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Development of *in vitro* immunization in murine and human hybridoma technology*

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Abstract: The development of *in vitro* immunization in murine and human hybridoma technology is reviewed. Special consideration is given to: technical aspects of *in vitro* immunization of mouse B cells; quality of antibodies produced by *in vitro* immunization; advantages of murine *in vitro* immunization; technical aspects of *in vitro* immunization of human B cells; and the advantages of human *in vitro* immunization and human monoclonal antibodies. Future developments likely to be made in this area of immunotechnology are outlined.

Keywords: In vitro *immunization; mouse hybridoma; human hybridoma; monoclonal antibodies.*

Introduction

Antigen-specific activation of murine and human B lymphocytes is dependent on an antigenic selection of cells carrying complementary membrane-bound receptors. This binding of antigen is a necessary, but not sufficient, first step in initiating the antigen-specific response. Study of the B cell activation process reveals the presence of three distinct steps: induction, proliferation and differentiation. The progression of resting B cells through the cell cycle to antibody-producing plasmacytes is furthermore regulated by a number of different cytokines that normally bind to receptors other than surface Ig [1].

In vitro immunization is a primary, antigen-specific activation of B cells in culture, subsequently used as fusion partners for the purpose of producing monoclonal antibodies [2]. The *in vitro* immunization process should therefore parallel the antigen-specific activation of B cells *in vivo*. The first successful attempt was reported by Luben and Mohler [3], using a system where the activation of mouse splenocytes was supported by lymphokines present in thymocyte conditioned medium. The subsequent development was mainly on murine *in vitro* immunization and the first attempts to study the effect of cytokines on *in vitro* immunization were recently reported [4, 5]. Despite major efforts in the past five years there is presently no *in vitro* immunization format for human B cells that can compete in efficiency and universal applicability with the murine system.

In the following sections, different aspects of murine *in vitro* immunization will be discussed together with some human *in vitro* immunization systems.

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Technical Aspects of *in vitro* Immunization of Mouse B Cells

Owing to ready availability of mouse spleen cells and myeloma cell lines for fusion experiments, *in vitro* immunization has mainly been developed and used for the production of mouse monoclonal antibodies.

Addition of a T cell dependent antigen to a culture of dissociated, unprimed mouse spleen cells does not activate specific B cells and no significant amounts of monoclonal antibodies could be produced from these cells [3, 4]. The *in vitro* immunization had to be supported by cytokines such as B cell growth factor (BCGF) [6], B cell differentiation factor (BCDF) [7], γ-interferon, interleukin 1 (IL-1) and interleukin 2 (IL-2). These cytokines were initially produced by a culture of young thymocytes [3] or by a mixed lymphocyte culture i.e. a culture of MHC-disparate lymphocytes [8–10]. The supernatant isolated from a mixed lymphocyte culture was already (1972) shown to be a source of the T cell replacing factor (TRF) [11]. It also contained IL-2 along with allogeneic helper factors (AHF), which are as yet undefined molecular entities acting directly on the B cells [12]. A number of reports on *in vitro* immunizations supported by lymphokines from mixed lymphocyte cultures have been published in the last 3–4 years [2] and a detailed description on how to produce these cytokines was published recently [13].

This early *in vitro* immunization system still functions well although better sources of the necessary lymphokines exist. In an attempt to study the requirements for different lymphokines during an *in vitro* immunization of mouse splenocytes a subline of the mouse thymoma cell line EL-4 was used [5]. Upon stimulation with a phorbol ester, this cell line produced cytokines such as IL-2 (50–100 U ml^{-1}) [14], BCGF [6, 15], BCDF [7], BCDFμ [16] and BCDFγ [17]. It is likely that culture supernatant from phorbol ester stimulated EL-4 thymoma cells contained enough essential factors for antigen-activated B cells to complete the subsequent proliferation and differentiation steps. Furthermore, the presence of factors secreted from isotype-specific regulatory T cells [17] enhanced the probability of obtaining monoclonal antibodies of the γ isotype. The development of an *in vitro* immunization system based on a combination of these factors was initially monitored by a filter immuno-plaque assay that processed several hundreds of samples in 1 day [18]. The optimal ratio of supernatants derived from a mixed lymphocyte culture and EL-4 culture was 33 : 25%. EL-4 derived lymphokines alone had the ability to support an *in vitro* immunization 2–3 times better than mixed lymphocyte culture derived lymphokines [5]. This system was then used to produce monoclonal antibodies against a variety of thymus dependent antigens including autologous antigens such as purified mouse albumin, mouse haemoglobin and mouse serum proteins. This clearly demonstrated one of the most important features with *in vitro* immunization; i.e. monoclonal antibodies could be produced against self-antigens and other weak immunogens against which it is normally impossible to evoke an immune response *in vivo*.

