

Generation and characterization of artificial cell surface constructs for studying T cell responses to drugs

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To my family

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I. SUMMARY

The vertebrate's immune system has evolved to protect the body from infectious agents which could be harmful. This protection is characterized by two major mechanisms: specificity and memory. The immune system is able to discriminate between "self" and "non self" based on differences in protein structure and amino acid sequence. However it is evident that also non peptide antigens as sugars, lipids, metals or drugs can be recognized.

Small molecular drugs (200-300 Dalton) like sulfamethoxazole or lidocaine can cause allergies and can bind noncovalently and extracellularly to MHC complexes and thereby activate drug-specific T lymphocytes in vitro. But the circumstances needed to activate such drug-specific T cells are not yet fully elucidated.

To further investigate the activation signals and the costimulation pattern of drug-specific T cells, a new model system of artificial cell constructs, using latex microspheres, was generated. These artificial cell constructs were intended to replace natural antigen presenting cells with their numerous ligands on the surface with well defined constructs that have only primary and / or secondary signal providing ligands on the surface.

Monoclonal antibodies against human MHC class II were produced with mouse hybridoma cell lines to purify human class II histocompatibility antigens from Epstein-Barr-Virus transformed human B lymphoblastoid cell lines by affinity chromatography. The purified molecules were successfully characterized by immunoblots, ELISA and flow cytometry analysis. MHC molecules were coated to latex beads alone or together with costimulation signal providing monoclonal antibodies and characterized by flow cytometry. Artificial cell constructs coated with a strong primary signal-providing monoclonal antibodies (anti-CD3) were generated and characterized to determine the functionality of the new model system with drug-specific T cells. Although the characterization of the MHC molecules was successfully done, latex beads displaying MHC molecules and costimulatory monoclonal antibody on their surface were, in the presence of the respective soluble drug antigen, not stimulatory for SMX or lidocaine-specific T cell clones. Still, latex microspheres coated with strong primary signal-providing monoclonal antibodies could stimulate T cell proliferation even in the absence of costimulatory factors. In conclusion, the use of the artificial "APC" in the antigen-specific stimulation of drug-specific T cells, however, awaits further experimentation.

ABBREVIATIONS

Ab	Antibody
APC	Antigen presenting cell
B-LCL	B lymphoblastoid cell line
BSA	Bovine serum albumin
CD	Cluster of determination
DOC	Deoxicorticosterone
cpm	Counts per minute
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
HBSS	Hank's balanced salt solution
HLA	Human leukocyte associated antigen
h	Hour(s)
IgG	Immunoglobuline G
IL	Interleukin
kD	Kilo Dalton
mAb	Monoclonal antibody
M	Molar
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
min	Minute(s)
NaAc	Sodium acetate
OD	Optical density
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline solution
rpm	Rounds per minute
RT	Room temperature
sec	Second(s)
SDS	Sodium dodecylsulfate

SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
SMX	Sulfamethoxazole
TCC	T cell clone
TCR	T cell receptor
Tris	Tris-(hydroxymethyl)-aminomethane

II. Introduction

1. Role of T cells in drug-induced allergies

T cells are a subset of lymphocytes defined by their development in the thymus and by heterodimeric receptors associated with the proteins of the CD3 complex. T cells recognize peptide antigens displayed on MHC molecules on antigen presenting cells (APC). Most T lymphocytes can either be grouped into CD8⁺ T cells that recognize endogenous peptide antigens presented on major histocompatibility complex (MHC) class I molecules or into CD4⁺

T cells that recognize exogenous peptide antigens on MHC class II molecules. MHC class II-restricted T cells express the coligand CD4 and MHC class I-restricted T cells the coligand CD8, which interact with the respective MHC molecules.

Nevertheless, studies showed that non-peptide antigens such as metal ions (Sinigaglia et al., 1995), lipids (Burk et al., 1995, Morita et al., 1995), or drugs (Brander et al., 1995, Mauri-Hellweg et al., 1995, Padovan et al., 1996, Zanni et al., 1997) can be presented by MHC or MHC-like molecules to T cells. There is ample evidence, that drug-specific T cells play a role in drug-induced allergies to β -lactam antibiotics, sulfamethoxazole, local anesthetics and carbamazepines (structures see Figure 1).

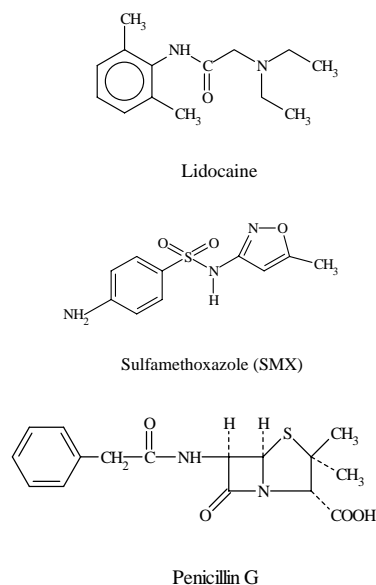


Figure 1. Chemical structures of drugs that are known to cause drug allergies.

As far as analyzed, most drug-specific T cell clones (TCC) obtained from allergic individuals are MHC-restricted CD4⁺ or CD8⁺, $\alpha\beta$ ⁺ T cells, although two $\gamma\delta$ ⁺ lidocaine-specific clones were described as well (Brander et al., 1995, Hertl et al., 1995, Mauri-Hellweg et al., 1995, Padovan et al., 1996, Zanni et al., 1997). Antigen recognition and costimulatory signals activate naive T cells to undergo clonal expansion and differentiation into effector and memory T cells. Antigen stimulation of effector

T cells leads to further clonal expansion and performance of effector functions such as cytokine secretion and cytolytic activity. In our hands, drug-specific T cells mainly produce a Th2/Th0-like cytokine pattern (IFN- γ , IL-4, and high levels of IL-5). Additionally, CD4 and CD8 positive drug-specific cytotoxic T cells could be isolated from several patients (von Greyerz et al., 1999).

Haptens are small (200-300 Dalton) molecules that can bind antibodies but cannot by themselves trigger adaptive immune response. Until a few years ago, T cells were thought to recognize haptens (like drugs or other chemicals) only after chemical coupling to a foreign carrier protein (Landsteiner et al., 1936). Hapten-carrier complexes would then be taken up, processed and presented by MHC class I or II molecules to T cells (for review see Martin and Weltzien, 1994). In drug allergy, a large body of experimental

evidence has led in the last years to several different models of antigen presentation by APC, depending on the chemical structure of the drug. Depending on the chemical reactivity of the allergy-inducing drugs, today different models of drug presentation by APC have been established (see Figure 2).

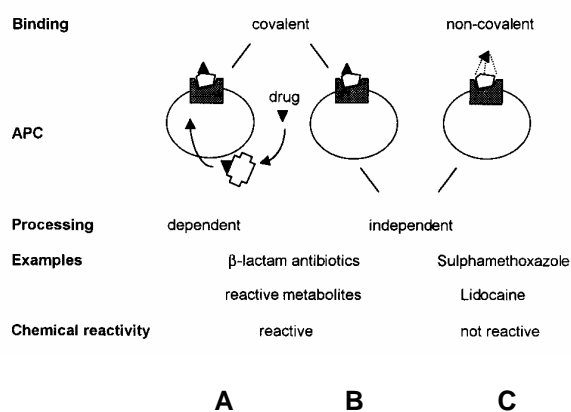


Figure 2. Pathways of drug presentation by APC.

The figure outlines the three until now postulated pathways of drug presentation to specific T cells.

(A) Chemically reactive drugs like β -lactam antibiotics or reactive metabolites of per se non-reactive compounds can covalently modify side chains of amino acids of serum or cellular proteins. After uptake and processing by APC, haptenated peptides can be presented as modified self-structures on MHC class I or class II molecules to specific T cells.

(B) Chemically reactive drugs also have the ability to covalently modify, in a processing-independent pathway, directly from outside MHC/peptide complexes on the surface of APC.

(C) Chemically non-reactive drugs like lidocaine or SMX are presented to specific T cells in a processing-independent way by undergoing an only labile association with preformed MHC/peptide complexes. In consequences, they form unstable trimolecular MHC/peptide/drug complexes. (from v. Greyerz et al., 1999)

β -lactam antibiotics such as penicillins or cephalosporins represent per se reactive drugs; their core structure, the β -lactam ring, opens spontaneously under physiological conditions. The formed penicilloyl can covalently bind to lysine residues of proteins (Schneider et al, 1966). Modification of soluble (i.e., penicilloyl-modified human serum albumin) or membrane-bound proteins leads to presentation of β -lactam-modified peptides to T cells (processing-dependent pathway). Alternatively, β -lactams appear to be able to bind directly to peptides within MHC molecules (processing-independent pathway) (Brander et al., 1995, Padovan et al., 1996).

However, most drugs are not chemically reactive per se. It was postulated that they gain chemical reactivity upon drug metabolism (Rieder et al., 1989). But it was showed recently that fixed APC were still capable of presenting non-

reactive drugs like sulfamethoxazole or lidocaine to drug-specific TCC. Strikingly, APC pretreated with drugs and washed were not stimulatory, suggesting a rather labile drug interaction with the MHC-peptide complex (Schnyder et al., 1997). This indicates that non-reactive drugs can be presented in a processing-independent and probably non-covalent way to activated T cells (Schnyder et al., 1997, Zanni et al., 1998).

Nevertheless, the molecular mechanisms of drug presentation by APC are not yet fully understood. We therefore intended to establish a new model system, in which APC were replaced by artificial cell constructs with defined molecules on their surface. Their inability to metabolize and process antigenic drugs might help to further elucidate the metabolism-independent way of drug presentation.

2. Components of T cell activation

Antigen-specific activation of T lymphocytes mainly results from interactions of the TCR with MHC-peptide complexes presented on the surfaces of APC. However, it has become clear that in many situations this antigen-specific signal must be augmented by antigen-non-specific

costimulation (Mueller et al., 1989, Lafferty et al., 1983). These costimulatory, or second signals may be provided by soluble factors (Raulet et al., 1982) as well as cell surface molecules (Janeway et al., 1994) that bind to costimulatory receptors on the T cell (see Figure 3).

Figure 3. Adhesion molecules and signal transducing molecules that can mediate interactions between activated CD4⁺ T cells (T) and activated B cells (B). Arrows show the receptors that can interact with each other (from Clark et al., 1994).

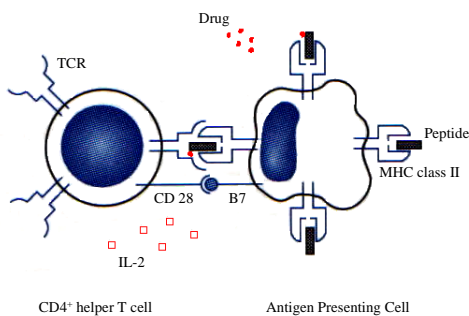
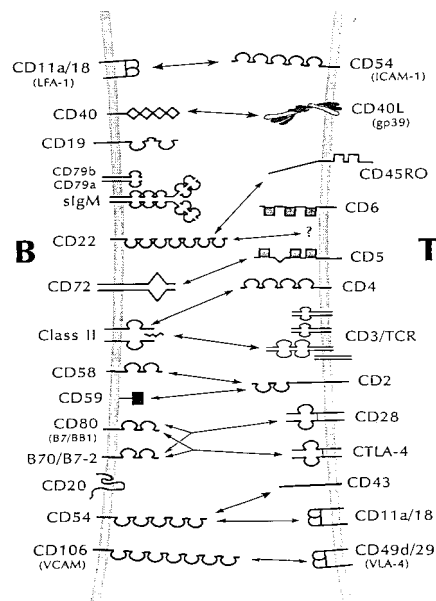


Figure 4. Model of an APC interacting with a T cell. The APC provides a first stimulation signal with the drug bound to MHC-peptide complex. Second signals are provided by B7-CD28 interaction and additionally by the soluble, autocrine T cell derived growth factor IL-2. (Adapted from Cellular and Molecular Immunology, Abbas et al., 1997).

Based on the broad relevance of the two-signal model in describing lymphocyte activation, it is not surprising that numerous molecules have been identified that can provide costimulation required for T cell activation. Of these molecules the B7 family members, B7-1 and B7-2 (CD80 and CD86 respectively), have been shown to be of central importance (June

et al., 1994). The B7 molecules, expressed on activated APC, have identified as the ligands for the costimulatory receptor CD28 (June et al., 1994, Linsley et al., 1994). Additionally, adhesion molecules provide a tight cell-cell contact leading to sufficient TCR triggering by the respective antigen (Figure 4).

Although the two-signal model is the base of T cell activation, signal one and two may be of different qualities and quantities and thus the response of the T cell may vary considerably. A weak primary activation signal, a defect or a weak costimulatory signal may eliminate a T cell from the pool of antigen-responsive lymphocytes either by promoting its death or by inducing a state of unresponsiveness called anergy (Schwarz, 1996).

Costimulatory signals play a crucial role in TCC activation but there are still open questions in the context of TCC activation with drug antigen like SMX or lidocaine. These drugs are known to bind very weakly to MHC molecules as they can be removed by simple washing processes. The unstable drug/peptide MHC complex is known to provide signal one. It is not yet clear, which costimulatory signals and adhesion molecules are indispensable for

enhancing a low-affinity drug/peptide MHC interaction sufficiently to activate a T cell via its TCR. B-LCL generally used as APC for the stimulation of drug-specific T cells express large amounts of B7 molecules as well as other costimulatory and adhesion molecules. Would MHC/drug complexes alone provide enough stimulus for T cell clones, which rely less on costimulation than naive T cells (Lenschow et al., 1996)? Could poor or absent costimulation lead to T cell unresponsiveness irrespective of the strength of signal one (Bachmann et al., 1999)?

