***In Vitro* Immunization of Mouse Spleen Cells and the Production of Monoclonal Antibodies ***

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The production of antibodies and lymphokines by immortalization of these functions in lymphoid cells can be achieved by somatic cell hybridization. The technology of immortalizing the ability of immune spleen cells to produce antibodies by fusing them with myeloma cells for the subsequent isolation of monoclonal antibodies is already well established. To further facilitate this technique and to introduce several advantages we have developed a new approach to the immunization procedure which allows the cells to be immunized in culture.

This *in vitro* immunization technique utilizes T cell replacing factors (TCRF) to support the antigen specific immunization of non-immune spleen cells. TCRF can be generated by thymocytes from 10 day old mice ^{1,2} or in a mixed lymphocyte culture ^{3,4} and they apparently mimic the action of soluble factors produced by T helper cells during an *in vivo* immunization. A lymphokine preparation with the ability to support an antigen specific immunization of mouse spleen cells *in vitro* was produced by a mixed thymocyte culture. Thymocytes from 5–6 week old BALB/c and C57Bl/6 mice were co-cultivated for 48 h at a cell density of $4\text{--}5 \times 10^6$ cells/ml of supplemented Dulbeccos modification of Eagles medium (s-DMEM) (streptomycin 50 $\mu\text{g}/\text{ml}$, penicillin 50 IU/ml, L-glutamine 4 mM, $\times 100$ non-essential amino acids 1 % (v/v)) containing 16 μM thymidine, 100 μM hypoxanthine, 50 μM 2-mercaptoethanol and 2 % (v/v) Quadroma type 100 rabbit serum (Diagnostic Biochemistry Inc., CA., USA).⁴ The medium was then collected by centrifugation at $800 \times g$ for 10 min, filtered (0.22 μm) and stored in 10 ml aliquots at -70°C for up to one year. Non-immunized BALB/c splenocytes (5×10^6 cells/ml) were then immunized in a 75 cm^2 tissue culture flask using s-DMEM containing 50 μM 2-mercaptoethanol and supplemented with 50 % (v/v) of supernatant from the mixed thymocyte culture together with fil-

tered antigen of choice. After five days in culture at 37°C , using an 8 % CO_2 and 92 % air gas phase, the cells were collected and used for cell hybridization.⁴

This *in vitro* immunization technique, briefly described above, has been used to specifically produce antigen-activated spleen cells subsequently used as parental cells to obtain specific hybridomas. Monoclonal antibodies have thus been produced against protein antigens and a hapten, such as human and sperm whale myoglobin, pig insulin and benzo[a]pyrene as well as against bacterial surface antigens (*Pseudomonas fragi*) using in *in vitro* immunized BALB/c cells and Sp2/0-Ag14 myeloma cells as fusion partners. Bovine serum albumin (BSA) was used as a protein carrier for benzo[a]pyrene at a ratio of 1:8. BSA-benzo[a]-pyrene, human myoglobin and pig insulin were used at a concentration of 50–100 $\mu\text{g}/\text{ml}$ during the immunization period. The specific efficiency ((no. of culture plate wells producing specific antibodies/no. culture plate wells exhibiting cell growth) $\times 100$) of hybridization experiments using spleen cells immunized in culture by these antigens was in the range of 20–30 %, which is comparable to when cells immunized *in vivo* were used. Monoclonal antibodies expressing both γ and μ isotypes could be isolated. To evaluate the relationship between the amount of antigen used in the immunization and the specific efficiency of each fusion, concentrations from 1 ng/ml up to 10 $\mu\text{g}/\text{ml}$ of sperm whale myoglobin were tested in the *in vitro* immunization. The immune spleen cells were then immortalized and the antigen specific response was recorded after three weeks when the hybrid cells were tested. A positive dose-response relation was recorded ranging from a specific efficiency from 3 up to 15 %, depending on the antigen concentration used in the immunization step. This means that at a concentration as low as 1 ng antigen/ml of culture medium 3 % of the culture wells that are exhibiting hybrid cell growth are also producing antigen specific antibodies.

The effect of TCRF on the outcome of the *in vitro* immunization was also tested. *Pseudomonas fragi* cells were used as immunogen (the ratio of spleen cells to bacterial cell was 1:8) and the immunizations were performed in medium supplemented with TCRF or medium lacking any exogenous TCRF. These two cell populations were then used as fusion partners in hybridization experiments with myeloma cells and a total dependence on the lymphokine preparation for the production of cell hybrids that produced specific antibodies was recorded. The specific efficiency of the hybrid cells produced using

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spleen cells immunized in the absence of TCRF was 0 % whereas the presence of TCRF during the immunization procedure yielded hybrid cells exhibiting a specific efficiency of 12 %. Cells that were immunized without the support of TCRF were hence unable to produce any specific immunoglobulins in the subsequent immortalization step illustrating the important role that lymphokines play during the immunization.

In summary, immunization in culture is an important technique which offers several advantages: (1) only a few nanograms of antigen are sometimes needed; (2) defined amounts of antigen and lymphokines are present during the immunization; (3) the immunization takes five days to perform. Furthermore, the normal cellular control of the immune response seems not to function in culture which results in the possibility of producing antibodies to highly conserved antigens.

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