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-IMMUNOPROPHYLACTIC APPROACH AGAINST CHEMICAL CARCINOGENESIS-

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Erklärung

Hiermit versichere ich, dass ich die wesentlichen Teile der vorliegenden kumulativen Dissertation „Immunoprophylactic approach against chemical carcinogenesis“ von mir selbstständig erarbeitet und keine anderen als die angegebenen Hilfsquellen verwendet wurden. Es wurden zwei Publikationen in Erstautorenschaft und eine in Ko-Autorenschaft sowie ein Manuskript für eine dritte Erstautorenschaft als kumulative Dissertation eingereicht, denen eine Einleitung in Form eines wissenschaftlichen Übersichtsartikels vorangestellt wurde. Diese Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt. Die Promotionsordnung ist mir in der gültigen Form bekannt.

Im Einzelnen besteht meine selbständige Forschungsarbeit aus:

- der Planung, Durchführung und Auswertung der Experimente, die in den folgenden Ergebnisdarstellungen resultieren: Kapitel 2 (Abbildung 2 und 3), Kapitel 3 (Abbildung 1-4, Tabelle 1-4), Kapitel 4 (Abbildung 1-5), Kapitel 5 (Abbildung 1-9).
- dem selbständigen Verfassen der Kapitel 1, 3 bis 5 sowie dem mit der Erstautorin Nathalie Grova gemeinsamen Verfassen des Kapitels 2 im Sinne einer geteilten Autorenschaft.
- Sowie dem Einreichen aller eingebrachten Publikationen (Kapitel 2-5) zur Veröffentlichung bei den entsprechenden Fachzeitschriften und Überarbeitung der Manuskripte im Review-Prozess.

Luxemburg 2011,

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Chapter 1

Integration of own findings in the context of current knowledge

Benzo[a]pyrene, an environmental pollutant

Benzo[a]pyrene (1,2-Benzpyrene, B[a]P) belongs to the group of polycyclic aromatic hydrocarbons (PAH) and is a ubiquitous environmental contaminant originating from both natural and anthropogenic sources. B[a]P is an air pollutant and food contaminant produced by incomplete combustion or pyrolysis of organic matter, for instance by vehicle exhaust emissions, heat and power generation, refuse burning, industrial processes, oil contamination by disposal or spills, cigarette smoke and domestic food preparation, such as smoking, drying, roasting, baking, frying or barbecuing (Hattemer-Frey and Travis, 1991; Hecht, 1999b; Phillips, 1999; Bostrom *et al.*, 2002). The emission of PAHs (including B[a]P, benzo(b)fluoranthene, benzo(k)fluoranthene and indeno(1,2,3-cd)pyrene) were estimated for France at 19 t in 2008 (source "Centre Interprofessionnel Technique d'Etudes de la Pollution Atmosphérique", (CITEPA) report April 2010), whereof 68% were attributed to residential/tertiary sources and 25% to road transport. Between 1990 and 2008, the emissions accounted a reduction of 52%. It can be assumed that the emissions are similar for other countries within Europe. Although it is possible to reduce anthropogenic sources of B[a]P and other PAH, it would be impossible for the general population, and especially for professionally exposed groups, to avoid the exposure to B[a]P, since B[a]P is a ubiquitous environmental and food contaminant. Professionally exposed groups including cooks, street workers, police officers, foundry and coke oven workers show an increased B[a]P intake compared to the general public (Omland *et al.*, 1994; Binkova *et al.*, 2007; Topinka *et al.*, 2007).

In food, vegetables are contaminated with PAHs through the deposition of airborne particles or when grown in contaminated soil. Meat, milk poultry and eggs do not normally contain high levels of PAH due to a rapid metabolism of these compounds in the species of origin. An exception is found in some marine organisms, such as mussels and lobsters, which are known to absorb and accumulate PAHs from contaminated water (Guillen *et al.*, 1997; Phillips, 1999). The European Food Safety Authority (EFSA) recently calculated the dietary exposure to PAHs for normal and high consumers based on data supplied by 17 European countries. Dietary exposure to B[a]P varied between 185 ng/day and 255 ng/day for normal population and over 700 ng/day for high consumers. The two highest contributors to the dietary exposure were to be cereals and seafood (EFSA, 2008).

Beside professionally exposed populations, cigarette smokers are particularly at risk of exposure to B[a]P and other carcinogens. According to the World Health Organisation (WHO), tobacco causes about five million premature deaths each year, and if current smoking patterns continue, it will cause over 10 million deaths each year by 2020. In most industrialised countries, tobacco smoking is responsible for 30% of cancer deaths (Shopland, 1995), which is largely linked to B[a]P (Denissenko *et al*., 1996). Remarkably, in spite of the rising anti-tobacco sentiments and improved cessation methods, there has been virtually no change in tobacco use since 1996, probably because of a hard-core population of smokers (Cancer Facts and Figures-1996, American Cancer Society, Atlanta (GA): American Cancer Society). Still one billion tobacco-consumers worldwide strain the national health care system by a high number of lung cancer cases. Tobacco smoke is the most common source of carcinogens in human, including PAHs and tobacco specific nitrosamines (i.e. 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone). Approximately 150 million deaths from tobacco use are projected worldwide for the period 2000–2024 if current smoking patterns persist; this number of deaths will not be much reduced unless a sizeable proportion of adults who are established smokers quit (source World Cancer Report 2008, WHO).

Benzo[a]pyrene uptake, absorption and distribution

Oral uptake. After oral B[a]P ingestion, at least 30% of the carcinogen absorbed from the gut but only 10% is bioavailable, most likely as a result of first-pass metabolism in the liver and hepatobiliary excretion of metabolites (Foth *et al*., 1988, Ermala *et al*., 1951). Induction of cytochrome P450 enzymes (monooxygenases) in the gut mucosa and liver may further decrease the systemic uptake of B[a]P. Orally uptaken B[a]P and its metabolites are detected in the gut, liver, lung, mammary gland and kidney and most other organs (Rahman *et al*., 1986; Yamazaki *et al*., 1987; van Schooten *et al*., 1997). Furthermore, adipose tissues may act as B[a]P deposits from which material is only slowly released (European Commission report 2002, Opinion of the Scientific Committee on Food on the risks to human health of Polycyclic Aromatic Hydrocarbons in food). Orally administrated B[a]P is also found in mammary tissues (Rees *et al*., 1971), and is detected in mouse embryo and foetuses, showing that it crosses the placenta (Neubert and Tapken, 1988).

Inhalation. Besides oral ingestion, B[a]P is also unavoidably inhaled by the general population, professionally exposed workers and of course tobacco smokers. Inhaled

B[a]P is mainly deposited (>80%) in the lung on the thin alveolar epithelium from where it is rapidly absorbed and diluted in the systemic circulation (Gerde *et al.*, 1997), exposing alveolar epithelium cells to relatively low tissue doses. In contrast, only a minor fraction is deposited on the thicker bronchiolar epithelium, and is rapidly absorbed into epithelial cells but is transported slowly into the circulating blood (Gerde *et al.*, 1997).

Metabolism of B[a]P. To exert its carcinogenicity B[a]P has to be metabolically activated (Miller and Ramos, 2001). B[a]P is a highly lipophilic compound with a low chemical reactivity until it is metabolically activated to electrophilic intermediates (Gehly *et al.*, 1979; Miller and Ramos, 2001). Multiple enzyme systems participate in the highly complex B[a]P metabolism (Figure 1). The P450 mono-oxygenase complex in the endoplasmatic reticulum initiates the reaction cascade by introducing oxygen at any position on the parent compound to form a variety of epoxides, of which 2,3-, 4,5-, 7,8-, and 9,10-isomers are the major forms (Hecht, 1999b). These epoxides can have one of three fates. First, they are converted by the microsomal epoxide hydrolase (EH) into 4,5-, 7,8- and 9,10-dihydrodiols. Second, the epoxides can spontaneously rearrange without enzyme participation into phenolic intermediates. Third, conjugates are formed chemically or are catalysed by glutathione S-transferase (GST) through covalent reaction with glutathione. Phenols and dihydrodiols can be further processed to water-soluble compounds by conjugation to either sulphate or glucuronide. B[a]P can also be converted by an alternative phase one pathway that involves one-electron oxidations to toxic radical cations that further react to become phenols and quinones. B[a]P-quinones participate in one-electron redox cycles, which lead to excessive oxidants which ultimately disrupt the antioxidant/oxidant balance in target cells to alter the redox status and induce cellular injury (Lin and Yang, 2007). The epoxide 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE) was identified as the ultimate reactive specie (Wislocki *et al.*, 1976; Jerina *et al.*, 1981) interacting with the N^2 of deoxyguanosine of the DNA and to form stable DNA adducts (Hanaoka *et al.*, 2002). DNA adducts were also found in organs that do not develop tumours, indicating that tissue-specific risk factors, such as the proliferative capacity, may also contribute to tumour induction (de Vries *et al.*, 1997).

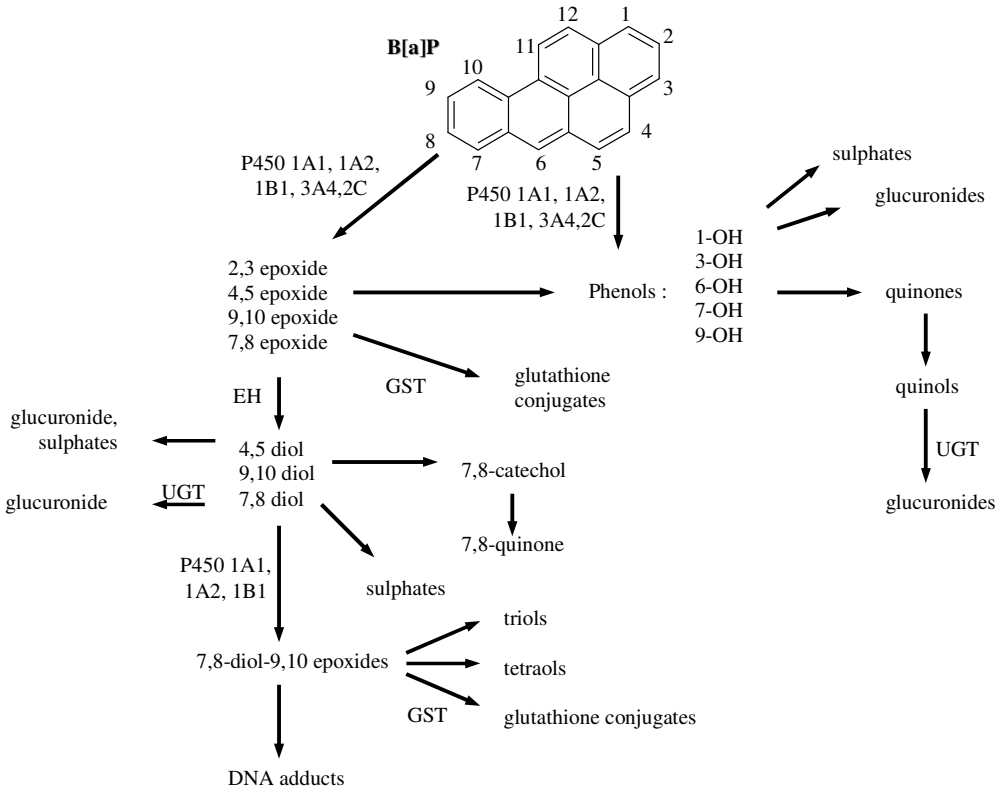

Figure 1: Metabolic pathways of B[a]P. EH = epoxide hydrolase; UGT = UDP-glucuronosyl transferase; GST = glutathione S-transferase; P450 = cytochrome P450; 1-OH, 3-OH = 1-hydroxy B[a]P, 3-hydroxy B[a]P, etc. (Adapted from Hecht, 1999).

B[a]P excretion. Upon oral B[a]P intake, 65-80% are secreted by hepatobiliary excretion into the faeces in form of metabolites such as 3-OH-B[a]P, 9-OH-B[a]P, B[a]P-3,6- and B[a]P-1,6-quinone, and trace amounts of 4,5- and 7,8-diol-B[a]P (Heidelberger and Weiss, 1951; O'Neill *et al.*, 1990a; O'Neill *et al.*, 1990b). This is also the case after intravenous injection of B[a]P in rats (Heidelberger and Weiss, 1951). Similarly, intravenous B[a]P injection into rats revealed that, after 24 hours, 65 % and 18% are excreted into the faeces or urine, respectively (Heidelberger and Weiss, 1951). Importantly, the gastrointestinal microflora has been shown to hydrolyse detoxified phase 2 B[a]P-conjugates leading to reabsorption of B[a]P metabolites by enterohepatic cycling (Renwick and Drasar, 1976; Chipman *et al.*, 1981).

Benzo[a]pyrene-induced toxicity

Animal studies have shown various toxicological effects of B[a]P, including haematological, reproductive and developmental toxicity as well as immunotoxicity. However, the carcinogenic and genotoxic potential of B[a]P has attracted most attention. These seemingly unrelated biological actions are believed to be mediated by the sustained activation of the arylhydrocarbon receptor (AhR) and the subsequent disturbance of cellular homeostasis (Figure 2). The AhR is a ligand dependent transcriptional regulator of genes involved in xenobiotic metabolism (e.g. *Cyp1a1, Cyp1a2, Cyp1b1, Gsta, Gstm, Nqo1, Ugt, Aldh-3*), oncogenes (e.g. *c-Fos, c-Jun. c-Erb-A, Bcl-2, Bax*), growth factors and receptors (e.g. *Pal-2, Il-2β, Tgf-α, Tnf-α*) (Lai *et al.*, 1996) and is probably implicated in the mediation of its toxicity. Shimizu and colleagues showed that B[a]P induced subcutaneous tumours in AhR-positive mice at the site of injection, which was abolished in AhR-deficient mice (Shimizu *et al.*, 2000).

Mechanisms of carcinogenicity. B[a]P is a very effective pulmonary carcinogen in human and in rodents (Perera, 1981; Likhachev *et al.*, 1992). The total dose experienced by a smoker in a lifetime is remarkably close to the lowest total dose shown to induce tumours in rats (Hecht, 1998). The ability of B[a]P to induce lung tumours is independent of its route of administration as it has been shown by local intratracheal instillation or inhalation (Thyssen *et al.*, 1981; Wolterbeek *et al.*, 1995) or when administrated systemically (Hecht *et al.*, 1994; Weyand *et al.*, 1995).

The AhR plays an important role in the B[a]P-induced carcinogenesis (Figure 2). Animal studies showed a significant correlation between the inducibility of the arylhydrocarbon hydroxylase activity and lung carcinogenesis induced by B[a]P (Ross *et al.*, 1995). Using cultured human lymphocytes, it has been shown that people expressing a high-inducibility phenotype had an increased risk of developing lung cancer (McLemore *et al.*, 1978).

Studies have demonstrated that mutant AhR cell lines and AhR null allele mice are relatively resistant to chromosomal damage induced by cigarette smoke, which are rich of PAHs. Furthermore, B[a]P induced tumours in wild type but not in the AhR null allele mice (Shimizu *et al.*, 2000), suggesting an important role of the AhR in B[a]P

induced carcinogenesis. This was strengthened by Dertinger *et al.* , who showed that an AhR antagonist does not influence CYP1A1 activity and thereby protects against B[a]P-mediated bone marrow cytotoxicity and genotoxicity suggesting a net potentiation effect of the AhR signalling on B[a]P induced genotoxicity (Dertinger *et al.*, 2001).

Apart from AhR activation, B[a]P mediated carcinogenicity can also be induced by its genotoxicity. Human lung and liver metabolically activate B[a]P to BPDE (Prough *et al.*, 1977). In human lung, DNA adducts of B[a]P have been detected (Hecht, 1996b; Boysen and Hecht, 2003). With 90% is the N^2 position of deoxyguanosine residues the primary target of BPDE, however it is also described that it acts at N^6 position of adenine, producing the minor N^6-deoxyadenosine adduct (Hanaoka *et al.*, 2002). Metabolic manipulations by isothiocyanites that decrease the formation of DNA adducts, without lowering levels of chemical exposure, have been shown to reduce the number of tumours (Hecht, 1996a; Hecht, 2001). Mechanistic studies have shown that the chemopreventive activity of isothiocyanates, such as phenethyl isothiocyanate and benzyl isothiocyanate, who modify the carcinogen metabolism specifically via inhibition of Phase 1 enzymes and/or induction of Phase 2 enzymes, result in increased carcinogen excretion or detoxification and decreased carcinogen DNA interactions (Hecht, 1999a).

BPDE adducts have been linked to G:C to T:A transversions in the *Tp53* gene at an unusual series of mutational hotspot codons in smoking-associated lung cancer (Hainaut and Pfeifer, 2001). Persistent mutations occurring in critical regions of oncogenes, such as *Ras* and *Myc* or tumour suppressor genes such as *Tp53*, can result in the aberration or loss of normal cellular growth-control mechanisms and subsequent cancer development (Osada and Takahashi, 2002).

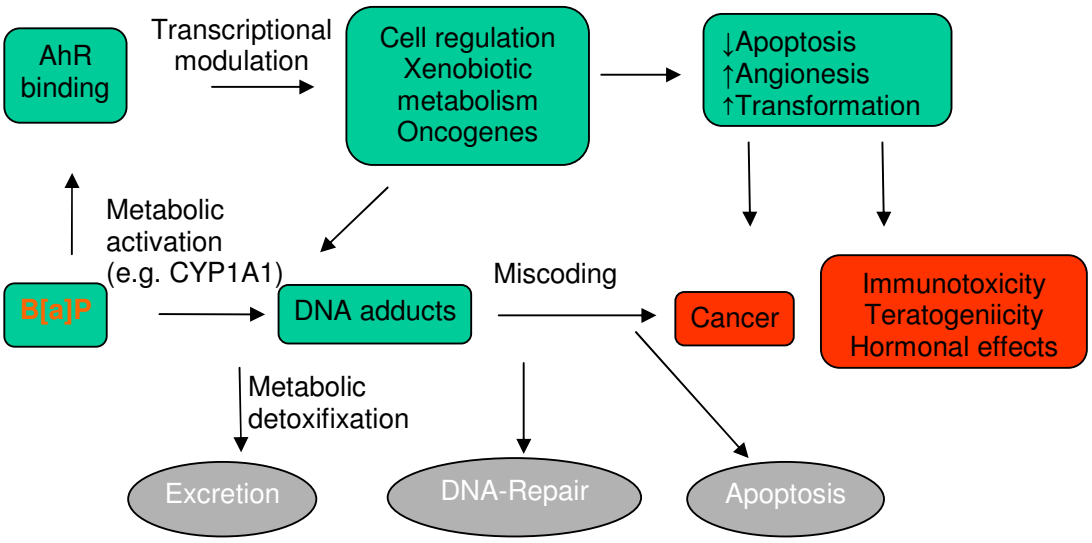

Figure 2: Mechanism of B[a]P induced toxicity. B[a]P has the potential to induce its toxic effects by two known mechanisms. The binding of B[a]P or its activated metabolites leads to a transcriptional modulation of genes involved in cell regulation, xenobiotic metabolism or oncogenes. In addition, the formation of DNA adducts by activated metabolites can initiate mutations which also ends up in tumour formation. The adduct formation can be increased by activation of genes which are essential for the metabolism of B[a]P via the AhR pathway. The metabolic detoxification, DNA repair and apoptosis are processes that protect from cancer formation.

Benzo[a]pyrene-induced immunotoxicity. It has been shown that AhR activation can influence PAH mediated immunosuppression (Near *et al.*, 1999). The diversity of PAH immunotoxic outcomes suggest that PAHs interfere with the lymphocyte programmed cell death/apoptosis machinery Quadri and colleagues for example demonstrated the induction of pre-B cell apoptosis (Quadri *et al.*, 2000). Among other PAHs, B[a]P inhibits murine T and B cell proliferation, alters T cell-related cytokine production and B cell-mediated antibody production (White and Holsapple, 1984; Blanton *et al.*, 1988; Pallardy *et al.*, 1989). It suppresses mitogenesis of human T lymphocytes (Mudzinski, 1993) and alters B cell lymphopoiesis through triggering pre-B lymphocyte apoptosis (Yamaguchi *et al.*, 1997). Besides lymphocytes, antigen presenting cells, such as macrophages and dendritic cells can also be affected by PAHs. Indeed, PAHs impair antigen presentation by mouse macrophages (Myers *et al.*, 1987), alter T cell-macrophage interactions (Myers *et al.*, 1988), and suppress

phagocytic activity of peritoneal macrophages (Tewari *et al.*, 1979). B[a]P also reduced esterase-positive macrophagic cell population in mouse spleen (Blanton *et al.*, 1986). Among the different splenic cell populations, murine splenic macrophages are considered to be the major cell type targeted by B[a]P, and have been demonstrated to metabolise PAHs, and to be responsible for the PAH-related suppression of the splenic humoral immune response (Ladics *et al.*, 1992a; Ladics *et al.*, 1992b).

The B[a]P induced immunotoxicity is most likely dependent on B[a]P metabolites. Both, Galvan and Uno, showed that CYP1B1 expression in the spleen and bone marrow is responsible for the metabolic activation of B[a]P, which results in immune damage (Galvan *et al.*, 2003; Uno *et al.*, 2006).

Hapten immunisation against low molecular weight compounds

Immunotherapy. For numerous low molecular weight compounds, it has been shown that antibodies are capable of inhibiting the physiological or pharmacological effects of the corresponding haptens (Colburn, 1980; Butler, 1982; Sullivan, 1986; Scherrmann *et al.*, 1989; Bismuth *et al.*, 1997). Major examples include snake and spider venoms, drugs with narrow therapeutic indices (e.g., digitalis glycosides), and endogenous ligands (e.g., cytokines, hormones). Several of these antibodies have been approved by the Food and Drug Administration (FDA) (Lobo *et al.*, 2004). In contrast, conjugate vaccines generating prophylactic hapten-specific antibodies have been less extensively investigated. Such an immunoprophylactic approach may be useful for vaccination against drugs of abuse (Kantak, 2003), including heroin, cocaine, and methamphetamine. Positive results have been reported from clinical trials with a vaccine against nicotine and cocaine (Kosten *et al.*, 2002). Nabi Pharmaceuticals has reached an agreement with the FDA for a pivotal Phase 3 clinical trial of a nicotine conjugate vaccine (NicVax®). This vaccine is dedicated to treat nicotine addiction and prevent smoking relapse by stopping nicotine passing the blood-brain barrier. GlaxoSmithKline Biologicals SA (GSK) and Nabi Biopharmaceuticals announced an exclusive worldwide option and licensing agreement for NicVAX® (www.nabi.com).