In order to address these questions, we aimed at generating artificial cell constructs providing signal one (MHC molecule and antigen) to which a defined costimulatory signal two in form of an anti-CD28 mAb could be titrated. (Lic. Equivalent)

3. Major histocompatibility complex molecules

The major histocompatibility complex is a cluster of genes on human chromosome 6 (Campbell et al., 1993), that encodes the MHC molecules. The MHC is structured in several genetic loci, and contains in humans amongst others HLA-A, HLA-B, HLA-C (class I) and HLA-DR, HLA-DP, HLA-DQ (class II) and more. HLA stands for human leukocyte-associated antigen, since these proteins were first observed on leukocytes. The two chains of class II molecules are encoded by different MHC genes, and, with a few exceptions, both class II chains are polymorphic. MHC class I molecules

present peptides generated in the cytosol to CD8⁺ T cells, and MHC class II molecules present peptides degraded in cellular vesicles to CD4⁺ T cells. In general, class I molecules are present on virtually all nucleated cells, whereas MHC class II molecules are mainly found on dendritic cells, B lymphocytes and macrophages that are often called professional APC. Professional APC have the potent ability to process and load endocytosed antigens onto MHC class II molecules. They express around 200,000 MHC class II molecules per cell on their surface (Valitutti et al., 1995).

All class I molecules are composed of a MHC-encoded α chain of about 44 kD and a non-MHC-encoded non-polymorphic chain of 12 kD called β_2 microglobulin (which is encoded by a gene on chromosome 15). All MHC class II molecules are composed of two non-covalently associated polypeptide chains and are $\alpha\beta$ heterodimers (Figure 5). In general, the two class II chains are similar to each other in overall structure (Brown et al., 1993, Engelhard et al., 1994). In class II molecules, both polypeptide chains contain N-linked oligosaccharide groups (the α -chain has one high-mannose and one complex carbohydrate moiety, whereas the β -chain has one complex carbohydrate). The α chain (32 to 34 kD) is slightly heavier than the β chain (29 to 32 kD) as a result of more extensive glycosylation.

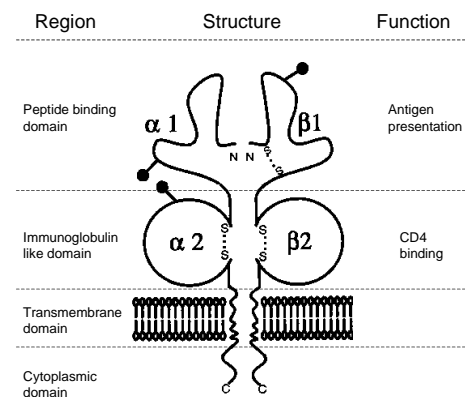


Figure 5. Schematic description of the $\alpha\beta$ heterodimeric MHC class II molecule.

N and C refer to the amino and carboxy-termini of the polypeptide chains, respectively; S-S, to intrachain disulfide bonds, and \cdot to carbohydrate. (Adapted from Einführung in das HLA-System, Wassmuth 1995, Cellular and Molecular Immunology, Abbas et al., 1997)

MHC class I and class II molecules each have a single peptide-binding site. This site consists of a deep groove between the α_1 and α_2 domains on class I and between α_1 and β_1 domain on class II. The groove can bind peptides with a length of about 10 amino acid residues on class I molecules and of 15 to 24 amino acids on class II molecules. The most striking feature of the MHC genes is their high level of polymorphism among individuals of the same species. Most of the polymorphic residues in MHC proteins are clustered in their antigen-binding grooves so that each polymorph binds a given antigenic fragment with a characteristic affinity. Epidemiological studies indicate that certain polymorphs of MHC genes are associated with increased or decreased susceptibilities to particular infectious and autoimmune diseases (Hill et al., 1991).

The association of antigenic peptides and MHC molecules is a saturable, low-affinity interaction ($K_d \approx 10^{-6}$ M) with a slow antigen binding rate and a very slow antigen releasing rate. Once

antigens are bound to MHC, they may stay for hours to many weeks (Lanzavecchia et al., 1992, Nelson et al., 1994). The extremely slow off rate of the MHC-peptide interaction allows peptide-MHC complexes to persist long enough to interact with T cells despite the low affinity of the interaction (Valitutti et al., 1997).

The exact molecular basis for the MHC-restricted recognition of drugs by specific TCR is not yet entirely clear. It has been demonstrated, that fixed APC were still capable of presenting non-reactive drugs like SMX or lidocaine to drug-specific TCC. Fixed APC pretreated with drugs and then washed were not stimulatory, suggesting a rather labile drug interaction with the MHC-peptide complex (Schnyder et al., 1997). However, it is still not known where such drugs, which do not form covalent hapten-carrier complexes, associate with the HLA molecules. It can be alone, with a peptide bound to MHC or even with both simultaneously.

4. From antigen presenting cells to artificial cell constructs

APC are cells that can process protein antigens and display them as peptide fragments on the cell surface together with molecules required for lymphocyte activation. The techniques of molecular biology have provided powerful tools for identifying the receptors and ligands on APC and for studying the structural basis of their contributions to lymphocyte activation. However, when the native or structurally modified gene products are expressed in a cell for functional studies, they are placed back into the complex milieu of a cell surface having numerous additional ligands that may contribute to the response.

One approach to attempt to determine the contribution of individual receptors is the use of specific antibodies crosslinking the relevant receptor (Dixon et al., 1989). In many cases this results in generation of a

transmembrane signal. Results of such experiments must be interpreted with caution, however. Antibodies typically bind with much higher affinity than the receptor binding to its native ligand and may not accurately mimic the signal generation that occurs under physiological conditions (Nunes et al, 1994). Furthermore, the potential contribution of the receptor-ligand interaction to adhesion cannot be assessed.

An excellent approach to defining contributions of an individual receptor is to study its interaction with the native ligand. This is straightforward for receptors that bind soluble ligands, like the IL-2 receptor, but is more difficult when the ligand is a membrane-bound protein that is recognized by the receptor in a potentially highly multivalent manner on a cell surface.

Table I. Properties of different supports for cell surface molecules.

Property	Medium used for the immobilization of the purified molecules			
	Latex microspheres	Liposomes	Planar membranes	Solid plastic support
Spherical shape	+	+	-	-
Uniform diameter	+	-	-	-
Usable in flow cytometry	+	-	-	-
Suitable for storage	+	-	+	+
pH stability	+	+	+	+
Recognition by T cells	++	++	+	+
Ease of manipulation	+++	-	+	+

The membrane protein must first be purified, requiring detergent to solubilize it from the membrane and maintain its solubility. In cases, where a mAb specific for the ligand is available, purification can usually be accomplished by immunoaffinity chromatography. Once purified, the protein must, in most cases, be displayed on a surface in order for effective recognition by lymphocytes to occur. A variety of means for accomplishing this have been used including incorporation into liposomes (Engelhard et al., 1978; Herrmann and Mescher, 1983), incorporation onto supported planar membranes (Nakanishi et al., 1983) and immobilization on plastic (Kane et al., 1989). Table I summarizes the properties of different

media for immobilization of purified molecules. One of the best method to imitate natural APC is the incorporation of purified membrane proteins, alone or along with other membrane or non-membrane proteins such as antibodies, onto cell-size latex microspheres for use in lymphocyte recognition and activation studies (Curtsinger et al., 1998). These artificial cell surface constructs are easily prepared and characterized, effectively present ligands to lymphocytes, and can be constructed to include multiple ligands at readily controlled surface densities (Curtsinger et al, 1997). We therefore were keen to adapt this system in order to study the requirements for activation of drug-specific T cells.

5. Aim of the study

The aim of the study was the generation and characterization of artificial cell constructs for studying T cell responses to drugs. In order to do this, a model system using immobilization of membrane and soluble molecules on latex microspheres was adapted. Thus, the following different artificial cell constructs mechanism were established. The first type of artificial cell constructs were coated with an anti-CD3 mAb and would be used to define the basic parameters of the model system such as the number of beads used in cell cultures or the optimal density of ligands on the surface. The next set of beads would consist of anti-CD28 mAb coated beads to test whether this

costimulation alone does activate TCC. There would be the possibility to purify human B7 molecules and to replace anti-CD28 mAb on the beads to get closer to physiological conditions in a further step (Deeths and Mescher, 1997). Finally, beads would be coated with HLA-DR molecules isolated from B-LCL together with or without anti-CD28 mAb as costimulatory molecules. Those beads, that lack any capacity to metabolize or process drug molecules, were pulsed with drug-antigen and cultured with drug-specific TCC. From this point on, the influences of costimulatory signals on drug-specific TCC can be elucidated in more detail.

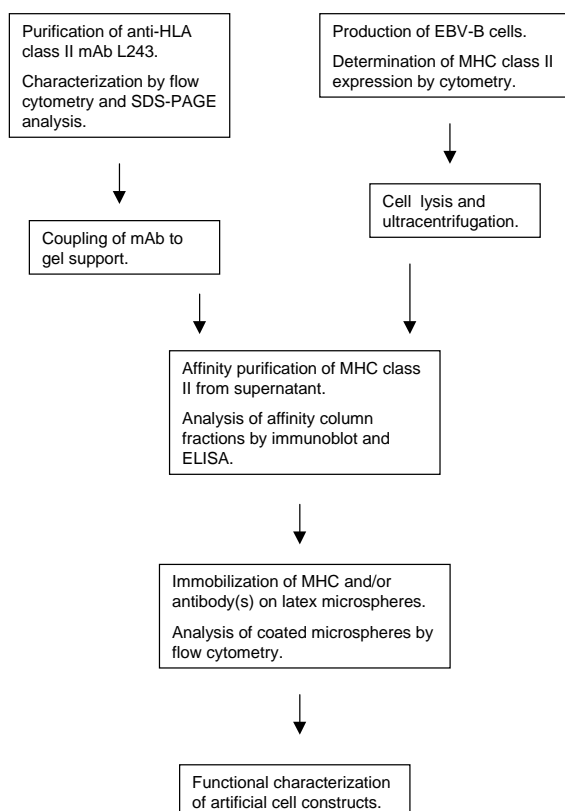


Figure 6. Flow chart of the main experimental steps.

Several methods for purifying MHC antigens by monoclonal affinity chromatography have been published. In principle, large amounts of cells were lysed with non-ionic detergent (Mescher et al., 1983, Curtsinger et al, 1998, Macklin et al, 1998) or hypotonic treatment (Weissmann et al., 1986, 1988). Then the lysate was cleared by ultracentrifugation and the solubilized membrane fraction applied to anti-MHC affinity columns. MHC molecules were either eluted by high salt (Mescher et al., 1983, Curtsinger et al, 1998) or by high pH (Gorga et al., 1987, Macklin et al, 1998). B-LCL were chosen as a

source for human HLA molecules and the well established anti-HLA-DR mAb L243 was used to prepare affinity columns. Purification of HLA molecules was then performed using mainly the method published by Macklin et al. (Macklin et al, 1998) with adaptations. The detergent solubilized, purified HLA-antigens as well as commercially available anti-CD3 mAb and anti-CD28 mAb were then used to generate artificial cell constructs.

Figure 6 shows a flow chart of the main experimental steps performed in the laboratory.

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III. Lizentiatsaequivalent

Generation and characterization of artificial cell surface constructs for studying T cell responses to drugs.

Steinemann, M.-A, von Greyerz, S., Britschgi, M., Pichler, W.J., Burkhart, C. (in preparation).

Generation and Characterization of Artificial Cell Surface Constructs for Studying T Cell Responses to Drugs

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ABSTRACT

T cells can recognize peptide and non-peptide antigens. Drugs are typical examples of non-peptide antigens. Drugs like sulfamethoxazole or lidocaine can be presented to specific human $\alpha\beta^+$ T cell clones by undergoing a non-covalent association with major histocompatibility complex-(MHC) peptide-complexes on human leukocyte antigen (HLA) -matched antigen presenting cells. However, an activation of T cell clones involves interaction between a number of different receptors on the T cell with their respective ligands on the antigen presenting cell. For studying the key mechanisms in activation of drug-specific-T cell clones by antigen presenting cells, a model system of artificial cell constructs was generated using latex beads.

Monoclonal antibodies specific for human MHC class II anti-HLA-DR molecules were produced and used to prepare immunoaffinity columns. HLA-DRB1*1/10 or HLA-DRB1*15 molecules were then purified from Epstein-Barr-Virus-transformed human B lymphoblastoid cell lines and characterized by immunoblots and ELISA. Affinity purified HLA-DR molecules as primary activation signal and anti-CD28 mAb as secondary activation signal, were immobilized on 5 μm diameter latex microspheres. Additionally, anti-CD3 mAb were coated onto a separate set of latex microspheres. Artificial cell constructs were characterized by flow cytometry and used to activate drug-specific T cell clones.

HLA-DR molecules were successfully purified from human B-LCL and characterized by FACS, ELISA and immunoblots. Latex beads were reproducibly coated with HLA-DR ligands or anti-CD3 mAb or anti-CD28 mAb and phenotypically characterized by FACS. Beads displaying HLA-DR molecules and anti-CD28 mAb on their surface were, in the presence of the respective soluble drug, not stimulatory for SMX or lidocaine-specific T cell clones. Still, latex microspheres coated with anti-CD3 mAb could stimulate T cell proliferation even in the absence of costimulatory molecules or IL-2. In conclusion, the use of the artificial "APC" in the antigen-specific stimulation of drug-specific T cells, however, awaits further experimentation.