A further improvement in increasing the number of hybridomas producing antigen-specific monoclonal antibodies was the addition of immune potentiators like bacterial peptidoglycan derivatives [19]. The addition of the adjuvant peptide *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MDP) significantly increased the number of plaque-forming cells obtained after an *in vitro* immunization (Table 1). A simple *in vitro* immunization system based on the sole addition of MDP was also reported to give a significantly higher number of glutamic acid decarboxylase-specific monoclonal antibodies compared with

Table 1
Effect of the adjuvant peptide *N*-acetylmuramyl-L-alanyl-D-isoglutamine on a murine *in vitro* immunization system, supported by lymphokines derived from mixed lymphocyte cultures and EL-4 thymoma cells [5]. The immunogens were 1 μg/ml of haemocyanin (KLH) or ovalbumin with an immunization period of 5 days. The number of plaque-forming cells was measured using the filter immuno-plaque assay [18]

	Number of plaque-forming cells/10^6 cells	
Concentration of MDP (μg/ml)	Ovalbumin	Hemocyanin
0	90	400
1	950	1250
10	1750	1490
50	2050	2120

those produced by *in vivo* immunization using the same antigen [20]. It seems, however, that this system only produced monoclonal antibodies of the μ isotype [21]; this was due most probably to the fact that no T cell derived lymphokines were present during the immunization step. The effect of adjuvant peptides is of general interest in these systems since they seem to enhance the *in vitro* immune response without simultaneous enhancement of mitogenicity in the cultured lymphocytes [22]. The desirable effect of MDP on murine splenocytes could not be found to the same degree in a human *in vitro* immunization system using peripheral blood lymphocytes (unpublished observations).

Recently another potentiator was reported to increase the yield of hybridomas producing monoclonal antibodies to baculovirus [23]. The addition of dextran sulphate increased the yield of specific hybridomas by approximately 20%, which was significantly lower compared with the effect of muramyl dipeptide. The general applicability of dextran sulphate for *in vitro* immunizations remains, however, to be seen.

A schematic presentation of the *in vitro* immunization system based on the presence of the above described T cell derived lymphokines and the biological response modifier, muramyl dipeptide, is outlined in Fig. 1. Use of this system results in a high probability of

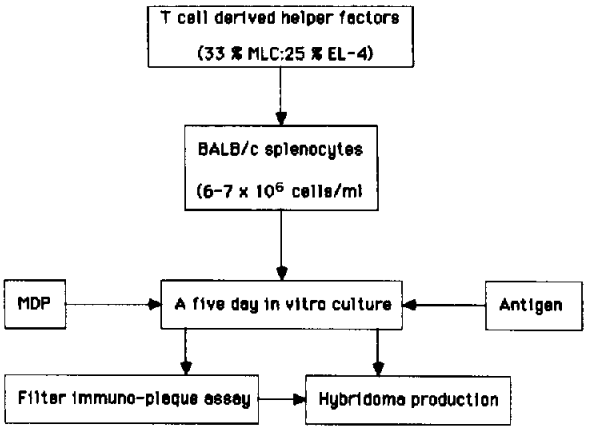

Figure 1
A schematic presentation of an *in vitro* immunization system for mouse splenocytes, as described in the text. MLC, mixed lymphocyte culture; MDP, muramyl dipeptide.

producing significant amounts of murine hybridomas that secrete antigen-specific monoclonal antibodies of the desired isotype (IgM or IgG, in a ratio of 10 : 2–3), against epitopes not able to elicit an *in vivo* immune response. An example of a typical composition of the *in vitro* immunization system is shown in Table 2.

Table 2
A typical composition of a murine *in vitro* immunization system. The final volume of this *in vitro* immunization culture is 30 ml with a final serum concentration of approximately 2%. For complete experimental details see ref. [5]

Medium/Cells	Antigen	Final concentration	Volume added
s-MLC	—	33%	10.0 ml
s-EL-4	—	25%	7.5 ml
s-DMEM	—	—	8.7 ml
50 mM 2-ME	—	50 μM	30 μl
*Splenocytes	—	6–7 × 10^6 ml	3.5 ml
—	100 μg/ml	1 μg/ml	300 μl

Abbreviations: s-MLC, supernatant from a mixed lymphocyte culture; s-EL-4, supernatant from phorbol ester-stimulated EL-4 thymoma cells; s-DMEM, DMEM medium supplemented with antibiotics, non-essential amino acids, L-glutamine; 2-ME, 2-mercaptoethanol.