Immunoprophylaxis against carcinogens. In 1937, the work of Creech and Franks provided the first evidence that immunisation of animals with dibenzanthracene protein conjugates results in the appearance of antibodies to the carcinogen and inhibits the development of dibenzanthracene-induced tumours (Creech and Franks, 1937). The possibility of active immunisation against specific carcinogens was proposed in 1996 by the induction of a humoral immunity towards 2-acetylaminofluorene in a mouse model (Verdina *et al.*, 1996). In 2001, Rasmussen *et al.* demonstrated that orally administrated, carcinogen-specific antibodies could sequester fed carcinogens, resulting in reduced gastrointestinal absorption and increased excretion (Rasmussen *et al.*, 2001). However, conjugate vaccines generating prophylactic hapten-specific antibodies have been barely investigated. A prophylactic immune strategy for blocking or preventing chemical carcinogenesis may be as useful as similar strategies used to prevent drug dependence; however, this has never been systematically studied. Our own previous work provides strong evidence in supporting this concept (De Buck *et al.*, 2005a; De Buck *et al.*, 2005b; De Buck and Muller, 2005; De Buck *et al.*, 2009).

The role of adjuvants in immunisation

To achieve an enhanced and prolonged immune response (humoral and cellular), potent immunogens must be administrated in combination with adjuvants, such as Freund's adjuvant, QS21, Montanide ISA-51, MF-59 etc. Existing adjuvants acting as an antigen delivery system or as immunopotentiators and can be divided into different mechanistic classes (Table 1). Nevertheless, their detailed mechanism of action remains unclear. While numerous adjuvants are already tested in animals, only a limited number is licensed by the FDA for human use. Aluminium salt/gel (alum) based adjuvants are commonly used, whereas AS04, a combination of aluminium hydroxide and monophosphoryl lipid A, as well as MF-59 (licensed in Europe), are only licensed for vaccines to prevent cervical cancer, caused by human papillomavirus or in vaccines against pandemic influenza, respectively.

Table1: Examples of mechanistic classes of adjuvants.

Antigen delivery systems	**Immunopotenciators**
Insoluble aluminium compounds	MPL and synthetic derivates
Calcium phosphate	MDP and derivates
Liposomes	Oligonucleotides
Virosomes™	Double stranded RNA
ISOMS®	PAMPs
Microparticles	Saponins
Emulsions	Small molecule immune potentiators
Virus-like particles and viral vectors	Cytokines and chemokines

MPL, Monophosphoryl Lipid A; MDP, N-acetyl-muramyl-L-alanyl-D-isoglutamine; PAMPs, pathogen-associated molecular patterns.

The role of carriers in hapten immunisation

B and T-cells largely vary in their ability to recognize antigenic components. B cells recognize proteins (both conformational determinants and determinants exposed by denaturation or proteolysis), nucleic acids, polysaccharides, lipids and small chemicals (haptens) in soluble form. In contrast, the majority of antigens for T cell antigens are processed proteins (Kaumaya *et al.*, 1990; Kobs-Conrad *et al.*, 1993). Antigen processing and presentation are processes that result from the fragmentation of proteins (proteolysis), the association of these peptides with the major histocompatibility complex (MHC) molecules, and the expression of peptide-MHC molecules at the cell surface, where they can be recognized by T cell receptors on T cells. To induce a potent immune response, the formulation of an effective immunogen should be composed of a B cell epitope that mimics the three-dimensional conformation of the antigen and a T cell epitope, which is produced during the antigen processing in antigen presenting cells, such as macrophages and dendritic cells. In particular, for low immunogenic compounds, like B[a]P, a conjugation to an immunogenic carrier (T cell epitope) is essential to induce antibodies against it. A number of “promiscuous” T cell epitopes from tetanus toxin, measles virus, hepatitis A and B virus, food-and-mouth disease virus and human

papillomavirus have been reported to be universally immunogenic (Demotz *et al.*, 1989; Panina-Bordignon *et al.*, 1989; Ho *et al.*, 1990; Partidos and Steward, 1990; Ivanov *et al.*, 1994; Doh *et al.*, 2003). To induce antibodies against small molecules, the conjugation of the chemical to a carrier protein, such as cholera B, tetanus toxoid and pseudomonas aeruginosa r-Exoprotein A was successfully for cocaine, heroin, morphine and nicotine respectively (Kosten *et al.*, 2002; Anton and Leff, 2006). Some of these conjugates have already been or are currently tested in clinical trials (Kosten *et al.*, 2002; Hatsukami *et al.*, 2005; Akbarzadeh *et al.*, 2007), indicating that a use of hapten-conjugates in humans is feasible.

Summary and concluding remarks

B[a]P is an environmental carcinogen and although it is possible to reduce anthropogenic sources, it would be impossible for the general population to avoid exposure to it. Since only metabolically activated B[a]P exerts carcinogenic properties, a prophylactic immunisation against B[a]P could potentially be beneficial for professionally exposed individuals, the population at large and smokers who cannot control their habit. By inducing antibodies against B[a]P using hapten carrier conjugates one might able to sequestrate B[a]P and its metabolites in the blood stream. A number of studies already indicated the use of an immunological approach against side effects of low molecular compounds, such as nicotine (Hasman and Holm, 2004; Mihaltan, 2007; Wagena *et al.*, 2008), heroin (Anton and Leff, 2006), cocaine (Martell *et al.*, 2005; LeSage *et al.*, 2006) but also against carcinogens (Peck and Peck, 1971; Silbart and Keren, 1989). Most of these studies are however designed to stop the addiction to the drug or its abuse by demonstrating that antibodies reduced the ability of the drug to cross the blood-brain barrier. The main difference compared to our approach is the prophylactic manner of immunisation to prevent carcinogenesis, which is difficult to prove in existing experimental animal models.

Reducing the number of lung cancer cases by an immunoprophylactic vaccination would considerably decrease the costs to the national healthcare systems worldwide. While our study focuses on B[a]P as a prototype PAH, the results may be in principle also applied to other chemical carcinogens and environmental toxins.

Research strategy of the thesis

General objective of the research thesis

The overall aim of this project was to explore the potential of a prophylactic immune strategy based on carcinogen-specific antibodies, to lower the risk of chemical carcinogens and to deliver a proof of concept for such an immunisation strategy. As a model for chemical carcinogens, we selected B[a]P for this study, the most studied PAH. Previous work done in our lab has already demonstrated that specific B[a]P antibodies protect cells *in vitro* against adverse effects of carcinogens. We showed that monoclonal anti-B[a]P antibodies were able to reduce the uptake of B[a]P into the cell as well as the transport across Caco-2 monolayers (De Buck *et al*., 2005a; De Buck *et al*., 2005b; De Buck and Muller, 2005). Consequently, the activity of B[a]P metabolising enzymes (CYP1A1) and subsequent DNA adduct formation was reduced. Based on these results, we further investigated the influence of antibodies on detrimental effects of B[a]P *in vivo,* which is part of this thesis.

Main findings and conclusions

In the present study, we developed B[a]P conjugate vaccines coupled to ovalbumin (OVA), tetanus toxoid (TT) and diphtheria toxoid (DT) and investigated the effect of the resulting specific antibodies on the bioavailability of this ubiquitous carcinogen and its metabolites. As shown in **chapter 2**, B[a]P-DT conjugates induced the most robust immune response. The antibodies reacted not only with B[a]P but also with the proximate carcinogen 7,8-diol-B[a]P. Antibodies modulated the bioavailability of B[a]P and its metabolic activation in a dose-dependent manner by sequestration in the blood. These findings indicate that protection by B[a]P specific antibodies is warranted.

To further demonstrate the protective effect of our immunoprophylactic strategy, we studied the modulation of the B[a]P-induced immunogenicity by specific antibodies. Most of the existing models for carcinogenesis use high concentrations of B[a]P to aim a short-term readout. However, high concentrations cannot be stoichiometrically matched by concentrations of specific antibodies that are normally reached after immunisation and are therefore inappropriate to assess whether antibodies may prevent B[a]P induced carcinogenesis and related detrimental effects. For this reason, we investigated the effect of B[a]P-specific antibodies on the pharmacokinetic and

immunotoxicity as well. Both parameters are already detectable at environmentally relevant concentrations of B[a]P, which can be easily matched by antibodies induced by active immunisation with B[a]P-carrier conjugates. In **chapter 3** we demonstrated that an active immunisation with B[a]P conjugates, based on carrier proteins used in licensed human vaccines (tetanus toxoid), increased levels of B[a]P and its metabolites in the blood by sequestration. B[a]P significantly suppressed the proliferative response of both T and B cells after a sub-acute administration of B[a]P, an effect that was completely reversed by vaccination. Furthermore, the immunotoxic effect of B[a]P on IFN-γ, IL-12, TNF-α production and reduced B cell activation was restored in immunised mice.

To further strengthen the potential use of a prophylactic immunisation against B[a]P in humans, we tested in **chapter 4** the potential compatibility of different adjuvants with their use in humans. We showed that all adjuvants tested induced specific antibodies against B[a]P and its carcinogenic metabolite 7,8-diol-B[a]P. The highest antibody levels were obtained with the adjuvants Quil A, MF-59 and Alum. Biological activity in terms of enhanced retention of B[a]P was confirmed in mice immunised with Quil A, Montanide, Alum and MF-59. Our findings demonstrate that a vaccination against B[a]P is feasible in combination with adjuvants licensed in humans.

To further improve our immunisation strategy, we tested the possibility of promiscuous T cell epitope peptides from tetanus toxoid as carrier to induce B[a]P specific antibodies. In **chapter 5** we demonstrated that a vaccination against B[a]P, using “promiscuous” T-helper cell epitopes as a carrier, is feasible. Furthermore, some tested peptide conjugates were more immunogenic than the whole protein conjugates, resulting in antibodies with increased specificity. In addition, a pre-exposure to the tetanus toxoid by vaccination, in respect to the high vaccination recovery rate for tetanus toxoid, did not negatively influence the immune response against B[a]P. These observations strengthen the possible applications of immunoprophylactic vaccinations against low molecular weight carcinogens in humans.

To follow up on these studies, we are currently aiming to demonstrate the long-term protection with our immunisation strategy as well as inhibition of B[a]P-mediated carcinogenesis. The effect on carcinogenesis will be assessed by using a XPA knockout mouse model. The *XPA* gene codes for the xeroderma pigmentosum group

A protein and is involved in the nucleotide excision DNA repair system. Mutations or deletions in this gene will result in increased DNA damages and thus increased mutation rates. In addition, we will to study the effect of our immunisation strategy on the mutation frequency and on DNA adduct formation as a measure for carcinogenesis (Ide *et al.*, 2000).

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Chapter 2

Modulation of carcinogen bioavailability by immunisation with benzo[a]pyrene-conjugate vaccines

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Abstract

Benzo[a]pyrene (B[a]P) conjugate vaccines based on ovalbumin, tetanus toxoid and diphtheria toxoid (DT) as carrier proteins were developed to investigate the effect of specific antibodies on the bioavailability of this ubiquitous carcinogen and its metabolites. After metabolic activation of this prototype carcinogen, B[a]P forms DNA adducts which initiate chemical carcinogenesis. B[a]P-DT conjugate induced the most robust immune response. The antibodies reacted not only with B[a]P but also with the proximate carcinogen 7,8-diol-B[a]P. Antibodies modulated the bioavailability of B[a]P and its metabolic activation in a dose dependent manner by sequestration in the blood. Our results showed that this immune prophylactic strategy influences the pharmacokinetic of B[a]P and further studies to investigate their effects on chemical carcinogenesis are warranted.

1. Introduction

Hapten-specific antibodies have been widely applied to reverse pharmacological effects of biologically active low molecular weight compounds (Butler and Beiser, 1973; Bismuth *et al.*, 1997; Silbart *et al.*, 1997). While in most of these studies antibodies have been explored as therapeutic agents, their potential as immunoprophylactic agents has received much less attention (Silbart *et al.*, 1988; Rasmussen and Silbart, 1998; Rasmussen *et al.*, 2001; De Buck *et al.*, 2005a; De Buck *et al.*, 2005b). In particular, carcinogen-specific antibodies have been proposed to reduce chemical carcinogenesis after occupational exposure or in cigarette smokers (De Buck and Muller, 2005). Antibodies against polycyclic aromatic hydrocarbons (PAHs) have also been found in humans exposed to high levels of these carcinogens (Harris *et al.*, 1985; Haugen *et al.*, 1986; Newman *et al.*, 1988; Newman *et al.*, 1990; Glushkov *et al.*, 1997; Glushkov *et al.*, 2004). However, the significance of such antibodies with respect to chemical carcinogenesis is poorly understood.

In a complex metabolic process, the majority of benzo[a]pyrene (B[a]P) is converted to detoxified endpoint metabolites. Phenols and dihydrodiols are further processed to water-soluble compounds by conjugation to either sulphate or glucuronides that can be excreted. In the same process, a small fraction of B[a]P is activated to the intermediate metabolite 7,8-diol which converts to the highly reactive 7,8-dihydroxy-9,10-epoxide-7,8,9,10-tetrahydro-B[a]P (PBDE) (Wislocki *et al.*, 1976; Jerina *et al.*, 1981). PBDE binds to deoxyguanosine, to form a DNA adduct associated with mutagenesis and tumourigenesis (Poirier, 2004).

Few attempts have been made *in vivo* or *in vitro* to understand the implications of antibodies for the metabolic activation of carcinogens and carcinogenesis. Recently, our *in vitro* studies demonstrated that antibodies might provide some protection against low doses of B[a]P, in the presence of an excess of antibodies (De Buck *et al.*, 2005b). Using a bicompartmental model of polarised intestinal cells (Caco-2), we showed that both luminal and serosal antibodies might reduce the carcinogenic process by preventing high local concentrations of B[a]P, which would overload DNA repair mechanisms in intestinal epithelia. Antibodies were shown to change the kinetic of carcinogen metabolism *in vitro* in both HepG2 cells and human peripheral blood cells. In these experiments, specific antibodies were shown to reduce both B[a]P-induced immunotoxicity and the induction of *cytochrome P450 1A1* (Cyp1a1),

a major risk factor for PAH carcinogenesis (De Buck *et al*., 2005b). In the current study, we developed a B[a]P-conjugate vaccine which was used to investigate whether specific antibodies modulate the bioavailability of B[a]P and its metabolites *in vivo*, using a mouse model.

2. Materials and methods

2.1. B[a]P conjugation to carrier proteins

A two-step zero-length cross-linking procedure using active esters was adopted for conjugating B[a]P butyric acid (B[a]P-BA), (Biochemical Institute of Environmental Carcinogens, Grosshansdorf, Germany) to ovalbumin (OVA, MW 45.000 g/mol, Sigma-Aldrich, Bornem, Belgium), purified diphtheria toxoid (DT, 63.000 g/mol, Serum Institute of India, Hadapsar, India) and purified tetanus toxoid (TT, 150.000 g/mol, Serum Institute of India).1 mL of protein (10 mg/mL) was reduced with 8 mL of dithiothreitol (DTT) (0.25 mg/mL, Sigma-Aldrich) in 100 mM PBS buffer pH 7 at 50°C for 15 min. The reduced proteins were alkylated by adding 1 mL of iodoacetamide (5 mg/mL, Sigma-Aldrich) in 100 mM PBS buffer pH 7.5 and stirring for 15 min at RT. 5 mL of the B[a]P-BA hapten (2 mg/mL in dimethylformamide, Sigma-Aldrich) was activated for 15 min at RT with a mixture of 2 mL of sulfo- *N*-hydroxysuccinimide (16 mg/mL, Pierce, Rockford, Illinois) in 100 mM 2-(N-morpholinoethanesulfonic acid (MES, Sigma-Aldrich) buffer pH 6 and 2 mL of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (5.5 mg/mL in 100 mM MES buffer pH 6). After activation, excess reagent was quenched at RT for 10 min with 100 µL of β-mercaptoethanol (20 mg/mL in 100 mM MES buffer pH 6, Sigma-Aldrich) and reacted over night with the alkylated protein. The bio-conjugate solutions were purified by dialysis against 50 mM ammonium bicarbonate buffer pH 7.8 using a 10 kDa cut-off amicon and quantified with a standard protein quantification kit (BC-Uptima, Amersham Biosciences, Diegem, Belgium).2 µg of bioconjugate were dissolved in 10 µL Nu-PAGE LDS sample buffer supplemented with 20 mM DTT and heated to 95°C for 5 min. Nu-PAGE 4 - 12% Bis-Tris precast gels (Invitrogen, Merelbeke, Belgium) were loaded with 10 µL of sample and run under denaturing conditions (MES SDS running buffer) using a Xcell II Novex electrophoresis. The natural fluorescence of B[a]P was sufficient to detect the bioconjugates in a standard UV transilluminator. B[a]P-protein conjugates were analysed in a linear mode in a sinapinic acid matrix (Bruker Daltonics, Leipzig, Germany) using a positive ion MALDI-TOF mass spectrometry (Bruker Daltonics ULTRAFLEX TOF/TOF).

2.2. Detection of specific antibodies after immunisation with B[a]P-protein conjugates

Groups of five female Balb/c mice (10 week old) were primed intraperitoneally (ip) on day 0 with 25 µg of B[a]P-OVA, B[a]P-DT or B[a]P-TT bioconjugates in 50% complete Freund's adjuvant (v/v) (Sigma-Aldrich). On days 14, 28 and 42, mice were boosted by ip injection with 25 µg of bioconjugate in 50% incomplete Freund's adjuvant (v/v) (Sigma-Aldrich). The "mock" immunised group was treated with buffer and adjuvant only (Figure 1). On days 14, 28, 42 and 56, B[a]P antibody levels were determined in 384-well microtiter plates (Greiner, Wemmel, Belgium) coated overnight at 4°C with 0.25 µM heterologous B[a]P-conjugate (B[a]P-OVA for -DT and -TT and B[a]P-DT for -OVA) in carbonate buffer (100 mM, pH 9.6). After washing, free binding sites were saturated at RT with 1% BSA. After 2 h, plates were washed again and diluted sera or specific mouse monoclonal antibody (P9E1R4 produced by immunisation with B[a]P-DT) was added for 90 min at room temperature. Binding was assessed by alkaline phosphatase-conjugated goat anti-mouse IgG (1/750 dilution, ImTec Diagnostics NV, Antwerp, Belgium) and the appropriate substrate (4-nitrophenyl phosphate disodium salt hexahydrate). Absorbance was measured at 405 nm (Spectromax Plus, Sopachem, Brussels, Belgium). Dilution curves (1/250 to 1/512000) of stock solution of different known concentrations (0.005, 0.010, 0.025, 0.050, 0.075, 0.1, 0.25, 0.50, 0.75 and 1 mg/mL) of B[a]P specific mab P9E1R4, were used to estimate absolute serum antibody concentrations. The dilution (1/32000) with the highest R^2-value was chosen to plot the linear standard curve. The same ELISA protocol was used for total IgG determination with the diluted purified mab or sera directly coated to plates.

2.3. Fine-specificity of antibodies by competition ELISA

The specificity of serum antibodies was determined using B[a]P-BA, B[a]P and its metabolites 7,8-diol-B[a]P, 1-OH-B[a]P, 3-OH-B[a]P and 9-OH-B[a]P (National Cancer Institute Chemical Carcinogen Reference Standard Repository, Midwest Research Institute, Kansas City, MO, USA) as competitors to inhibit antibody binding to coated heterologous B[a]P-conjugate. For these competition experiments, the optimal amount of coating antigen and the serum dilution were determined by indirect ELISA as described above. The minimal amount of antigen required for saturation

was coated on 384-well microtiter plates. Sera dilutions were determined to obtain 70% of saturation and to give a signal of 1.0 to 1.5 OD in the absence of competitor (highest signal). Dilution series of the above competitors were mixed 1:1 with the diluted serum to final concentrations of 0 to 1024 µM. No competition (highest signal) and 100% competition (background signal) were determined using no competitor or B[a]P-BA as competitor. The difference between the two values corresponds to the dynamic range of the assay (Δ OD max). For each competitor concentration, the percent inhibition of antibody binding was determined using the following formula:

$$\% \text{ binding inhibition} = 100 - [(OD_{- \text{ competitor}} - OD_{+ \text{ competitor}}) * 100 / (\Delta \text{ OD max})]$$

From the resulting inhibition curves, the 50% inhibition concentration of each competitor (IC_{50}) was determined.

2.4. Distribution of [^{3}H]-B[a]P in immunised mice after a single injection

Two weeks after a complete immunisation schedule with B[a]P-conjugates, each animal received a single ip injection of [^{3}H]-B[a]P (0.1 µg/kg, 2.67 10^4 Bq/mouse) diluted in 200 µL of vegetable oil (ISIO 4, Lesieur, Asnières-sur-Seine, France). Then, mice were placed in individual metabolic cages (Uno Roestvastaal, Netherlands) and urine, faeces and several organs were collected 24 h later and stored at – 20°C before analysis. EDTA-blood (500 µL) was obtained by retro-orbital bleeding and stored at 4°C until analysis (Figure 1). 500 µL of acetate buffer (0.2 M, pH 5.6), 5 µL of sulfatase (32 units, Sigma Aldrich) and 5 µL of ß-glucuronidase (622 units, from *Helix pomatia* juice, Sigma Aldrich) were added to blood samples (500 µL). The hydrolysis of conjugated metabolites was carried out at 37°C for 1 h and was followed by a liquid - liquid extraction of B[a]P and its metabolites with 500 µL ethyl acetate and 500 µL cyclohexane (Biosolve, Valkenswaard, Netherlands). After stirring (60 min at 1400 rpm) and centrifugation (3000 rpm, 15 min, 4°C), supernatant was collected and 2 mL of Hionic Fluor scintillation liquid (Perkin Elmer, Zaventem, Belgium) was added. Urine was directly counted after adding scintillation liquid (200 µL urine + 3 mL Hisafe II scintillation liquid per vial, Perkin Elmer). Pre-hydrated faeces (20 mg) or tissues (100 mg) were solubilised in Soluene-350 according to Perkin Elmer procedure and previously described (Grova *et al.*, 2002). Radioactivity was measured by liquid scintillation counting (2 min) using a 1209 LKB ß-counter (Wallac, Zaventem, Belgium). All samples were corrected for quenching of

radioactivity. Radioactivity was expressed in Bq or Bq/g accounting for the effective counting efficiency.