1. INTRODUCTION

Over the years, T cell involvement in drug allergic reactions has been well established (de Weck, 1983, Hertl et al., 1992, Pichler et al., 1998). Still, the molecular mechanism which leads to the recognition of some drug molecules with a molecular weight of 200-300 Daltons has not yet been fully elucidated and understood. Examples of such drug molecules are sulfamethoxazole (SMX) (Padovan et al., 1996, Zanni et al., 1998) or lidocaine (Zanni et al., 1997), both bind non-covalently to MHC molecules and need not to be processed by antigen presenting cells (APC) (Schnyder et al., 1997, Horton et al., 1998). Drug-specific $\alpha\beta^+$ T cell clones (TCC) from several drug allergic patients have been generated over the past years. Most of these TCC were of the $CD4^+$ phenotype but almost every cloning procedure revealed also drug-specific $CD8^+$ clones (Mauri-Hellweg et al., 1995, Zanni et al., 1997).

Antigen-specific activation of T lymphocytes mainly results from interactions of the TCR with MHC-peptide complexes presented on the surfaces of APC. However, it has become clear that in many situations this antigen-specific activation signal must be augmented by antigen-non-specific costimulation (Lafferty et al., 1983, Mueller et al., 1989). These costimulatory, or second signals may be provided by soluble factors (Raulet et al., 1982) as well as cell surface molecules (Janeway et al., 1994) that bind to distinct receptors on the T cell.

For $CD4^+$ T cells, the primary activation signal is provided by MHC class II molecule-antigen complexes. MHC class II molecules are mainly found on dendritic cells, B lymphocytes and macrophages that are often called professional APC. Professional APC have the potent ability to process and load endocytosed antigens onto MHC class II molecules. Such a cell expresses 50,000 to 200,000 MHC class II molecules on their surface (Valitutti et al., 1995). MHC class II molecules are $\alpha\beta$ -heterodimers and have a single peptide-binding site which consists of a deep groove between the α_1 and β_1 domain.

The group of Mescher et al. established a model system for the study of receptor-ligand contributions in lymphocyte activation. The model system consists of latex microspheres which can be coated with soluble and non-soluble proteins in different densities and with up to three different ligands. The fundamental advantage of this

model system is the reduction of a complex cell surface of an APC with its multiple ligands to a simple cell-like construct with only the desired molecules on the surface. Mescher et al. were able to activate TCC with different types of antigen and costimulatory factors. Surface contact requirements for activation of cytotoxic T lymphocytes were investigated with murine MHC class I H-2K^b molecules immobilized on latex microspheres, incubated with alloantigen specific TCC in the presence of Concanavalin A (Con A)-stimulated rat lymphocyte supernatant. A cytotoxic activation of the TCC was observed with latex microspheres > 3 µm diameter (Mescher, 1992).

Receptor-ligand contributions to lymphocyte activation were studied with murine MHC class I H-2K^b and D^b molecules from C57BL/6 mice coated to latex microspheres. Immobilized MHC molecules on artificial cell constructs were pulsed with ova₂₅₇₋₂₆₄ peptide to form antigen complexes and could stimulate ova₂₅₇₋₂₆₄-specific TCC from OT-1 transgenic mice in the presence of exogenously added IL-2 (Curtsinger 1997).

Mouse MHC class I molecules immobilized onto latex microspheres were incubated with memory T cells or naive cells in the presence of 5 U/ml human rIL-2 and could generate an allogeneic response by memory, but not naive, CD8⁺ T cells (Curtsinger et al., 1998).

Anti-TCR mAb or murine class I H-2K^b and B7-1 immobilized on 5 µm diameter latex microspheres revealed, that B7-1 dependent costimulation results in qualitatively and quantitatively different responses by CD4⁺ and CD8⁺ T cells (Deeths et al., 1997).

A crucial question in drug-induced allergies caused by small drug molecules like SMX or lidocaine is the manner, in which antigen is presented to the drug-specific T cells and what costimulation is necessary to activate the drug-specific T cells. To investigate the antigen presentation mechanism, the above described model system of artificial cell constructs, that lack any capacity to metabolize or process drug molecules was adapted. Thus this model system might be an ideal tool for the analysis of metabolism and processing-dependence of drug presentation and for the determination of the influence of costimulatory signals on T cell activation.

2. MATERIAL AND METHODS

2.1 Cells

The anti-HLA-DR monoclonal Ab L243 was produced by the mouse B cell hybridoma HB55 (American Type Culture Collection, Rockville, MD) (Acolla et al., 1981, Cresswell et al., 1988).

MHC class II molecules were isolated from the following B-lymphoblastoid cell lines (B-LCL): B-LCL OF was derived from a patient suffering from lidocaine drug allergy (Zanni et al., 1997). The MHC-phenotype of B-LCL OF was: HLA-A2, A26, B7/x, DRB1*1501-1503/x. B-LCL KaVo was derived from a healthy individual. The MHC phenotype of KaVo was: HLA-A1, A11, B51, B8, DRB1*0301/1001.

The L1210 mouse lymphoblast cell line (CCL-219, American Type Culture Collection, Rockville, MD) does not express human class II and was used as a negative control in immunoblots, ELISA and flow cytometry.

For functional characterization of artificial cell constructs different SMX-specific and lidocaine-specific TCC were used: the TCC SMX #3, 9.5 and Z1.1. were isolated from a SMX-allergic patient and recognized SMX in the context of DRB1*1001 molecules (Schnyder et al., 1997, von Greyerz et al, 1999, Burkhart et al., manuscript in preparation). OFB12 was isolated from a lidocaine-allergic patient and recognized the drug in the context of DRB1*1501-03 molecules (Zanni et al, 1998).

2.2 Culture media

Culture medium for EBV-transformed B cells, L1210 and HB55 was RPMI 1640 supplemented with 10% pooled heat-inactivated FCS (Seromed, Fakola, Basel, Switzerland), 25 mM HEPES buffer, 2 mM L-glutamine (Seromed, Fakola, Basel, Switzerland). Culture medium for T cell assays (R9) consisted of RPMI 1640 supplemented with 10% pooled heat-inactivated human AB serum (Swiss Red Cross, Bern, Switzerland) and 25 mM HEPES buffer, 2 mM L-glutamine, 25 µg/ml transferrin (Biotest, Dreieich, Germany), 100 µg/ml streptomycin, 100 U/ml penicillin. Medium for culturing TCC was R9 enriched with 50 U/ml human recombinant IL-2 (obtained from Dr. D. Wrann, Sandoz Research Institute, Vienna, Austria).

2.3 Purification of anti-HLA-DR antibodies.

The HLA-DR $\alpha\beta$ chain-specific mAb L243 (IgG2a) secreted by the mouse hybridoma HB55 was used for preparation of affinity columns and as primary mAb in immunoblots and flow cytometry. mAb were purified from supernatant of cell cultures growing at a density of 2×10^5 cells/ml. Supernatant was subjected to centrifugation at $5000 \times g$, then filtered through a $0.22 \mu\text{m}$ sterile filter. Finally, 0.02% NaN_3 was added and the supernatant was stored at -20°C until further use. Cell-free, sterile-filtered supernatant was applied to 1.5 ml protein G ("Ultra link immobilized protein G", Pierce, Socochim, Lausanne, Switzerland). In general, 1.5 l of supernatant were passed at RT over the column at a flow rate of 50 ml/h. Then the column was washed with 15 column volumes of PBS until the absorbance of the flowthrough measured at 280 nm wavelength (Lambda2, Perkin-Elmer, Switzerland) was at base level. Subsequently, mAb were eluted with elution buffer consisting of 0.01 M glycine, pH 2.5 at RT and collected in 2 ml fractions. The pH of the fractions was immediately raised to neutral with 200 μl Tris, pH 8.3. Protein content of fractions was determined by measuring absorbance at 280 nm. mAb containing fractions were pooled and 0.02% NaN_3 was added. The final concentration of the pooled mAb containing fractions was determined by absorbance at 280 nm wavelength. Pooled mAb was stored at a concentration of 0.6-1.0 mg/ml at $2-6^\circ \text{C}$ in the dark.

2.4 Preparation of affinity columns

Two different methods were used to prepare anti-HLA-DR affinity columns. The purified L243 mAb was either coupled to Affigel Hydrazide resin (BioRad, Glattbrugg, Switzerland) according to the manufacturer's protocol. Briefly, 16 mg of L243 mAb were dialyzed against a 1,000 fold excess of 1 M NaAc, 1.5 M NaCl at pH 5.5 (coupling buffer Hz) at 4°C overnight in the dark. mAb were then oxidized with 2.5 mg/ml NaIO_4 for 1 h at RT in the dark. Oxidized mAb was desalted on a 1×10 cm Sephadex G25 (Fluka, Basel, Switzerland) column and dissolved in coupling buffer Hz. The oxidized and desalted L243 Ab was coupled to Affigel Hydrazide resin overnight at RT, poured into a 1×10 cm column and washed with 15 resin volumes of PBS. Alternatively, the

purified L243 mAb was coupled to CNBr-activated sepharose 4 fast flow (Pharmacia Biotech, Sweden). Briefly, 20 mg of L243 mAb were diluted in 0.1 M NaHCO₃, 0.5 M NaCl, pH 8,3 (coupling buffer CNBr) at a ratio of 0.5:1 coupling buffer:CNBr sepharose. Sepharose was washed 12 x with one sepharose volume ice cold 1 mM HCl, and twice with 0.1 M NaHCO₃, 0.5 M NaCl, pH 8.3. The washed sepharose and the mAb were mixed and coupled over 4 h at RT. Uncoupled groups of the CNBr-activated sepharose were then blocked with 5 sepharose volumes of Tris buffer for 2 h at RT. The resin was then washed using alternate low and high pH (pH 3 and 9). Of each solution 6 x 2 resin volumes were applied. The column was finally equilibrated with 15 column volumes of PBS.

The coupling efficiency of both the affigel hydrazide resin and the CNBr-activated sepharose was determined by monitoring absorbance at 280 nm wavelength. Aliquots of the coupling mixture were measured before, during, and after coupling reaction with UV-spectrophotometer.

2.5 Purification of HLA-DR molecules

HLA-DR molecules were purified from the EBV-transformed B-LCL OF and B-LCL KaVo, according to a method described for purification of DRB1*0101 (Macklin et al., 1998). B-LCL cultures with a density of 2×10^5 cells/ml were applied to centrifugation at 5,000 x g for 30 min. Pellets were taken up in HBSS and centrifuged again at 5,000 x g for 20 min. Supernatant was discarded and the pellets were frozen at -80° C until further use. Aliquots of 10- 25 g of frozen cell pellets were lysed in 60 ml lysis buffer (10 mM Tris, pH 8.0, 150 mM NaCl containing 0,5% polidocanol (polyoxyethylene-9-lauryl ether)) at 4 °C. One tablet protease inhibitor (CompleteTM protease inhibitor tablets, Boehringer Mannheim, Mannheim, Germany) was added to 10 ml of lysis buffer. Cell lysis was performed on a shaker at 4 °C in the dark until the cell pellet was completely solved. The lysed cell extract was clarified by ultracentrifugation at 100,000 x g for 60 minutes in a Beckmann L8-MUC ultracentrifuge using a fixed angle rotor Ti50. The clarified supernatant was then applied to a 5 ml sepharose precolumn. The flowthrough of the precolumn was immediately applied to the immunoaffinity column. The flow rate was 0.2 ml/min. Flowthrough of the affinity column was monitored by measurement of the absorbance at 280 nm wavelength. After loading, the affinity column was washed with 10 mM Tris, pH 8.0, 0.5 M NaCl containing 0.1% polidocanol

until the absorbance at 280 nm reached baseline and then with 10 mM Tris, pH 8.0. 0.1% polidocanol until absorbance at 280 nm reached baseline. The bound material was eluted with 50 mM sodium phosphate, pH 11.5 containing 0.1% polidocanol. The pH of the 0.5 ml or 1.5 ml fractions was immediately adjusted to pH 7.5 with 33 % vol. of 0.5 N HCl. Protein concentration was calculated from the measured absorbance at 280 nm using the molar extinction coefficient of $6.68 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$, based on the tryptophan and tyrosine content of HLA-DR molecules. (Gill et al., 1989). Fractions were stored in the presence of 0.02% NaN₃ at 4 °C in the dark.

2.6 Electrophoretic analysis of column eluates

Purity of mAb L243 and protein content of affinity column fractions were analyzed by SDS-PAGE. Gels were run as described previously (Laemmli et al., 1970) with modifications. For protein separation, 12% SDS-PAGE gels of 10 x 7 cm were run at 200 V in a Mini-Protean II gel chamber (BioRad, Glattbrugg, Switzerland) over 30 min at RT. The following markers were used: RPN 755, RPN 756, RPN 800 (Amersham Life Science, Buckinghamshire, UK) and Kaleidoscope prestained standards (BioRad, Glattbrugg, Switzerland). Gels with samples of eluted fractions from affinity column were further analyzed by immunoblotting (see below), gels of mAb fractions were stained with coomassie blue staining. For coomassie blue staining, gels were prewashed 2 x 10 min with 100 ml deionized H₂O and then stained with 15 ml Gelcode (GELCODE Blue Stain Reagent, Pierce, Socochim, Lausanne, Switzerland) for 1 h at RT on a shaker. After staining, gels were incubated for 3 h in 3x100 ml deionized H₂O to enhance the protein bands.