*The concentration of splenocytes was adjusted to 50–60 × 10^6 cells/ml in s-DMEM containing 10% rabbit serum.

Quality of Antibodies Produced by *in vitro* Immunization

Monoclonal antibodies are often characterized in regard to parameters such as isotype, specificity and affinity constant. At present monoclonal antibodies have been produced against approximately 40 different protein antigens, haptens and cells using the *in vitro* immunization technique. Table 3 summarizes all the monoclonal antibodies derived from *in vitro* immunizations that to this date (December 1986) could be retrieved from literature data bases.

The majority of monoclonal antibodies produced from *in vitro* immunizations have the IgM isotype. The ratio of γ to μ isotype was, however, 1 : 3; this ratio is high and ensures in most cases the possibility of producing IgG antibodies from *in vitro* immunizations [2]. Only one report compared the specificity of monoclonal antibodies produced from *in vivo* and *in vitro* immunized splenocytes [37]. The cross-reactivity of different anti-digoxin antibodies was compared and it was concluded that antibodies derived from *in vitro* immunization were similar in cross-reactivity pattern to the monoclonal antibodies derived from *in vivo* immunizations. The experience of the authors of the present paper supports this fact i.e. individual monoclonal antibodies derived from *in vitro* immunizations do not exhibit a broader pattern of cross-reactivity as compared with antibodies derived from *in vivo* immunizations. *In vitro* immunizations can, however, give rise to specificities that cannot be obtained by *in vivo* immunizations (Dahmus and Borrebaeck, manuscript in preparation). This is for example, attributed to the fact that subunits in a multiprotomeric molecule that are immunodominant *in vivo* do not have this property *in vitro*. This means that monoclonal antibodies obtained from *in vitro* immunizations might exhibit a broader repertoire of specificities compared with antibodies derived from *in vivo* immunizations.

Table 3
A summary of all antigens that have been used in *in vitro* immunizations for the production of mouse monoclonal antibodies

Antigens	Reference
Protein/peptides	
Osteoclast activating factor	3
Hypothalamic growth hormone-releasing factor	24
Pig insulin	9
Human myoglobin	9
Bovine testes calmodulin	25
Vasoactive polypeptide	26
Fibroblast growth regulator	27
Bovine glutamic acid decarboxylase	20
Sperm whale myoglobin	4
Nonhistone chromosomal protein (NHCP/sc-1)	28
Vimentin	28
Hen oviduct progesterone receptor	29
Calmodulin dependent phosphatase	30
Rabbit poxvirus RNA polymerase	31
Dopamin β-monooxygenase	32
Bovine insulin	33
Porcine ACTH	33
Mouse albumin	5
Mouse hemoglobin	5
Rabbit actin	5
Calf thymus RNA polymerase	5*
Human thyroglobulin	34
Thyroglobulin synthetic peptide (1–19)	35
Bovine histones	36
Glucose transporter	36
Keyhole limpet hemocyanin	36
Haptens	
Benzo(a)pyrene	9
Digoxin	37
DNP	†
Glycolipids/carbohydrates	
Yeast mannan	38
Sulfatoxygalactosylglycerolipid	39
Asialo-GM1	‡
Bacterial/viral/parasite antigens	
Streptococcal group A polysaccharide	40
Rabies virus	41
Toxoplasma gondii	41
Baculovirus	23
Cells	
Sheep red blood cells	42
RAW117 lymphosarcoma	8
AML cells	43
ALL cells	44
Pseudomonas fragi AT182	4

*M. Dahmus and C. A. K. Borrebaeck, manuscript in preparation.
†M. Wallèn, B. Mattiasson and C. A. K. Borrebaeck, unpublished data.
‡T. Kalland, Z. Hossein and C. A. K. Borrebaeck, unpublished data.

At present no comparison has been made between the affinity constants that can be obtained from antibodies derived from *in vivo* and *in vitro* immunizations. The affinity maturation that takes place *in vivo* during an immunization process is due to antigenic selection of high affinity B cells and to somatic mutation. *In vitro*, however, only antigenic selection can contribute in obtaining high affinity antibodies. The immunization time is normally 4–6 days which is too short for somatic mutation to contribute significantly to the yield of high affinity antibodies. Preliminary experiments using low doses of DNP-KLH indicate that it was possible to obtain similar affinity constants from antibodies derived from *in vitro* immunization compared with those from antibodies derived from *in vivo* immunizations (Wallen, Mattiasson and Borrebaeck, unpublished data). The range of affinity constants obtainable in antibodies derived from *in vitro* immunizations still remains to be determined.

