CLINICAL APPLICATIONS OF MONOCLONAL ANTIBODIES

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PREFACE

Immunology has come a long way in the hundred or so years since the general concepts were first enuciated by Metchnikoff, Ehrlich, Von Behring and others. One of the landmarks in this progress was the invention and development of monoclonal antibody secreting hybridomas by Milstein and his co-workers in Cambridge. Unlike most modern inventions of this importance that of monoclonal antibody production was made available to the scientific community throughout the world unimpeded by patent protection. This may explain the unusual rapidity with which it has been applied to the benefit of mankind in general. This book, representing as it does the proceedings of the first International Symposium to be held on the clinical applications of monoclonal antibodies, shows just how much has been achieved within the space of little more than a decade. The enormous promise of monoclonal antibody technology, which became apparent soon after its discovery, has already progressed a long way towards fulfillment. The contributors to this volume, all of whom are actively engaged in monoclonal antibody development and application, represent the state of the art.

Professor Vincent Marks

INTRODUCTION

It has been some twelve years since the pioneering experiments of Köhler and Milstein led to the discovery of monoclonal antibodies. Single molecular species antibodies with desired specificities could be produced by the fusion of antibody - producing cells with neoplastic cells. The original aims of Köhler and Milstein were to study the size of the antibody repertoire and to assess the contribution of somatic mutation to antibody diversity. The applications of these single monoclonal antibodies were not completely obvious then, but it was clear they would be of significant value.

In fact we now look back on the production of monoclonal antibodies as a most exciting and tremendous discovery which has led to a vast range of applications including analytical, diagnostic and therapeutic spanning all the chemical, biological and medical sciences.

Applications of monoclonals in research are worldwide and they have clearly been extensively studied for use in immunoassay and immunopurification of a wide range of industrially and clinically important chemicals, biochemicals and microbiological materials. Clinical applications also include immunocytochemistry, imaging, sensors, diagnosis and therapy in man and in the veterinary fields.

A number of advances in the technology of the production of monoclonal antibodies have occurred and these are highlighted in this book. The production of human monoclonal antibodies in very large quantities, has become increasingly important since they are required for human therapeutic application. Mouse or rat monoclonals remain perfectly suitable for analytical, diagnostic, and research applications.

We can now also look forward to a new, second generation of monoclonal antibodies which involve new technologies including molecular biology. It is now possible to modify the antibody molecule extensively, for example (1) hybrid antibodies have arms of different specificity thus enabling them to be used as a bridge between two different chemical structures; (2) chimeric antibodies which are produced by genetic engineering allowing the V-region mouse genes to be linked to the C-region human genes; these antibodies are less immunogenic in man and are much less likely to produce side-effects in human therapy; (3) CDR (complementary determining region) graft antibodies have only the amino acid residues from the mouse as the antigen-binding site i.e., the hypervariable amino acids of the V domains,

whilst the rest of the molecule can be human. Clearly with these new antibodies come new applications and safer human therapy.

This book represents the state of the art in the research into, and the value of, monoclonal antibodies in clinical and veterinary applications. The book provides background to the development of certain topics and highlights current areas of progress. This book will be of great interest to research workers in the field and clinicians in general, but is accessible to the non-specialist with an interest in these topics and hence it has broad educational value.

We are grateful to the many eminent scientists and clinicians who contributed to the meeting with presentations and discussions, and to the secretariat and graduate students of the University of Surrey Biochemistry Department who helped in a variety of roles to ensure the success of the Symposium. In particular the editors would like to give special thanks to Peter Goldfarb, Jan McCall and Ann Hanson for all their help.

The Symposium organisers are also most grateful to our sponsors including Celltech Ltd., Cetus Corporation and Serono Diagnostics Ltd. Finally the editors wish to record their thanks to the staff of Plenum Press and Medimedia Ltd. who gave us every encouragement to realise this project.