2.7 Identification of HLA-DR protein by immunoblotting

In order to identify HLA-DR molecules in eluted fractions from affinity chromatography the proteins of SDS-PAGE gels (see above) were transferred to a nitrocellulose membrane (Trans-Blot Transfer Medium, BioRad, Glattbrugg, Switzerland) during 2 h at a constant current of 180 mA in a Mini-Protean II western blot chamber. The transfer occurred in 4 °C precooled 25 mM Tris, 192 mM glycine, 20% methanole (v/v) at pH 8.1-8.3. Constant temperature during the transfer was provided by a cooling unit in the transfer chamber. The membrane was blocked after the

transfer for 2 h with 100 ml blocking buffer (BB), containing 0.5% BSA, and 1% milkpowder in PBS. The primary mAb L243 was added (0.3 µg/ml) in 10 ml BB enriched with 30 µl Tween-20 (BB+) and the membrane was incubated at 4 °C overnight on a shaker. After extensive washing with PBS containing 0.05% Tween-20, the membrane was incubated with the secondary HRPO-labeled goat-anti-mouse mAb (Dako, Glostrup, Denmark) (0.2 µg/ml) in 10 ml BB+ for 2 h at RT on a shaker. The membrane was again washed with PBS/0.05% Tween-20 and finally in pure PBS. The washed membrane was incubated with 12 ml of chemiluminescent substrate (Pierce, Socochim, Lausanne, Switzerland) for 5 min at RT. The blotted membrane was placed in a plastic cover and exposed against enhanced autoradiographic film (RX X-RAY film, Fuji, Switzerland) for 1 s to 30 min. The exposed film was developed in a Kodak RP X-OMAT Model M6B (Kodak, Lausanne, Switzerland) (Walker et al., 1995).

2.8 Qualitative and quantitative analysis of purified HLA-DR molecules by ELISA

Additionally, affinity-purified HLA-DR molecules were characterized by standard ELISA technique. Affinity-purified HLA-DR molecules were kept in the elution buffer containing 0.3% polidocanol. For coating MHC-molecules on microtiter plates (Immunoplate F96, NUNC, Life Technologies, Basel, Switzerland), aliquots of 100 µl HLA-DR molecules (corresponding to 125 µg protein of OF and 140 µg protein of KaVo) in elution buffer were added to 100 µl coating buffer (30% 1 M Na₂CO₃, 70% 1 M NaHCO₃, pH 9.6). The prediluted HLA-DR molecules were titrated by serial twofold dilutions in coating buffer. Plates were incubated overnight at 4 °C and then washed with PBS containing 0.05% Tween-20 and the supernatant was discarded. Unbound sites on the plastic were blocked by addition of 200 µl of blocking buffer (1% milkpowder and 0.5% BSA in PBS) and incubated for 60 min at RT. As primary mAb, 0.5 µg/ml anti-HLA-DR mAb L243 diluted in 10 ml blocking buffer was added and the plates were incubated for 60 min at RT. Plates were washed and supernatant was discarded. Unbound Ab was removed by washing with PBS containing 0.05% Tween-20. Second step HRPO-labeled goat-anti-mouse mAb (Dako, Glostrup, Denmark) diluted at 0.5 µg /ml in blocking buffer was added and the plates were again incubated for 60 min at RT. After extensive washing with PBS containing 0.05% Tween-20, 200 µl of substrate was added (0.05 M Na₂PO₄, 0.025 M citric acid, 800 µg/ml O-

phenylenediamine hydrochloride (Calbiochem, La Jolla, CA), 0.15% H₂O₂). The reaction was stopped by addition of 2 N H₂SO₄ during the linear phase of color development. Absorbance at 490 nm wavelength was then determined using a microtiter ELISA plate reader (Molecular Devices, Basel, Switzerland).

2.9 Immobilization of proteins on latex microspheres

Affinity purified MHC class II HLA-DR molecules and mAb for costimulation were immobilized on sulfate polystyrene latex microspheres with a diameter of 5 µm (Interfacial Dynamics, Portland, OR). Latex microspheres were stored at 4 °C in distilled water until use. For immobilization, the beads were diluted into PBS to a concentration of 10⁷ beads/ml at RT. The mAb and/or the HLA-DR molecules to be immobilized were added to the diluted beads and the mixture was vortexed immediately. Membrane proteins in polidocanol containing buffer were added to the bead suspension with sufficient dilution with PBS to yield a final detergent concentration of less than 0.05%. The amount of protein added depended on the desired final surface density and was determined empirically by phenotypical analysis of coated latex microspheres by flow cytometry (see below). For HLA-DR molecules, the range of immobilized protein was 24-29 µg per 10⁷ beads and for immobilized antibody 1 µg per 10⁷ beads. The following mAb were used for immobilization: mouse-anti-CD28, (IgG2a), (Becton Dickinson, Rutherford, NJ); and the anti-CD3 mAb OKT3 (Orthoclone OKT3[®], Janssen-Cilag, Beerse, Belgium). After addition of the protein to the bead suspension, the mixture was placed on a rotator and incubated for 20 min at 4 °C. After the incubation period, 1% BSA in PBS was added at one-fourth of the volume of the reaction mixture and incubation was continued for additional 30 min at 4 °C on a rotator to block all remaining sites on the beads. The beads were then washed with 3 x 1 ml of sterile PBS or tissue culture medium. Finally, coated latex microspheres were resuspended in 1 ml of the desired medium. Beads were counted using a hemacytometer and characterized by flow cytometry (see below).

2.10 Phenotypical analysis of latex microspheres and cells by immunofluorescence

Coated latex microspheres were analyzed by flow cytometry to determine the coating efficiency and the number of HLA-DR ligands on the surface. Cells were analyzed to determine the amount of physiologically expressed MHC class II molecules (see also below).

For direct immunofluorescence, $1-2 \times 10^5$ cells or latex microspheres were washed once with 3 ml of PBS, 1% FCS, 0.02% NaN_3 (FACS buffer), at 4 °C and resuspended in 150 μl of the same buffer. Cells or microspheres were then incubated for 30 min at 4 °C with one of the following mAb: 10 $\mu\text{g}/\text{ml}$ of FITC- or PE labeled anti-HLA-DR mAb (Becton Dickinson, Rutherford, NJ) for detection of HLA-DR molecules, and or FITC- or PE labeled goat-anti-mouse mAb (Dako, Glostrup, Denmark) for detection of coated anti-CD3 and anti-CD28 mAb. After washing with FACS buffer, cells or beads were fixed by PBS/0.5% paraformaldehyde.

For indirect immunofluorescence staining, $1-2 \times 10^5$ cells or latex microspheres were washed once with 10 ml of FACS buffer, at 4 °C and resuspended in 150 μl of the same buffer. Cells were then incubated with 10 $\mu\text{g}/\text{ml}$ of mAb L243 for 30 min at 4 °C. After washing with FACS buffer, cells or microspheres were resuspended in 150 μl FACS buffer and subsequently incubated with 10 $\mu\text{g}/\text{ml}$ of FITC- or PE labeled goat-anti-mouse mAb for 30 min at 4 °C. Finally cells or latex microspheres were fixed as described above.

Cells and latex microspheres were analyzed on an EPICS XL II flow cytometer using System II Software version 3.0 (Coulter, Hialeah, FL) and on a FACSCalibur using Cell-Quest Software version 3.2.1 (Becton Dickinson, Basel, Switzerland).

2.11 Quantification of HLA-DR ligands on cells / microspheres

The absolute number of HLA-DR molecules expressed on the surface of APC and immobilized on latex microspheres, were estimated by reference to a standard curve of beads coated with known amounts of mouse antibodies (Quifikit, Dako, Glostrup, Denmark). Briefly, aliquots of 1.0 to 2.0×10^5 cells or latex microspheres were washed once with FACS buffer at 4 °C and resuspended in 150 μl of the same buffer. Cells or microspheres were then incubated for 30 min at 4 °C with 10 $\mu\text{g}/\text{ml}$ of mouse-anti-HLA-DR mAb L243, washed with FACS buffer at 4 °C and resuspended in 150 μl of

the same buffer. Aliquots of 100 μl "setup" beads (two populations of beads, blank beads and beads with a high number of mAb molecules) and "calibration" beads (five populations of beads bearing different numbers of mAb) were washed with FACS buffer at 4 °C and resuspended in 150 μl of the same buffer. Thereafter, a secondary FITC- or PE-labeled goat-anti-mouse mAb (10 $\mu\text{g}/\text{ml}$) was added to the samples, calibration and setup beads and they were incubated for 30 min at 4 °C. The samples were analyzed by flow cytometry and the number of ligands was calculated by reference to the standard curve obtained from the analysis of the precoated calibration beads.

2.12 Functional lymphocyte activation assays with artificial cell constructs

Artificial cell constructs were used as APC in proliferation assays with drug-specific TCC. In order to determine the proliferative capacity of the TCC to the different stimuli, 5×10^4 TCC were incubated either with 1×10^5 ligand-coated beads or 1×10^5 autologous irradiated (6000 rad) B-LCL in the presence or absence of the respective drug in 200 μl R9 in U-bottom microtiter plates (Falcon, Lincoln Park, NJ). The following drug concentrations were used: lidocaine : 100 $\mu\text{g}/\text{ml}$, SMX : 200 $\mu\text{g}/\text{ml}$. In all experiments IL-2 was added at different concentrations. Cultures were pulsed after 48 h with [^3H]thymidine (0.5 μCi) for 8 h, and cells were then harvested onto glass fiber disks and counted in a microplate beta-counter (Inotech Filter Counting System INB 384, Inotech, Dottikon, Switzerland).

3. RESULTS AND DISCUSSION

3.1 Purification and characterization of monoclonal antibody L243

L243 is a mAb which is produced by the mouse hybridoma cells HB55 and is specific for HLA-DR molecules (Lampson et al., 1980). Purification of an anti-HLA-DR mAb is a prerequisite for the preparation of anti-HLA-DR affinity columns. Additionally, the purified mAb can be used for flow cytometry and as primary Ab in immunoblots (see below). Anti-HLA-DR mAb was purified from cell culture supernatant of HB55 cells on a sepharose protein G column. From 1 liter of HB55 supernatant 3 mg purified antibody could be obtained as measured by absorbance at 280 nm wavelength. Totally 15 l supernatant were purified on the same protein G column and yielded in a total amount of 45 mg mAb.

Every batch of purified mAb was tested by SDS-PAGE analysis for its purity and protein content. Protein electrophoresis was performed as described in Materials and Methods. Figure 1 shows a representative gel from a purified antibody batch. Non-reduced and non-boiled L243 mAb showed a typical band at around 200 kD, which corresponds to the mass of an intact antibody. Reduction with β -mercaptoethanol resulted in typical bands at around 25 kD for the Immunoglobuline light chain and 70 kD for the heavy chain. The comparison of the purified mAb with a commercially obtained isotype control mAb (mouse IgG2a) revealed similar bands for both mAb.

Additionally, the purified mAb was tested by flow cytometry for its specific binding to HLA-DR molecules. Samples of B-LCL KaVo or HLA-DR negative L1210 cells were stained with serial dilutions of L243 mAb and a FITC-labeled goat-anti-mouse mAb as second step reagent. Stainings were compared to those obtained with a commercially available FITC-labeled L243 mAb. Figure 2 shows, that protein G purified anti-HLA-DR L243 mAb stain positively B-LCL KaVo. The mean fluorescence values ranged from 4,100 for a positive staining to 0.4 for the negative control. Titration of the mAb showed a decrease of fluorescence intensity. Furthermore, the mAb was tested by flow cytometry for its specific binding to B-LCL OF and similar results as for B-LCL KaVo were obtained (data not shown).

3.2 Purification and characterization of HLA-DR molecules

Drug presented on HLA class II molecules would provide the primary activation signals for specific CD4⁺ T cell clones. HLA-DR molecules were isolated from EBV-transformed B cells expressing either HLA-DRB1*0301/1001 molecules (B-LCL KaVo) or HLA-DRB1*1501-03 (B-LCL OF). Cells were cultivated up to a concentration of 1x10⁶ cells per ml and then harvested by centrifugation. Cells were lysed with the non-ionic detergent polidocanol and subjected to ultracentrifugation. HLA-DR molecules were then purified from the detergent soluble fraction by immunoaffinity chromatography. Two different affinity chromatography columns with mAb L243 were prepared. In one, the mAb was bound to CNBr-activated sepharose, in the other to BioRad hydrazide affinity resin. Bound HLA-DR molecules were eluted from affinity columns by a increase of the pH to 11.5.

Several purifications with small amounts (≈5 g) of cells were performed until a satisfactory protein yield was obtained. Finally, two purification of HLA-DR molecules were performed from 20 g B-LCL KaVo and two from 15 g B-LCL OF using a sepharose affinity column. Figure 3 shows the elution diagrams of anti-HLA-DR affinity chromatography columns of one purification from OF and of one purification from KaVo cells. The UV absorbance diagram of the B-LCL OF assay shows a peak from fraction 5-6 (max. OD: 0.63) and the B-LCL KaVo shows a peak from fraction 6-21 (max. OD: 0.73). Later used fractions are indicated by arrows or stars. Absorbance at 280 nm of the collected fractions was measured and the protein content calculated with the following formula (Voet and Voet, 1995):

$$m \text{ [g]} = \frac{\text{OD}_{280} \times M_r \text{ [mol]}}{\epsilon_r \text{ [M}^{-1} \text{ cm}^{-1}] \times l \text{ [cm]}}$$

protein mass: m; absorption: OD; M_r: mol weight; ε_r: molar extinction coefficient; l:
length of the cuvette

For HLA-DR molecules: ε_r = 66,800, M_r = 66,000, l = 0.5

The approximate yield of protein 1 g of cells was calculated as around 690 μg for B-LCL KaVo and 490 μg for B-LCL OF. Colorimetric quantification of protein yields

with BioRad protein assay was not reliable. This can be due to interferences of the detergent polidocanol.