2.5. B[a]P metabolism in immunised mice after sub-acute exposure

After a complete immunisation schedule with B[a]P-DT, animals were randomly separated into 4 groups of 5 mice that were given 2 ip injections per week of 0, 0.2, 2 and 25 mg/kg B[a]P solubilised in vegetable oil (10 mL/kg) for a 4 week period (Figure1). 20 control mice, mock immunised with adjuvant only, were given the same B[a]P injections. Blood samples were obtained by retro-orbital bleeding on day 1 (before the first injection of B[a]P) and on days 3, 8, 15, 22 and 29 after the first injection of B[a]P. The last blood sample (day 29) was taken 3 days after the last B[a]P injection. Sera were stored at –20°C until analysis of B[a]P, its metabolites and specific antibodies. B[a]P-specific IgG and total IgG was measured by indirect ELISA as described above.

2.6. HPLC-analysis of B[a]P and its metabolites in immunised mice

B[a]P and its metabolites 7,8-diol-B[a]P, 1-OH-B[a]P, 3-OH-B[a]P and 9-OH-B[a]P were extracted from sera as previously described (Grova *et al*., 2008) . The separation of B[a]P and metabolites were performed on an Agilent 1100 Series Liquid Chromatograph, equipped with a fluorescence detector on a reversed-phase Vydac C18 column (250 mm x 4.6, I.D., 5 µm particle size, Alltech, Lokeren, Belgium). 500 µL of samples were loaded on a Vydac C18 guard column (4.6, I.D., 5 µm particle size) by an initial 2 min isocratic elution with 20% acetonitrile in water 0.1% TFA at 1.5 mL/min. PAHs were eluted by acetonitrile/water gradient: 0-5 min by 20% acetonitrile, 5-10 min a linear gradient from 20% to 60% acetonitrile, 10-20 min a linear gradient from 60% to 100%, 20-22 min 100% acetonitrile, 22-24 min linear gradient to 20% acetonitrile and 24-30 min 20% acetonitrile. B[a]P and its metabolites were identified and quantified by comparing retention times and peak areas with standards (Grova *et al*., 2008). Compounds were eluted in the following order at the following excitation and emission wavelengths: 7,8-diol-B[a]P at 370-410 nm and 9-OH-B[a]P, 1-OH-B[a]P, 3-OH-B[a]P, B[a]P at 380-430 nm.

2.7. Statistical analysis

The results were analysed using a one way analysis of variance (ANOVA) and a two way ANOVA procedure for one independent factor and repeated measurements. Both are followed by planned contrasts with Student-Newman-Keuls correction. The relationship between the level of B[a]P recovered and the serum titers of individual immunised mice was tested by linear regression test (Figure 5). Statistical analyses were performed using the Sigma Stat for Windows software (Erkrath, Germany). Differences were considered to be significant when $p<0.05$.

3. Results

3.1. B[a]P conjugation to carrier proteins

The small synthetic hapten B[a]P-BA was coupled to the large carrier proteins OVA, DT or TT using a standard active ester procedure (Figure 2A) (Grabarek and Gergely, 1990). Conjugations were performed with at least 120-fold molar excess of hapten over the protein carboxylic reactive groups. Molecular weights of proteins and B[a]P conjugates were determined by MALDI–TOF. A typical mass difference between B[a]P-DT (63950 Da) and DT (61350 Da) was 2.6 kDa corresponding to approximately 8 hapten molecules (322.4 Da) per carrier molecule (Figure 2B). Similar numbers of B[a]P hapten per protein were estimated for OVA and TT (data not shown). The conjugates were also analysed using a 4-12% gradient Nu-PAGE gel electrophoresis taking advantage of the natural fluorescence of B[a]P (Figure 2C). The gel was observed on a 310 nm UV-transilluminator of which the emission overlaps with the 297 nm excitation maximum of B[a]P. The conjugates were visible on the gel as blue bands (404-428 nm) corresponding to the specific emission wavelength of B[a]P of 380-430 nm.

3.2. Detection of specific antibodies after immunisation with B[a]P-conjugates

The induction of specific antibodies in mice was followed for 2 weeks after each injection of the hapten conjugates B[a]P-OVA, B[a]P-DT or B[a]P-TT (Figure 3A-C). Within two weeks after the first injection, carrier-specific antibodies appeared (data not shown). Using heterologous B[a]P-conjugates, the serum of each mouse was titrated 2 weeks after each injection, as shown in Figure 3A for mice immunised 4 times with B[a]P-DT. In order to obtain some estimate of the antibody response in absolute concentrations, a standard curve was prepared using dilutions of purified B[a]P specific monoclonal antibody (P9E1R4) (0.005 to 1 mg/mL) (Figure 3B). Significant levels of B[a]P-specific antibodies were detected 2 weeks after the first boost with B[a]P-DT and only after the second boost with B[a]P-TT and B[a]P-OVA (Figure 3C). After a complete immunisation schedule with B[a]P-DT or B[a]P-TT, specific antibody levels were significantly higher ($p<0.01$, 0.54 ± 0.17 and 0.51 ± 0.17 mg/mL respectively) than after immunisation with B[a]P-OVA (0.13 ± 0.08 mg/mL) (Figure 3C).

3.3. Modulation of [^{3}H]-B[a]P distribution in immunised mice after a single injection

Two weeks after a complete immunisation schedule with B[a]P-conjugates (Figure 1), animals received a single injection of radiolabeled B[a]P (0.1 µg/kg). 24 h later, [^{3}H]-B[a]P in the blood was on average 20, 14 and 10 fold higher in B[a]P-DT ($p<0.001$), B[a]P-TT ($p<0.001$) and B[a]P-OVA immunised mice ($p<0.01$) than in the mock immunised group (Figure 4A). In contrast, immunised animals showed only a maximal 1.9-fold increase in recoverable [^{3}H]-B[a]P levels in the solid tissues tested (Figure 4B-E). The difference compared to mock immunised animals was highest in the spleen ($p<0.05$; Figure 4B) and liver ($p<0.05$; Figure 4C), while there was no difference in the brain. Animals immunised with OVA conjugate showed no enhanced retention of [^{3}H]-B[a]P in any of the four organs tested (Figure 4B-E). Thus, overall recovery of [^{3}H]-B[a]P in organs was somewhat (but significantly) higher in B[a]P-TT and B[a]P-DT mice than in control mice and the B[a]P-OVA group (Figure 4H). In faeces, the level of radioactivity recovered was 56% lower in mice immunised with B[a]P-DT and B[a]P-TT than in mock controls while B[a]P-OVA had no effect (Figure 4G). Excretion of radioactive B[a]P in the urine was essentially the same in both immunised and naive control mice (Figure 4F). The effect of antibodies on recoverable [^{3}H]-B[a]P was also confirmed when antibody levels of individual mice were considered. Specific antibodies of all mice correlated significantly ($R^2=0.73$, $p<0.001$) with the level of [^{3}H]-B[a]P recovered in the blood (Figure 5A) and in the liver ($R^2=0.60$, $p<0.001$; Figure 5B) after 24 h. For both, the recovery of [^{3}H]-B[a]P was maximal above an antibody concentration of 0.4 mg/mL (2.67 µM)

3.4. Persistence of B[a]P specific antibodies after sub-acute B[a]P exposure

After a complete immunisation schedule with B[a]P-DT (Figure 1), groups of 5 animals were given 2 injections of 0.2, 2 and 25 mg/kg B[a]P per week for 4 weeks ("sub-acute B[a]P exposure"). Control mice received only diluent. Levels of B[a]P antibodies were determined 14 days after the last boost, i.e. one day before the first of the 8 injections of B[a]P. Before B[a]P injection mean OD values of B[a]P antibodies in the four actively immunised groups ranged between 0.863 ± 0.26 to 1.013 ± 0.24 (dilution of 1/500) corresponding to a serum antibody concentration of about 0.22 ± 0.02 to 0.29 ± 0.05 mg/mL (data not shown). In the immunised groups

that were not exposed to B[a]P or exposed to low levels of B[a]P (0.2 and 2 mg/kg), antibody levels showed a 14.3 to 22.8% increase during the four week observation period (Figure 6A). Thus, after sub-acute exposure of up to 2 mg/kg of B[a]P, essentially no effect of B[a]P on relative levels of specific antibodies was observed. Even after 8 injections of a large excess of B[a]P (25 mg/kg), specific antibody levels showed only a slight reduction of 20% on day 29. Similarly, the effect of B[a]P on total IgG was measured. On day 1, the level of total IgG was significantly higher in animals immunised with B[a]P-DT than in mock immunised controls probably as a result of polyclonal activation induced by the carrier itself ($p<0.05$). A 1.5-1.8 fold increase of total IgG was observed in mock immunised mice during the one-month of B[a]P administration ($p<0.05$) whereas essentially no change (-7 to +10% variation) was observed in B[a]P-DT animals (Figure 6B). This increase of total IgG in mock immunised mice seems to be the result of unspecific immune stimulation of the adjuvant.

3.5. Modulation of fine-specificity of antibodies by sub-acute B[a]P exposure

In order to test the relative capacity of the sera to bind the metabolites, the latter were added as competitors to the sera in competitive ELISA. All metabolites inhibited antibody binding i.e. cross-reacted with the sera albeit with different IC_{50} (day 1, Figure 7A). After sub-acute administration of B[a]P (0.2 to 25 mg/kg), the ratio of IC_{50} on day 29 versus day 1 revealed some shifts in the binding efficiency. A decrease in competitive binding (higher IC_{50}) was observed in most control mice for B[a]P, 7,8-diol-B[a]P and 1-OH-B[a]P, but it was only significant for the 9-OH-B[a]P ($p<0.05$; data not shown). Conversely, a significant ($p<0.05$) decrease of the IC ratio revealed an increase in competitive binding of the 3-OH-B[a]P irrespective of the dose used (Figure 7B).

3.6. Modulation of B[a]P metabolism by specific antibodies

After a complete immunisation schedule with B[a]P-DT, the animals were subjected to the above sub-acute B[a]P exposure. The recovery of B[a]P and its metabolites was analysed in the serum. Specific antibodies in immunised mice increase the concentration of recovered B[a]P over the whole observation period irrespective of

the B[a]P dose (Figure 8A, F, K). For example at 2 mg/kg, B[a]P recovery was increased up to 1.4-, 1.3- and 1.4-fold on days 3, 15 and 29, respectively but significance was only seen on days 3 and 29 (but is not very obvious on Figure 8F as a result of the log scale). Even after exposure with 25 mg/kg, the recovery of B[a]P was still 1.4-fold increased (Figure 8K). Moreover, antibodies modulated some B[a]P metabolites at least for 29 days. The recovery of phenol end-point metabolites (in particular 3-OH-B[a]P, and 1-OH-B[a]P to a lesser extent) in immunised mice injected with 0.2 mg/kg of B[a]P was significantly increased at most time points (Figure 8B, C). 3-OH-B[a]P recovery was increased up to 12- and 165-fold on days 3 and 15 and even after 30 days, the recovery of 3-OH-B[a]P was still 36-fold increased. After the injection of 10-fold higher dose of B[a]P (2 mg/kg), overall levels of phenol metabolites recovered were significantly higher on day 3 in immunised mice, and differences in recoverable phenol metabolites between immunised and naïve mice were similar to those associated with the lower B[a]P dose (Figure 8G-I, 8B-D). These differences were strongest for the major phenol metabolites 3-OH-B[a]P and 1-OH-B[a]P and weak for 9-OH-B[a]P and were dose dependent, as shown for day 3 (Figure 8Q, R, S). Similarly, in immunised mice the recovery of the intermediate metabolite 7,8-diol-B[a]P was increased at least on day 3 (Figure 8E, J, T). At the lowest concentration 7,8-diol-B[a]P was detectable only in immunised mice but at the intermediate concentration, the recovery of this intermediate metabolite was dramatically enhanced ($P<0.001$) (Figure 8J). As expected, immunisation had little or no effect on metabolites after injection of 25 mg/kg (Figure 8L-O).

4. Discussion

Although natural or induced antibodies against environmental carcinogens have been described (Harris *et al.*, 1985; Haugen *et al.*, 1986; Newman *et al.*, 1988; Lee and Strickland, 1993), biological consequences of such antibodies have rarely been investigated (Glushkov, 2003; De Buck and Muller, 2005). Many experimental carcinogenesis models conveniently use high doses of carcinogens mostly given as single bolus. Such models may provide simple surrogate end points, but they poorly reflect chronic exposure to the low levels found in the environment. Moreover, these excessive doses of carcinogens cannot be matched by equimolar concentration of antibody levels normally obtained by immunisation. In contrast, the much lower levels of B[a]P found in the general population, in professionally exposed individuals or self-exposed smokers, can be matched by the large molar excess of antibodies normally found after vaccination. However, the low concentrations of B[a]P do not permit to directly measure chemical carcinogenesis or DNA adduct-formation. Therefore, we measured other surrogate markers to assess the effect of antibodies on the pharmacokinetic and pharmacodynamic of B[a]P.

Here, we describe conjugate vaccines based on B[a]P butyric acid and immunogenic carrier proteins, which induce specific antibodies that redistribute low concentrations of B[a]P in blood and tissues of immunised mice (Figure 4A-E). The B[a]P-DT conjugate induced the most robust immune response (Figure 3 and 5). Assuming a serum IgG concentration of about 12 mg/mL (Harlow and Lane, 1988; Junghans, 1997) B[a]P-specific antibodies represented approximately 2.8 to 5.4% of the serum IgG that corresponds to a strong antibody response (Janoff *et al.*, 1991; Hieda *et al.*, 1999; Hieda *et al.*, 2000; Pentel *et al.*, 2000). On a molar level of binding sites, these antibody titers can bind 9-17 nmol or 2.2-4.6 µg B[a]P corresponding to a concentration of 0.1-0.2 mg/kg body weight (assuming an extracellular distribution volume of a mouse of 2 mL, and average body weight of 20 g).

Animals immunised with B[a]P-DT conjugate and challenged with an environmentally relevant concentration of B[a]P (0.1 µg/kg) also had the highest recovery of [^{3}H]-B[a]P in the blood and in solid tissues after a bolus injection (Figure 4). At least in the blood and the liver, this effect was antibody dose-dependent (Figure 5). For both, the recovery of [^{3}H]-B[a]P was maximal above an antibody concentration of 0.4 mg/mL (2.67 µM). Thus, at a molar ratio of specific antibodies to B[a]P of 666, an antibody-dependent redistribution of B[a]P can be observed. We have previously shown that

specific antibodies restrict cellular uptake of B[a]P (De Buck *et al.*, 2005b). Here, we show that *in vivo* this translates into higher levels of B[a]P in the blood and tissues and a reduced faecal excretion by the enterohepatic pathway.

The higher levels of B[a]P recovered in solid tissues of immunised mice cannot be explained by the increased B[a]P concentration in organ blood. For instance, the blood in the liver accounts for about 10% of the total blood volume, corresponding to only 40 Bq of B[a]P. Similarly, the radioactivity associated with the organ blood of the spleen and kidney (2 to 2.5% of the total blood volume) is negligible (less than 10 Bq). Thus, in immunised mice higher levels B[a]P-antibody complexes seem to be sequestrated in the solid tissues. These results are in agreement with Johansson et al. (1996) who showed a higher recovery of radioactive dinitrophenol in the liver (+20%), spleen (+50%) and lung (+50%) in rat passively immunised with anti-dinitrophenol antibody (Johansson *et al.*, 1996).

In the liver, one of the main organs of B[a]P metabolism, antibodies seem to modulate cellular uptake of B[a]P. The reduced enterohepatic circulation and / or biliary excretion suggest that antibodies reduce B[a]P uptake by parenchymal cells. On the other hand, it is well known that in general antigen-antibody complexes are avidly internalised by the Kupffer cells of the reticuloendothelial (RE) system (Skogh *et al.*, 1985; Skogh *et al.*, 1988; Johansson *et al.*, 1996). Differential uptake of B[a]P by parenchymal and RE cells would have important consequences for processing of B[a]P and its metabolites. RE cells have considerably (13-fold) lower cytochrome P450 activity than parenchymal cells, but only two fold lower phase II enzyme activities. Thus, RE cells have a lower ability to oxidize xenobiotics to DNA adduct forming precursor and a greater relative ability to conjugate metabolites to inactivated species (Steinberg *et al.*, 1987; Zhong *et al.*, 1994). This is in agreement with Galati et al. (Galati *et al.*, 2000) and Verdina et al. (Verdina *et al.*, 1996) who elicited antibodies against B[a]P-diol-epoxide (BPDE) and 2-acetylaminofluorene (2-AAF) and found a strong reduction in B[a]P- and 2 AAF-DNA adducts in the liver of mice either after a single injection of B[a]P or after chronic administration of 2-AAF in diet for 4 weeks.

In our previous studies, we showed that antibodies can also modulate metabolic activation *in vitro* (De Buck *et al.*, 2005a; De Buck *et al.*, 2005b). Here, immunised mice were sub-acutely exposed to different doses of B[a]P (0.2 to 25 mg/kg) in order to determine whether antibodies may provide some longer-term effects. Our

metabolism studies showed that essentially all metabolites were enhanced in the sera and that this effect was strongest after 24 h, but it was still measurable after day 15 and even day 29. As the antibodies react not only with B[a]P but also with its metabolites (particularly 7.8-diol-B[a]P; Figure 7A), the higher metabolite concentrations correspond to a redistribution of these from the intracellular space to the bloodstream as we have observed for the recovery of radioactive B[a]P. The repartitioning was also strongest at the lower concentrations of B[a]P (0.2 mg/kg and 2 mg/kg), i.e. at the highest molar excess of antibody. In order to detect the metabolites, the injection of relatively high doses of B[a]P was necessary: the 0.2 mg/kg B[a]P is 25 to 50 times higher than the level of exposure in smokers, in heavy consumers of smoked or grilled meat and fish, or in individuals with heavy occupational exposure (Menzie *et al.*, 1992; IPCS, 1998) whereas the 25 mg/kg dose is more relevant for the toxic range of exposure (IPCS, 1998). At the 50-100 times lower doses of B[a]P to which the general population is exposed, the antibody effects can be expected to be even stronger but limitations of analytical sensitivity do not allow experimental readouts at such low concentrations of B[a]P.

The B[a]P immune sera showed high binding capacities with IC_{50} values for B[a]P and most metabolites of 4-24 µM (Figure 7A). These IC_{50} values are approximately 4 times higher than those reported for the commercial monoclonal antibody against B[a]P (mab13, 0.6 - 5.8 µM) using a similar competitive ELISA. (De Buck *et al.*, 2005b). Interestingly, there was a measurable shift in antibody specificity towards 3-OH-B[a]P (Figure 7B) the major endpoint metabolite in human and rodents after the sub-acute B[a]P exposure (Jacob and Grimmer, 1996; Hollender *et al.*, 2000). This specificity shift could perhaps be explained by recent observations (Sugihara and James, 2003) of covalent binding of 3-OH-B[a]P to blood proteins.

A sustained immune response is normally supported by pathogen-specific T helper cells, which are boosted by a wild-type infection. However, B[a]P and probably DNA adducts do not provide the T cell epitopes necessary to boost a B[a]P conjugate vaccine induced immune response. Surprisingly, our results showed little effect of a large excess of B[a]P (300 fold) given every 3 days over one month on specific antibody levels. High concentrations of free B[a]P did not deplete the vaccine-induced antibodies nor upset the specific or total antibody homeostasis. This suggests that the antibody response may be boosted by protein or DNA adducts of B[a]P. Thus, the absence of a T cell epitope in the free B[a]P hapten does not undermine the concept of a vaccination strategy.

In light of our *in vitro* studies (De Buck *et al*., 2005a; De Buck *et al*., 2005b), the data presented here suggest that antibodies may protect against detrimental effects of carcinogens in several ways: (i) redistribution of B[a]P and its harmful metabolites by partial sequestration in the extracellular space; (ii) reducing residence times in susceptible tissues; (iii) preferential targeting of B[a]P to RE cells rather than parenchymal cells (iv) our *in vitro* studies also suggest that these effects may result in reduced intracellular peak concentration and reduced cytochrome P450 induction, a major risk factor of environmental carcinogenesis (De Buck *et al*., 2005b). (v) Antibody cross reactivity with the proximate carcinogen 7,8-diol-B[a]P provides a second chance for interrupting metabolic activation by sequestration.

In conclusion, these results demonstrate that our candidate vaccine based on carrier proteins that are used in licensed human vaccines, induce specific antibodies with measurable pharmacokinetic and pharmacodynamic effects. The antibodies modify the bioavailability of B[a]P *in vivo* and interfere with the metabolic activation both at the level of the pro-carcinogen B[a]P and its metabolites, even if the sequestered carcinogen cannot be directly eliminated from the host organism. Thus, antibodies against carcinogens clearly affect the host response to chemical carcinogens. Further studies are warranted to investigate whether the observed effects may provide some protection against environmental carcinogenesis.

Acknowledgements

We are grateful to Gilles Laverny for his excellent technical assistance. We acknowledge the support of the Fond National de la Recherche (Tobavac project, FNR: 01/04/11), and the CRP-Santé and the Ministry of Health of Luxembourg for the continued support.

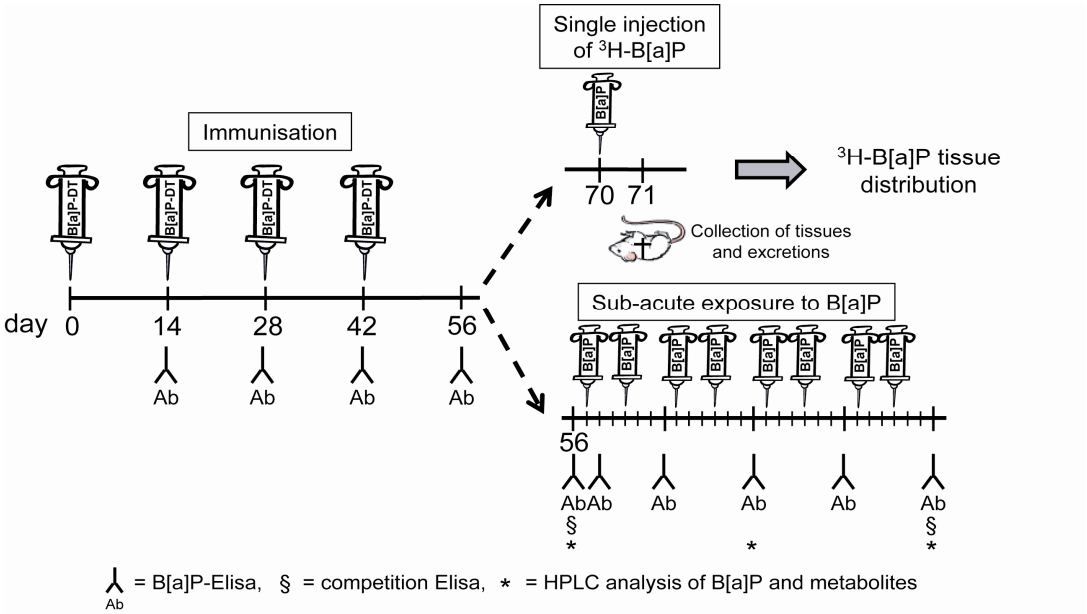

Figure 1: Experimental design. After immunisation with B[a]P-DT, mice were either given a single injection of [^{3}H]-B[a]P to investigate B[a]P tissue distribution or they were given 8 injections over 4 weeks ("sub-acute exposure to B[a]P", no Antibody-titer determination before day 56 for these mice) to analyse (i) the persistence of specific antibodies, (ii) a shift in antibody fine specificity and (iii) B[a]P metabolism by HPLC. For further details, see Materials and Methods.