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July 1988

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THE POTENTIAL OF ELECTROFUSION FOR HYBRIDOMA PRODUCTION

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INTRODUCTION

The potential of monoclonal antibodies as diagnostic and therapeutic agents has now been widely accepted. Improvements in murine hybridoma formation protocols are being continuously developed by almost every grou of investigators actively involved in the generation of monoclonal antibodies. However, the preparation of human hybridomas is still generally assumed to be more difficult than that of murine hybrids. Apar from the chromosomal instability of human-human hybridomas (Foung et al, 1982) the choice of fusion agent may be an important factor (Gravekamp et al, 1985). Polyethyleneglycol (PEG) normally used in cell fusion has a number of disadvantages which might result in drawbacks in the production of a broad spectrum of hybrid cells.

The electrofusion technique pioneered by Zimmermann and co-workers (see review articles of Zimmermann, 1982, 1986, 1987) may therefore be an efficient alternative to PEG-induced cell fusion, in particular in the production of human hybrids. The electrofusion technique is based on the application of physical (vectorial) forces which allow precise control an monitoring of the fusion process. Visualization and identification of th hybrids under the microscope offers the possibility of avoiding the use o selective mutants as fusion partners and, in consequence, employment of selection media. This aspect is of great relevance in human hybrid production because the number of available tumor cell lines that have to be tested for hybrid selection is limited by the requirement of HAT-sensitivity.

Electrofusion can be universally applied to all living cells. The field conditions found optimal for two fusion partners can therefore be applied, with appropriate modifications to the fusion of cells of other species, provided that the biology and the biophysics of these cells are taken into account (Zimmermann and Urnovitz, 1987).

The present article reviews our knowledge on electrofusion and emphasis is given to recent results which may pave the way to the production of human hybrids on a large scale using this new technology.

PRINCIPLES OF ELECTROFUSION

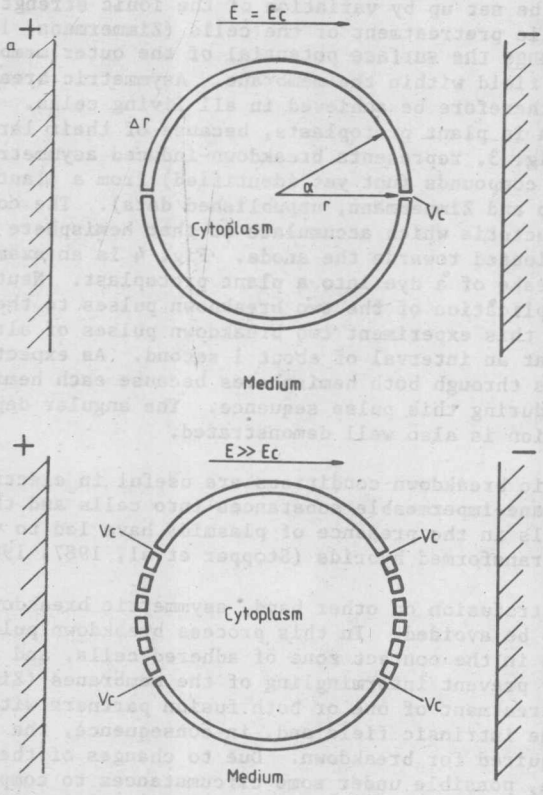
Electrofusion is based on the temporary permeabilization of the cell membrane in response to electrical breakdown. This phenomenon was discovered in 1973 (Zimmermann et al, 1973, 1974) and was first used for the electroinjection of membrane-impermeable substances (such as dyes, drugs, particles, proteins and DNA) into freely suspended cells without deterioration of cellular functions or membrane integrity (see e.g. Zimmermann et al, 1981, Zimmermann, 1986).

