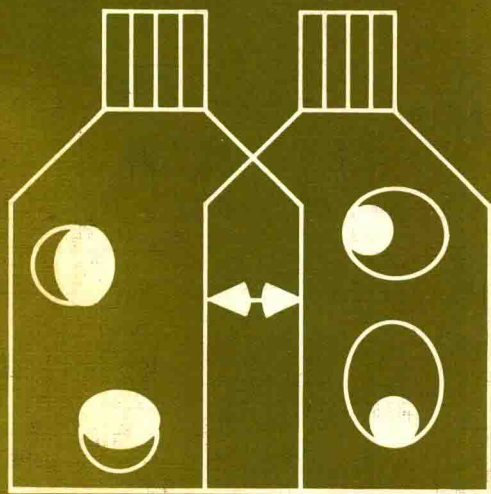


In Vitro Immunization in Hybridoma Technology

Edited by

Carl A.K. Borrebaeck

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In Vitro Immunization in Hybridoma Technology

**Proceedings of the International Symposium on In Vitro Immunization
in Hybridoma Technology, Tylösand, Sweden, September 7-8, 1987**

Edited by

Carl A.K. Borrebaeck

*Associate Professor of Immunotechnology,
Department of Biotechnology, Lund University, Sweden*



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PREFACE

The introduction of monoclonal antibodies in 1975 has stimulated and promoted research in a number of areas; one important and exciting field is the application of therapeutic antibodies *in vivo*. The most difficult technological problem in the production of therapeutically valuable antibodies has been to obtain human monoclonal antibodies. An important reason for this is obvious, since very few antigens can be used to immunize patients for the production of immune lymphocytes. From this point of view, *in vitro* immunization, i.e. an antigen-specific activation of B cells in culture, has the potential of overcoming this problem.

In vitro immunization, in conjunction with monoclonal antibodies, has been an area of immunotechnology research for almost ten years. During this period of time murine *in vitro* immunization systems have emerged that can be used to efficiently produce mouse monoclonal antibodies. The meeting in Tylösand described the most thoroughly investigated procedures. Although several important advantages were obvious when using *in vitro* immunization for the production of mouse monoclonal antibodies, e.g. very small amounts of the immunogen were needed, "new" specificities could be obtained, etc, the single most important factor was still the possibility to produce human monoclonal antibodies against any antigen of choice. Human B cells have, however, until now defied major efforts to be used for the production of monoclonal antibodies by the same technique. From the meeting it was clear that the technology of *in vitro* immunizing human B cells has taken several major steps towards systems as simple as those described for mouse cells.

The most frequent questions raised at the meeting concerned evidence for an *in vitro*-induced immune response, the presence of natural antibodies, and the affinities of the produced monoclonal antibodies. Evidence was presented that clearly showed that an immune response of both mouse and human B cells was obtained *in vitro*. Furthermore, a consensus was reached that specifically induced antibodies and natural antibodies could be discriminated by stringent test methods.

From studies made with mouse monoclonal antibodies it was also reported that IgM monoclonal antibodies produced by *in vitro* or *in vivo* immunization had very similar affinities. It still remains uncertain what affinities can be obtained in IgG monoclonal antibodies produced by *in vitro* immunizations.

Finally, I would like to thank Ann-Britt Madsen of the Symposium Secretariat and our sponsors, who all contributed to the success of this meeting and the present book, which fulfills its goal of presenting the state of the art of this area of hybridoma technology.

Lund

Carl A.K. Borrebaeck

October, 1987

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PART 1

IN VITRO IMMUNIZATION OF MOUSE B
LYMPHOCYTES

DEVELOPMENT OF AN *IN VITRO* IMMUNIZATION TECHNIQUE FOR THE PRODUCTION OF MURINE MONOCLONAL ANTIBODIES USING SMALL AMOUNTS OF ANTIGEN AND WEAK IMMUNOGENS.