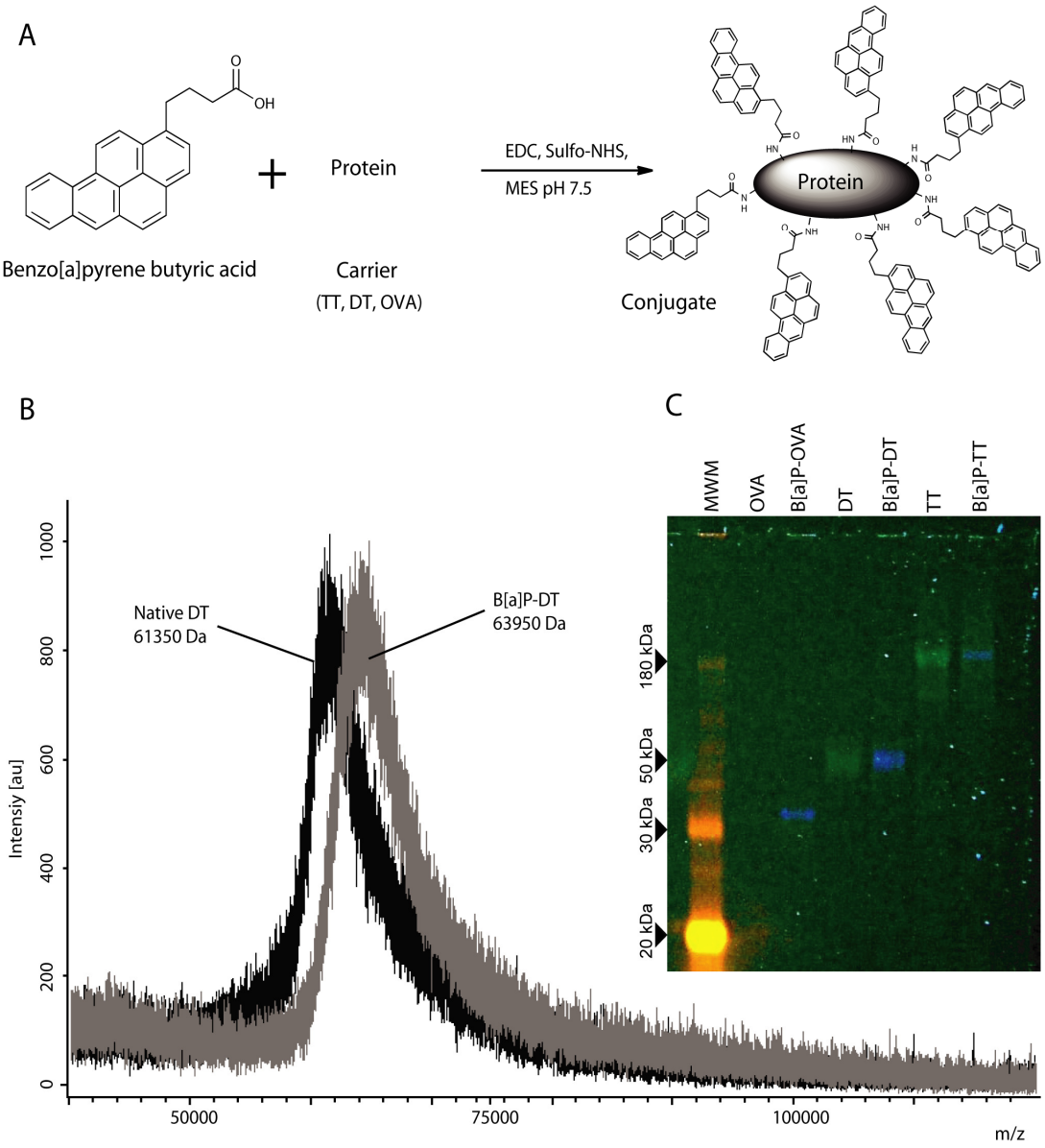

Figure 2: (A) B[a]P conjugate formation via O-acylisourea intermediates ;(B) MALDI–TOF mass spectrograms of B[a]P-DT and native DT; (C) 4-12% gradient Nu-PAGE gel electrophoresis of free (i.e. unconjugated) and B[a]P-conjugated carrier proteins. In lane DT and TT bands correspond to green autofluorescence of unconjugated carrier proteins. In lane B[a]P-DT and B[a]P-TT the blue fluorescence of the bands with a slightly higher molecular weight corresponds to the autofluorescence of B[a]P.

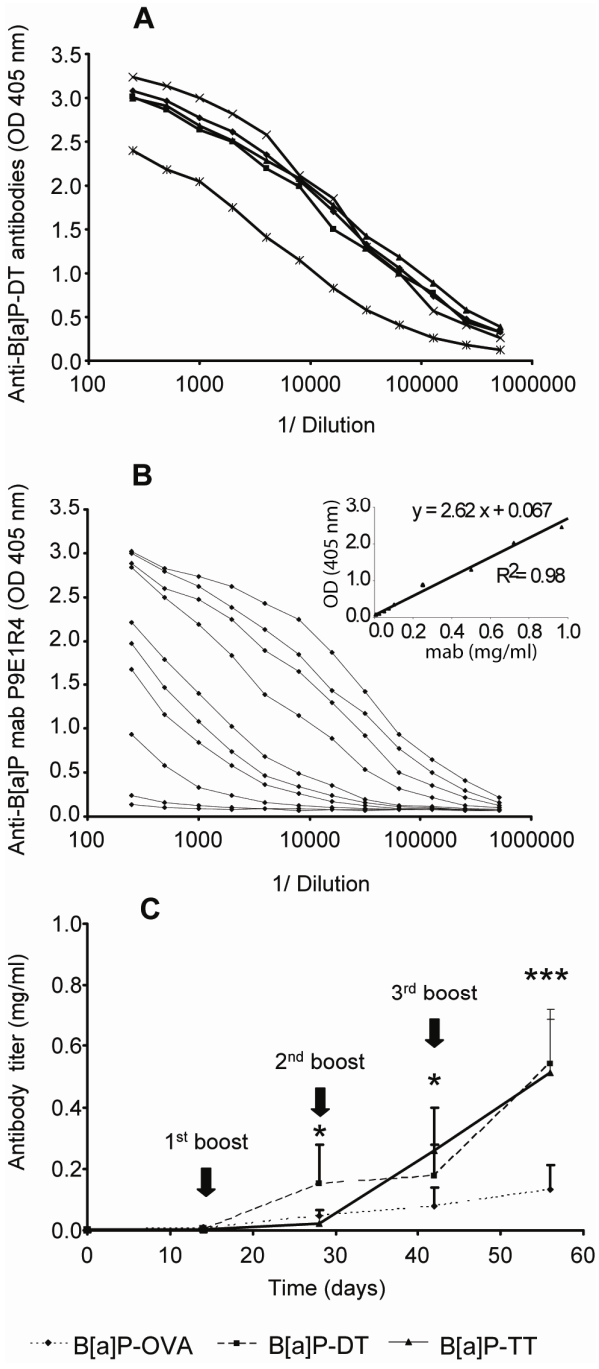

Figure 3: (A) Titration of serum antibodies of individual mice immunised with B[a]P-DT conjugates two weeks after the 3rd boost by indirect ELISA. (B) To estimate absolute antibody concentrations a standard curve (insert) was used, that is based on dilution curves (1/250 to 1/512000) of different concentrations (0.005-1 mg/mL, represented by a line for each concentration in panel B) of purified monoclonal antibody (mab P9E1R4 against B[a]P). 1/32000 dilution was chosen to plot the standard curve (insert, R^2=0.98). (C) B[a]P specific antibody titers after each injection of B[a]P-OVA, B[a]P-DT or B[a]P-TT by ELISA using heterologous conjugates for plate coating. Results are expressed as mean ± SE of 5 mice per group. Two way ANOVA procedure was applied for 1 independent factor (B[a]P conjugates) and repeated measurements (bleeding days).* $p<0.05$ *** $p<0.001$, statistically significant difference from B[a]P-OVA treated animals (Student-Newman-Keuls-t test for multiple comparisons).

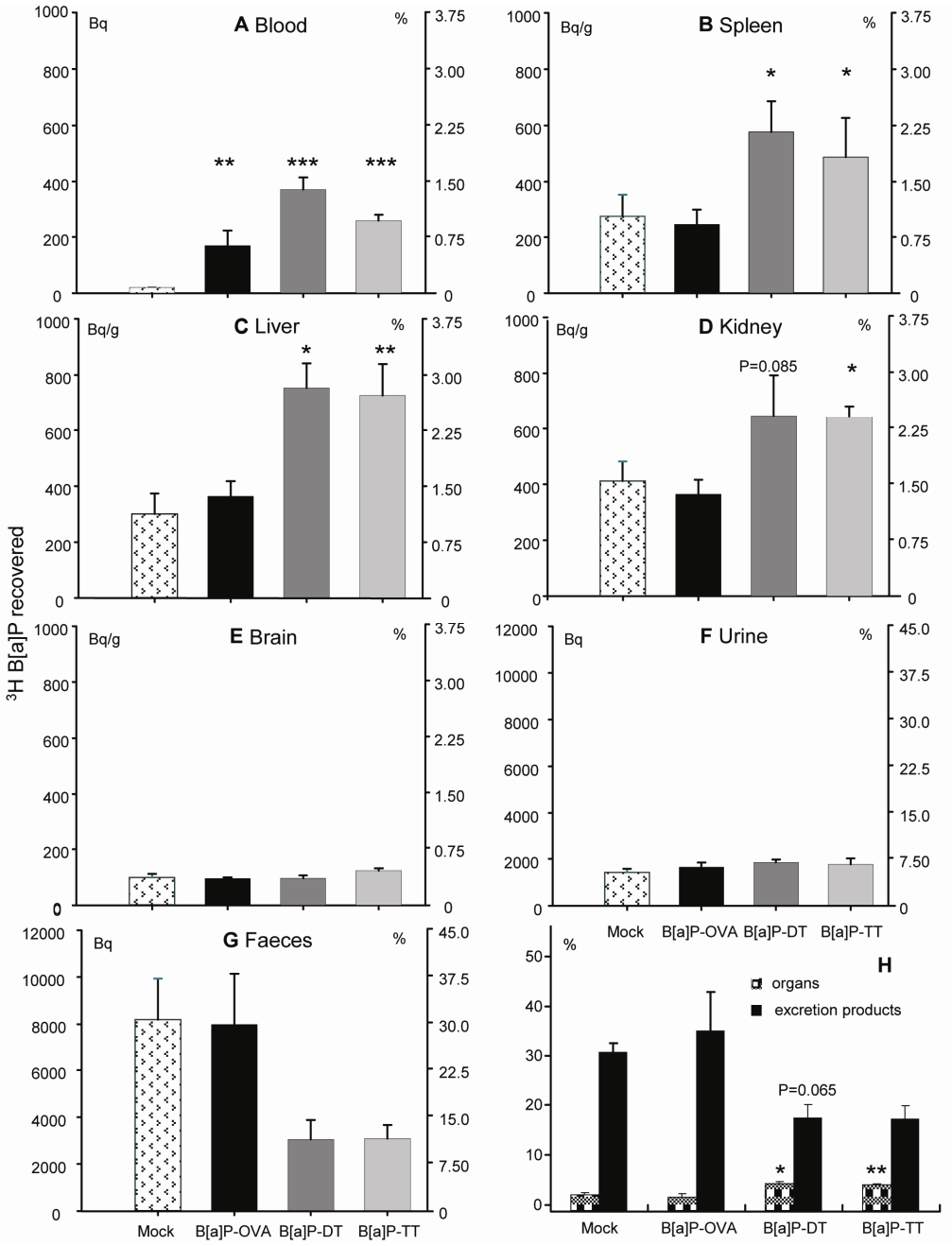

Figure 4: [^{3}H]-B[a]P (in Bq/g or Bq and percent of total radioactivity) recovered in blood (A), tissues (B-E) and excretion products (F, G) 24 h after a single ip injection of [^{3}H]-B[a]P (0.1 µg/kg) in mice immunised with B[a]P-OVA, B[a]P-DT or B[a]P-TT or mock immunised mice. Percent of total radioactivity recovered after 24 h in organs and excretion products (urine and faeces, panel H). Results are expressed as mean ± SE of 5 mice per group. * $p<0.05$; ** $p<0.01$ and *** $p<0.001$, significant difference from controls (Student-Newman-Keuls-t test for multiple comparisons).

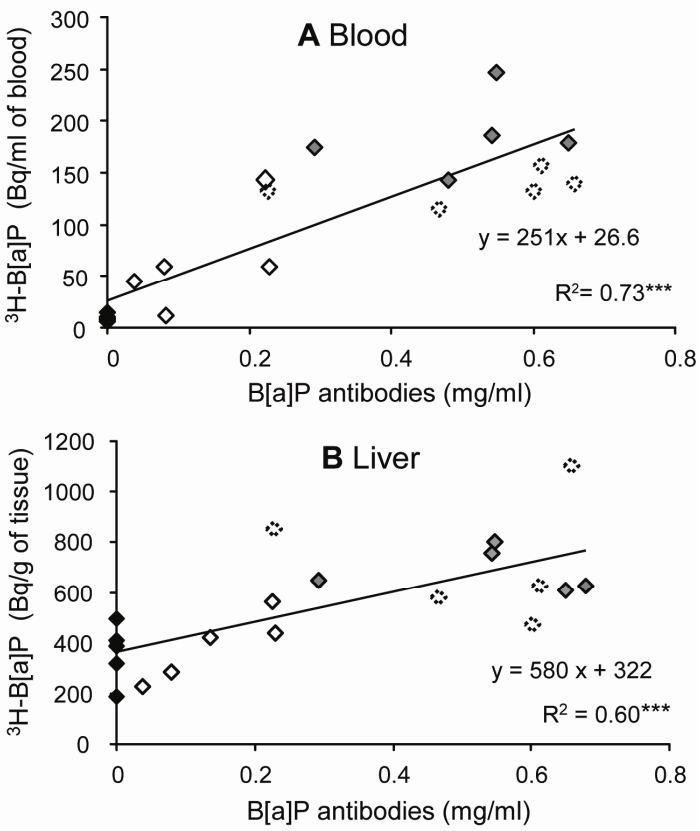

Figure 5: Correlation between the serum titers of individual mice mock immunised (◆) or immunised with B[a]P-OVA (◇), B[a]P- DT (◈) or B[a]P-TT (◌) conjugates and the concentration of [^{3}H]-B[a]P (Bq/mL or Bq/g) recovered in their blood (A) or liver (B) after a single ip injection of 0.1 µg/kg of [^{3}H]-B[a]P. The significant relationship between the two variables was estimated by linear regression (*** $p<0.001$).

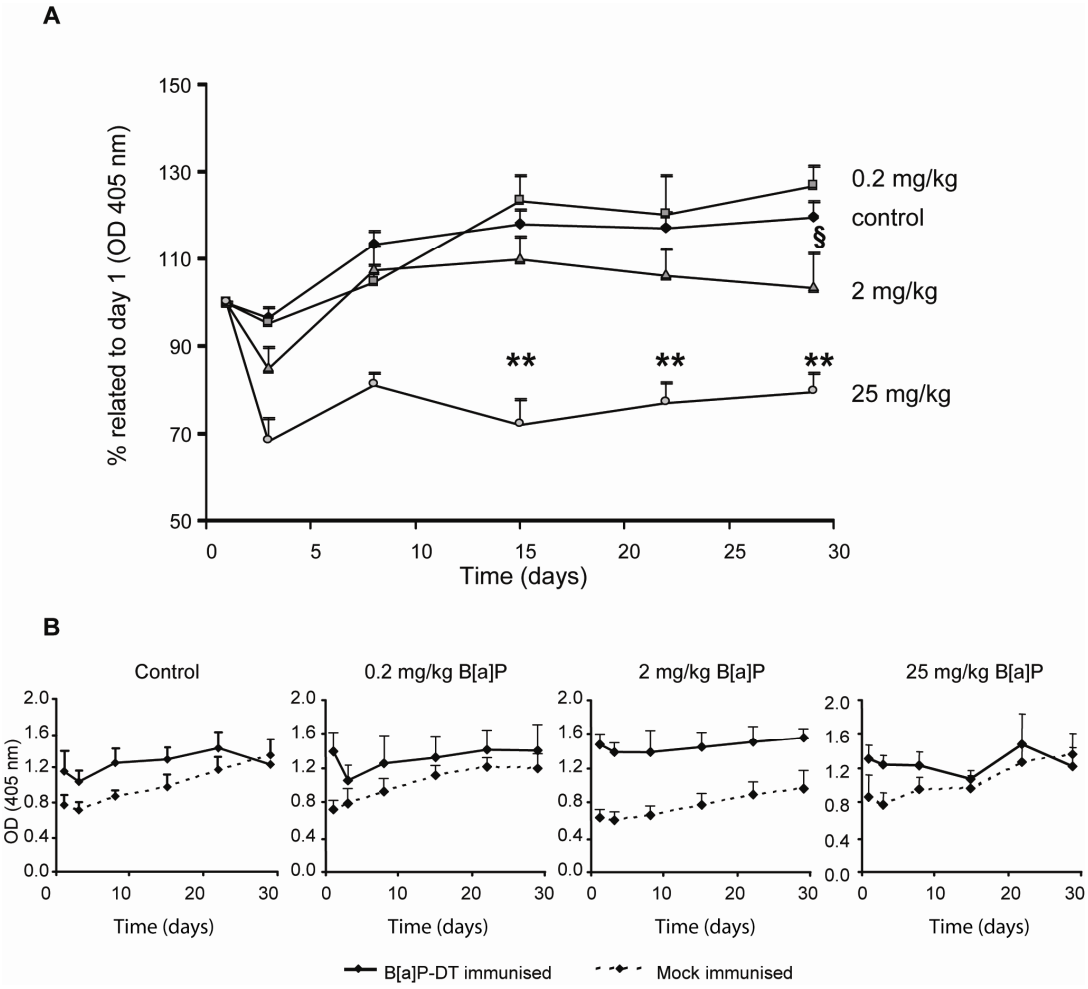

Figure 6: Kinetics of B[a]P-specific antibodies (A) and total IgG (B) in mouse sera (1:500 (A); 1/100000 (B)) during sub-acute exposures to B[a]P (0.2 to 25 mg/kg, 8 injections over 4 weeks) of mice immunised with B[a]P-DT. Specific IgG and total IgG were measured by indirect ELISA. Values are mean ± SE of 5 mice per group and are expressed in percentage OD of day 1 (A) or in OD value (B). Two way ANOVA procedure was applied for 1 independent factor (B[a]P dose (A) or IgG levels (B) and repeated measurement (bleeding day). (A) § $p<0.05$ ** $p<0.01$, statistically significant difference to control for 2 mg/kg and 25mg/kg treated animals respectively (Student-Newman-Keuls-t test for multiple comparisons). (B) Mock immunised mice showed slight but significant lower IgG levels compared to B[a]-DT immunised mice ($p<0.05$ for all concentrations and time points except for day 15 (25 mg/kg), day 21 (25 mg/kg) and day 29 (25 mg/kg and control)).

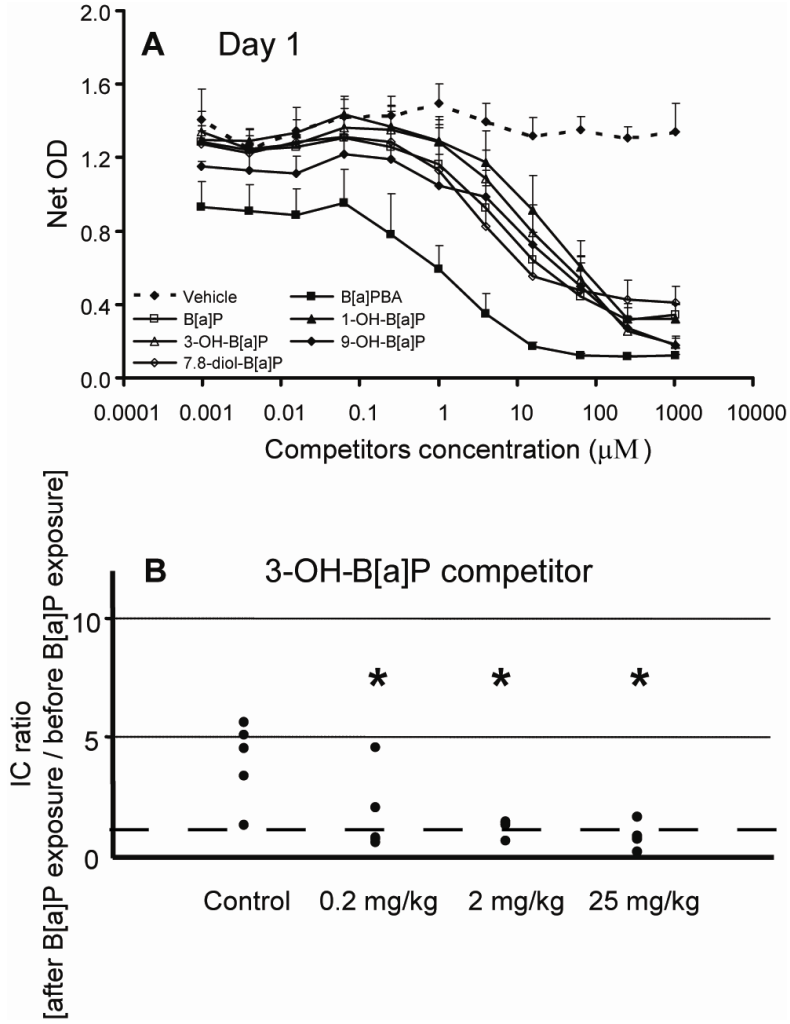

Figure 7: Competition ELISA profile of sera from immunised mice (n=5) before B[a]P injections (day 1; panel A). B[a]P and its metabolites were used as competitors to compete for binding of specific antibodies to B[a]P-OVA as the coated antigen. B[a]P-BA was used as the reference (100% competition) to determine the IC_{50} value (mM) of each competitor. Values are mean ± SE of 5 mice per group. (B) Binding competition of the 3-OH-B[a]P was measured also in all immunised mice after sub-acute administrations of B[a]P (0 to 25 mg/kg; 29 days). Data are expressed as the IC ratio on day 29 versus day 1 (before sub-acute administration of B[a]P). Dashed line corresponds to ratio 1. A one way ANOVA test was applied followed by a Student Newman-Keuls-t test for multiple comparisons. * $p < 0.05$, statistically significant difference from mock animals.