Breakdown Conditions

Experimentally, electrical breakdown is observed when cells are exposed to a field pulse of high intensity (some kV/cm) and short duration (in the nano-to microsecond range). Due to charge separation a potential difference is built up across the membrane which is superimposed on the intrinsic membrane potential - or more precisely on the intrinsic electric field within the membrane. Breakdown occurs when the vectorial sum of the intrinsic and the induced potentials exceeds the breakdown voltage of the biological membrane which is of the order of 1 V (at room temperature). The generated potential difference is proportional to the field strength, to the radius of the cell, and to the cosine of the angle between a given membrane site and the field vector (Zimmermann, 1982, 1986) (Fig. 1). The radius-dependence of the generated potential difference has the important consequence that breakdown of differently sized cells requires different critical field strengths a priori. Electrofusion of lymphocytes and myeloma cells suffers from this problem because the average volume of non-activated lymphocytes is about 120 $(\mu m)^3$, whilst that of myeloma cells about 1200 $(\mu m)^3$. Since lymphocytes stimulated by antigens either in vitro of in vivo exhibit larger volumes (up to 600 μm^3) than inactivated ones the breakdown conditions of these lymphocytes will be more comparable to those of myeloma cells with the consequence that fusion and subsequent hybridization is preferentially induced between antibody producing lymphocytes and myeloma cells. The radius-dependence can, therefore, be used for physical selection of stimulated lymphocytes in the presence of a large amount of non-stimulated ones provided that appropriate field and media conditions are used (see below and ref. Jeltsch and Zimmermann, 1979).

The angular dependence of the generated potential difference across the cell membrane determines the size of the permeabilized area of a given cell once the breakdown voltage is exceeded. As the external electric field strength is increased the electrical breakdown potential is first reached at membrane sites oriented in the field direction and then at membrane sites with progressively increasing angle when supracritical field strengths are applied. Membrane breakdown cannot occur perpendicular to the field vector because cosine 90° is zero. The more the critical field strength is exceeded the greater the area of disturbance of the membrane. In electroinjection work larger molecules can be admitted into freely suspended cells under these conditions (e.g. Stopper et al, 1987). On the other hand, in electrofusion large field-induced pertubations in the contact zone of adhered cells facilitate fusion of differently sized cells and accelerate the intermingling process.

The superposition of the generated potential difference on the intrinsic membrane field can lead to an asymmetric breakdown under some circumstances (Zimmermann and Stopper, 1987). As shown schematically in Fig. 2 the vectors of the generated and intrinsic field within the membrane are parallel in one hemisphere and antiparallel in the other one. In this figure it is assumed that the cell interior is negatively charged with respect to the external solution, as is the case for lymphocytes and myeloma cells. If the intrinsic membrane potential or field is



$V_c = 1{,}5 \cdot r \cdot E_c \cdot \cos \alpha$

Fig. 1. Symmetric breakdown
Representation of a spherical cell with radius, r, exposed to an external field, E.
a) The breakdown voltage, Vc, will be attained first at membrane sites oriented in the direction of the field vector because of the angular dependence of the generated membrane potential (see the cosine α term in the equation). The field strength required for breakdown at these sites is donated by Ec. b) When supracritical field strengths E > Ec are applied the breakdown voltage is also exceeded at positions on the membrane oriented at a certain angle, α, to the field vector.
For the purposes of illustration only, the formation of "pores" is shown to indicate where breakdown has occurred even though there is evidence that pores are not generated (Zimmermann, 1986). Note that it is assumed in this figure that the intrinsic membrane potential is zero or negligibly small compared to the potential generated by the external field (taken from Zimmermann, 1986).