S.A. MÖLLER and C.A.K. BORREBAECK

Department of Biotechnology, University of Lund, P.O.Box 124, S-221 00 Lund
(Sweden)

SUMMARY

The antigen-specific activation of B lymphocytes for the purpose of developing an *in vitro* immunization technique applicable in hybridoma technology has been studied in our laboratory for seven years. Non-immunized splenocytes have been stimulated *in vitro* with antigen and supporting T-cell derived lymphokines isolated from mixed lymphocyte cultures and/or the murine thymoma cell line EL-4. Optimal culture conditions were evaluated by determining the number of specific antibody secreting cells and by somatic cell hybridization.

The data presented here demonstrates that an antigen-dependent immunization can be performed *in vitro* by regulating the activation and maturation of antigen-specific B lymphocytes, using growth and differentiation inducing factors. This *in vitro* immunization technique has been used for the production of monoclonal antibodies of both IgM and IgG isotypes, against weak immunogens and low doses of antigen.

INTRODUCTION

The humoral immune response to antigens implicates the activation and proliferation of specific immunoglobulin-expressing B lymphocytes and their subsequent maturation to antibody producing cells. *In vitro* immunization is defined as a primary, antigen-specific activation of cultured B cells resulting in a clonal expansion and maturation of specific lymphocytes.

Stimulation of *in vitro* cultured mouse splenocytes using sheep red blood cells as immunogen for the purpose of initiating a humoral response was first demonstrated over 20 years ago by Mishell and Dutton (ref. 1) and Marbrook (ref. 2). The introduction of hybridoma technology (ref. 3) made it possible to combine

in vitro immunization with cell hybridization to immortalize antigen-specific B cells for the production of monoclonal antibodies. The first successful attempt to produce monoclonal antibodies using *in vitro* immunized mouse splenocytes was reported 1978 (ref. 4) using sheep red blood cells as immunogen, and two years later using a T cell dependent protein antigen (ref. 5). Since then some 30 papers have been published describing monoclonal antibodies obtained against a variety of antigens using *in vitro* immunization of murine spleen cells (reviewed in refs. 6-7). This paper summarizes the work done in our laboratory to develop a general technique for murine *in vitro* immunization (refs. 8-12).

REQUIREMENTS FOR B CELL ACTIVATION

Since the first reported *in vitro* stimulations (refs. 1-2), several hundred papers have been published describing the soluble factors (lymphokines) and different cell types that are involved in the regulation of proliferation and differentiation of *in vitro* cultured B cells (reviewed in refs. 13, 14).

A B cell response to a thymus-dependent antigen normally requires cooperation between helper T-cells, accessory cells i.e. macrophages, monocytes, dendritic cells etc., and B-cells in a MHC-restricted fashion. The specific activation is dependent on an antigenic selection of B cells carrying complementary membrane bound immunoglobulins. This is a necessary but not sufficient first step to initiate the antigen-specific response. Dissection of the B cell activation process reveals the presence of three distinct steps; induction/activation, proliferation, and differentiation. The requirements of soluble factors vary in these steps and the overall progression of resting B cells through the cell cycle to antibody producing plasmacytes is regulated by a number of different cytokines that normally bind to receptors other than surface bound immunoglobulins (ref. 15). It has recently become evident that several of the individual lymphokines acting on B cells possess both proliferation and differentiation-inducing abilities. In summary, IL-1 and IL-2 together with BSF-1 (BCGF-I, IL-4) (ref. 16) are involved in the proliferative stage of both murine and human B cells (ref. 14), whereas interferon gamma, BSF-1, BSF-2 (IL-5) (ref. 17) and BCDF are some of the factors involved in the B cell differentiation.

SOURCES OF FACTORS FOR ANTIGEN-SPECIFIC B CELL ACTIVATION

The *in vitro* immunization process should parallel the antigen-specific activation of B cells *in vivo*. Consequently, a combination of T cell derived lymphokines had to be used. For this purpose we prepared supernatants from mixed lymphocyte cultures (MLC) (refs. 8-10) and from a phorbol ester (PMA) stimulated subline of murine EL-4 thymoma cells (refs. 11, 12).