Advantages of Murine *in vitro* Immunization

The major advantages of murine *in vitro* immunization, compared with *in vivo* immunization can be summarized as follows. First, the immunization process takes 5 days *in vitro*, instead of usually several weeks *in vivo*. Second, the normal cellular control of the immune response to self-antigens or conserved structures does not function *in vitro* [5]. This results in elicitation of strong responses against weak immunogens against which it is normally impossible to evoke an immune response *in vivo*. Third, extremely low amounts of immunogen are needed. Normally less than 1 μg of the antigen is sufficient and there are several cases where an amount of only one or a few ng has been used.

Technical Aspects of *in vitro* Immunization of Human B Cells

Sheep red blood cells were the first immunogens to which human monoclonal antibodies were produced using *in vitro* immunization of tonsillar lymphocytes [45]. Although red blood cells have been useful for analysis of the B cell response they are not a representative immunogen. Studies with mouse B cells have shown that antibody production against soluble protein antigens involved carrier-specific and MHC-restricted T helper cells, which were not required for an antigen-specific activation of B cells against red blood cells [46]. *In vitro* immunization systems with sheep red blood cells as immunogen could only be used for the production of monoclonal antibodies against red blood cell-bound haptens and not soluble protein antigens [47]. The *in vitro* immune response against sheep red blood cells was also argued recently not to be a true primary antigenic stimulation [48]. Nevertheless, the simple *in vitro* immunization system described by Strike *et al.* [45] has been employed to produce human monoclonal antibodies against human prostatic acid phosphatase using an allogeneic culture of spleen cells [48]. The system was based only on the presence of PWM (1 : 10 000) and antigen (100 ng–2 μg ml^{-1}) during an incubation period of 5–8 days [48]. Other parameters found to affect an *in vitro* immunization of human B cells were recently reported to include IL-2, serum source, antigen concentration and removal of $OKT8^+$ T cells [49]. At this early stage in development, human *in vitro* immunization experiments have, however, generated several contradictory findings. Some investigators found human serum to be necessary whereas other systems do not function without foetal calf serum [45, 47, 49, 50]; the removal of $CD8^+$ T suppressor cells have been reported to be

essential [49] whereas other investigators have found no effect when this cell population was removed [50].

An *in vitro* immunization system that has been reported to support an antigen-specific activation of human B cells against a peptide was, however, developed by Ho *et al.* [50]. In this case a human monoclonal antibody from a mouse × human hybridoma was produced using nylon-separated human spleen cells and culture supernatant from lectin (PHA) stimulated lymphocytes. The antigen used was bombesin, a tetradecapeptide (molecular weight 1592), conjugated to tetanus toxoid. *In vitro* immunized splenocytes were fused with a mouse myeloma (NS-1) after a 6-day cultivation period. Approximately 1% of the wells produced human monoclonal antibodies against bombesin and 60% of the wells produced antibodies against tetanus toxoid. The isotype of the anti-bombesin antibodies were all IgM whereas antibodies against tetanus toxoid were of both IgG and IgM isotype. Success in the production of anti-bombesin antibodies may stem from the fact that most organ donors are *in vivo* sensitized to tetanus toxoid. Their spleen cells therefore contain *in vivo* sensitized carrier-specific T helper cells which provides necessary antigen-specific signals during the *in vitro* immunization. A large amount of IgG antibodies specific to tetanus toxoid was also isolated, which supports the fact that donors were *in vivo* primed to this antigen. Recently, the same *in vitro* immunization system was reported to support an antigen-specific activation of human spleen cells against two human breast tumour lines [51], without any involvement of tetanus toxoid. Several IgM antibodies reacting with the tumour cell lines were isolated.

None of these *in vitro* immunization systems [48–51] could support the specific activation of human peripheral lymphocytes. Human peripheral blood lymphocytes are, however, the most easily accessible cells to be used for *in vitro* immunization purposes. An *in vitro* immunization system that supports the antigen-specific activation of human blood lymphocytes against T cell dependent antigens is therefore desirable. Recently, haemocyanin was used as antigen to develop such a system [52]. To obtain a specific response *in vitro* the peripheral lymphocytes had to be separated into B, T, accessory and dendritic cells. These cells were activated separately and reconstituted to give a population with a B : T ratio of 1 : 2. If induction was supported by MHC-restricted, radioresistant T cells this cell population could be antigen-specifically activated. The immunization had also to be supported by cytokines (BCGF, BCDF, IL–2, γ-IFN) [52].