sufficiently high, breakdown will occur in the membrane of one hemisphere and only in the other one at much higher field strengths. Asymmetric breakdown related to only one hemisphere minimizes irreversible side effects within the cell and thus increases the viability of the field-treated cells. This is because the current flowing through the cell interior once breakdown in one hemisphere has occurred is restricted by the

intact membrane of the other hemisphere. Optimum conditions for asymmetric breakdown can be set up by variation of the ionic strength of the solution and by enzymatic pretreatment of the cells (Zimmermann, 1986). Both procedures change the surface potential of the outer membrane and therefore the intrinsic field within the membrane. Asymmetric breakdown of the cell membrane can therefore be achieved in all living cells. This phenomenon was first seen in plant protoplasts, because of their large size (Mehrle et al, 1985). Fig. 3. represents breakdown-induced asymmetric release of intracellular compounds (not yet identified) from a plant protoplast (Mehrle, Hampp and Zimmermann, unpublished data). The compounds attract chemotactic bacteria which accumulate at that hemisphere which was originally oriented towards the anode. Fig. 4 is an example of double asymmetric uptake of a dye into a plant protoplast. Neutral red was added before the application of the two breakdown pulses to the external solution. In this experiment two breakdown pulses of alternating polarity were applied at an interval of about 1 second. As expected, the uptake of dye now occurs through both hemispheres because each hemisphere is oriented to the anode during this pulse sequence. The angular dependence of the permeabilization is also well demonstrated.

Asymmetric breakdown conditions are useful in electroinjection of foreign membrane-impermeable substances into cells and their application to mammalian cells in the presence of plasmids have led to very high yields of genetically transformed hybrids (Stopper et al, 1987, 1988).

For electrofusion on other hand, asymmetric breakdown conditions have apparently to be avoided. In this process breakdown pulses are applied to the membranes in the contact zone of adhered cells, and asymmetric breakdown may prevent intermingling of the membranes (Zimmermann, 1986). However, pretreatment of one or both fusion partners with digestive enzymes may change the intrinsic field and, in consequence, the external field strengths required for breakdown. Due to changes of the intrinsic field it is, therefore, possible under some circumstances to compensate partly the radius-dependence of the breakdown voltage. Electrofusion of different sized cells is facilitated by pronase pretreatment as was observed for lymphocytes and myeloma cells (Vienken and Zimmermann, 1982). However, it has to be noted that pronase may affect the viability of the hybrids if it is taken up during the fusion process through the permeabilized areas.

Resealing Properties

The electrical breakdown of the cell membrane occurs very rapidly (in less than 50 ns) and is reversible provided that the pulses are not too long and do not exceed a critical intensity which leads to an unfavorable ratio of the permeabilized area to the total surface (Zimmermann, 1986). Under these conditions the pertubations in the membrane introduced by the field pulse reseal, so that the original electrical properties and impermeability of the membrane are restored. Resealing proceeds more rapidly with increasing temperature. In contrast to electroinjection, electrofusion of aligned cells is normally carried out at room temperature, provided that weakly conducting solutions are used during pulse application. At this temperature, the resealing of the field-induced membrane pertubations in the cell chain termini is still fast enough to avoid substantial loss of intracellular substances into the external medium, and slow enough to allow intermingling of the membranes of adhered cells in a chain. Performance of electrofusion at higher temperatures would facilitate the competitive process of resealing of the individual adhered membranes in the contact zone resulting in lower fusion and hybrid yield.

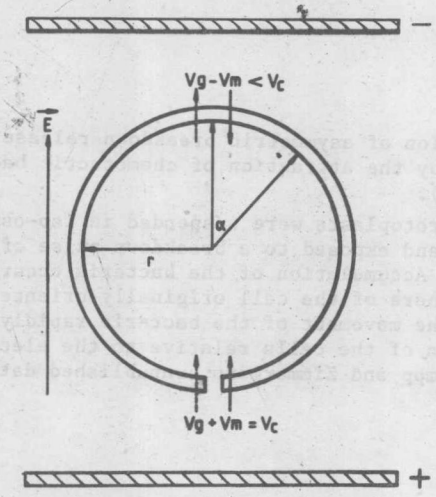


Fig. 2. Asymmetric breakdown
Superimposition of the membrane potential, Vg, generated by an external field, E, on the intrinsic membrane potential, Vm, (which is normally of the order of -100 mV, inside negative). The field vectors are parallel in the hemisphere facing the anode and antiparallel in the other one. As indicated, breakdown will first occur in the membrane of the cell hemisphere oriented to the anode (taken from Zimmermann, 1986).