The quantification of the purified protein was a major problem encountered in the affinity purification of MHC molecules. Comparing our protein yields with those of others, we found that this was 25-30% of the amount reported by Gorga et al. (Gorga et al., 1987). His laboratory had purified 1.2 mg HLA-DR molecules per gram of human B-LCL LG-2 with L243 mAb affinity columns. Other investigators have obtained 0.24 mg MHC protein from 10^{10} murine cells P815 or EL4 cells, corresponding to about 1.2 $\mu\text{g/g}$ cells (Kane et al., 1989) or 0.9 mg from 10^{10} murine cells RDM-4, corresponding to 4.5 $\mu\text{g/g}$ cells (Herrman and Mescher, 1979).

In order to compare the experimental with the theoretical yield, the theoretical yield of HLA-DR molecules was calculated as follows: In 1 ml B-LCL of cell cultures are about 1×10^6 cells. If 1 liter cell culture is centrifuged the pellet weighs about 2 g, corresponding to 1×10^9 cells. With approximately 200,000 HLA-DR molecules per B-LCL (Valitutti et al., 1995), 1 g cells contains about 2×10^{14} molecules. The weight of 1 mol HLA-DR molecules is approximately 66 kD. This results in a theoretical yield of about 20 μg HLA-DR molecules per g of lysed cells.

Our protein yield is below the yield that was obtained by Gorga et al., Herrmann et al. and Kane et al.. A possible quantitation error may lie in the detergent used by us, as polidocanol is known to influence UV-measuring methods through its molecular structure; it could be the result of different cell species (mouse vs. human), different elution protocols (high pH vs. salt) or might be an intrinsic characteristic of the HLA-DR molecules expressed on the B-LCL used by us. Finally, our elution diagram does not exclude that our column eluates can be contaminated with other proteins.

The isolated proteins were subjected to SDS-PAGE. Since there were no commercially available HLA-DR molecules for use as positive control, B-LCL cell lysate served as positive control. Lysates of L1210 cells, which do not express HLA class II molecules were used as negative control. Applied protein of the column eluates was below the detection limit of 0.1–1.0 μg protein per band in a coomassie staining of SDS-PAGE gels.

The shown fractions of B-LCL OF (Figure 3 A) were visualized by silver staining. Silver staining detects quantities of 1-10 ng protein per band (BioRad, Glattbrugg, Switzerland) and only at around 66 kD bands were seen. 14 fractions of Figure 3 A

subjected to silver stain silver stain detected protein. This means that in a ml are 67-670 ng and in 14x1.5ml 1,407-14,070 ng or 0.09-0.9 µg HLA-DR per gram of cells (data not shown).

Therefore, purified HLA-DR molecules were characterized by immunoblots. Immunoblots were performed with 15 µl of column fractions, 2 µl cell lysates of B-LCL KaVo or OF respectively as positive control and L1210 lysate as negative control. L243 mAb was used as primary Ab, secondary Ab was HRPO-labeled goat-anti-mouse Ab. SDS-PAGE gels were blotted to a membrane and then exposed to luminol chemiluminescence fluid and later to x-ray film. Non-boiled proteins applied to immunoblots resulted in characteristic bands at about 66 kD (Figure 4). Boiling of the sample resulted in denaturation and no bands were visible.

Immunoblotting detects protein quantities in the lower pictogram aerea per band (Pierce, Socochim, Switzerland). When immunoblots of fractions of affigel hydrazide columns and sepharose columns from B-LCL KaVo were compared, the hydrazide column showed intensive bands at about 180 kD. It could not be elucidated what protein species was represented by those bands or if it were artifacts and therefore the affigel column was not further used (data not shown). The obtained results of the immunoblots were compared to the results published by Gorga (Gorga et al., 1987) and protein bands appeared at the same size aerea.

The obtained results of coomassie stainings and immunoblots and silver stainings indicate, that the protein content of the bands of 15 µl column eluates applied to the stainings per lane, was 1-10 ng.

In a second approach, purified HLA-DR molecules were quantified by direct coating of HLA-DR molecules of B-LCL KaVo (fraction 14, Figure 3) or B-LCL OF (fraction 5, Figure 3) to ELISA plates. Prediluted samples were of HLA-DR molecules were coated in a serial twofold dilution. Then, HLA-DR molecules were labeled with mAb L243 as first antibody which was detected by HRPO-labeled goat-anti-mouse second antibody. After addition of the substrate, chemiluminescence was measured at 490 nm. As negative control, coated protein was labeled with secondary mAb only. Figure 5 demonstrates that detergent-solubilized, affinity purified MHC-antigens can bind concentration dependent and reproducible to solid plastic surfaces. For sample OF a maximal binding was detected at 1/8 dilution, for KaVo at 1/16 dilution. Both OD values decrease then to 0 according to the dilution. Immobilization of protein on plastic

probably involves partial denaturation to exposed hydrophobic regions which bind to the plastic (Beutler et al, 1987). However, the binding of MHC antigens to plastic neither requires nor results in sufficient denaturation to cause loss of determinants recognized by the specific mAb. In our hands, increase of the HLA-DR protein concentration did not result in maximal binding or a plateau, suggesting a negative influence of the non-ionic detergent polidocanol used for MHC protein solubilization on the binding of the membrane proteins to the plastic surface. Interestingly, the results obtained by Kane et al. reach a maximal binding at increasing protein concentrations of the murine MHC solubilized in 0.5 % DOC containing buffer.

3.3 Immobilization of HLA-DR molecules and monoclonal antibodies on latex microspheres and phenotypic analysis by FACS

Affinity purified HLA-DR molecules and commercially obtained mAb were used to generate artificial APC. The following types of artificial cell constructs were generated by immobilization of different ligands: Firstly, latex microspheres were coated with the anti-CD3 mAb Otk3 and/or the mAb anti-CD28 Leu-28. Second, affinity purified HLA-DR molecules from B-LCL OF (fraction 5, Figure 3 A) and B-LCL KaVo (fraction 15, Figure 3 B) were immobilized either alone or together with anti-CD28 mAb on latex microspheres. Of HLA-DR containing fractions, an amount of 20 μ l was coated corresponding to 25 μ g protein of OF and 28 μ g of KaVo protein after UV measuring. This amount has been reported as optimal for membrane proteins (Curtsinger et al., 1997). Uncoated and coated latex microspheres were then characterized by flow cytometry with PE labeled anti-HLA-DR mAb (L243) and FITC labeled anti-mouse mAb (Figure 6-8). The mean fluorescence intensity (MFI) of bound antibodies was used as a measure for the efficiency of ligand immobilization. Coating of latex microspheres with membrane and soluble protein occurred in a manner, that the HLA-DR-specific and the mouse IgG2a-specific labeled mAb could still recognize their determinants on the coated protein (see Figure 8). Single mouse mAb coated to latex microspheres resulted in MFI values in the range of 6,400-9,500 (see Figure 7, right column). Beads coated with HLA-DR molecules had MFI values around 170 (Figure 8, middle column). MFI 170 is 17 times higher than the values of uncoated beads (Figure 7).

This strikingly different MFI of mAb and HLA-DR molecules immobilized on latex microspheres may have different reasons. HLA-DR molecules are detergent solubilized

and the detergent concentration might inhibit the coating process (Kane et al., 1989, Curtsinger et al., 1997). HLA-DR molecules may occupy more space than mAb on the beads which might result in a lower density. Another problem could be, that the mAb concentration was known but the concentration of HLA-DR protein was calculated from OD₂₈₀ values. Eventually, the different staining intensity may be a result of the fact that FITC-labeled mAb were used for the detection of the immobilized mAb and PE-labeled mAb for the detection of immobilized HLA-DR molecules on latex microspheres.

Immobilization of soluble protein (mAb) or membrane proteins (HLA-DR) onto 5 µm diameter latex microspheres proved to be reproducible and reliable and, therefore, seems to be an adequate method for generation of artificial APC. For a further improvement of the coating efficiency, especially of HLA-DR molecules, the detergent concentration of the buffer should be kept as low as possible but sufficiently high to retain the membrane proteins in solution.

3.4 Efficiency of immobilization of different quantities of purified HLA-DR molecules on latex microspheres

To test which was the optimal amount of HLA-DR protein for immobilization, different amounts of HLA-DR protein (fraction 16, Figure 3 B) from B-LCL KaVo were coated onto latex microspheres. The coupling efficiency was then measured by flow cytometry with PE-labeled anti-HLA-DR mAb (Figure 9). Beads coated with 20 µl of fraction 16, corresponding to 26 µg protein showed a maximal binding of HLA-DR specific mAb. Lower concentrations were less efficient, while at higher amounts immobilization was possibly hindered by the detergent concentration (0.3%) of the elution buffer.

3.5 Comparison of coating efficiency after simultaneous and sequential incubation of ligands

Comparison of the MFI values obtained for immobilized anti-CD28 mAb in the presence or absence of HLA-DR molecules revealed a MFI of 9,400 for anti-CD28 mAb and 50 for HLA-DR molecules (compare Figure 7 with Figure 8). This may be explained by the probability that simultaneous incubation of mAb and HLA-DR molecules with latex microspheres could prevent HLA-DR molecules from optimal

coating. We therefore wondered if sequential coating of HLA-DR molecules and anti-CD28 mAb would increase the amount of bound HLA-DR protein.

HLA-DR molecules (from fraction 15, Figure 3 B) and anti-CD28 mAb were immobilized on latex microspheres simultaneously or sequentially and unbound sites on the beads were then blocked with BSA. In the sequential incubation, HLA-DR molecules were added first. Coated latex microspheres were characterized by flow cytometry with PE-labeled anti-HLA-DR mAb (L243) and FITC-labeled anti-mouse mAb.

Using simultaneous incubation the MFI value for HLA-DR molecules was 105 and for anti-CD28 546. With sequential incubation the MFI value for HLA-DR molecules was 79 and for anti-CD28 548 (Figure 10). Therefore, there was no significant difference in the amount of coated ligands. Similar findings were made by other investigators (Curtsinger et al., 1997) for membrane molecules such as MHC class I and B7-1 and mAb such as anti-CD28.

3.6 Quantification of HLA-DR molecules on cells and microspheres

In order to evaluate the maximal amount of HLA-DR molecules that could be theoretically purified from B-LCL and the amount of HLA-DR molecules finally immobilized on latex microspheres, a system was adapted that is generally used to quantify TCR molecules on the surface of T cells (Quifikit, Dako, Glostrup, Denmark). In this assay, the sample is first labeled with a primary mouse mAb directed against the antigen of interest. Then, the samples are labeled with FITC-conjugated secondary anti-mouse mAb and compared with the staining of cell-sized beads displaying a standardized amount of mouse mAb on their surface.

The amount of HLA-DR molecules on B-LCL was calculated from flow cytometry data by reference to a standard curve of beads coated with known amounts of mouse Ig. In our hands, B-LCL UNO express approximately 100,000 HLA-DR molecules, B-LCL OF 453,000 molecules and B-LCL KaVo 361,000 molecules on their surface. This is concordant with the number of HLA-DR molecules expressed on B-LCL of about 200,000 as reported in literature surface (Valitutti et al., 1995). In contrast, latex microspheres could be coated only with about 2,500-7,000 HLA-DR molecules. It must be taken into account that the surface of a B cell is around 6 times larger than the surface of a latex microsphere when comparing the amount of coated ligands. But even

if the difference of size is considered, latex microspheres have less than the half the density of HLA-DR molecules on their surface as B-LCL.

The main problem in adaptation of the Quifikit method for latex microspheres was that "setup" and "calibration" beads of the kit are approximately of cell size (diameter of a B cell: 12 μm , of a latex microsphere: 5 μm). Thus, calibration beads and latex beads could not be measured with identical settings of forward scatter and side scatter and therefore the results obtained should be considered with care. Additionally, TCC express TCR in a more uniform density than B-LCL express MHC molecules. TCC are more homogeneous in size and morphology in contrast to B-LCL which can vary in cell size and morphology considerably depending on the cell "history".

3.7 Functional analysis of artificial cell constructs with TCC

In order to evaluate the ability of coated latex microspheres to present antigen to T cells, TCC specific for SMX or lidocaine were coincubated with coated beads or with B-LCL cells plus antigen as described in Materials and Methods.

Firstly, the DR10-restricted TCC SMX #3 or Z1.1 were each incubated with B-LCL KaVo which express HLA-DRB1*1001 in the presence of varying concentrations SMX. In a separate experiment, TCC OF B12 was incubated with autologous B-LCL and graded concentrations of lidocaine. For all TCC, a concentration dependent proliferation to drug antigen was observed (representative results see Figure 11).

Next, beads coated with anti-CD3 mAb, anti-CD28 mAb or both were incubated with TCC in the presence and absence of IL-2. Proliferation increased with increasing concentrations of IL-2 for clone Z1.1 and SMX #3. Anti-CD3 mAb coated beads could activate TCC even in the absence of IL-2. In contrast, anti-CD28 mAb coated beads were not able to activate TCC. Beads coated with both anti-CD3 and anti-CD28 were similar in their stimulating capacity to beads with anti-CD3 mAb beads only (representative results see Figure 12 B).