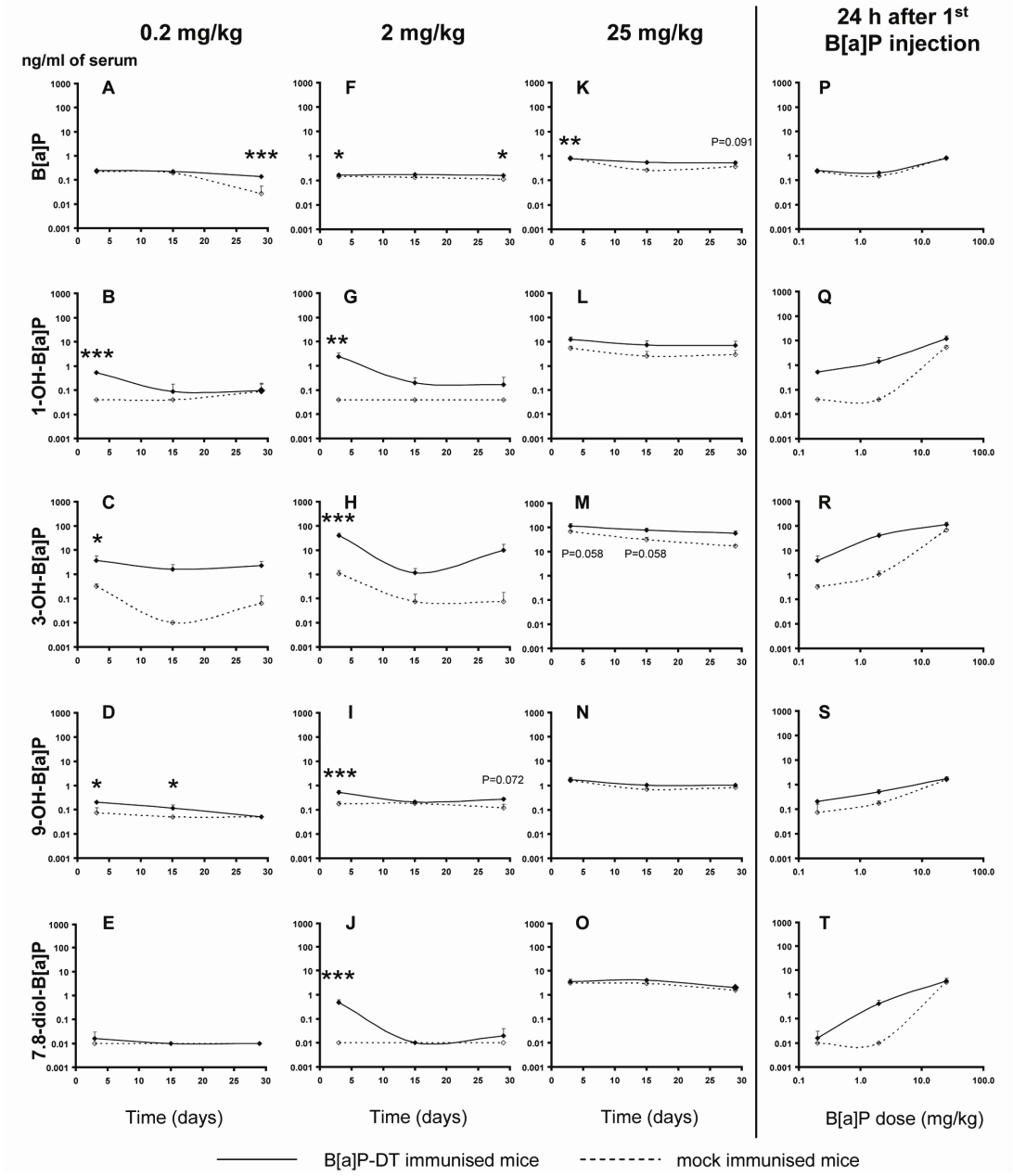

Figure 8: Recovery of B[a]P and its metabolites from serum of immunised mice compared to control mice sub-acutely exposed to B[a]P (0.2 to 25 mg/kg) by HPLC analysis. Values are mean ± SE of 5 mice per group and represented on a log scale. Two way ANOVA procedure was applied for 1 independent factor (treatment: mock or B[a]P-DT immunisation) and repeated measurement (bleeding day). * $p<0.05$ ** $p<0.01$ *** $p<0.001$, statistically significant difference from mock animals (Student-Newman-Keuls-t test for multiple comparisons). p-values between 0.05 and 0.1 represent a tendency.

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Chapter 3

Modulation of benzo[a]pyrene induced immunotoxicity in mice actively immunized with a B[a]P-diphtheria toxoid conjugate

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Abstract

Benzo[a]pyrene (B[a]P) is a small molecular weight carcinogen and the prototype of polycyclic aromatic hydrocarbons (PAHs). While these compounds are primarily known for their carcinogenicity, B[a]P and its metabolites are also toxic for mammalian immune cells. To develop a prophylactic immune strategy against detrimental effects of B[a]P, we have immunized mice with a B[a]P-diphtheria toxoid conjugate vaccine. We showed that high levels of antibodies against B[a]P and its metabolites modulate the redistribution of these PAHs in the blood. After immunization, increased levels of B[a]P and its metabolites were recovered in the blood. B[a]P significantly suppressed the proliferative response of both T and B cells after a sub-acute administration, an effect that was completely reversed by vaccination. In immunized mice also the immunotoxic effect of B[a]P on IFN-γ, IL-12, TNF-α production and the reduced B cell activation was restored. Finally, our results showed that specific antibodies inhibited the induction of Cyp1a1 by B[a]P in lymphocytes and Cyp1b1 in the liver, enzymes that are known to convert the procarcinogen B[a]P to the ultimate DNA-adduct forming metabolite, a major risk factor of chemical carcinogenesis. Thus, we demonstrate that vaccination with a B[a]P conjugate vaccine based on a carrier protein used in licensed human vaccines reduces immunotoxicity and possibly other detrimental effects associated with B[a]P.

1. Introduction

Benzo[a]pyrene (B[a]P) the prototype polycyclic aromatic hydrocarbon (PAH) is an environmental pollutant emanating from both natural and anthropogenic sources. B[a]P is produced during incomplete combustion, pyrolysis of organic matter, during industrial processes and during household activities such as cooking, barbequing and smoking (Hattemer-Frey and Travis, 1991; Hecht, 1999; Phillips, 1999). It is taken up by ingestion of contaminated food such as grilled and smoked meat, and by inhalation of contaminated air and cigarette smoke (Lambré and Muller, 2004). Although it should be possible to reduce anthropogenic sources of B[a]P, it would be difficult for the general population and in particular for risk groups to avoid exposure to this ubiquitous compound.

B[a]P is a procarcinogen, which is well known to induce tumours in a number of organs, both in animal models and humans (Thyssen *et al.*, 1981; Wolterbeek *et al.*, 1995). In a complex metabolic process, the majority of B[a]P is converted to detoxified end point metabolites. Phenols and dihydrodiols are further processed to water-soluble compounds by phase 2 enzymes (e.g. sulfatase and glutathione S-transferase) by conjugation to either sulphate or glucuronides that can be excreted. In the same process, a small fraction of B[a]P is activated to the intermediate metabolite 7,8-diol-B[a]P which converts it to the highly reactive 7,8-dihydroxy-9,10-epoxide-7,8,9,10-tetrahydro-B[a]P (PBDE) (Wislocki *et al.*, 1976; Jerina *et al.*, 1981) the ultimate DNA-adduct forming carcinogen responsible for mutagenesis and tumourigenesis (Poirier, 2004). Antibodies have been used to detect B[a]P DNA adducts in various tissues (Santella *et al.*, 1985; Zhang *et al.*, 1998; John *et al.*, 2009; Sagiv *et al.*, 2009). In iron foundry workers adducts in white blood cells correlated with the degree of exposure to B[a]P (Perera *et al.*, 1988). The cytochrome P450 enzymes Cyp1a1 and 1b1 play a pivotal role in this activation/detoxification pathway of B[a]P (Baird *et al.*, 2005; Arlt *et al.*, 2008). While B[a]P is mainly metabolized in the liver, immune cells such as murine spleen macrophages and human PBMC have also been implicated in PAH activation (Ladics *et al.*, 1992; Baron *et al.*, 1998; Hanaoka *et al.*, 2002).

B[a]P and its metabolites (Rahman *et al.*, 1986; van Schooten *et al.*, 1997) are detected in most organs independent of the route of administration (Yamazaki *et al.*, 1987). While B[a]P is primarily known for its carcinogenicity, it is also toxic for immune cells. B[a]P has been shown to immunosuppress cytotoxic T cell responses,

alloantigen-specific mixed-lymphocyte reactions, mitogen-induced T cell and B cell proliferative responses, and cytokine production. Human PBMC seem to be even more sensitive to B[a]P than mouse spleen cells (Mudzinski, 1993; Davila *et al.*, 1996). Activation of the aryl hydrocarbon receptor (AhR) plays an important but not exclusive role in immunotoxicity. In human macrophages, activation of the AhR by B[a]P reduced IL-8 secretion (Podechard *et al.*, 2008). B[a]P also suppressed B cell lymphopoiesis in bone marrow cultures and induced preB cell apoptosis via the AhR signalling pathway. AhR ligation also upregulates cytochrome P450 enzymes (Andrieux *et al.*, 2004; Schreck *et al.*, 2009) and is thus intimately linked to B[a]P activation and carcinogenesis (Gelboin, 1980; Buters *et al.*, 1999). Thus, activation of the AhR and the cytochrome P450 enzymes are implicated in both immunotoxicity and chemical carcinogenesis of B[a]P.

Immunoprophylactic approaches against nicotine (Hasman and Holm, 2004; Mihaltan, 2007; Wagena *et al.*, 2008) and cocaine (Martell *et al.*, 2005; LeSage *et al.*, 2006) have already been explored but only few attempts have been made to immunize against chemical carcinogenesis (Peck and Peck, 1971; Moolten *et al.*, 1978a; Moolten *et al.*, 1978b; Silbart and Keren, 1989).

We have previously shown *in vitro* that monoclonal antibodies raised against B[a]P can decrease cellular uptake of B[a]P in a variety of cells (e.g. HepG2 and PBMC) and thus modulate its subsequent metabolic conversion. In these experiments, antibody cross-reactivity with the proximate carcinogen 7,8-diol-B[a]P provided a second chance for interrupting metabolic activation by sequestration in the extracellular space of this intermediate metabolite generated intracellularly (De Buck *et al.*, 2005). Although reversible, antibody binding was associated with a beneficial biological effect *in vitro*, such as the reversal of B[a]P-induced immunotoxicity and the induction of cytochrome P450, a major risk factor for PAH carcinogenesis (De Buck *et al.*, 2005).

Nevertheless, few attempts have been made to explore potentially beneficial effects of such antibodies *in vivo* (Moolten *et al.*, 1978b; Grova *et al.*, 2009). Here we investigate in a mouse model, whether a B[a]P conjugate vaccine induces specific antibodies that modulate immunotoxic effects of B[a]P to provide proof of principle for an immunoprophylactic vaccination against this polycyclic aromatic hydrocarbon, and discuss potential effects on its carcinogenicity.

2. Material and methods

2.1. Synthesis of B[a]P-haptenconjugates

B[a]P was derivatised to Benzo[a]pyrenyl Butyric Acid (B[a]P-BA) as described before (Grabarek and Gergely, 1990). B[a]P-BA was coupled to diphtheria toxoid (DT, MW 63.000 g/mol, a generous gift from the Serum Institute of India, Hadaspar, India) or ovalbumin (OVA, MW 45.000 g/mol, Sigma-Aldrich, Bornem, BE) using a standard mixed ester activation of carboxyl groups(Grova *et al.*, 2009).

2.2. Animal treatments

Balb/c mice (10 week olds, female) were obtained from Harlan (Horst, NL) and acclimatized for 1 week. Animals were kept under timed 12 h light/dark cycles at 22 ± 2 °C and 40 ± 5% relative humidity. Food and water were available *ad libitum*. Water, food and oil were tested according to NF ISO 15302 by Micropolluant Technologie (Thionville, FR) to confirm that all matrices were B[a]P free down to a detection limit of 1 ng/g of fat matter and 10 ng/l of water. All animal experiments were done in compliance with the rules of the European Communities Council Directive of 24 November 1986 (86/609/EEC). Two groups of 30 mice were primed intraperitoneally (ip) with 30 µg of B[a]P-DT bioconjugates on day 0, emulsified in 50% complete Freund's adjuvant (CFA, v/v in PBS, Sigma Aldrich, Bornem, BE). On days 14 and 28 mice were boosted ip with the same antigen emulsified in 50% incomplete Freund's adjuvant (IFA, v/v in PBS, Sigma-Aldrich, Bornem, BE). Two mock immunized groups (n=30) were primed with 50% CFA and boosted with 50% IFA (v/v in PBS) alone. After a complete immunization schedule, mice were ip injected for 11 days with 2 mg/kg/day B[a]P (>97% purity, Sigma-Aldrich, Bornem, BE) diluted in 200 µl of commercial vegetable oil (ISIO 4, Lesieur, Asnières-sur-Seine, FR). The B[a]P stock solution in oil was freshly prepared weekly. Control animals received vegetable oil alone. Animals were anesthetized 2 h after the last B[a]P injection and blood was drawn by retro-orbital bleeding and collected in heparin tubes. Spleen and liver were dissected and directly used for analysis.

2.3. Detection of specific antibodies after immunizations with B[a]P-DT conjugates

On day 38 B[a]P antibody levels were determined in 384-well microtiter plates (Greiner, Wemmel, BE) coated overnight at 4 °C with 0.25 µM B[a]P-OVA in carbonate buffer (100 mM, pH 9.6). After washing, free binding sites were saturated with 1% BSA at RT. After 2 h, plates were washed again and diluted serum or carcinogen-specific mouse monoclonal antibody (P9E1R4 produced by immunization with B[a]P-DT, (Grova *et al*., 2009) was incubated for 90 min at RT. Binding was assessed by alkaline phosphatase-conjugated goat anti-mouse IgG (1/750 dilution, ImTec Diagnostics NV, Antwerp, BE) and the appropriate substrate (4-nitrophenyl phosphate disodium salt hexahydrate). Absorbance was measured at 405 nm (Spectromax Plus, Sopachem, Brussels, BE). Dilution curves (1/200 to 1/100000) of stock solution of different known concentrations (0.025, 0.050, 0.075, 0.1, 0.25, and 0.50 mg/ml) of B[a]P specific mab P9E1R4, were used to estimate absolute serum antibody concentrations. The dilution (1/25000) with the highest R^2-value was chosen to plot the linear standard curve (Figure1).

2.4. HPLC-analysis of B[a]P and its metabolites in immunized mice

B[a]P and its metabolites 7,8-diol-B[a]P, 1-OH-B[a]P, 3-OH-B[a]P and 9-OH-B[a]P were extracted from blood as previously described (Grova *et al*., 2008). The separation of B[a]P and metabolites were performed on an Agilent 1100 Series Liquid Chromatograph, equipped with a fluorescence detector on a reversed-phase Vydac C18 column (250 mm x 4.6, I.D., 5 µm particle size, Alltech, Lokeren, BE). 500 µl of sample was loaded on a Vydac C18 guard column (4.6, I.D., 5 µm particle size) by an initial 2 min isocratic elution with 20% acetonitrile in water 0.1% TFA at 1.5 ml/min. PAHs were eluted by acetonitrile/water gradient: 0-5 min by 20% acetonitrile, 5-10 min a linear gradient from 20% to 60% acetonitrile, 10-20 min a linear gradient from 60% to 100%, 20-22 min 100% acetonitrile, 22-24 min linear gradient to 20% acetonitrile and 24-30 min 20% acetonitrile. B[a]P and its metabolites were identified and quantified by comparing retention times and peak areas with standards (Grova *et al*., 2008). Compounds were eluted in the following order at the following excitation and emission wavelengths: 7,8-diol-B[a]P at 370-410 nm and 9-OH-B[a]P, 1-OH-B[a]P, 3-OH-B[a]P, B[a]P at 380-430 nm.

2.5. Quantification of Cyp1a1 and Cyb1b1 mRNA by quantitative real-time PCR

mRNA was purified from 10 mg of homogenized liver tissues and 10^6 splenocytes using a μMACS^{TM} mRNA isolation kit (Miltenyi Biotech, Utrecht, NE). First-strand synthesis of total cDNA was carried out at 42 °C for 50 min using 200 U Superscript II RT (Invitrogen, Merelbeke, BE) and 2.5 mM dT_{20} primer in a 30 μl reaction containing 250 mM Tris–HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$, 10 mM dithiothreitol, 40 U RNaseOUT ^{TM} (Invitrogen, Merelbeke, BE) and 500 mM deoxynucleoside triphosphates (dNTPs). Quantitative amplification of cDNA by PCR was performed on an Opticon 2 (MJ Research, Bio-Rad, Nazareth, BE) using 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 200 mM dNTPs, 1x SYBR-Green (Cambrex, Verviers, BE) and 2.5 U Platinum TaqDNA polymerase (Invitrogen, Merelbeke, BE) (35 cycles: 96 °C, 20 s; annealing, 20 s; 72 °C, 20 s), with a final elongation step for 10 min at 72 °C (Table 1). Specific pCR4-TOPO clones were used to generate absolute calibration curves. Plasmids were sequenced using 100 nM M13 primers and the BigDye 3.1 terminator cycle sequencing reagent (Applied Biosystems, Nieuwerkerk, NL) to ensure the correct sequence insert. Plasmid DNA was linearised using Not I (NEB, Hitchin, UK) and quantified using PicoGreen (Molecular Probes, Leuven, BE). The stability of the three housekeeping genes (*ß-Actin*, *Gapdh* and *Ppia*) in liver and spleen was measured as described previously (Schote *et al.*, 2007). All qRT PCR results of *Cyp1a1* and *Cyp1b1* gene expression are expressed as the number of copies of the gene per copies of *Ppia*.

2.6. Quantification of glutathione-S- transferase (Gsta4) mRNA by TaqMan PCR

Real-time PCR was performed on an ABI PRISM 7000 sequence detection system by using TaqMan Expression Assays for *Gsta4* (NM_010357.3) and TaqMan Fast Universal PCR Master Mix (Applied Biosystems, Lennik, BE) according to manufacturers' specifications. Thermal cycler conditions were as follows: 2 min at 50 °C, 20 s at 95 °C, followed by 2-step PCR for 40 cycles of 3 s at 95 °C followed by 30 s at 60 °C. All PCRs were performed in triplicates in independent PCR runs. Mean values of these repeated measurements were used for Δc_t calculation and *Ppia* as housekeeping gene.

2.7. Preparation of liver microsomes

Microsomes were prepared by differential centrifugation using an Optima L-100XP ultracentrifuge (Analis, Gent, BE). Briefly, liver samples were homogenized in buffer (20 mM Trizma® Base, 250 mM $MgCl_2$, 1 mM EDTA, pH 7.4) using a Elvehjem Potter. The lysate was centrifuged to remove the nuclei and mitochondria using a Heraeus 3047 rotor (600 xg, 10 min, 4 °C) and a Type 50.4 Ti rotor (10,000 xg, 10 min, 4 °C), respectively. The supernatant was again centrifuged at 170,000 xg for 40 min at 4 °C to sediment microsomes. Microsomal pellets as well as the cytosolic fraction (supernatant) were stored at -80 °C until use. The protein concentration was determined using the DC protein assay with BSA as protein standard (Bio-Rad, Nazareth, BE).

2.8. Determination of EROD activity

Cyp1a1/1b1 enzyme activity in splenocytes and liver microsomes was determined using ethoxyresorufin as specific substrate. Ethoxyresorufin and resorufin (Sigma-Aldrich, Bornem, BE) stock solutions were prepared as 10 mM stock solutions in dimethyl sulfoxide (DMSO) and stored at 4 °C in the dark. $1.2x10^6$ splenocytes were homogenized in buffer (20 mM Trizma® Base, 250 mM $MgCl_2$, 1 mM EDTA, pH 7.4) using a Elvehjem Potter. Frozen microsomes were thawed on ice and gently mixed. 200 µl reaction mixtures containing 0.1 M Tris-HCl buffer (5 mM $MgCl_2$, pH 7.4), 10 µM ethoxyresorufin and 40 µg microsomes ($1.2x10^6$ splenocytes respectively) were preincubated for 5 min at 37 °C. The reaction was initiated by adding 0.2 µmol NADPH (Sigma-Aldrich, Bornem, BE) and incubated for 30 min at 37 °C. The samples were quantified by measuring fluorescence using a Tecan Genius Plus plate reader equipped with a 535 nm ± 20 nm excitation and 590 ± 20 nm emission filters (Tecan Benelux, Mechelen, BE). A standard curve of resorufin (Sigma-Aldrich, Bornem, BE) was used (0.02 µg – 0.2 µg) to determine the specific enzyme activity expressed as pmol/mg protein/min. To distinguish between the activity of Cyp1a1 and 1b1, the latter was selectively inhibited by homoeriodictyol (Carl Roth, Karlsruhe, GE). The homoeriodictyol stock solution (1 mM) was prepared in absolute ethanol and stored at -20 °C. 200 µl reaction mixtures containing 0.1 M Tris-HCl buffer (5 mM $MgCl_2$, pH 7.4) 10 µM ethoxyresorufin, 1.5 µM homoeroiodictyol and 40 µg

microsomes ($1.2x10^6$ splenocytes respectively) were preincubated for 5 min at 37 °C. The reaction was initiated by adding 0.2 µmol NADPH (Sigma-Aldrich, Bornem, BE) and incubated for 30 min at 37 °C. 0.64 pmol Cyp1a1 and 1b1 supersomes served as control (BD-Gentest, Erembodegen, BE).

