SESSION 5 : EMERGING TECHNIQUES

Influence of tissue microenvironments on immune responses *in vitro* with special reference to immunoglobulin and cytokine production

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During the past decade, our knowledge of the cellular and molecular events associated with key immunological responses has been greatly advanced by the use of isolated subpopulations of immunocompetent cells, cloned cell lines, and recombinant derived cytokines. Valuable as these studies have been, they do not truly reflect the complex integrative events which take place in both primary and secondary lymphoid tissue both *in vivo* and *in vitro*. In order to address this problem, we have developed a tissue explant procedure based on that previously used by others to study T cell maturation in the thymus [1]. This involves culturing uniform slices of lymphoid tissue in a sponge culture system [2]. Using this technique we have observed marked differences in both immunoglobulin and cytokine secretion between explants and suspensions of human spleen. In brief, explants (mitogen stimulated or otherwise) consistently secrete higher levels of immunoglobulin, IL-1ß, IL-6, IL-8, and IL-11 and exhibit much lower proliferation than suspensions of the same tissue. Mitogen stimulated suspensions, on the other hand, secrete higher levels of IL-2, IL-4, IL-10, and TNF(*alpha*) than do explants. These differences are also observed at the mRNA and intracellular cytokine level. Additional studies reveal that the immunoglobulin and cytokine secretion observed is largely due to the *de novo* synthesis of these molecules and not as a result of spontaneous secretion of preformed products. Furthermore, immunoglobulin secretion in both explants and suspensions can be inhibited by the addition of specific antibodies to IL-1ß, IL-6, and TNF(*alpha*). Preliminary studies indicate that close interaction between B cells and stromal cells within explants accounts for some of the observed differences. In this overview lecture, the basic technique will be described, the differences noted in the performance of explants summarized and their possible basis and relevance discussed.

References

1. Jenkinson EJ, Anderson G. *Current Opin Immunol* 1994; **6:** 293 - 297
2. Hoffmann P, Skibinski G, James K. *J Immunol Meth* 1995; **179:** 37 - 49

Comparison of human hybridoma production after EBV transformation or pokeweed mitogen stimulation of PBLs

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The generation of human hybridomas using B lymphocytes and the K6H6/B5 heteromyeloma (mouse x human) requires that the lymphocytes be in active division. Because most lymphocytes in the normal B-cell repertoire are in a resting state, polyclonal B-cell activators (PBA) are generally used before fusion. The two PDAs most commonly used are EBV and pokeweed mitogen (PWM). The infection of lymphocytes with EBV results in blast formation, Ig secretion and, ultimately, immortalization of the cells. PWM induces blastogenesis resulting in the increase of mitotically active B lymphocytes. The objective of this study was to compare the efficacy of the fusion of PBLs after either EBV transformation or stimulation with PWM. PBLs from 3 different patients immunized with a cancer vaccine were separated by gradient centrifugation, and the resultant lymphocytes were treated with EBV or PWM. EBV transformation was accomplished by exposing the lymphocytes to the supernatant of the B95-8 marmoset lymphoblastoid cell line. After overnight incubation, cells were washed and kept in culture until fully transformed. PWM stimulation was achieved by incubation of lymphocytes in the presence of PWM (10 µg/ml) for 48 h. EBV transformed or PWM stimulated PBL were fused with K6H6/B5 cells using poly(ethylene glycol) (1500) at a 1:1 ratio. The cells were then placed at 5 x 10^4 cells per well in RPMI-1640 media (+15% FBS, 1% hybridoma cloning factor, ORIGEN®) followed by HAT/ouabain selection. Hybridomas producing antibodies reactive with the specific antigen (radioimmunoassay) were cloned by limiting dilution. Fusions after EBV transformation showed growth in 99-100% of the plated wells (hybrid frequency of 400/10^7 lymphocytes), with 90-100% of the hybrids producing Igs, and these were mainly IgM (%IgG/%IgM: 25.3/99, 0.7/100, 0/99). The cloning efficiency was 78-96%, with 16-65% of the clones remaining Ig producers. Fusions after PWM stimulation exhibited growth in 67-97% of the plated wells (hybrid frequency of 256-388/10^7 lymphocytes) with 71-80% of the hybrids producing Igs from both classes (% IgG/%IgM: 50/38, 64/55, 87/72). The cloning efficiency was 64-74% with 8-50% of the clones remaining Ig producers. Although it is possible to generate stable hybridomas from both EBV and PWM stimulatory systems, the EBV transformed lymphocytes gave higher hybrid frequency and better cloning efficiency with a larger number of hybrids remaining Ig producers as compared to the PWM stimulated lymphocytes.

