3d shows only a single peak and a slight shoulder (arrow), which probably represents non-fused B-lymphocytes. In addition, due to the absence of the elevated plateau on the right of the curve, it can be argued that there must be a new cell population. This population can be attributed to hybridised cells.

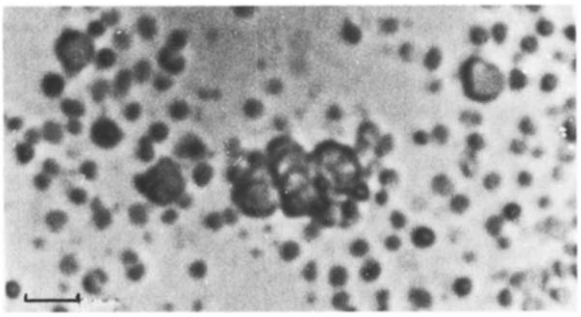

Fig.2. Hybridoma cells obtained by electro-fusion and cultivated on a feederlayer of human macrophages with DMEM and 20% FCS went through asynchronous phases of division during the course of 3 weeks. First division of the hybridoma cells was observed after 4 days. Bar: 20 μm.

However, one cannot rule out the possibility that this peak may be the result of myeloma cells with a low DNA content fusing with one another. This possibility could be disregarded by measurement of the DNA content as a function of the cell size. This relationship is shown in fig.3e for the mixture and in fig.3f for the hybridoma cells. A comparison of the two figures reveals the presence of a population which is not detectable in the original cell populations. This population not only exhibits a totally different volume distribution from its parent cells, but is also characterised by a more homogeneous DNA distribution. It therefore seems reasonable to assume that this population can be attributed to hybridised cells. The volume of the hybrids indicates that fusion between 2 cells occurred, if the wide distribution in volume of the