Interestingly, our TCC were activated by anti-CD3 mAb in the absence of IL-2, whereas in a similar experiment the anti-TCR V β 8 mAb F23.1 only provided TCC activation if costimulatory signals were delivered in the form of IL-2 or coimmobilized B7-1 molecules (Deeths et al., 1997). This finding may be explained by the fact that anti-CD3 mAb binds proximal to the cell surface, directly to the signal transducing CD3 receptor whereas mAb F23.1 binds distal to the TCR.

Finally, beads coated with HLA-DR molecules and beads coated with both, HLA-DR molecules and anti-CD28 mAb were used as artificial "APC" for drug presentation. This drug presenting capacity was tested by incubation with drug-specific T cells. T cell clones Z1.1 or SMX #3, were incubated with beads in the presence of SMX and different concentrations of IL-2. Alternatively, clone OF B12 was incubated with coated beads, lidocaine and IL-2. Only a IL-2 dependent proliferation of the drug-specific T cells was observed (representative results see Figure 12).

The failure of human HLA-DR molecules coated beads in our hands to stimulate drug-specific TCC could have various reasons. Firstly, it could be attributed to our purification method. Our HLA-DR binding studies indicate, that the detergent polidocanol interferes with the coating process on plastic (Figure 5) as well as with the coating process to latex beads (Figure 9). However Kane and Mescher demonstrated that there is no interference with the detergent Triton X-100 (Kane and Mescher, 1989). As polidocanol interferes with the coating process, a low density of HLA-DR on the beads can be the consequence. This leads to few MHC-antigen-complexes and a poor TCR stimulation is the result. In addition, HLA-DR derived from heterozygous B-LCL KaVo consisted of DRB1*0301/1001 and the effective number of TCC stimulating molecules is in fact lower as it is in the case of the homozygous B-LCL OF. It may well be that the amount of HLA-DR molecules on the beads was too low to reach the threshold of T cell activation.

HLA-DR was eluted at high pH and immediately neutralized in the collected fractions. It can not be excluded that the high pH affected the HLA-DR molecule or that the time between the collection and the neutralization of the fractions was not short enough to prevent a destruction of some parts of the molecular structure. It seems, that only the antigen binding site on HLA-DR was affected of the destruction. The mAb L243 bound well to its epitope on the $\alpha\beta$ chains and detected the HLA-DR molecules.

Basically, the beads can function as matrix for stimulating molecules as shown with Okt3 mAb (anti-CD3). Anti-CD3 mAb provides a stronger activation signal to T cells than do the MHC-antigen complexes. Antibodies typically bind with much higher affinity than the receptor binding to its native ligand and they may not accurately mimic the signal generation that occurs under physiological conditions (Nunes et al, 1994).

As the drugs bind labile to HLA molecules, a high density for stimulation of TCC is required as a consequence. It is possible that the T cell stimulation did not occur even if

the antigen epitope on HLA-DR was still functionally but that the T cell activation threshold was simply not reached.

MHC-coated microspheres have been previously used mainly coated with murine MHC class I (Goldstein et al., 1987, Mescher, 1992, Curtsinger et al.,1998). and class II molecules (Deeths and Mescher 1997). The failure of human HLA-DR molecules coated beads in our hands to stimulate drug-specific TCC could simply be due to the intrinsic difference between human and murine MHC molecules.

In order to improve the validity of the model system, studies are in process using beads coated with HLA-DR molecules and incubated with alloantigen-specific TCC. Further improvement of the latex microsphere system could be achieved with beads coated with HLA-DR molecules and pulsed with peptide to stimulate HLA-restricted, peptide-specific TCC. Peptides have a higher affinity to MHC than SMX and lidocaine and therefore more MHC molecules can trigger the T cells. This results in a lower activation threshold requirement or a lower density of MHC on the beads.

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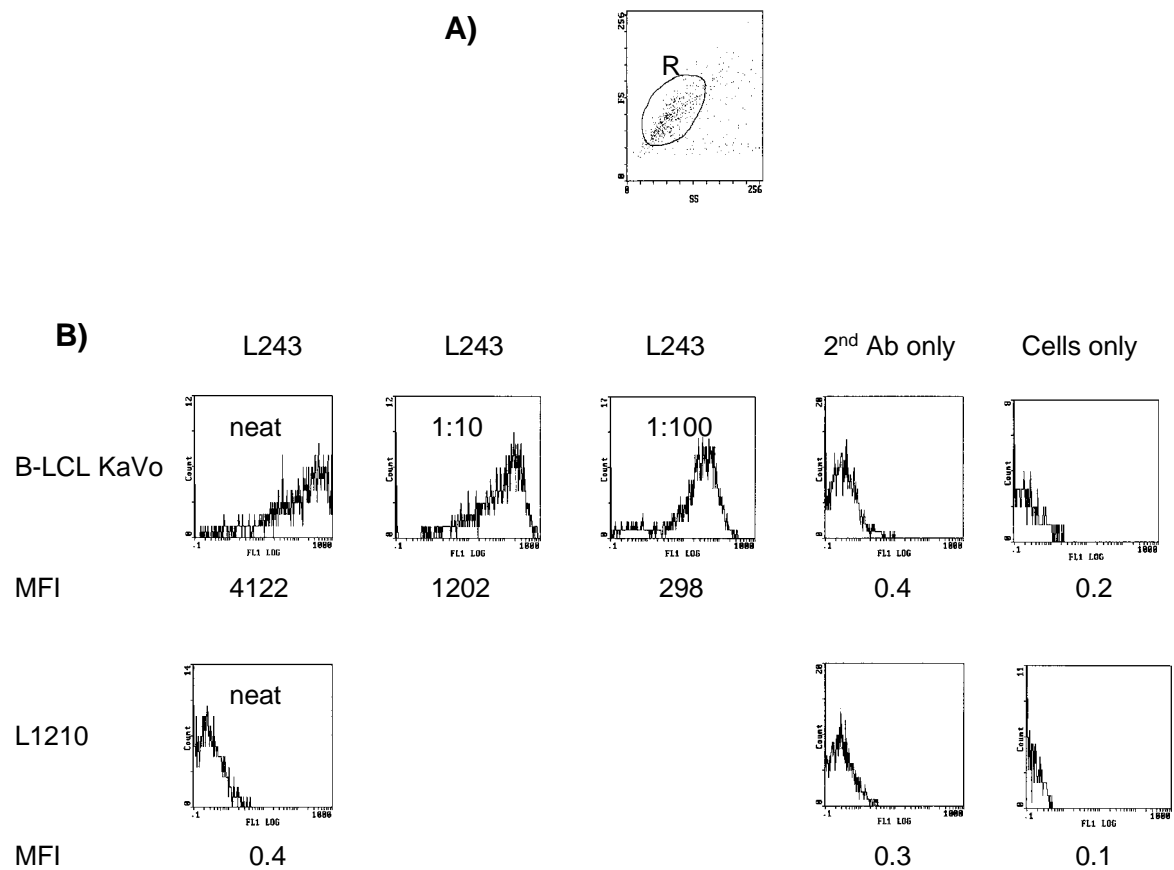


Figure 2. Characterization of mAb L243 by flow cytometry.

A) Forward versus side scatter of B-LCL KaVo. For further analysis gates were set on region R.

B) B-LCL KaVo (top row) or L1210 cells (bottom row) were incubated with the indicated dilutions of anti-DR mAb L243 followed by FITC-labeled goat-anti-mouse mAb. Values of the mean channel fluorescence intensity (MFI) are indicated below each histogram.

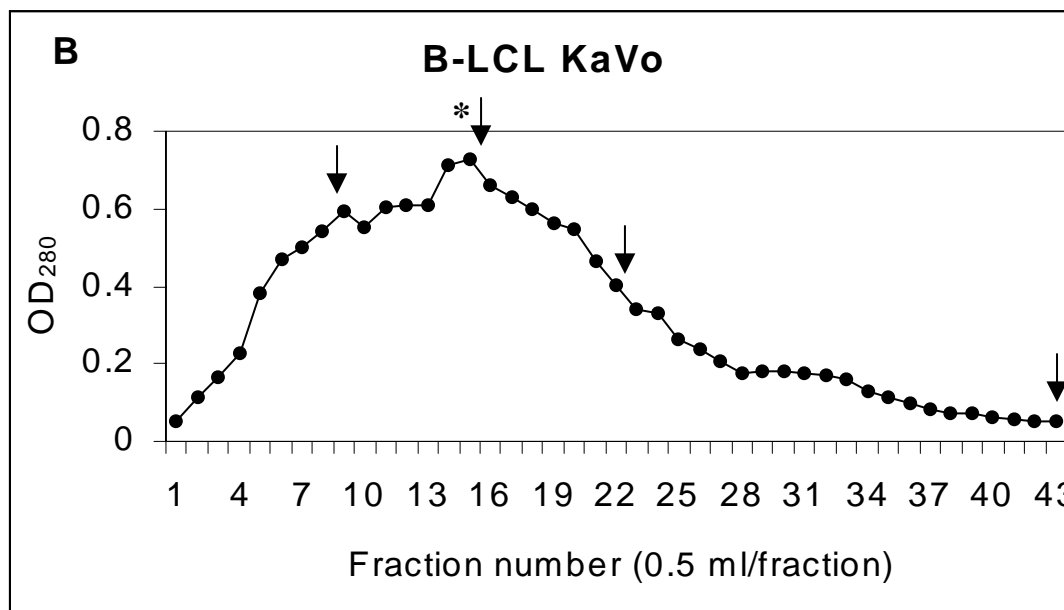
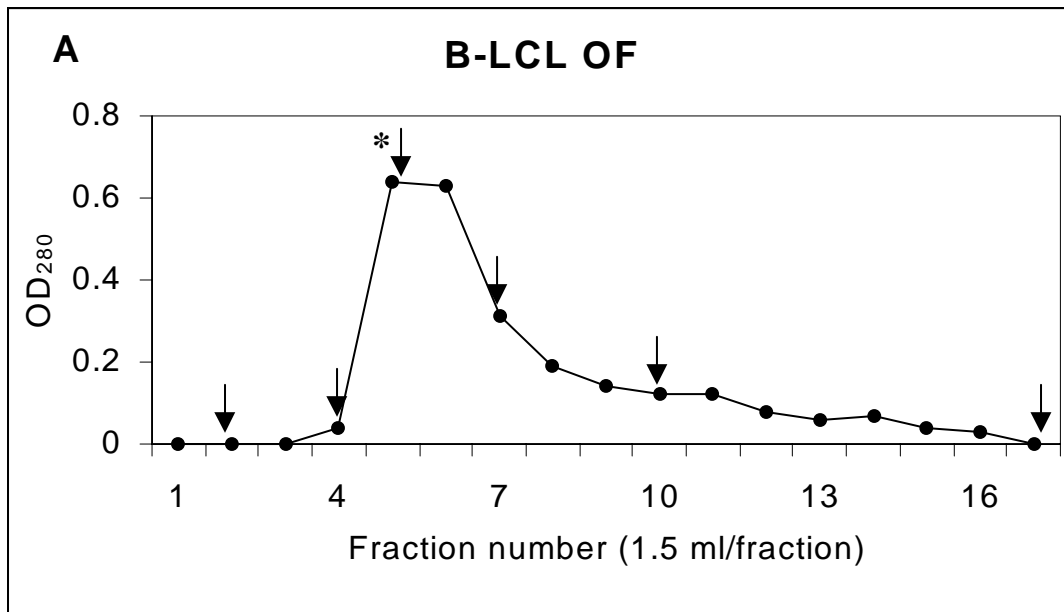


Figure 3. Elution diagrams of anti-HLA-DR affinity chromatography columns. EBV- transformed B cells were lysed and MHC class II was purified from lysate of B-LCL OF (**A**) and of B-LCL KaVo (**B**) by immunoaffinity column consisting of mAb L243 coupled to CNBr-activated sepharose. Absorbance at 280 nm of the collected fractions was measured. Arrows indicate fractions further analyzed by immunoblots. Stars indicate fractions used for immobilization on latex microspheres and ELISA.