2.9. Determination of GST activity

The enzymatic activity of glutathione-S-transferase (GST) was determined with a colorimetric kit (Gentaur, Brussels, BE) based on the GST-catalyzed reaction between glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) a substrate of the α-, µ- and π-classes of GST isoenzymes (Pushparajah *et al.*, 2008). Briefly, 10 µl CDNB was pre-incubated for 5 min at RT with 90 µl GST assay buffer. The reaction was initiated by adding 100 µl of cytosolic fraction from the above microsome preparation to the pre-incubation mixture. Absorbance was measured every minute at 340 nm using a plate reader (Spectromax Plus, Sopachem, Brussels, BE) until 5 time points were obtained. A standard curve of GST standard was used (0.02 µg – 0.2 µg) to determine the specific enzyme activity expressed as nmol/mg protein/min.

2.10. Mitogen stimulation of PBMC and splenocytes

Spleens were mechanically disrupted, washed and lymphocytes were isolated by Ficoll gradient centrifugation (Ficoll-Paque PLUS, Amersham Biosciences, Diegem, BE). PBMC were also isolated by Ficoll gradient from 900 µl of heparinised blood diluted in 1 ml of Hanks Balance Salt Solution (HBSS, Biowhittaker, Verviers, BE). After several washing steps, cell counts and viability were determined on a nucleocounter (Chemometec, Allerød, DK). $2x10^6$ cells/ml were re-suspended in pre-warmed RPMI-1640 medium (37 ºC) supplemented with 10% fetal bovine serum (v/v), 100 IU/ml penicillin and 100 µg/ml streptomycin. Cells were plated in 96-well plates (2 x1 0^5 cells/well) and incubated for 1 h prior stimulation with 50 µl phytohemagglutinin (50 µg/ml, PHA, Sigma-Aldrich, Bornem, BE) or lipopolysaccharide (30 µg/ml, LPS, Sigma-Aldrich, Bornem, BE) from *E. coli* as mitogen. PBMC and splenocytes of each animal were cultured in triplicates for 48 h in a humidified (95%) atmosphere with 5% CO_2. To assess the proliferation rate cells were pulsed with 0.5 µCi of [^{3}H]-thymidine (Amersham, Pharmacia Biotech, Diegem,

BE) for 18 h and afterwards harvested onto glass filters using a MicroBeta FilterMate-96 Harvester (Perkin-Elmer, Zaventem, BE), dried and counted in a liquid scintillation counter (1450 Microbeta Trilux, Perkin-Elmer, Zaventem, BE). Aliquots of cell culture supernatants were collected and stored at -20 °C for determination of cytokine release.

2.11. Cytokine release in PBMC and splenocytes

Tumour necrosis factor alpha (TNF-α) and interferon gamma (INF-γ) release were determined by BD™ OptEIA Elisa kits (BD Biosciences**,** Erembodegem, BE). Briefly, 100 µl of the 1/250 diluted capture antibody (v/v carbonate buffer, pH 9.5) was incubated overnight at 4 °C in 96-well microtiter plates. After washing, free binding sites were blocked with 200 µl/well blocking buffer for 1 h at RT. After washing, 100 µl diluted cell culture supernatant (1/75 and 1/150, v/v for TNFα and INF-γ, respectively) was incubated for 2 h at RT. Binding was assessed by biotinylated anti-mouse TNF-α and INF-γ antibodies (1/250 diluted), a streptavidin horseradish peroxidase conjugated antibody (1/250 diluted) and the appropriate substrate (3,3',5,5' tetramethylbenzidine). Absorbance was measured at 450 nm – 570 nm (Spectromax Plus, Sopachem, Brussels, BE).

IL-12 was measured in cell culture supernatants by ELISA kits (Mabtech, Hamburg, GE). 2 µg/ml IL-12 capture antibody (200 µl) was coated of overnight at 4 °C in 96-well microtiter plates (v/v PBS, pH 7.4). After washing, free binding sites were blocked for 1 h at RT with 200 µl/well PBS containing 0.1% BSA and 0.05% Tween-20. After washing, 100 µl diluted cell culture supernatant (1/10 v/v) was incubated for 2 h at RT. Binding was assessed by 100 µl/well biotinylated anti-mouse IL-12 antibody (0.1 µg/ml respectively), and 2nd step antibodies as described above. Dilution curves of recombinant mouse TNF-α, INF-γ and IL-12 (3-200 pg/ml) were used to estimate absolute antibody concentrations.

2.12. T-cell-dependent antibody response assay

Isolated splenocytes (2x10^5 cells/well) were seeded in 96-well plates and stimulated with 100 µl Keyhole Limpet Hemocyanin (KLH, 100 µg/ml, Sigma Aldrich, Bornem,

BE) for 4 days, to activate B-cells. T-cell-depended IgM antibody levels were assessed in supernatants using ELISA plates coated overnight with KLH. Plates were incubated with serial dilutions of diluted supernatants (1/250 in PBS). After washing, free binding sites were saturated for 2 h with 1% BSA at RT. After washing, binding was assessed by alkaline phosphatase-conjugated goat anti-mouse IgM (1/500 dilution, ImTec Diagnostics NV, Antwerp, BE) and the appropriate substrate (4-nitrophenyl phosphate disodium salt hexahydrate). Absorbance was measured at 405 nm (Spectromax Plus, Sopachem, Brussels, BE). Dilution curves of purified mouse IgM anti-KLH ($1.5\ 10^{-4}$ – 25.0 µg/ml, BD Biosciences**,** Erembodegem, BE) were used to estimate absolute serum antibody concentrations.

2.13. Statistical analysis

The results were analyzed using a one way analysis of variance followed by planned contrasts with Student-Newman-Keuls correction. Given that most data did not follow a Gaussian distribution, non-parametric statistical procedures were used. Data from controls were compared to those of B[a]P-exposed animals using a Kruskal-Wallis test followed by a Dunn's procedure for post-hoc comparisons. The statistical analysis was performed using the Sigma Stat software for Windows (Erkrath, GE). Differences were considered to be significant when $p<0.05$.

3. Results

3.1. Detection of specific antibodies after immunization with B[a]P-DT conjugates

Levels of B[a]P antibodies were determined 14 days after completing the immunization schedule with B[a]P-DT and were compared to a standard curve of dilutions of known concentrations of purified B[a]P specific monoclonal antibody (P9E1R4, 0.025 to 0.5 mg/ml) to estimate antibody concentrations (Figs. 1A and 1B insert). Mean OD values of B[a]P antibodies in the two actively immunized groups of 30 mice each ranged between 1.246 ± 0.12 and 1.429 ± 0.16 (dilution of 1/25,000) corresponding to a serum antibody concentration of about 0.16 ± 0.07 and 0.19 ± 0.09 mg/ml (Figure 1B). Assuming a serum IgG concentration of about 12 mg/ml (Harlow and Lane, 1988; Junghans, 1997), B[a]P-specific antibodies represented approximately 1.3 to 1.6% of the serum IgG, which correspond to a usual antibody response (Janoff *et al.*, 1991; Hieda *et al.*, 1999; Hieda *et al.*, 2000; Pentel *et al.*, 2000). The 2 groups of mice were further used for sub-acute B[a]P exposure experiments. A similar number of mice was mock immunized and produced no detectable levels of B[a]P antibodies (data not shown).

3.2. Modulation of B[a]P metabolism by specific antibodies

3 weeks after a complete immunization schedule with B[a]P-DT, animals were given a daily injection of 2 mg/kg B[a]P for 11 days ("sub-acute B[a]P exposure"). Control mice received diluent only. The recovery of B[a]P and its metabolites was analyzed in the serum. Specific antibodies in immunized mice increased the concentration of recovered B[a]P about 5-fold by the end of the exposure period ($p<0.01$; Table 4). Antibodies also modulated the recovery of B[a]P metabolites in particular 3-OH and to a lesser extent 1-OH and 9-OH-B[a]P by 2.6 (1-OH-B[a]P) to 6.3 fold (3-OH-B[a]P) ($p<0.05$, Table 4). In addition, the recovery of 7,8-diol-B[a]P, an intermediate metabolite was enhanced (6.35-fold; $P<0.05$). Specific antibodies increased the total recovery of B[a]P and metabolites by 6-fold ($p<0.01$) but the relative distribution between B[a]P and its metabolites was unaffected (Table 4). Also concentrations of B[a]P metabolites recovered correlated with specific antibodies at the level of individual mice (R^2=0.57 to 0.80, Figure 2).

3.3. Cyp1a1, Cyp1b1 and Gsta4 mRNA expression by quantitative real-time PCR

Since B[a]P metabolites (in particular 7,8 diol-B[a]P) are responsible for immunotoxicity (Kawabata and White, 1987; Carlson *et al.*, 2004), the induction of *Cyp1a1* and to a lesser extent *Cyp1b1* expression (by B[a]P and its metabolites) is critical. As expected *Cyp1a1* and *1b1* were differentially expressed in liver and spleen, both constitutively and in response to B[a]P by AhR activation (Choudhary *et al.*, 2005). Table 2 shows that sub-acute administrations of B[a]P induced a ~30-fold increase in *Cyp1a1* mRNA expression in liver but specific antibodies had no effect on up-regulation. B[a]P increased *Cyp1b1* mRNA expression by 5 fold, and B[a]P specific antibodies reversed about 55% of this effect (Table 2). Both *Cyp1a1* and *Cyp1b1* expression in splenocytes was increased by B[a]P (2-fold, $p<0.05$, Table 2), but specific antibodies prevented up-regulation only of *Cyp1a1* expression: There was no statistical difference between Mock/B[a]P and B[a]P-DT/B[a]P groups. B[a]P-induced expression of *Gsta4*, was low but significant and reproducible in the liver and this up-regulation was prevented by B[a]P specific antibodies ($p<0.05$, Table 2). In splenocytes, there was no measurable effect of B[a]P on *Gsta4* expression.

3.4. Cyp1a1, Cyp1b1 and GST activity

At the (low) concentration of B[a]P used, the induced increase in activity of Cyp1a1 and 1b1 was relatively weak but significant enough (Cyp1a1/1b1 1.9-fold, $p<0.05$, Table 2) to further investigate the effect of antibodies against B[a]P. Specific antibodies had no apparent influence on the combined Cyp1a1 and 1b1 activity in the liver. The activity of each of the two enzymes was assessed by using the Cyp1b1-selective inhibitor homoeriodictyol. Cyp1b1 activity determined by subtraction was reduced in mice immunized against B[a]P compared to mock immunized mice; no effect of specific antibodies on Cyp1a1 activity was observed (Table 2). B [a] P (Table 2) did not measurably induce the cytosolic GST activity in liver microsomes when the broadly reactive substrate CDNB was used (see Materials and Methods). Cyp1a1, 1b1 and GST activity were tested in splenocytes but levels were below detection limits (Table 2), in the crude cell lysate.

3.5. Mitogen stimulation of PBMC and splenocytes

The attenuation of B[a]P induced immunotoxicity was also evaluated in mice actively immunized with B[a]P-DT. 60 min after the last B[a]P injection, PBMC and splenocytes were collected and treated with LPS and PHA for 66 h to assess mitogen responsiveness (Figs. 3A and B). As expected, the overall response to the mitogens was stronger in spleen cells than in PBMC and spleen cells containing more B cells and macrophages responded better to LPS than PHA. Sub-acute exposure of B[a]P did not effect cell counts and viability (data not shown) but suppressed the proliferative response of both B (-33% to -67%, Figure 3) and T cells (-36% to -74%, Figure 3) to LPS and PHA, respectively, although the immunotoxic effect of B[a]P on PHA-stimulated PBMC was only marginal ($p=0.106$). Figure 3 also shows that B[a]P-induced inhibition of proliferation in both PBMC and splenocytes is reversed in immunized mice. Thus, specific antibodies protect both B and T cells against B[a]P-associated immunotoxicity. Cytokine levels were also measured in culture supernatants. B[a]P specific antibodies completely restored B[a]P-inhibited release of IFN-γ in PBMC and splenocytes stimulated with PHA (-45%, $p<0.05$, Table 3). PHA-induced TNF-α was reduced slightly by B[a]P, both in PBMC and splenocytes (- 17%, $p<0.05$) and this decrease was also reversed in B[a]P-DT immunized mice (Table 3). Specific antibodies also reversed inhibition of TNF-α by B[a]P in splenocytes stimulated with LPS (Table 3, $p<0.05$). No effect of B[a]P on TNF-α release from LPS-stimulated PBMC was observed. Only a tendency to a reduction of IL-12 was observed in LPS-stimulated PBMC and splenocytes in response to B[a]P. In both, PBMC and splenocytes of immunized animals this immunosuppressive effect of B[a]P seemed to be reversed (Table 3).

3.6. T-cell-dependent antibody response

T-cell-dependent antibody response is one of the most sensitive parameters of immunotoxicity. Therefore, the protective effect of an immunoprophylactic strategy was also investigated by stimulating B cells with Keyhole Limpet Hemocyanin (KLH) as an antigen. B[a]P-DT and mock immunized mice were exposed to B[a]P using our standard sub-acute protocol. Two hours after the last injection, spleen cells were explanted and co-cultured with KLH. B cell activity was significantly reduced in mice

that were exposed to B[a]P (-15%, $p<0.05$) as measured by levels of anti-KLH IgM produced *in vitro* (Figure 4). Remarkably, B cells of mice vaccinated against this B[a]P were protected against B[a]P associated immunotoxic effect *in vivo*.

4. Discussion

In this study, we explored the protective effect of specific antibodies on biological effects of B[a]P. Host concentrations of B[a]P caused by environmental or self exposure are very low and usually do not lead to short-term measurable biological effects. Therefore, most experimental models use much higher concentrations of B[a]P to obtain a short-term readout. However, these high concentrations cannot be stoichiometrically matched by concentrations of specific antibodies that are normally reached after immunization and are therefore inappropriate to test whether antibodies may influence biological effects of B[a]P in animals. In contrast, the relevant low host concentrations resulting from environmental exposure can be matched by specific antibodies and immunosuppression is sensitive to those low concentrations (0.1 nM) early after sub-acute administration of B[a]P (Hardin *et al.*, 1992).

Using a B[a]P-DT conjugate vaccine we induced specific antibodies in mice, which attenuated immunotoxic effects even of high environmental concentrations of B[a]P (2 mg/kg). Active immunization reversed or attenuated the effect of B[a]P on the proliferation of peripheral blood and spleen lymphocytes and concomitant cytokine secretion (TNF-α, IFN-γ and perhaps IL-12 a potent inducer of IFN-γ, Table 3). Both the responses to LPS and PHA were restored, indicating that both B and T cells were protected (Figs. 3A and B). In B cells, specific antibodies also restored antigen-dependent antibody responses using KLH as an antigen (Figure 4). These results demonstrate a beneficial effect of specific antibodies against B[a]P to reduce B[a]P-induced immunotoxicity on the humoral and cellular mediated immunity**.**

Several authors suggested that B[a]P and in particular its activated metabolite 7,8-diol-B[a]P (Kawabata and White, 1989; Carlson *et al.*, 2004), exert their immunotoxic effects by binding to a specific aryl hydrocarbon receptor (AhR). Ligation of AhR also up-regulates the phase 1 enzymes cytochrome P450 responsible both for B[a]P activation to adduct forming metabolites and the generation of immunotoxic metabolites. The importance of this activating step was demonstrated in murine and human B cells where B[a]P-induced immunotoxicity was prevented by α-naphthoflavone, an agent blocking the AhR as well as Cyp1a1 and 1b1 activity (Mudzinski, 1993; Davila *et al.*, 1996; Salas and Burchiel, 1998). Our data indicate that in the liver antibodies reduced B[a]P-induced *Cyp1b1* up-regulation both at the

mRNA and protein activity level but had no effect on B[a]P-induced *Cyp1a1* levels (Table 2). The liver, like most other tissues (e.g. lung; kidney), expresses low constitutive levels of *Cyp1a1*, but these are highly sensitive to induction by low levels of B[a]P (Galvan *et al.*, 2003). In contrast, *Cyp1b1* is constitutively expressed mainly in extra-hepatic tissues e.g. bone marrow cells (Heidel *et al.*, 1998; Galvan *et al.*, 2003) and its expression in the liver is not very sensitive to B[a]P (Shimada *et al.*, 2003). Thus in the liver, antibodies seem to reduce B[a]P concentrations enough to prevent the induction of *Cyp1b1* but not *Cyp1a1* which is sensitive to lower B[a]P concentrations. In spleen cells, both constitutive and induced levels of P450 enzymes are low and high levels of B[a]P are necessary for induction. Thus, in contrast to the liver, in splenocytes B[a]P-specific antibodies completely reversed B[a]P-induced up-regulation of *Cyp1a1***,** but in this organ the immunization had no preventive effect on the expression of *Cyp1b1*.

We also showed at least at mRNA level that B[a]P induces *Gsta4* in the liver (in contrast to the spleen) and that this induction was also reversed by B[a]P-specific antibodies. If the effect was not visible at the activity level GSTA4 this may be due to a broader reactivity of the substrate with all α-, μ- and π-classes of GST (Pushparajah *et al.*, 2008). B[a]P has been found to deplete glutathione (Yuan *et al.*, 1994; Romero *et al.*, 1997), resulting in an increased formation of 7,8-diol-B[a]P, a precursor of the ultimate carcinogen. Thus, sequestration of B[a]P and its metabolites in the blood may also reduce glutathione depletion by preventing peak concentrations of free circulating metabolites. Together these results indicate that specific antibodies reduce cytochrome P450 activity and influence the detoxification/activation pathways by restoring the balance between phase I and phase II metabolizing enzymes. Thus, one explanation of the protective effect of antibodies is a reduced B[a]P metabolism. Our metabolism studies showed that all metabolites (in particular the 7,8-dio- B[a]P) were several fold increased in the serum. This can be explained by cross-reactivity of antibodies with both B[a]P and its metabolites (De Buck *et al.*, 2005). Their relative concentrations remained unchanged (Table 4) and the correlation between specific antibody titers and B[a]P-metabolites recovered in the serum (Figure 2) suggesting that the increase in the recovery of B[a]P and its metabolites in blood of B[a]P-DT immunized mice is more likely the consequence of B[a]P/metabolite sequestration by binding to the antibodies than increased metabolism of B[a]P.

Pathways leading to B[a]P associated immunotoxicity such as activation of the aryl hydrocarbon receptor (AhR) and of cytochrome P450 enzymes partially overlap with those triggering chemical carcinogenesis (Burchiel *et al.*, 2001; Burchiel and Luster, 2001). Several lines of evidence suggest that tissue specific expression and inducibility of *Cyp1a1* and *Cyp1b1* by B[a]P govern the sensitivity to B[a]P-toxicity and also carcinogenicity (Liang *et al.*, 1997; Galvan *et al.*, 2003; Peter Guengerich *et al.*, 2003; Galvan *et al.*, 2005). Thus, our results suggest that beyond modulating immunotoxicity an immunoprophylactic strategy may have some beneficial effect also on carcinogenesis. Because most models of carcinogenicity are based on relatively high doses of PAH that are difficult to match with stoichiometric concentrations of antibodies, such effects on chemical carcinogenesis are difficult to demonstrate in animal models. However, concentrations reached by high environmental exposure with long-term carcinogenic effects can easily be matched by equivalent concentrations of specific antibodies. On a molar level of binding sites, the antibody levels obtained by our immunization strategy in mice can bind 2.8 nmol or 0.7 µg B[a]P corresponding to a concentration of 30 µg/kg body weight (assuming a blood volume in mouse of 2 ml, and average body weight of 20 g). Assuming a daily mean uptake of B[a]P even for highly exposed individuals (e.g. coke oven workers) of 2.8 µg/kg/day (Lewtas *et al.*, 1997), these concentrations can be matched by antibodies induced by immunization.

However, based on current cancer theories, the Office of Environmental Health Hazard-Assessment (OEHHA) treats B[a]P carcinogenesis as a non-threshold phenomenon, meaning that even the long-term exposure to low concentrations of B[a]P is associated with a certain risk of cancer. From this perspective, an immune-intervention against chemical carcinogenesis could be in principle sensible at least for individuals with genetic risk factors or with a high risk of exposure such as street workers, police officers, fire fighters, coal mining workers, cooks or smokers etc. Since most individuals even in the general population cannot escape exposure to B[a]P, a formal distinction between pre- or post-exposure intervention does not apply. Clinical studies are necessary to identify which risk groups may benefit, and under which conditions, from such an intervention.

In conclusion, our study provides proof of principle that vaccination with a B[a]P conjugate vaccine based on carrier protein used in licensed human vaccines reduces immunotoxicity induced by environmentally relevant concentrations of B[a]P. Furthermore, to some extent, this is also proof of principle for other low molecular

weight compounds with similar physical properties e.g. 2-acetylaminofluorene (AAF), dimethylbenzanthracene (DMBA), and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Our data revealed that this effect is due to the sequestration of B[a]P and 7,8-diol-B[a]P, and by blocking induction of enzymes that convert the procarcinogen B[a]P to the ultimate DNA-adduct forming metabolite.

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Table 1: Primer sequences and quantitative RT-PCR conditions.

Gene	Sequence	Product length (bp)	$[Mg^{2+}]$ (mM)	[Primers] (μM)	Annealing Tm (°C)
Ppia NM_008907	Fwd 5'-CAA-GAC-TGA-ATG-GCT-GGA-TGG-C Rev 5'-CAG-GAC-ATT-GCG-AGC-AGA-TGG	235	3	0.5	59
Cyp1a1 NM_009992	Fwd 5'-TCT-GAG-CAA-TGA-GTT-TGG-GGA-GG Rev 5'-ACA-GCT-GGA-CAT-TGG-CAT-TCT-CG	251	2	1.0	62
Cyp1b1 NM_009994	Fwd 5'-TTG-ACC-CCA-TAG-GAA-ACT-GC Rev 5'-GCT-GTC-TCT-TGG-TAG-GAG-GA	113	2	0.75	59

Table 2: mRNA expression of selected genes and their enzymatic activity in liver and splenocytes of B[a]P-DT or mock immunized mice subsequently exposed sub-acutely (or not) to B[a]P. [1]

	Gene/Enzyme	Liver				Splenocytes			
		Mock	B[a]P-DT	Mock / B[a]P	B[a]P-DT / B[a]P	Mock	B[a]P-DT	Mock / B[a]P	B[a]P-DT / B[a]P
mRNA expression	***Cyp1a1*** (nb copy of Cyp1a1/10^3 copy of ppia)	0.19 ± 0.04	0.33 ± 0.05	5.66 ± 1.22*	8.78 ± 1.02*	0.04 ± 0.01	0.03 ± 0.01	0.08 ± 0.01*	0.04 ± 0.01
	Cyp1b1 (nb copy of Cyp1b1/10^3 copy of ppia)	0.28 ± 0.01	0.34 ± 0.04	1.43 ± 0.25*	0.65 ± 0.08*	0.08 ± 0.02	0.03 ± 0.01	0.14 ± 0.03*	0.34 ± 0.07*
	Gsta 4 (ΔC_t)	2.55± 0.10	2.65± 0.07	3.00± 0.11*	2.52± 0.10	8.21 ± 0.13	8.07 ± 0.16	7.83 ± 0.19	7.51 ± 0.29
Protein activity	**Cyp1a1+Cyp1b1** (pmol/min/mg)	16.88 ± 3.04	19.77 ± 2.77	31.43 ± 3.07*	30.18 ± 3.48*	n.d.	n.d.	n.d.	n.d.
	Cyp1a1 (pmol/min/mg)	11.56 ± 1.57	13.82 ± 1.59	20.33 ± 2.13*	21.24 ± 2.96*	n.d.	n.d.	n.d.	n.d.
	Cyp1b1 (pmol/min/mg)	5.32 ± 1.55	5.95 ± 1.47	11.09 ± 1.20*	8.94 ± 1.35	n.d.	n.d.	n.d.	n.d.
	Cytosolic Gst (nmol/min/mg)	266 ± 10.0	253 ± 10.6	267 ± 8.1	238 ± 11.5	n.a.	n.a.	n.a.	n.a.