The effect of large granular lymphocytes was subsequently found to be crucial for *in vitro* immunization of unseparated human peripheral lymphocytes [53]. This cell population contains natural suppressor and killer cells [54] that have a strong negative influence on the number of inducable antigen-specific plaque-forming cells that can be detected after *in vitro* immunization. Removal of these cells has yielded an *in vitro* immunization procedure that the present authors have used to produce efficiently human × human or mouse × human hybridomas secreting specific antibodies against haemocyanin, digoxin, and PB1 (a recombinant gp120 fragment of HIV) [53].

Advantages of Human *in vitro* Immunization and Human Monoclonal Antibodies

Human *in vitro* immunization and human monoclonal antibodies [2] have several advantages.

Very few antigens can be used for *in vivo* immmunization of patients or volunteers; the advent of *in vitro* immunization is therefore crucial for universal applicability of the human hybridoma technology.

In most species, a vigorous immune response has been observed after *in vivo* administration of xenogeneic monoclonal antibodies. The immune response against therapeutic doses of human monoclonal antibodies is expected to be considerably lower.

Immunization *in vitro* of human lymphocytes against alloantigens (e.g. human tumour cells) will give rise to a different repertoire of antibody specificities compared with immunization across a xenogeneic barrier (i.e. immunization of mice with human tumour cells). This increases the possibility of obtaining a human monoclonal antibody to a tumour-associated antigen instead of predominantly to blood group antigens and the strong transplantation antigens.

Finally the carbohydrate sequence of human monoclonal antibodies is most probably more compatible with human Fc receptor-bearing effector cells (e.g. monocytes, K cells) than that of mouse monoclonal antibodies. This implies that human monoclonal antibodies may have a higher therapeutic value since a tumouricidal effect is a net effect of several functional mechanisms.

Future Developments

The research in this area of immunotechnology will be directed to the optimization and further development of human *in vitro* immunization systems. There are, however, other areas that will contribute to the accessibility of in particular human monoclonal antibodies. Areas of interest for human hybridomas include molecular genetics of immunoglobulin genes, DNA-transfection and electrofusion.

Because of the difficulties in producing human monoclonal antibodies, mouse variable regions have been joined to human constant regions and expressed in mouse myelomas [55, 56]. The resulting chimeric antibodies were synthesized, assembled and secreted by the myeloma cells and retained their antigen-binding and effector functions. The course of genetically manipulated antibodies took a step further by the work of Jones and coworkers [57]. They "humanised" mouse monoclonal antibodies by transplanting only the complementary determining regions (CDR1–3) of the mouse antibody into the framework region of a human antibody. Thus a human monoclonal antibody bearing mouse CDR1–3 was produced. Several questions have to be answered before the universal applicability of this approach can be evaluated. How important are the specific interactions between CDRs and framework determinants that are lost during transplantation? What knowledge is there on the immmunogenicity and pharmacokinetics of such mouse–human chimeric antibodies?; How easy will this technique be to perform routinely? It is clear, however, that recombinant-DNA technology will enable isotypes to be changed and affinities to be increased by site-directed mutagenesis of monoclonal antibodies. It follows that human monoclonal antibodies produced by *in vitro* immunizations can be engineered for a certain application and less concern has to be taken about the initial isotype and the affinity of the antibody.

At present, the majority of human fusion partners do not express a myeloma phenotype, but are rather lymphoblastoid cell lines; this is one of the reasons why human hydridomas normally produce significantly lower amounts of monoclonal antibodies compared with mouse hybridomas. To circumvent the apparent lack of human fusion partners, comparable to the existing mouse plasmacytomas, DNA transfection may be used to immortalize human *in vitro* immunized B lymphocytes. Jonak and coworkers [58] reported the transfer of DNA from the human leukaemia cell line Reh. Mouse splenocytes were transformed by an amplified c-myc gene transferred from the Reh cells.

To become fully transformed the transfected cell line might, however, require incorporation of an additional oncogene. The oncogenes can be transferred into the B cells by calcium precipitation, protoplast fusion, electroporation or by oncogene sequences in retroviral vectors.

Finally, to increase the frequency of conventional hybrid formation, electrofusion has shown some promise since the fusion frequency was estimated to be at least one order of magnitude better than that of polyethylene-induced cell fusions. *In vitro* immunization of human B cells normally takes 6–7 days. During this time each proliferating B cell undergoes approximately 7–8 cell divisions and develops into clones of roughly 250 cells, assuming a generation time of 20 h. Depending on the clonotype frequency of B cells specific for a certain antigen, an estimation of the theoretical attainable number of antigen-specific B cells might be about 10^5, after a 6–7 day culture of 10^8 cells. Therefore, an attractive approach to combat difficulties with low progenitor frequencies involves antigenic selection of the specific B cells and utilization of high frequency electrofusion techniques [59, 60].

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