The MLC-derived lymphokines were produced by co-cultivating thymocytes from BALB/c and C57Bl/6 at 5×10^6 cells/ml for 48 h, whereafter the supernatant was harvested. The supportive effect of MLC supernatants harvested after a stimulation period of 24, 72, and 96 h was evaluated in *in vitro* immunizations; they gave, however, a poorer induction of specific antibody secreting cells as compared to when supernatant from the 48 h MLC was used. Furthermore, lymphokines were prepared from the EL-4 thymoma cell line by stimulation with 10 ng PMA/ml for 30-40 h, in medium containing 1% rabbit serum.

Supernatant from a mixed lymphocyte culture (sMLC) contains TRF (ref. 18), BCGF (ref. 19), IL-2 and allogeneic helper factors (AHF) (ref. 20), whereas the EL-4 derived supernatant (sEL-4) contains B cell growth and differentiation inducing factors (BSF-1, BCDF) (refs. 13, 21-23) and IL-2 (ref. 24). Factors with isotype regulatory effects are also present in sEL-4, e.g. BCDF μ which induces IgM secretion in BCL1 cells (ref. 25) and BCDF-gamma which significantly increases the secretion of IgG1 from LPS stimulated mouse splenic B cells (ref. 26).

ASSAYS FOR B CELL ACTIVATION AND DIFFERENTIATION

In order to evaluate the effect of MLC-derived lymphokines on cells stimulated *in vitro* their volumes were measured using a Coulter Counter with an attached Channelyzer (ref. 10). Fig 1. compares the size distribution profiles of mouse splenocytes immunized *in vitro* against myoglobin for 5 days, in the presence or absence of MLC. It can clearly be seen that MLC provided the necessary support for the activation of the spleen cells into large blast cells. The hatched area indicates the size profile of the new blast cell population obtained on day 5.

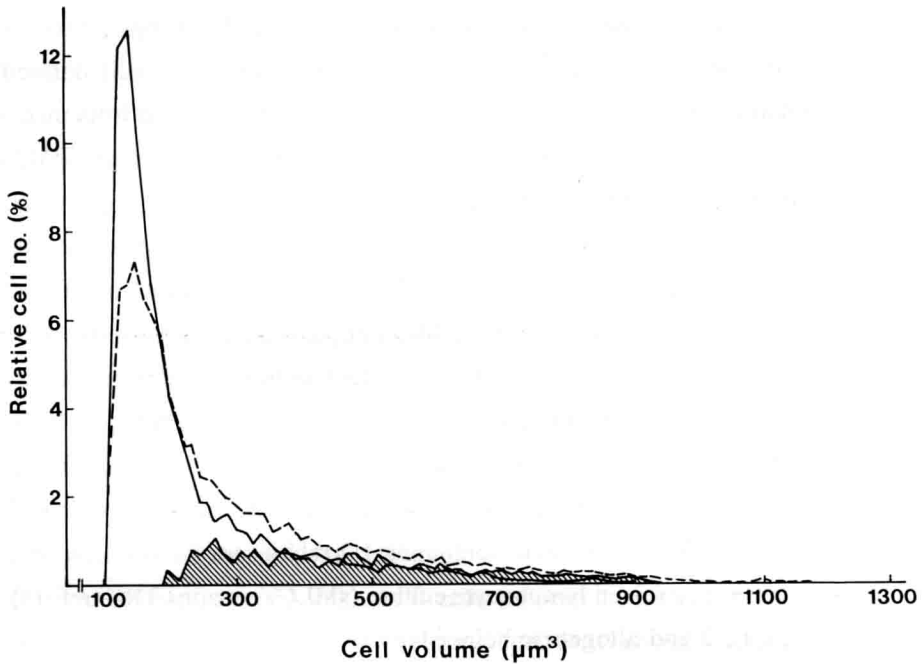


Fig. 1. Cell volumes of *in vitro* immunized mouse spleen cells on day 5. The immunization was performed in the presence (- - -) or absence (—) of MLC derived lymphokines. Ten μg myoglobin/ml was used as antigen. The hatched area indicates the difference in cell volume between the two cultures.