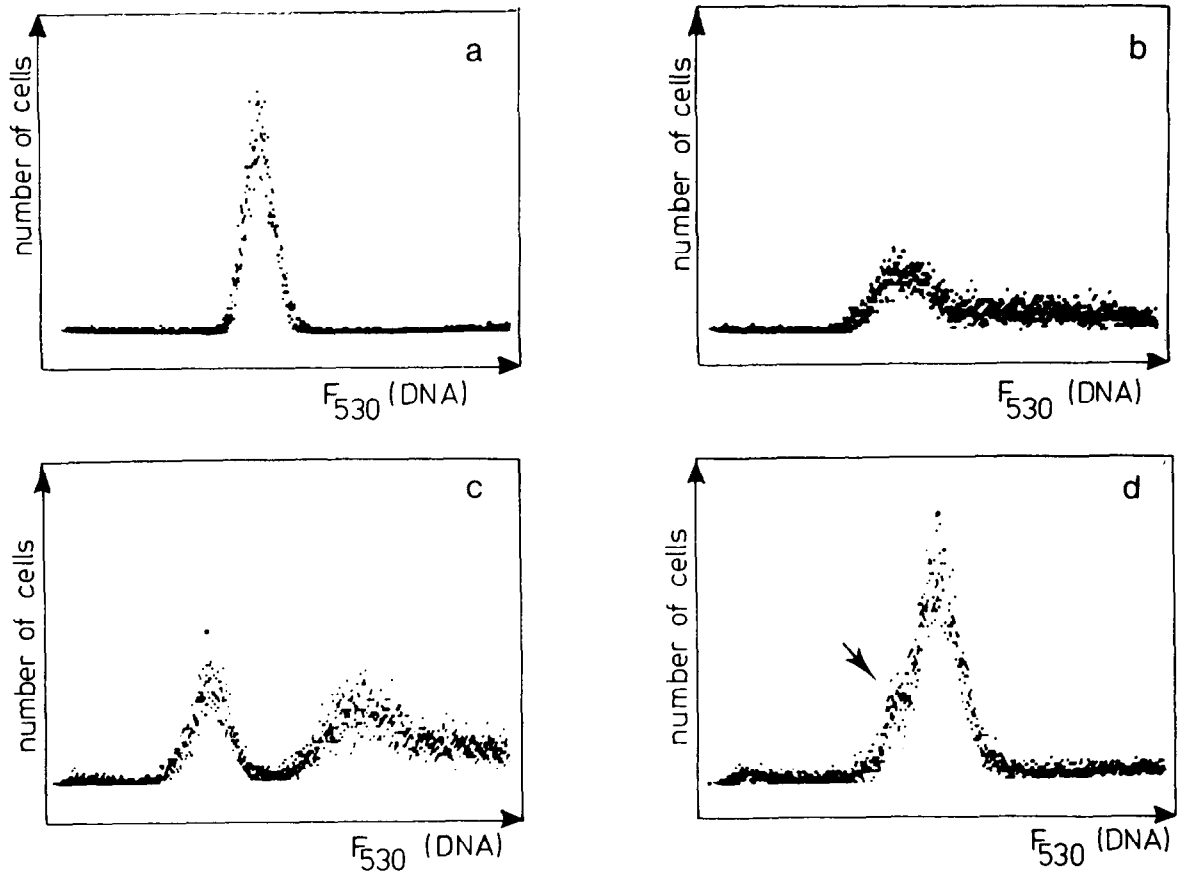

Fig.3.(a–d) Cytofluorographs showing the relative DNA content of different cell populations: (a) B-lymphocytes; (b) L363 myeloma cells; (c) a mixture of B-lymphocytes and L363 myeloma cells; (d) hybridoma cells taken 10 h after electro-fusion. The position of each dot indicates the intensity of green fluorescence (DNA-content) for a single cell. For details see text.

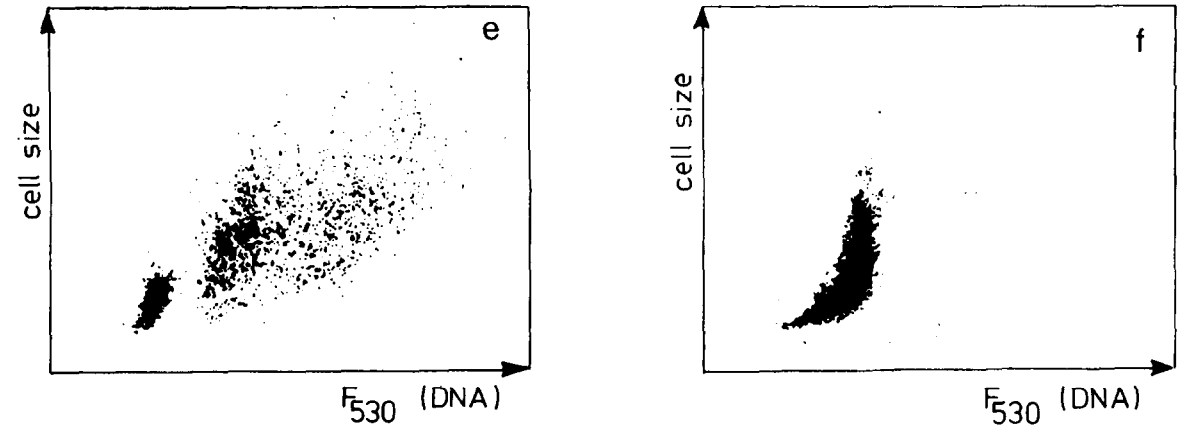

Fig.3.(e–f) Cytofluorographs representing the relative DNA content (green fluorescence intensity) as a function of the cell size: (e) mixture of B-lymphocytes and L 363 myeloma cells; (f) hybridoma cells, taken 10 h after electro-fusion. For details see text.

myeloma cells is considered.

There are no indications of a cell-fragmentation or cell constituents (fig.3f) in the semi-quantitative assessment of fluorescence. This finding correlates well with the trypan blue exclusion test.

Further evidence of the formation of hybridoma cells using the electrical field technique was obtained with the aid of the indirect immunofluorescence technique. Hybridoma cells which have been cultivated for three weeks exhibited a positive fluorescence reaction with monoclonal anti-Ia sera. Fused L363 myeloma cells, on the other hand, exhibit no fluorescence with anti-Ia sera.

Therefore, we can conclude that this cell fusion technique offers the possibility of hybridisation of human lymphocytes producing antibodies of a given specificity with a non-immunoglobulin secreting myeloma cell line. The high yield of hybridised cells as well as the possibility of direct visualisation of the cell fusion can be regarded as reasonable prerequisites for the preparation of human monoclonal antibodies of a given specificity.

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