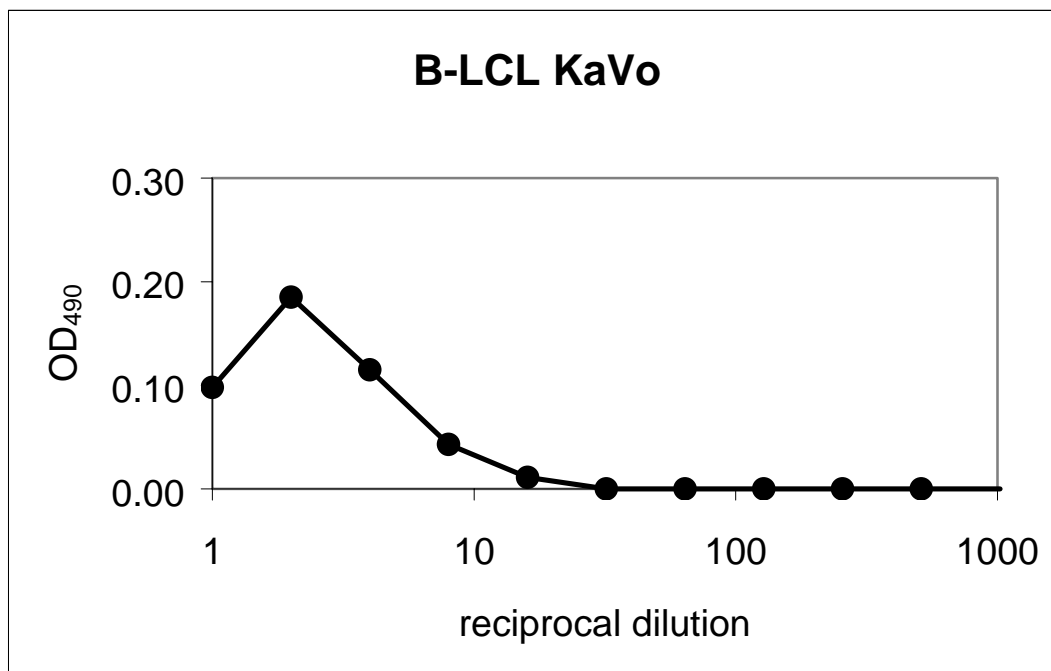
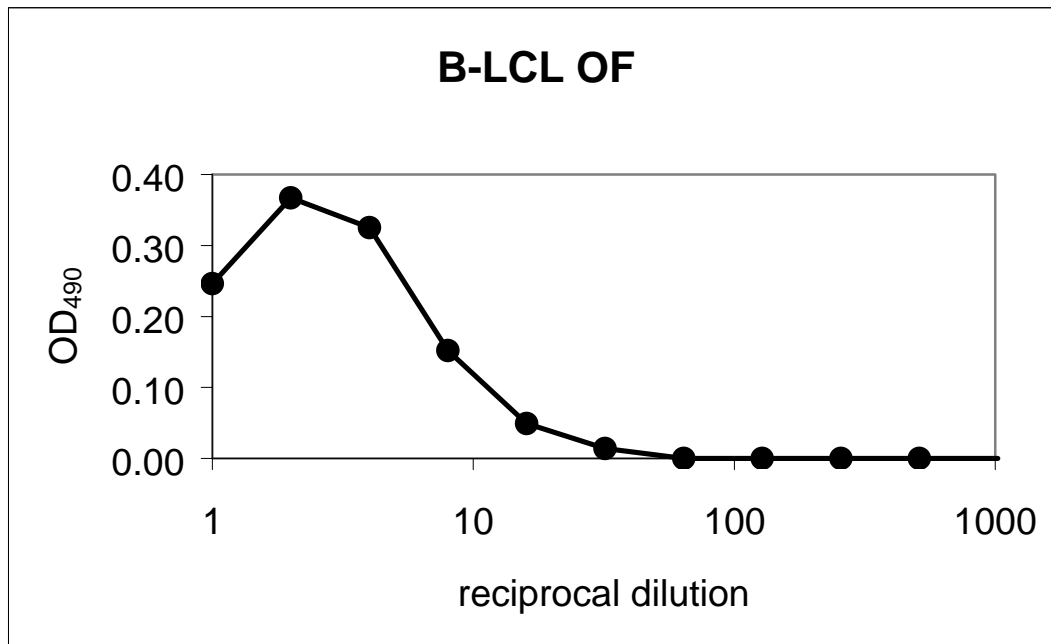


Figure 5. Direct ELISA of affinity-purified MHC molecules. Serial twofold dilutions of affinity-purified MHC molecules were coated onto ELISA plates. The wells were then assayed by direct ELISA-technique as described in Material and Methods. Mean values of duplicates are indicated. 140 μ g protein of B-LCL KaVo (fraction 14, Figure 3) was 8-fold prediluted and 140 μ g protein of B-LCL OF (fraction 5, Figure 3) was 4-fold prediluted and then coated.

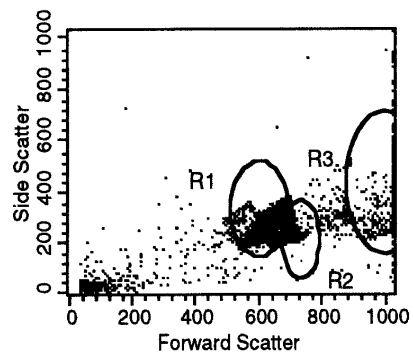


Figure 6. Characterization of microspheres by flow cytometry. Forward versus side scatter profile of 5 μm latex microspheres. Single beads were contained within R1 and R2 and aggregates of beads within R3. Gate R1 was used for phenotypic analysis.

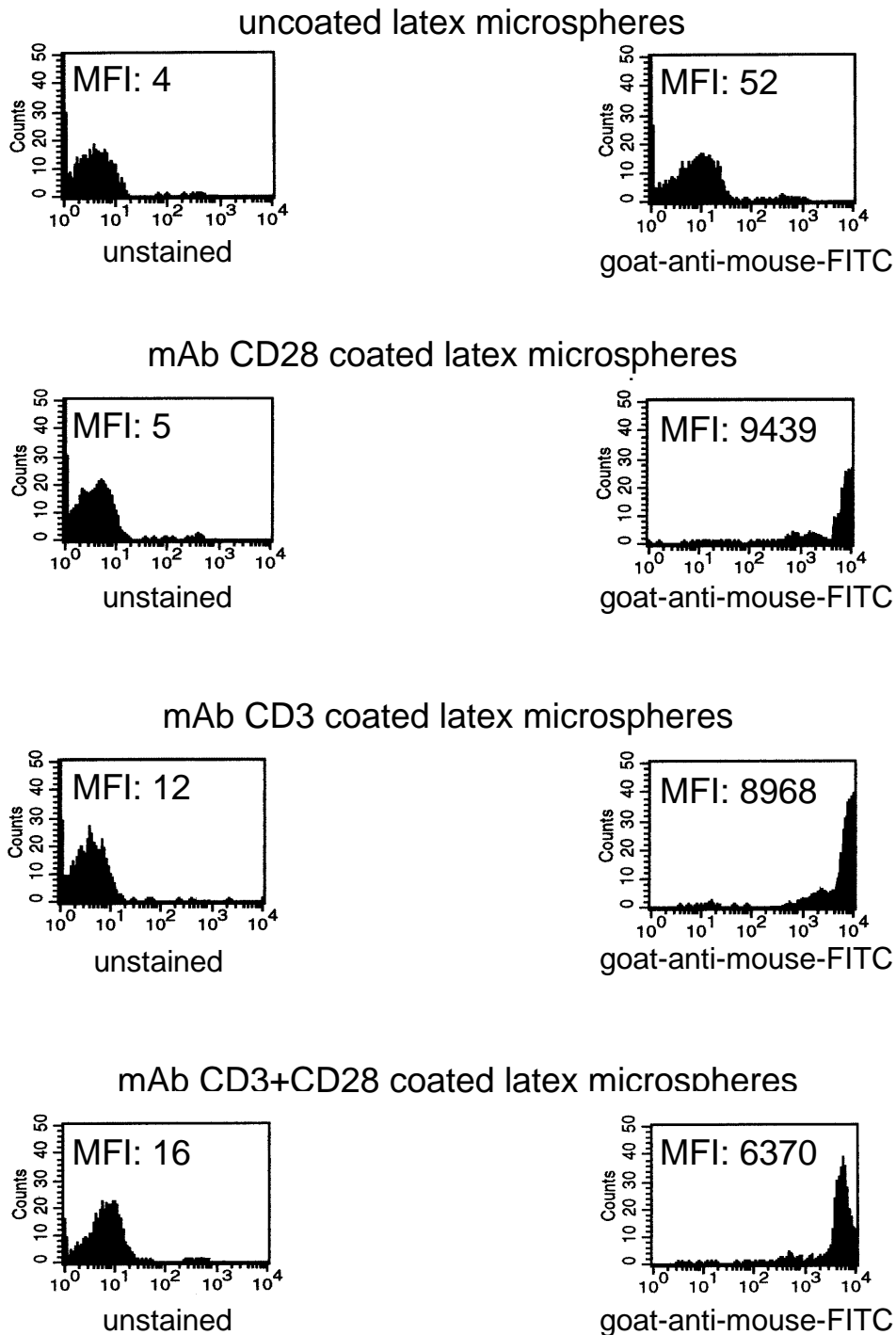
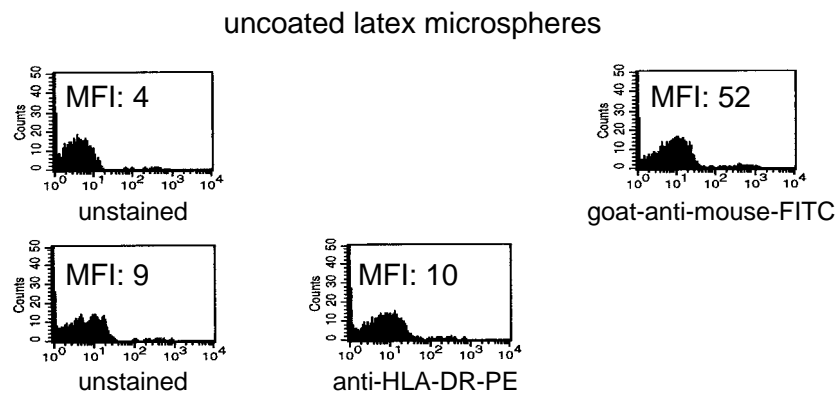
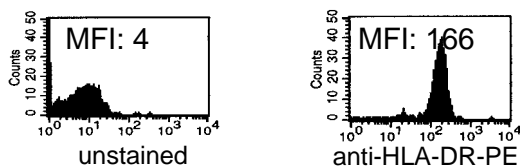


Figure 7. Characterization of mAb-coated latex microspheres by flow cytometry.

The indicated mAb were immobilized on latex microspheres as described in Materials and Methods. Thereafter, latex microspheres were stained either with FITC-labeled goat-anti-mouse mAb (right column) or left untreated (left column). Values of the MFI are indicated in the histograms.



HLA-DR of B-LCL KaVo coated latex microspheres



HLA-DR of B-LCL KaVo + mouse-anti-CD28 mAb coated latex microspheres

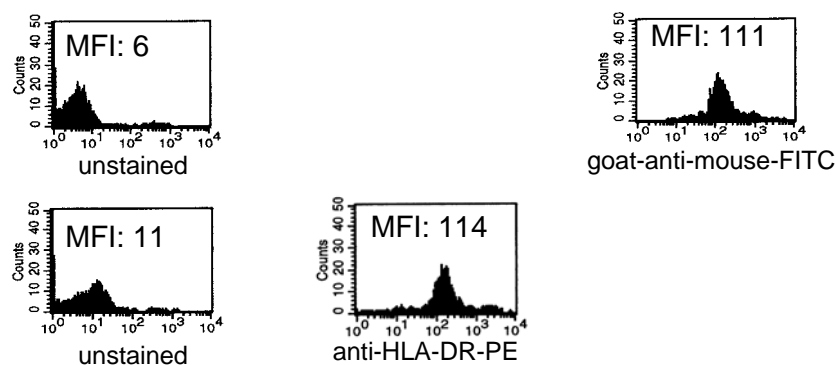


Figure 8. Characterization of MHC molecules-coated latex microspheres by flow cytometry.

Latex microspheres were coated with either HLA-DR molecules alone or with HLA-DR molecules and mouse-anti-CD28 mAb as described in Materials and Methods. Thereafter, latex microspheres were stained either with PE-labeled anti-HLA-DR mAb (middle column), FITC-labeled goat-anti-mouse mAb (right column) or left untreated (left column). Values of the MFI are indicated in the histograms.