[1] Results are expressed as mean ± SE of 5-10 mice.
n.d. not detectable, n.a. not analyzed.
*p< 0.05 statistical significant difference between mock and B[a]P-DT immunized groups (Dunn's procedure for post-hoc comparisons).

Table 3: Cytokine release in cell culture supernatants of isolated PBMC and splenocytes stimulated *in vitro* either with LPS or with PHA.

Cytokines	Conditions	PBMC				Splenocytes			
		Mock	B[a]P-DT	Mock / B[a]P	B[a]P-DT / B[a]P	Mock	B[a]P-DT	Mock / B[a]P	B[a]P-DT / B[a]P
IFN-γ (ng/ml)	PHA	8.68 ± 0.60	9.17 ± 0.75	4.70 ± 0.84 ***	9.48 ± 0.75	10.99 ± 0.44	9.80 ± 0.53	6.66 ± 0.57 *	9.47 ± 0.44
TNF-α (ng/ml)	LPS	5.30 ± 0.28	5.00 ± 0.31	4.93 ± 0.35	4.56 ± 0.33	3.81 ± 0.82	3.59 ± 0.24	2.83 ± 0.55*	3.39 ± 0.09
	PHA	1.65 ± 0.20	1.72 ± 0.11	1.37 ± 0.12 *	1.98 ± 0.15	1.43 ± 0.08	1.58 ± 0.14	1.17 ±0.07 *	1.52 ± 0.04
IL-12 $_{tot}$ (ng/ml)	LPS	2.69 ± 0.69	2.93 ± 0.63	2.17 ± 0.21	2.67 ± 0.25	0.76 ± 0.05	0.79 ± 0.11	0.69 ± 0.09	0.78 ± 0.07

Animals were either B[a]P-DT or mock immunized and subsequently exposed sub-acutely (or not) to B[a]P. Results are expressed as mean ± SE of 10-20 mice.
***p< 0.001, *p< 0.05 statistical significant difference between mock and B[a]P-DT immunized groups. (Dunn's procedure for post-hoc comparisons)

Table 4: Recovery of B[a]P and its metabolites (ng/ml) in the serum of B[a]P-DT or control mock immunized mice sub-acutely exposed to B[a]P.

Metabolites	Mock / B[a]P		B[a]P-DT / B[a]P	
	ng/ml ± SE	% total recovery	ng/ml ± SE	% total recovery
7,8-diol-B[a]P	3.744 ± 0.463	21.65	23.777 ± 10.501*	23.53
9-OH-B[a]P	0.203 ± 0.028	1.18	0.843 ± 0.223*	0.84
1-OH-B[a]P	2.070 ± 0.205	11.97	5.500 ± 0.944*	5.44
3-OH-B[a]P	11.093 ± 0.844	64.15	70.212 ± 18.406**	69.48
B[a]P	0.183 ± 0.021	1.06	0.890 ± 0.392**	0.88
Total	17.293 ± 1.359	100	102.029 ± 26.568**	100

Percentage values represent the contribution of each metabolite/B[a]P to the total recovered. Results are expressed as mean ± SE of 5.
*$p<0.05$ **$p<0.01$, statistically significant difference between B[a]P-DT and mock immunized mice (Mann-Whitney U-test for multiple comparisons).

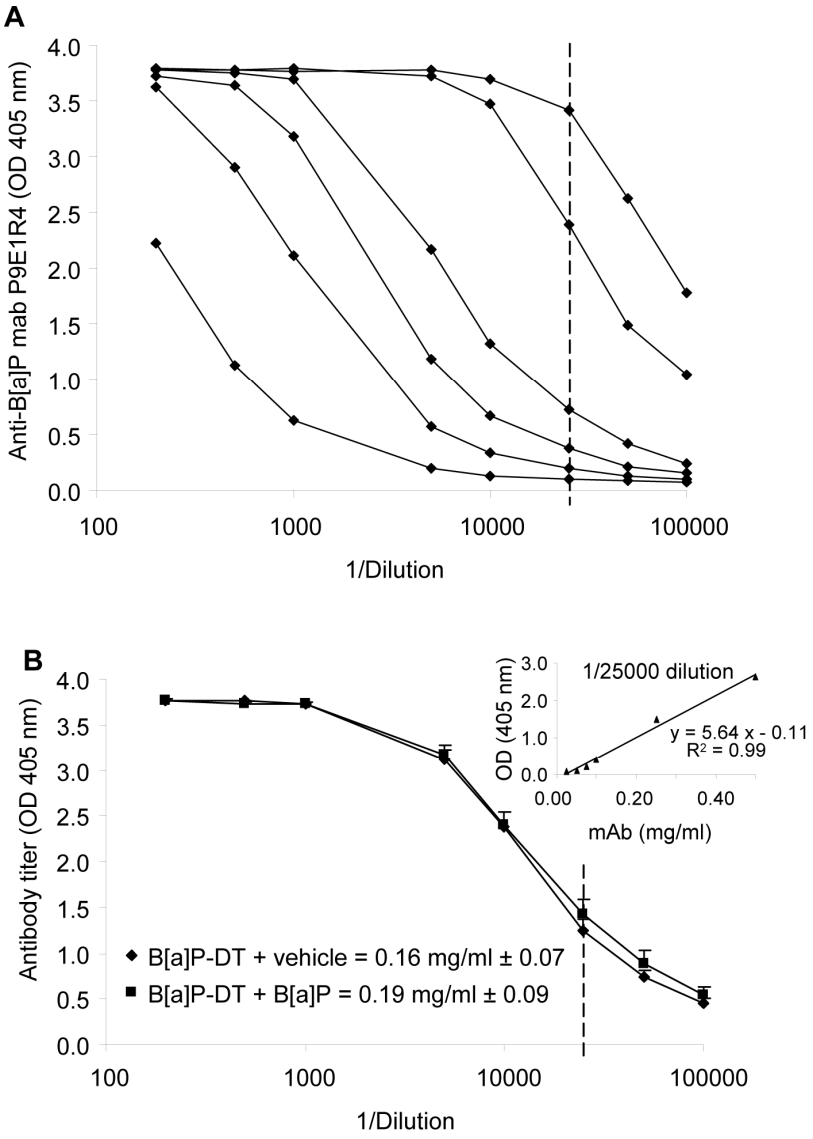

Figure 1: Anti-B[a]P antibody concentration determined by indirect ELISA. (A) To estimate absolute antibody concentrations a standard curve (insert panel B) was used, that is based on dilution curves (1/200 to 1/100000) of different known concentrations (0.005-0.5 mg/mL, represented by a line for each concentration in panel A) of purified monoclonal antibody (mab P9E1R4 against B[a]P). 1/25000 dilution (dashed line) was chosen to plot the standard curve (insert B, R^2=0.99). (B) Titration of serum antibodies and estimated serum concentration of mice immunized against B[a]P-DT two weeks after the third boost by indirect ELISA. Values are presented as mean ± SE of 30 mice per group.

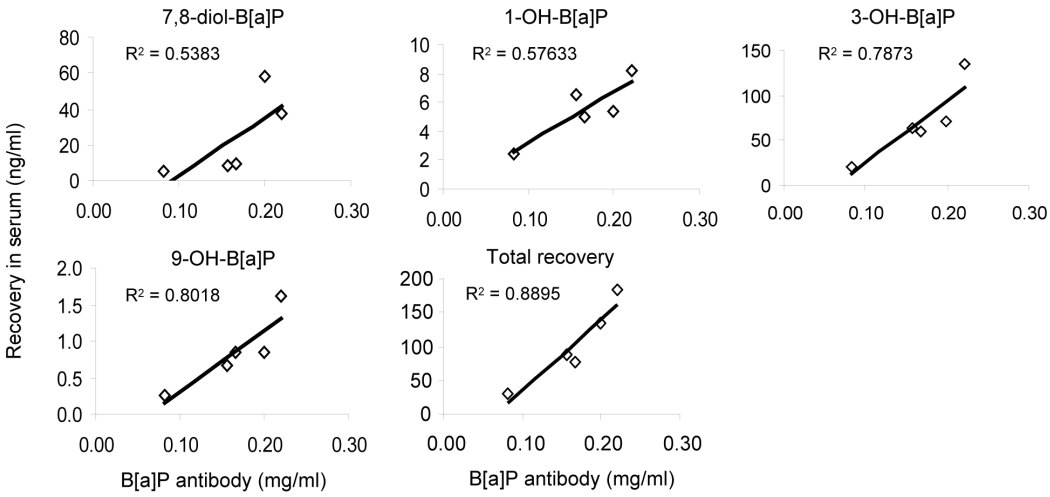

Figure 2: Correlation (linear regression) between serum titers of B[a]P specific antibodies in individual mice immunized with B[a]P-DT and the concentration of B[a]P/metabolites recovered in the serum.

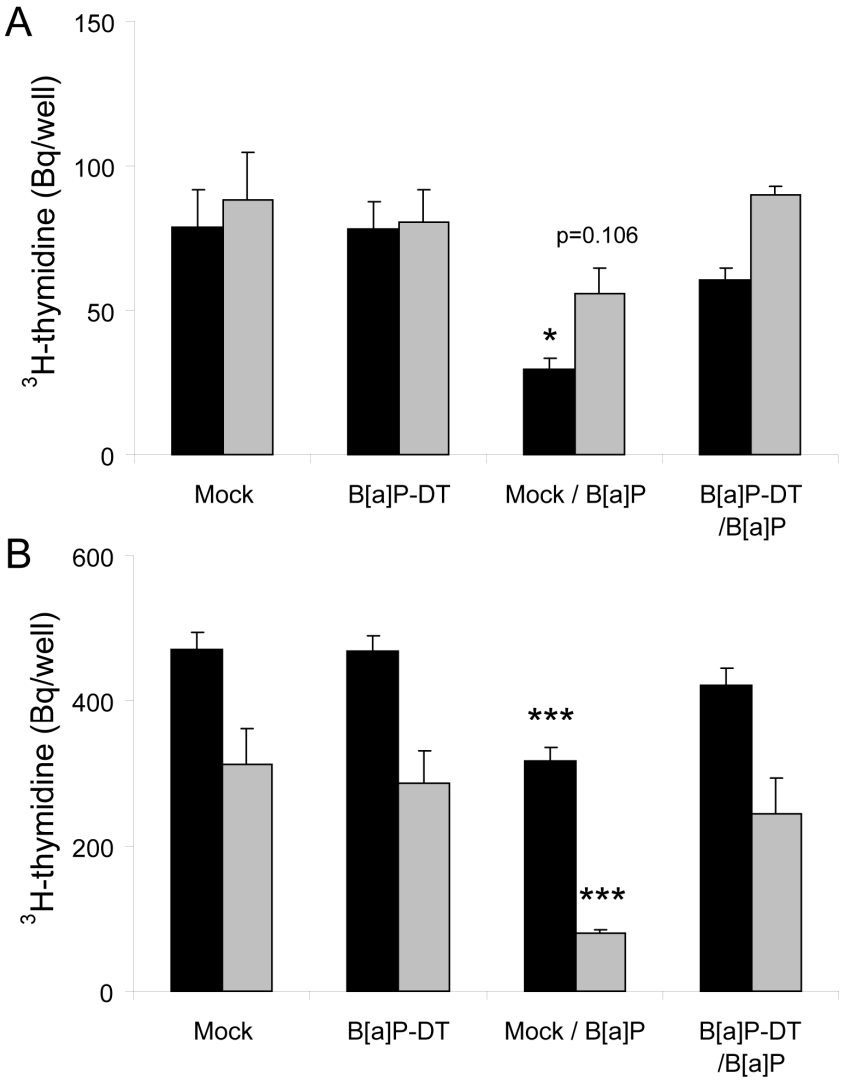

Figure 3: Proliferation of mouse PBMC (A) or splenocytes (B) stimulated with 30 µg/ml LPS (closed bars) or 50 µg/ml PHA (gray bars). Mice were either B[a]P-DT or mock immunized and were subsequently sub-acutely exposed to B[a]P. Values are expressed as mean ± SE of 20 mice per group, one way ANOVA procedure was applied, *$p<0.05$ ***$p<0.001$, statistically significant difference between B[a]P-DT and mock immunized mice (Student-Newman-Keuls-t test for multiple comparisons).

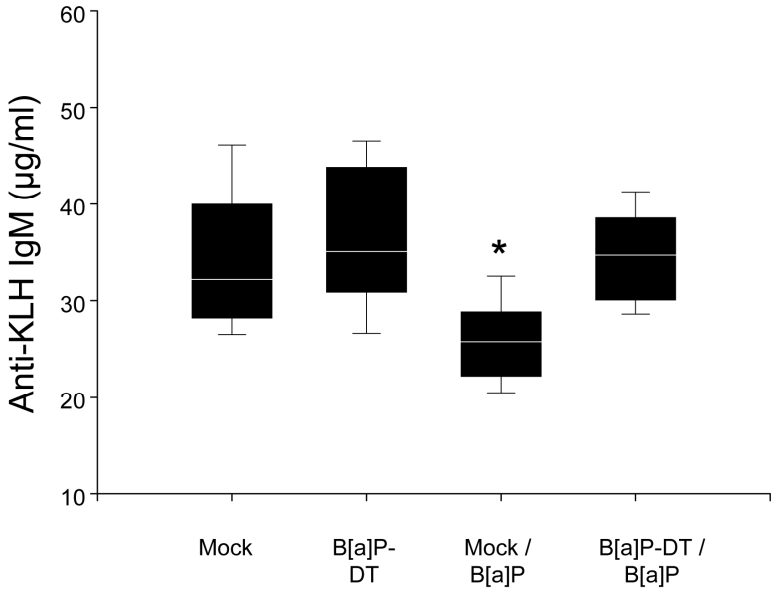

Figure 4: T-cell dependent anti-KLH IgM response in mouse splenocytes (KLH assay). Mice were either B[a]P-DT or mock immunized and were subsequently sub-acutely exposed to B[a]P. O.D. values of serial dilutions of culture supernatant were converted to absolute concentration of anti-KLH IgM using a standard curve prepared with serial dilutions of a purified commercial anti-KLH IgM antibody, as described in Figure 1. Values are expressed as mean ± SE of 20 mice per group, one way ANOVA procedure was applied, *$p<0.05$, statistically significant difference from mock immunized animals (Student-Newman-Keuls-t test for multiple comparisons).

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Chapter 4

Evaluation of adjuvants for a candidate conjugate vaccine against benzo[a]pyrene

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Abstract

We have recently developed an experimental vaccine based on benzo[a]pyrene (B[a]P) conjugated to tetanus toxoid as a carrier protein. In combination with Freund adjuvant, this vaccine induces high levels of B[a]P-specific antibodies to protect against detrimental effects of this carcinogen. Here we evaluate this conjugate vaccine by replacing Freund adjuvant by adjuvants that are potentially compatible with their use in humans. We showed that all adjuvants tested induced specific antibodies against B[a]P and 7,8-diol-B[a]P, its carcinogenic metabolite. The best antibody levels were obtained with Quil A, MF-59 and Alum. Biological activity in terms of enhanced retention of B[a]P was confirmed in mice immunised with Quil A, Montanide, Alum and MF-59. Our findings demonstrate that a vaccination against B[a]P is feasible in combination with adjuvants licensed in humans.

1. Introduction

Benzo[a]pyrene (B[a]P, Figure 1A) the prototype polycyclic aromatic hydrocarbon (PAH) is an environmental pollutant emanating from both natural and anthropogenic sources including industrial processes, cooking, barbecuing and cigarette smoking (Hattemer-Frey and Travis, 1991; Hecht, 1999; Phillips, 1999). Its main uptake is by ingestion of contaminated food, by inhalation of contaminated air and cigarette smoke (Lambré and Muller, 2004). Even if it were possible to reduce anthropogenic sources of B[a]P, it would be impossible for the general population and in particular professional and other risk groups to avoid exposure to this ubiquitous compound.

B[a]P is a procarcinogen, which is well known to induce tumours in a number of organs, both in animal models and humans (Thyssen *et al.*, 1981; Wolterbeek *et al.*, 1995). Although natural antibodies against environmental carcinogens have been described (Harris *et al.*, 1985; Haugen *et al.*, 1986; Newman *et al.*, 1988; Lee and Strickland, 1993; Galati *et al.*, 2001), the biological consequences of such antibodies have only recently been investigated (Glushkov, 2003; De Buck and Muller, 2005). Hapten-specific antibodies against biologically active low molecular weight compounds have been widely applied as therapeutic agents to reverse their pharmacological effects (de Villiers *et al.*; Butler and Beiser, 1973; Colburn, 1980; Butler, 1982; Sullivan, 1986; Scherrmann *et al.*, 1989; Bismuth *et al.*, 1997; Silbart *et al.*, 1997; Oliver *et al.*, 2007), but their use as immunoprophylactic agents has only recently gained some interest (Silbart and Keren, 1989; Rasmussen *et al.*, 2001; De Buck *et al.*, 2005a; De Buck *et al.*, 2005b; De Buck and Muller, 2005; De Buck *et al.*, 2009; Grova *et al.*, 2009; Schellenberger *et al.*, 2009). Several candidate vaccines against nicotine and cotinine, its major metabolite, have been developed and are in clinical trials (de Villiers *et al.*; Hatsukami *et al.*, 2005; Maurer *et al.*, 2005; Oliver *et al.*, 2007; Cornuz *et al.*, 2008; Wagena *et al.*, 2008).

We have developed an experimental vaccine based on B[a]P conjugated to tetanus toxoid (TT) or diphtheria toxoid (DT) as carrier proteins that induces high levels of antibodies against this carcinogen (Grova *et al.*, 2009). Our earlier work demonstrated that antibodies derived against low molecular weight carcinogens protect cells *in vitro* by a number of expected and unexpected mechanisms against adverse effects of carcinogens (De Buck *et al.*, 2005a; De Buck *et al.*, 2005b; De Buck and Muller, 2005; De Buck *et al.*, 2009). We demonstrated in a bicompartment model of polarised intestinal cells (Caco-2) that antibodies have the potential to

reduce the absorptive transport (De Buck *et al.*, 2005a). The biological relevance of antibodies was shown by modulating the B[a]P metabolism and DNA adduct-formation *in vitro* (De Buck *et al.*, 2005b). *In vivo* our immune prophylactic strategy was protective against B[a]P-induced immunotoxicity and neurotoxicity (Schellenberger *et al.*, 2009). These animal-studies showed that specific antibodies modulate the pharmacokinetic and pharmacodynamic of environmentally relevant low concentrations of B[a]P and reduce detrimental biological effects of B[a]P (Grova *et al.*, 2009; Schellenberger *et al.*, 2009). These results also suggested that specific antibodies might reduce the risk of chemical carcinogenesis. In the above animal studies, mice were immunised with the B[a]P-carrier conjugate emulsified in complete/incomplete Freund's adjuvant. Complete Freund's adjuvant (CFA) is an oil based adjuvant consisting of mycobacteria, paraffin oil and mannide monooleate. It is normally combined with Freund's incomplete adjuvant (IFA), an oil based adjuvant without bacterial extracts. Its excessive inflammatory effects prohibit its use in humans and limit its applications in animals.

Alum is the most widely used adjuvant in humans either as aluminium hydroxide or as aluminium phosphate. It is thought to act as a slow release system of the absorbed antigen (Singh *et al.*, 2006). MF-59 is an oil-in-water emulsion containing squalene, Tween-80 and Span-85 and was first used in a seasonal influenza vaccine (Fluas ®) and is licensed for human use in more than 20 countries (Podda and Del Giudice, 2003). MF-59 has also been tested in clinical trials with several different antigens (HIV gp120/gp140, HBV) (Nitayaphan *et al.*, 2000; Schultze *et al.*, 2008). Microparticles prepared from biodegradable and biocompatible polymers such as poly lactide co-glycosides (PLG) represent a new approach as vaccine adjuvant (Okada and Toguchi, 1995). These polymers have been widely used in humans as a drug delivery system and have an excellent safety record but are not yet licensed as vaccine adjuvants (Okada and Toguchi, 1995). A common problem with the encapsulation of antigens into microparticles is the denaturation or degradation of the antigen during its encapsulation. The Montanide Incomplete Seppic Adjuvants (ISAs, trademarked by SEPPIC, Paris, France) are a series of adjuvants composed of a variety of oils, different emulsifiers and immunomodulators, which can be formulated as water/oil emulsions ready to use with an aqueous solution of antigen. Montanide ISA 50V is commercial available and licensed for veterinary applications. The use of saponin in experimental vaccines has been known for more than 30 years and has been effectively used as an adjuvant in experimental vaccines, e.g. against *Taenia ovis* and *Echinococcus granulus* (Ebbesen *et al.*, 1976; Lightowlers *et al.*, 1996;

Lightowlers, 2006). Quil A is a highly refined form of saponin isolated from the bark of the South American soaptree *Quillaja saponaria Molina*, containing triterpene glycosides (Dalsgaard, 1974; Kensil, 1996). The adjuvant activity of Quil A is thought to be mediated through an aldehyde group on the saponins' triterpene aglycone forming a Schiff base with amino groups of costimulatory T cell receptors (Kensil, 1996).

The aim of this study was to demonstrate the feasibility of an immunoprophylactic strategy against B[a]P by replacing Freund adjuvant with adjuvants that are potentially compatible with their use in human. By showing that Quil A, MF-59 and Alum induced high levels of B[a]P-specific antibodies, we provide further support to our concept of a prophylactic immunisation against B[a]P modulating the pharmacokinetic of B[a]P.