Synthesis of immunotoxins by Chinese hamster ovary cells

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For more than 15 years immunotoxins have been used as possible therapeutic agents for the treatment of cancer, AIDs and autoimmune diseases because of their highly selective action. Especially during the last 5 years better insight has been gained into the basic aspects of the immunotherapy resulting in a lot of chemically coupled immunotoxin conjugates which are currently being tested in clinical phase 1 and 2 trials (Vitetta *et al. TIBS* 1993; **14:** 148; Ghetie *et al. Current Opin. Immunol.* 1994; **6:** 707). Since tens of milligrammes are needed for the treatment of patients, the problem of efficient production remains. The objective of our work is to produce large amounts of uniform, correctly assembled immunotoxins, which can only be obtained by a genetic coupling between the antibody and the toxin. Monovalent immunotoxins, consisting of scFv fragments linked to the toxin part, can be made in *E. coli* because this organism is naturally resistant to toxins of bacterial origin and some plant toxins. However, because of its low secretion capacity, the overexpressed immunotoxins are often found in insoluble inclusion bodies in the cytoplasm. Our purpose is to develop a model system in which bivalent immunotoxins directed against the human marker placental alkaline phosphatase (hPLAP) can be produced very efficiently. It has been suggested that the internalization of a bivalent immunotoxin is much more efficient than that of a monovalent immunotoxin (Ledbetter *et al. J Immunol* 1986; **136:** 3945). This led us to focus on the development of bivalent immunotoxins in mammalian cells. Up to now, immunotoxins have never been produced in mammalian cells because of the sensitivity to the toxin part. For the expression of an immunotoxin consisting of the *Pseudomonas aeruginosa* exotoxin A (ETA), we have chosen the CHO RPE40 cell line as toxin partner which is resistant to the ETA toxin. Due to a furin mutation this cell line cannot cleave the ETA toxin necessary for an active toxin formation. However, we found that constitutive expression of the ETA immunotoxin is highly toxic and expression could only be obtained with an inducible expression system which makes use of a tetracyclin controlled hybrid transactivator. Secretion of the product was extremely low and most of the immunotoxin remained inside the cells. In further experiments we will investigate if the cellular association of the immunotoxin is due to misfolding or sterical hindrance of the fusion complex. A second immunotoxin consisting of the pokeweed antiviral protein (PAP) coupled to the hPLAP specific antibody with a furin sensitive linker was secreted at 0.1 µg/ml but still caused toxicity for the recombinant CHO cells. In the future, we will select for mutants which can give a better secretion of the PAP immunotoxin.

Incorporation of T cell line in fusion partner to generate human MAbs from *S. aureus* stimulated B lymphocytes

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To obtain human MAbs a prefusion stimulation of human B lymphocytes is essential. This is usually carried out by EBV transformation. For clinical use of resultant HuMAbs, removal contaminant EBV DNA would be essential. Alternatively, a non-transforming mitogen-like formalinized *Staphylococcus aureus* (FSTA) can be used for stimulation of B cells. FSTA stimulation of B cells is T cell independent; however, T cell help is needed for antibody secretion. Thus T cell lines were incorporated in fusion partners to obtain HuMAbs reactive with rabies virus. Fusion of T cell lines with mouse/human heterohybrid or with hematopoietic precursor cell line has been tried. Jurkat-4 cells were fused with mouse x human heterohybrid C54D253. Hybrid cells were selected by panning on anti-CD4 MAb. A clone (IIID4) was made HAT sensitive and fused with FSTA stimulated human B lymphocytes. The fusion efficiency was very low and only one IgM secreting clone (G3) with unknown reactivity was obtained. It was decided to include precursor of both T and B cells within the same fusion partner, in order to accommodate both T and B cells. Human hematopoietic precursor cells are $CD34^+$. KG1a cell line which is $CD34^+$ was fused with HuT cells ($CD4^+$ human T cell lymphoma cell line). Hybrid cells (434 AM) were selected by panning on both anti-CD4 and anti-CD34 MAbs. HAT sensitive derivative of 434 AM cells when fused with FSTA stimulated human B lymphocytes obtained from rabies vaccinated individuals resulted in generation of 125 hybrids out of which 27 were reactive to rabies virus in ELISA. After initial growth phase, these hybrids, however, ceased to grow indicating lack of myeloma-type secretory capabilities. Further, 434 AM cells were fused with mouse myeloma SP2/0. Resultant cells named $SNA4^+$ were used to obtain HuMAbs reactive with rabies virus. Twelve hybrids secreting HuMAbs reactive with rabies virus were obtained. A clone B1G10 secreting IgG type neutralizing antibody to rabies virus has been grown as ascitic tumor in pristane primer irradiated NMRI nude mice. This HuMAb shows both *in vivo* and *in vitro* neutralization against rabies virus and is reactive with rabies virus glycoprotein in Western blot analysis.