Membrane Contact

Close membrane contact is a pre-requisite for fusion. Without such contact, only solute exchange between the cell interior and the external medium occurs. This field-induced exchange can be used - as mentioned above - for entrapment of membrane-impermeable substances such as drugs, proteins and DNA (so-called electroinjection, electropermeabilization or electroporation).

Controlled membrane contact can be achieved by various physical forces. In the standard electrofusion technique a non-uniform alternating electric field of low strength (V/cm range) and, therefore, of long duration (seconds to minutes) is applied.

In an inhomogeneous electrical field the forces acting on the induced positive and negative charges within the cells are different due to the field inhomogeneity; thus a net force arises that induces migration of the cells (Pethig, 1979, Pohl, 1979). The movement of a particle in a non-uniform field is independent of the polarity of the field and is therefore also observed in an alternating field (usual frequency range 10 kHz to 5 MHz) provided that poorly conducting media are used in order to avoid turbulence due to local heat development. This phenomenon which has been known since the 1920s (see literature quoted in Zimmermann, 1982, Pethig, 1979) has been termed dielectrophoresis.

Depending on the differences between the dielectric properties of the medium and the cells (which vary with the frequency of the alternating field) movement of the cells or particles into the direction of either highest or lowest field gradients is observed. These effects are therefore termed positive and negative dielectrophoresis, respectively. Particles approaching each other during their movement towards the regions of highest

Fig. 3. Demonstration of asymmetric breakdown release of intracellular compounds by the attraction of chemotactic bacteria (Pseudomonas aeruginosa).

Oat leaf protoplasts were suspended in iso-osmotic sorbitol solutions and exposed to a breakdown pulse of 800 V/cm and 50μs duration. Accumulation of the bacteria occurs asymmetrically at the hemisphere of the cell originally oriented to the anode. However, the movement of the bacteria rapidly changes the orientation of the cells relative to the electrodes (taken from Mehrle, Hampp and Zimmermann, unpublished data).

Fig. 4. Symmetric uptake of neutral red into an oat protoplast exposed to two breakdown pulses of 450 V/cm strength and 50 μs duration applied in opposing directions.

In each pulse, breakdown occurred essentially only in that hemisphere which faces the anode (see Fig. 2). However, the change of polarity of the field between the pulses resulted in symmetric uptake of the dye (Mehrle, Hampp and Zimmermann, unpublished data).

Fig. 5. Alignment of bacteria (Pseudomonas aeruginosa) close to the "pole" regions and contact zones of oat protoplasts aligned in a slightly non-uniform alternating field between two cylindrical electrodes (frequency 1MHz, field strength 200 V/cm, iso-osmotic mannitol solution). Taken from Mehrle et al, 1988).

Fig. 6. Formation of concentric rings of polystyrene beads (2.5 µm diameter) around oat protoplasts aligned in the non-uniform alternating field (field strength 240 V/cm, frequency 1 MHz) between two cylindrical electrodes.

In contrast to the bacteria in Fig. 5, the beads show negative dielectrophoresis at this frequency (taken from Mehrle et al, 1988).

Fig. 7. Electrofusion between aligned oat protoplasts.

Field conditions: application of an alternating field of 150 V/cm strength and 1 MHz frequency for 60 s followed by 1 breakdown pulse of 1 kV/cm strength and 20 µs duration. Photographs were taken just before application of the breakdown pulse (a), after 10 s (b) and 10 min after pulse application (c).

Fig. 10. Nuclear membrane fusion in Ehrlich ascites tumor cells after dielectrophoretic alignment and subsequent application of breakdown pulses.

Field conditions: alternating field of 220 V/cm strength and 1.7 MHz frequency followed by 2 pulse trains each of 3 pulses of 5 kV/cm strength and 15 µs duration. Nuclei were Giemsa stained after fixation with Carnoy's fixative. 520 x magnification, oil immersion.