To evaluate the *in vitro* immunization we needed an assay that enumerated the number of antigen-specific activated B cells. Thus, a plaque assay was developed which determines the number of antibody secreting cells (ref. 27). This filter immuno-plaque assay (FIPA) is based on enzyme immunoassay principles and needs only small amounts of antigen to detect isotype-specific antibody secreting cells. The technique utilizes microtiter plates which have their plastic bottom replaced by a nitrocellulose membrane (millititer plates). Briefly, the antigen is adsorbed onto the nitrocellulose membrane, whereafter the wells are washed. Cells are added to the antigen-coated wells and incubated for 1-3 h, whereafter the cells are discarded and the wells washed extensively. Enzyme conjugated to anti-immunoglobulin antibodies are added, and after an 1 h incubation the plate is washed and developed by the addition of an enzyme substrate. The product of the enzymatic reaction is

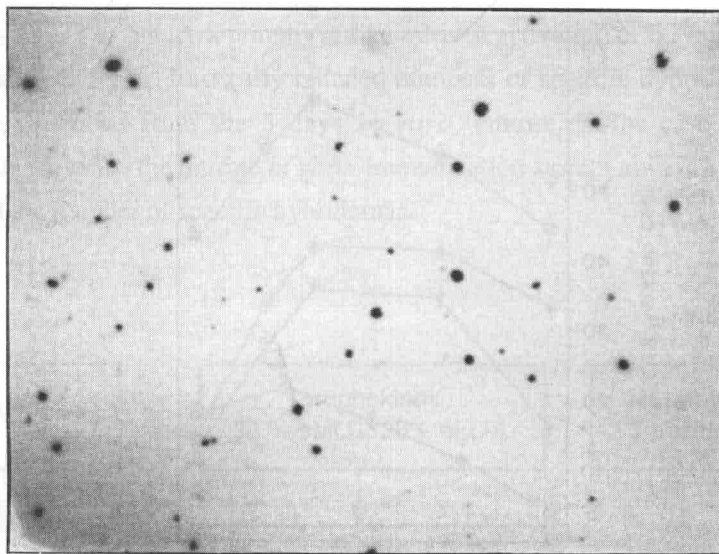


Fig. 2. Each plaque represents an antigen-specific antibody producing cell.

insoluble and appears as blue plaques, each indicating an antibody-secreting cell (see Fig. 2). However, caution should be taken when assessing the assay since batch variations in the quality of nitrocelluloses have been shown to drastically affect the nonspecific binding of proteins and cell debris, thereby making the interpretation of the results difficult (unpublished results).

DEVELOPMENT OF THE MURINE *IN VITRO* IMMUNIZATION SYSTEM

The filter immuno plaque assay was used to evaluate the optimal lymphokine support for *in vitro* immunization of splenic cells. Non-immune spleen cells from 5-7 week old BALB/c mice were immunized in culture for 5 days with the antigen ovalbumin. Bovine serum albumin (BSA) was used as a control antigen in the plaque assay. Fig. 3 shows the synergistic effect that different concentrations of MLC- and EL-4-derived lymphokines had on the number of antigen-specific plaque-forming cells (PFC) after an immunization in culture. There was a positive dose-response relationship between the amount of supportive lymphokines and the number of antigen-specific PFC that was recorded. A maximum was obtained using 33% MLC supernatant together with 25% EL-4 supernatant. The addition of EL-4-derived lymphokines to the *in vitro* immunization resulted in a more than three-fold