An assay for detection of antigens in immune complexes

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A simple dot blot assay is developed for the quantitative determination of antigens as a part of their complexes with the IgG antibodies. The assay could be used in immunological and clinical laboratories without sophisticated equipment. The procedure is based on the ability of staphylococccal Proteins A and G to adsorb IgG immune complexes as well as free IgG. The Sepharose beads with immobilized Proteins A (or G) are mixed with serum or with other solutions containing IgG immune complexes. After washing, the beads are transferred to nitrocellulose membrane. The antigen immobilized on the membrane after the elution is detected by corresponding antibodies labelled with biotin or with ^{125}I. The elution performed directly on the membrane surface allows one to obtain a higher local concentration of eluted immune complexes and as a consequence a higher sensitivity of the assay. The acid elution disrupts antigen-antibody complexes and facilitates the detection of antigens. The validity of this procedure was proved by the determination of avidin, DNA and low molecular weight antigens in their complexes with the corresponding IgG antibodies. The assay was used to follow the effect of various treatments of lupus erythematosus. The values of DNA in immune complexes were in parallel with the changes in the clinical manifestations of the disease (Nezlin R, Mozes E. *J Immunol Meth* 1995; **184:** 273; Nezlin R. In Lefkovits I, *Immunological Methods Manual* 1996; Academic Press: San Diego). The assay can be applied for detection of any substance in a complex mixture after addition of corresponding polyclonal antibodies to form specific immune complexes.

Production of antigen specific human MAbs by human lymphocytes engrafted in normal strains of mice

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The use of lethal total body irradiation (TBI) followed by radioprotection with SCID bone marrow provides a general approach to achieve engraftment of human antibody producing B cells in normal strains of mice. Following transplantation of human peripheral blood lymphocytes (PBL) in such chimeric recipients, a marked engraftment of human T and B cells as well as significant amounts of human immunoglobulin could be detected. Human CD45+ cells were detected in all internal organs including the spleen. Total human Ig in mice sera reached an average of 5 mg/ml 14 days after transplantation. We have used this system to develop human MAbs to HBV as well as to human γ-interferon by immunizing these mice with hepatitis B vaccine (Engerix-B) or human γ-interferon conjugated to tetanus toxoid. A strong specific human antibody response to HNsAg was detected after engrafting human PBL from donors positive for anti-HBs antibodies following immunization with the hepatitus vaccine. The anti-HBs specific antibody population was 10^2 - 10^4 higher than in the donors, indicating induction of a strong memory immune response. Specific immune response to human γ-interferon was induced using PBL from normal donors. In this case, the enrichment of the specific antibodies in mice sera was about 10-fold higher than in the donors' sera. Splenic B cells were harvested from these mice, then fused to human/mouse heteromyeloma cells to generate hybridoma clones secreting human MAbs. We have isolated several stable clones producing different high affinity MAbs (6 - 1 x 10^{-9} M). All clones were analysed for their specificity, IgG subclass, secretion levels, and biological functions to evaluate their potential therapeutic use. Our data show that the chimeric mouse system is a powerful tool for developing fully human MAbs for cancer therapy, viral and autoimmune diseases.

Antigen-specific human monoclonal antibodies produced by mice engineered with human Ig YACs

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We generated human antibody-producing mice by introducing megabase-sized, germline configuration segments of the human heavy and kappa light chain loci, contained on yeast artificial chromosomes (YACs), into the germline of mice engineered by gene targetting to be deficient in functional mouse immunoglobulin (Ig) genes. These mouse strains produce significant levels of fully human IgM and IgG antibodies with diverse repertoire. Upon immunization, these mice can mount an antigen-specific human antibody response against different human antigens, and can be used to generate mouse hybridomas secreting antigen-specific fully human monoclonal antibodies. The utilization of these mice to generate high affinity and specificity therapeutic fully human MAbs such as antibodies against human IL-8, will be presented.