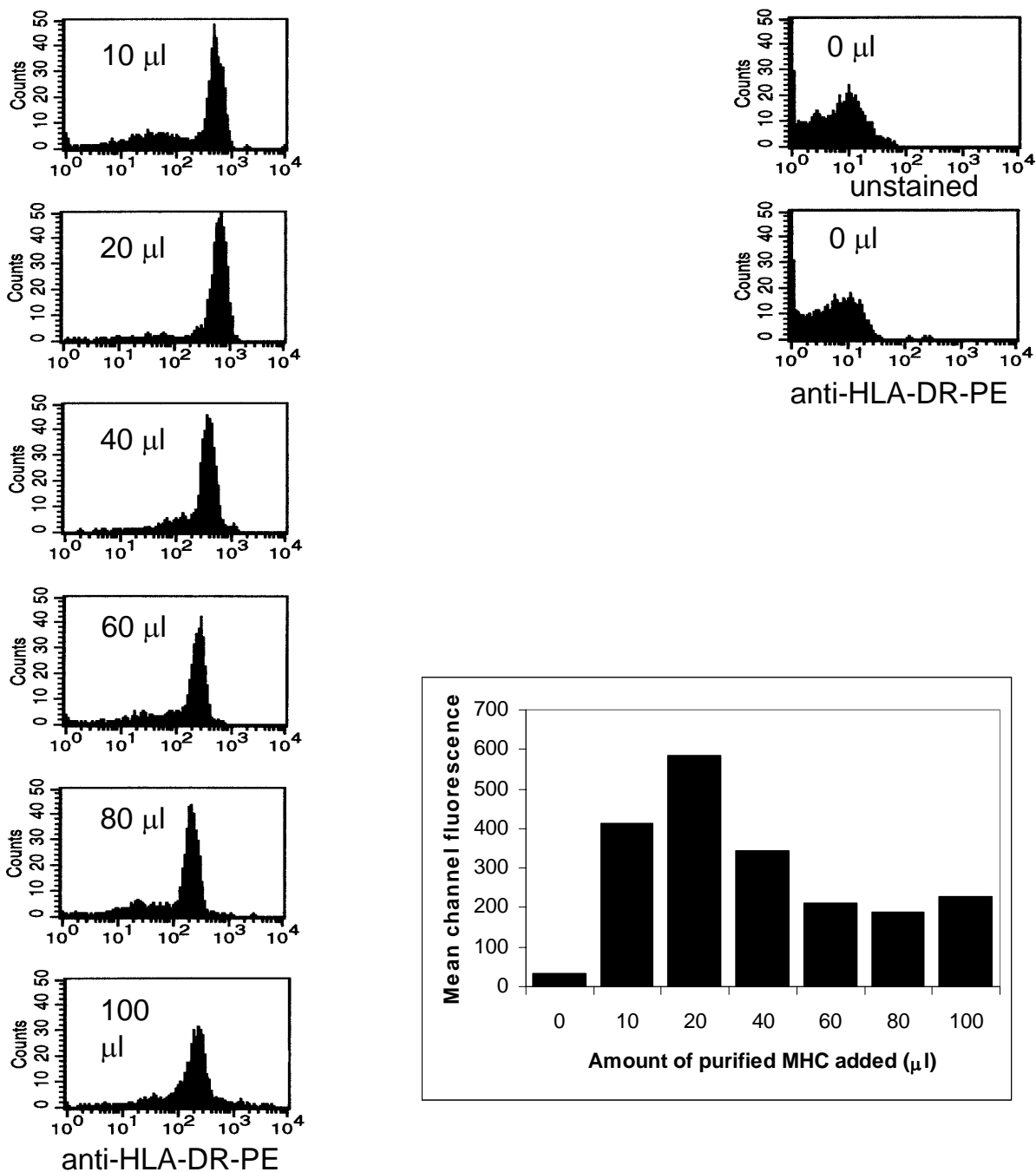
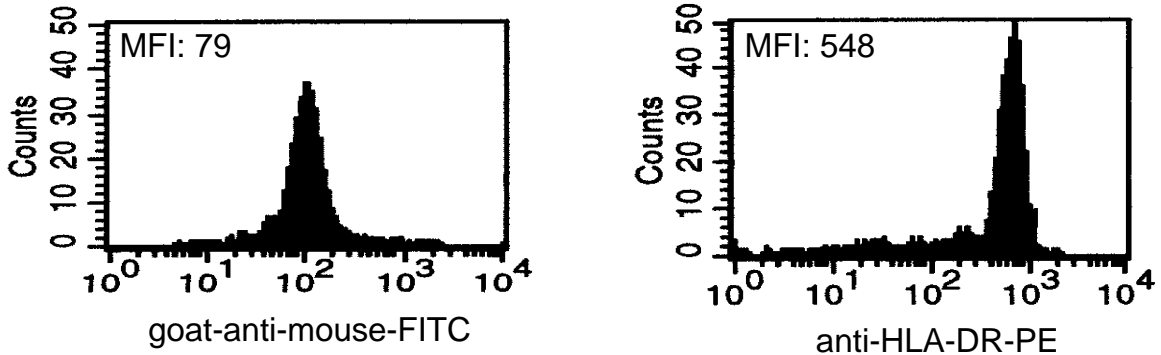


Figure 9. Optimizing of the coating efficiency for HLA-DR molecules of B-LCL KaVo on latex microspheres. Aliquots of latex microspheres coated with different amounts of MHC molecules were stained with PE-labeled anti-HLA-DR mAb. The amount of purified HLA-DR used for the coating procedure is indicated in the histograms. The values of the mean channel fluorescence intensity obtained for the different aliquots are given in the bar diagram.

Sequential immobilization of HLA-DR molecules and anti-CD28 mAb onto latex microspheres.



Simultaneous immobilization of HLA-DR molecules and anti-CD28 mAb onto latex microspheres.

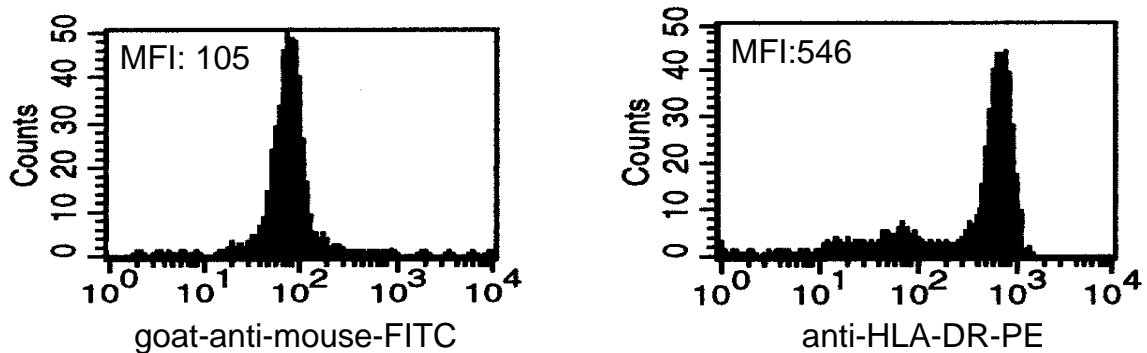
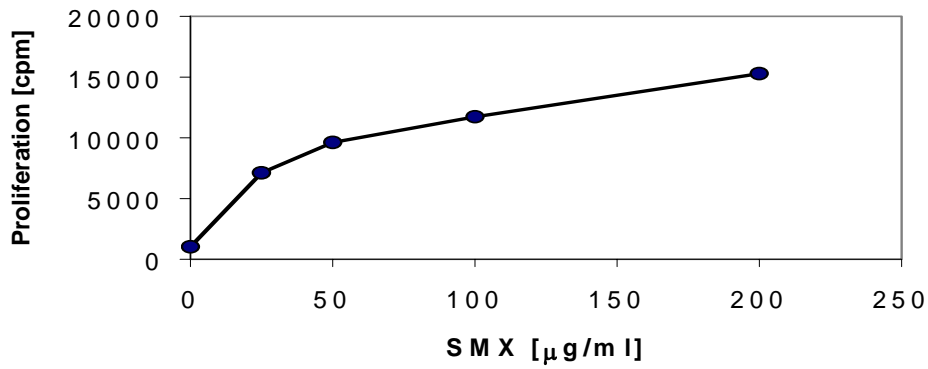
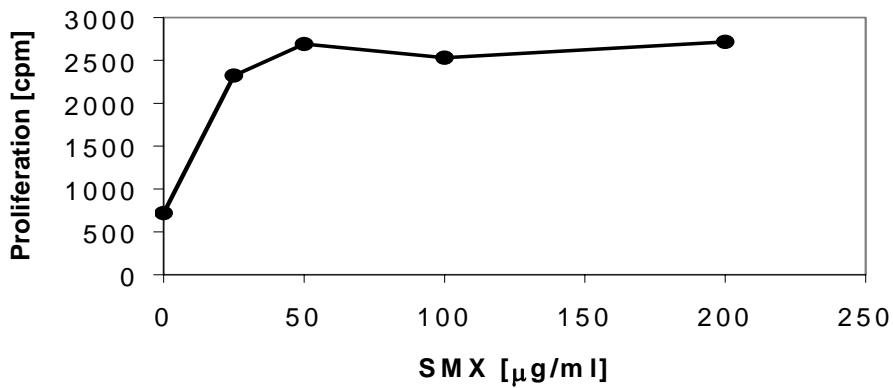


Figure 10. Comparison of the efficiency of sequential and simultaneous coating of mAb and HLA-DR molecules to latex microspheres. Latex microspheres were coated either sequentially (upper row) or simultaneously (lower row) with 20 μ l of fraction 15, Figure 3, corresponding to 28 μ g protein of B-LCL KaVo and 1 μ g anti-CD28 mAb per 10⁷ beads as described in Materials and Methods. Then, latex microspheres were stained either with FITC-labeled goat-anti-mouse mAb (left column) or with PE-labeled anti-HLA-DR mAb (right column). Values of the mean channel fluorescence intensity are indicated in the histograms.

Z1.1



SMX #3



OF B12

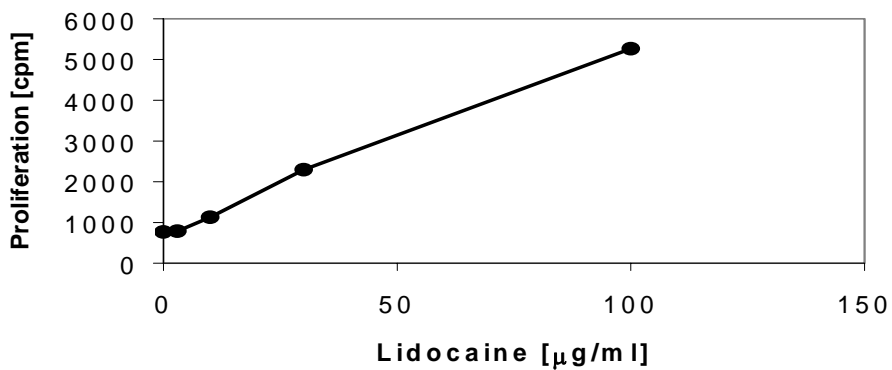


Figure 11. Concentration-dependent proliferation of TCC Z1.1 or SMX #3 to SMX and TCC OF B12 to lidocaine.

T cells were stimulated with indicated concentrations of the drug SMX in the presence of the B-LCL KaVo (Z1.1, SMX #3) or of the B-LCL OF (OF B12) as described in Materials and Methods. Proliferative response was determined after 48 hrs by [^3H]-thymidine incorporation.

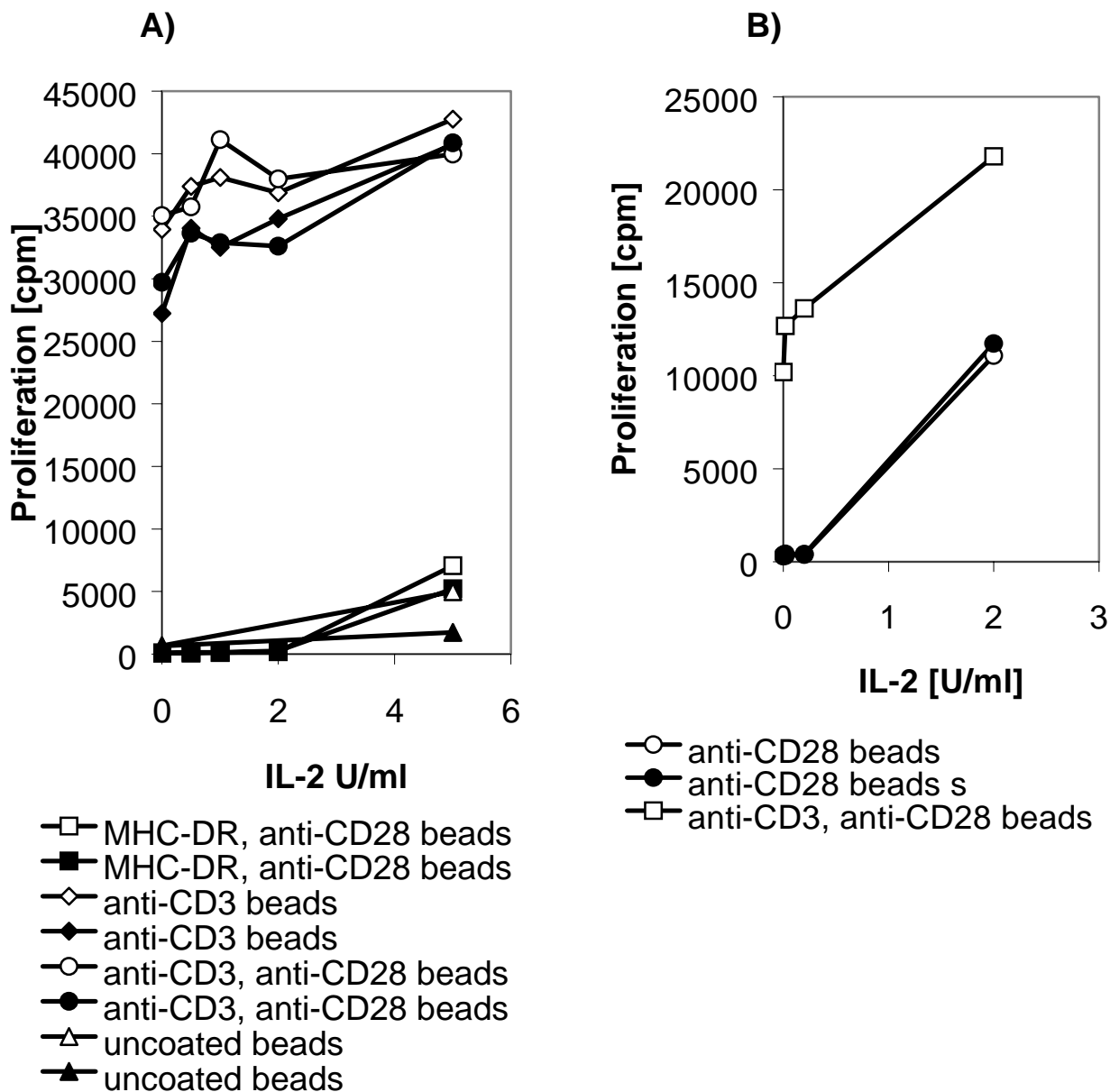


Figure 12. TCC stimulating activity of latex microspheres.

A) TCC SMX #3 or **B)** TCC Z1.1 were stimulated with different types of latex microspheres in the presence (full symbols) or absence (empty symbols) of SMX and various concentrations of IL-2 as described in Materials and Methods. Proliferative response was determined after 48 hrs by [³H]-thymidine incorporation.

IV. Curriculum vitae

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