2. Material and Methods

2. 1. Animals

Balb/c mice (10 week olds, female) were obtained from Harlan (Horst, The Netherlands) and acclimatised for 1 week. Animals were kept under timed 12 h light/dark cycles at 22 ± 2 °C and 40 ± 5% relative humidity. Food and water were available *ad libitum*. Water, food and oil were tested according to NF ISO 15302 by Micropolluants Technologie (Thionville, France) to confirm that all matrices were B[a]P free down to a detection limit of 1 ng/g of fat matter and 10 ng/L of water. All animal experiments were done in compliance with the rules of the European Communities Council Directive of 24 November 1986 (86/609/EEC) and following national regulations.

2.2. B[a]P conjugation to carrier proteins

B[a]P was derivatised to benzo[a]pyrenyl butyric acid (B[a]P-BA, Figure 1) as described before (Grabarek and Gergely, 1990) and conjugated to ovalbumin (OVA, MW 45.000 g/mol, Sigma-Aldrich, Bornem, Belgium) and purified tetanus toxoid (TT, 150.000 g/mol, Serum Institute of India) as described previously using a standard mixed ester activation of carboxyl groups (Grova *et al.*, 2009).

2.3. Preparation of adjuvants

100 µL Montanide ISA 50V (gift from Seppic, Paris, France), 100 µL Freund adjuvant (Sigma- Aldrich) and 25 µg Quil A (Brenntag-Biosector Frederikssund, Denmark) were emulsified in 100 µL PBS containing 25 µg B[a]P-TT. MF-59, an emulsion consisting of 5% v/v squalene, 0.5% v/v Tween-80 and 0.5% v/v Span-85 in water was prepared by homogenisation using a Pro 200 Homogenizer (Pro Scientific, Oxford, USA) at maximal speed. The day before immunisation, 100 µL MF-59 emulsion or 16.5 µL Alum (Alhydrogel 2%, Brenntag-Biosector Frederikssund, Denmark) were combined with 25 µg B[a]P-TT in 200 µL and 100 µL PBS respectively and incubated over night at 4°C under continuous rotation. The alum-protein suspension was 1:2 diluted in PBS prior to injection.

Resomer® RG503 a poly (D,L-lactide-co-glycolide) polymer (PLG) was a kind gift from Boehringer Ingelheim (Ingelheim, Germany). Anionic microparticles were prepared by a solvent evaporation technique (Xie *et al*., 2005). Briefly, microparticles were prepared by homogenising 10 mL of 6% w/v polymer solution in methylene chloride with 2.5 mL PBS, using the above homogenizer at maximal speed to form a water in oil emulsion. This was added to 50 mL of water containing 6 µg/mL dioctyl sulfosuccinate sodium salt (DSS) and homogenised at maximal speed for 25 min in an ice bath. The water in oil in water emulsion was stirred at 1000 rpm overnight at room temperature to evaporate the methylene chloride. DSS concentration in resulting particle solution was 0.05%. The particle size was determined to be 3-7 µm. PLG content of the suspension was determined by weighting 1mL lyophilised aliquots in triplicate. To adsorb B[a]P-TT to PLG particles, a suspension containing 200 mg PLG was incubated overnight with 2 mg protein conjugate in a total volume of 16 mL PBS (pH 7) at 4°C. For mock immunisation, PLG particles were incubated without protein. Prior to lyophilisation, formulation stabilizers (final concentration 3% manitol and 4% sucrose) were added. PLG loaded particles were aliquoted to 200 µg protein/tube and lyophilised in an Alpha 2-4 lyophilisator (Christ, Osterode am Harz, Germany). Particles were stored at room temperature in a desiccator and resuspended in PBS before injection.

2.4. Immunisation against B[a]P

Groups of six female Balb/c mice (10 week old) were immunised against B[a]P using 25 µg of B[a]P-TT conjugates with six adjuvant formulations (PLG particles, Alum, Quil A, Montanide, MF-59, CFV/IFA). Except for Quil A which was given subcutaneously (sc, 100 µL), all vaccines were ip injected (200 µL). On days 15, 29 and 43, mice were boosted. The “mock” immunised group was treated with buffer and adjuvant only. On days 11, 25, 39 and 53, mice were bled from the retro orbital plexus and antibody titers were analysed by ELISA. Animals were weighted before and after immunisation. There was no difference in weight observed between the different groups.

2.5. Detection of specific antibodies after immunisation with B[a]P-protein conjugates

B[a]P antibody levels were determined in 384-well microtiter plates (Greiner, Wemmel, Belgium) as described previously (Schellenberger *et al*., 2009). Briefly, microtiter plates were coated overnight at 4°C with 0.25 µM heterologous B[a]P-conjugate (B[a]P-OVA) in carbonate buffer (100 mM, pH 9.6). After washing, free binding sites were saturated at room temperature with 1% BSA. After 2 h, plates were washed again and serial three-fold dilutions of sera (Figure 2B) or specific mouse monoclonal antibody (P9E1R4 produced by immunisation with B[a]P-diphtheria toxoid, Figure 2A) were added and incubated for 90 min at room temperature. Dilutions were done in dilution buffer consisting of 0.1% Tween-20 w/v, 1% BSA, 15 mM Trizma-Acetate, 136 mM sodium chloride, 2 mM potassium chloride. Binding was assessed by alkaline phosphatase-conjugated goat anti-mouse IgG (1/750 dilution, ImTec Diagnostics NV, Antwerp, Belgium) and 4-nitrophenyl phosphate disodium salt hexahydrate. Absorbance was measured at 405 nm (Spectromax Plus, Sopachem, Brussels, Belgium). Antibody levels were expressed as endpoint titers corresponding to five-fold the background of dilution-buffer without serum. Absolute antibody concentrations were estimated using a B[a]P specific monoclonal antibody of known concentrations as a standard curve as described previously (Schellenberger *et al*., 2009). Dilution curves (1/200 to 1/ 437,400**)** of 0.025, 0.050, 0.1, 0.25, 0.50, and 1 mg/mL of B[a]P-specific mab P9E1R4 were prepared. The dilution (1/16200) with the highest R^2-value (0.956) was chosen to plot the linear standard curve (insert Fig 2A).

2.6. Fine-specificity of antibodies by competition ELISA

The specificity of serum antibodies was determined by competition ELISA as described previously (Grova *et al*., 2009). B[a]P and 7,8-diol-B[a]P (National Cancer Institute Chemical Carcinogen Reference Standard Repository, Midwest Research Institute, Kansas City, MO, USA) were used as competitors to inhibit antibody binding to coated heterologous B[a]P-conjugate (B[a]P-OVA). For these competition experiments, the optimal amount of coating antigen and the serum dilution were determined by indirect ELISA as described above. The minimal amount of antigen required for saturation was coated on 384-well microtiter plates. Sera dilutions were

determined to obtain 70% of saturation in the absence of competitor (highest signal) (Figure 3A). Dilution series of the above competitors were mixed 1:1 with the diluted serum to final concentrations of 0 to 1024 µM (Figure 3B). No competition (highest signal) and 100% competition (background signal) were determined using no competitor. The difference between the two values corresponds to the dynamic range of the assay (Δ OD max). For each competitor concentration, the percent binding of antibody was determined using the following formula (Figure 3B):

$$\% \text{ binding} = [(OD_{-\text{ test}} - OD_{+\text{ background}}) * 100 / (\Delta \text{ OD max})]$$

From the resulting inhibition curves, the 50% inhibition concentration of each competitor (IC_{50}) was determined (Figure 3C). In mice without specific antibodies, the IC_{50} was set to 1 mM.

2.7. [^{3}H]-B[a]P recovery in immunised mice after a single injection

Two weeks after a complete immunisation schedule with B[a]P-conjugates, each animal received a single ip injection of [^{3}H]-B[a]P (2 µg/kg, 6.7 10^4 Bq/mouse) diluted in 200 µL of vegetable oil (ISIO 4, Lesieur, Asnières-sur-Seine, France). 24 h later, EDTA-blood (500 µL) was obtained by decapitation and immediately further processed. Samples were analysed for [^{3}H]-B[a]P recovery as described previously (Grova *et al.*, 2009). Briefly, 500 µL of acetate buffer (0.2 M, pH 5.6), 5 µL of sulfatase (32 units, Sigma Aldrich) and 5 µL of ß-glucuronidase (622 units, from *Helix pomatia* juice, Sigma Aldrich) were added to 500 µL blood. The hydrolysis of conjugated metabolites was carried out at 37°C for 1 h and was followed by a liquid - liquid extraction of [^{3}H]-B[a]P and its metabolites with 500 µL ethyl acetate and 500 µL cyclohexane (Biosolve, Valkenswaard, Netherlands). After stirring (60 min at 1400 rpm) and centrifugation (3000 rpm, 15 min, 4°C), supernatant was collected and 2 mL of Ultimagold scintillation liquid (Perkin Elmer, Zaventem, Belgium) was added. Radioactivity was measured by liquid scintillation counting (2 min) using a 1450 MicroBeta TriLux (Perkin Elmer, Zaventem, Belgium). All samples were corrected for quenching of radioactivity. Radioactivity was expressed in Bq.

3. Results

3.1. Antibody titer

Animals were given four injections of B[a]P-TT conjugate vaccine combined with different adjuvants. Ten days after each injection, antibody titers were tested by indirect ELISA. Each serum was titrated in two-fold dilutions to estimate endpoint titers (Figure 2). All adjuvants induced specific IgG antibodies against conjugated B[a]P, but levels of antibodies varied between adjuvant formulations. Quil A ($p < 0.001$), MF-59 ($p < 0.05$) and Alum induced significantly higher antibody levels than Freund adjuvant after the complete immunisation schedule (Figure 2C, bleeding 4). Quil A induced already significantly higher antibody levels after two injections (bleeding 2) and MF-59 after the third injection (bleeding 3). In contrast, Montanide and PLG induced lower endpoint titers than Freund adjuvant. Absolute concentrations of antibodies determined with a standard curve produced with known concentrations of a monoclonal antibody (Figure 2A and B, see Materials and Methods and ref. (Grova *et al.*, 2009)), were 349 µg/mL, 329 µg/mL, 231 µg/mL, 156 µg/mL, 117 µg/mL and 88 µg/mL for Quil A, MF-59, Alum, CFA/IFA, Montanide and PLG respectively, after a complete immunisation schedule (bleeding 4). A similar number of mice was mock immunised and produced no detectable levels of antibodies (endpoint titers < 200, data not shown).

3.2. Antibody specificity

The antibody specificity was further tested by competition ELISA using B[a]P and 7,8-diol-B[a]P as competitors (Figure 3). Both competitors competed with serum binding to B[a]P-OVA of immunised mice albeit with different IC_{50} values. Median IC_{50} values for B[a]P as competitor ranged from 4.2-36 µM (Figure 3C). Lowest IC_{50} values (corresponding to best competition) were observed in Quil A and Montanide immunised mice, intermediate values for PLG and Alum, and highest values were detected for Freund adjuvant. In general, IC_{50} values for 7,8-diol-B[a]P were higher than those of B[a]P, but the overall pattern was similar, there was a strong correlation (R^2=0.847, p=0.009) between IC_{50} values of B[a]P and 7,8-diol-B[a]P (Figure 3D). Best results were again obtained with Montanide, Quil A and Alum with a median IC_{50} of 2.8, 19.5 and 29.5 µM, respectively (Figure3C). IC_{50} values of 7,8-diol-B[a]P were

much higher in mice immunised with Freund adjuvant (1000 µM) and MF-59 (356 µM).

3.3. [^{3}H]-B[a]P recovery in immunised mice after a single injection

To test the biological effect of the antibodies *in vivo*, 2 µg/kg [^{3}H]-B[a]P were injected ip (intraperitoneal) and the blood analysed 24 hours later for [^{3}H] recovery. In all immunised mice there was a significant increase of retained [^{3}H]-B[a]P compared to mock immunised mice ($p<0.05$) (Figure 4, Figure 5A and B). For Quil A, Montanide and MF-59 the recovery in immunised animals was on average 3.4 to 3.75 times higher than in mock immunised mice (control). For PLG and Freund adjuvant the retention was only 1.6 times higher (Figure 4).

4. Discussion

In combination with B[a]P-TT conjugate several adjuvants induced higher levels of specific antibodies than CFA/IFA (Figure 2, Figure 5A and C). While Quil A, MF-59 and Alum gave high antibody titers against a heterologous B[a]P-conjugate, Montanide, Quil A and Alum induced antibodies with the best specificity to the procarcinogen and its metabolite 7,8-diol-B[a]P (Figure 3C, Figure 5B and C). Thus, Montanide induced antibodies with the best specificity but levels of specific antibodies were 3 times lower than those of MF-59 (Figure 5C). In contrast, MF-59 induced antibody levels that were only second to Quil A but the IC_{50} of B[a]P and 7,8-diol-B[a]P were 7 and 125 times higher than those of Montanide (Figure 5C). Quil A gave the highest antibody titer and was only second in terms of specificity with a good IC_{50} to both B[a]P and 7,8-diol-B[a]P (Figure 5C). Considering the quality and quantity of the induced antibodies (Figure 5), Quil A would perhaps be the best choice for an adjuvant in a vaccine against B[a]P. Although this adjuvant is not always described without side effects (Petrovsky and Aguilar, 2004; Sun *et al.*, 2009). Alum, which is licensed in humans, was intermediate in terms of quantity and quality of antibodies.

To induce hapten-specific antibodies the B[a]P was derivatised in position 1 or 3 by butyric acid (Figure 1) and conjugated to the carrier protein TT. Thus antibodies bind probably mainly to the aromatic rings 3 to 5 (Figure 1). Nevertheless the diol in position 7 and 8 of the 5^{th} ring only partially reduced antibody binding to 7,8-diol-B[a]P (Figure 3C), suggesting that also the unstable ultimate carcinogen 7,8-diol-9,10-epoxide-B[a]P (Figure 1) could be recognised and sequestrated by the antibodies. We have previously shown that antibodies binding to 7,8-diol-B[a]P provide additional protection by reducing cellular re-uptake and its conversion to the ultimate carcinogen 7,8-diol-9,10-epoxide-B[a]P (De Buck *et al.*, 2005b). Despite the strong correlation between the IC_{50} for B[a]P and 7,8-diol-B[a]P, Montanide seemed to induce antibodies with some selectivity for the activated metabolite 7,8-diol-B[a]P (Figure 3D).

Assuming a serum IgG concentration of about 12 mg/mL (Harlow and Lane, 1988; Junghans, 1997), B[a]P specific antibodies represent up to 3 % (mean of Quil A, corresponding to < 349 µg/mL) of the total mouse IgG. Some mice have developed as much as 500 µg/mL of specific antibodies. These levels of antibodies are typical for a specific immune response (Janoff *et al.*, 1991; Hieda *et al.*, 1999; Hieda *et al.*,

2000; Pentel *et al.*, 2000). On a molar level of binding sites, these antibodies can bind up to 9.3 nmol or 2.3 µg B[a]P corresponding to a concentration of 0.12 mg/kg body weight (assuming a blood volume of a mouse of 2 mL and an average body weight of 20g). Assuming a daily mean uptake of B[a]P even for highly exposed individuals (e.g. coke oven workers) of 2.8 µg/kg/day (Lewtas *et al.*, 1997), and a B[a]P organ concentration of 0.32 µg/kg e.g. in the liver (Graf *et al.*, 1975), these concentrations can be easily matched by antibodies induced by our immunisation.

The challenge of studying the effect of a vaccination against low molecular compounds such as B[a]P is that usually high concentrations (e.g. 13 mg/kg, de Vries *et al.*, 1997) of B[a]P are used to obtain an experimental read out, but such concentrations cannot be stoichiometrically matched by antibodies induced by active immunisation. At such high doses of B[a]P no protective effect of the immunisation can be expected. However, concentrations reached by environmental or self-exposure can be easily matched by the above antibody concentrations. Therefore, we evaluated the biological effect of B[a]P-specific antibodies induced by the different adjuvants by analysing the retention of [^{3}H]-B[a]P after a single injection in immunised mice. An increased retention of B[a]P and its metabolites was shown for all adjuvants (Figure 4). Quil A and Montanide were most efficient in capturing B[a]P and its metabolites in the blood, followed by Alum and MF-59, two adjuvants which are already used in human licensed vaccines (Podda and Del Giudice, 2003). All of these adjuvants were better than CFA/IFA and PLG. We have shown before that an increase in retention reduces intracellular peak concentrations and therefore reduces metabolism and activation to its ultimate carcinogenic metabolite by cytochrome P450 enzymes in sensitive organs (De Buck *et al.*, 2005b; Schellenberger *et al.*, 2009). We have shown that this reduces pharmacological and/or toxic effects of B[a]P such as immuno and neurotoxicity (Schellenberger *et al.*, 2009). Furthermore, a reduction in B[a]P metabolism and activation by antibodies as shown before (Schellenberger *et al.*, 2009) may prevent overloading of DNA repair mechanisms and preventing mutagenesis in sensitive tissues.

In conclusion, our findings demonstrate that a vaccination against B[a]P with a tetanus toxoid conjugate and an adjuvant compatible with human use induces levels of specific antibodies with significant biological activity. Tetanus toxoid is the common antigen used in combination with alum to vaccinate against tetanus. We have previously shown that prior vaccination with a tetanus toxoid vaccine does not affect the ability of our conjugate vaccine to induce antibodies against B[a]P (unpublished

data). Our results with alum showed that the B[a]P-tetanus toxoid conjugate could probably be used in combination with the anti-tetanus vaccine, at least in high risk groups.

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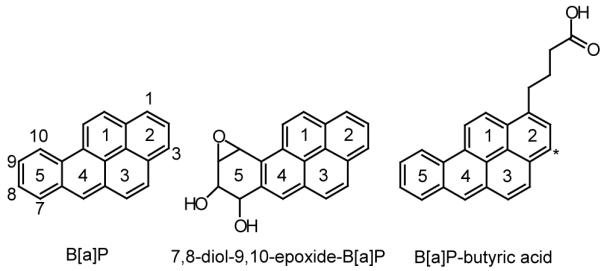

Figure 1: Chemical structure and numbering of aromatic rings of B[a]P, 7,8-diol-9,10-epoxide-B[a]P and the B[a]P-butyric acid used for conjugation to a carrier protein. The B[a]P butyric acid used for the conjugation is a mixture of 1 and 3-butyric acid substituted B[a]P (indicated by *).

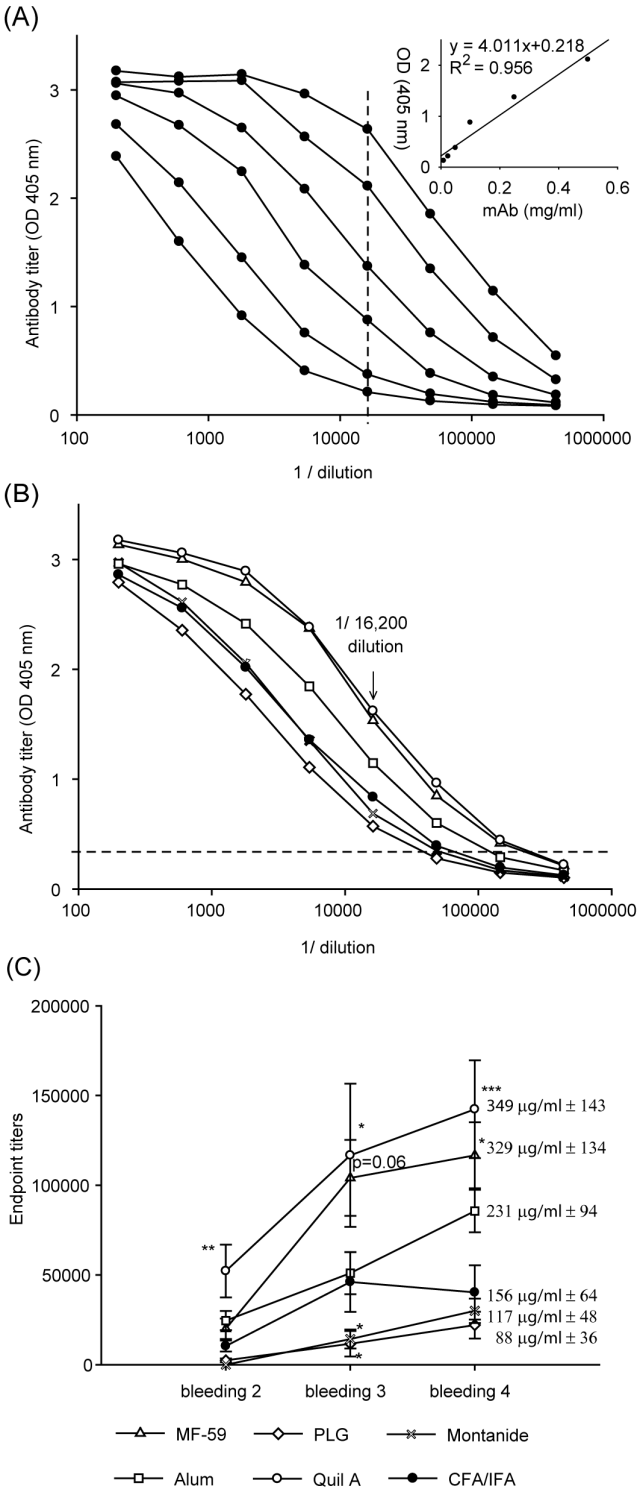

Figure 2: Quantification of B[a]P specific antibodies. (A) To estimate absolute antibody concentrations a standard curve (insert A) was used, that is based on dilution curves (1/200-1/437,400) of different known concentrations (0.025-1 mg/mL, represented by a line for each concentration in panel A) of purified monoclonal antibody against B[a]P (P9E1R4). 1/16,200 dilution (dashed line) was chosen to plot the standard curve (panel A, R^2 = 0.956) and to calculate the antibody concentration. (B) Titration of serum (shown for fourth bleeding, 10 days after last injection) of mice immunised with B[a]P-TT, used to determine endpoint titers and absolute antibody concentrations. Dashed line shows the OD at five fold the background used to determine the endpoint titers. The arrow indicates the 1/16,200 dilution used to determine the absolute antibody concentration with the standard curve shown in panel A. (C) Anti B[a]P endpoint titers on day 25 (bleeding 2), 39 (bleeding 3) and 53 (bleeding 4), 10 days after each injection. Values are expressed as mean ± S.E. of 6 mice per group. The absolute antibody concentrations (mean ± S.E.) correspond to the fourth bleeding. Two way ANOVA procedure was applied for one independent factor (B[a]P conjugates) and

repeated measurements (bleeding days).* $p<0.05$ *** $p<0.001$, statistically significant difference in mean endpoint titers from mice immunised with Freund adjuvant (Bonferoni t- post hoc test).

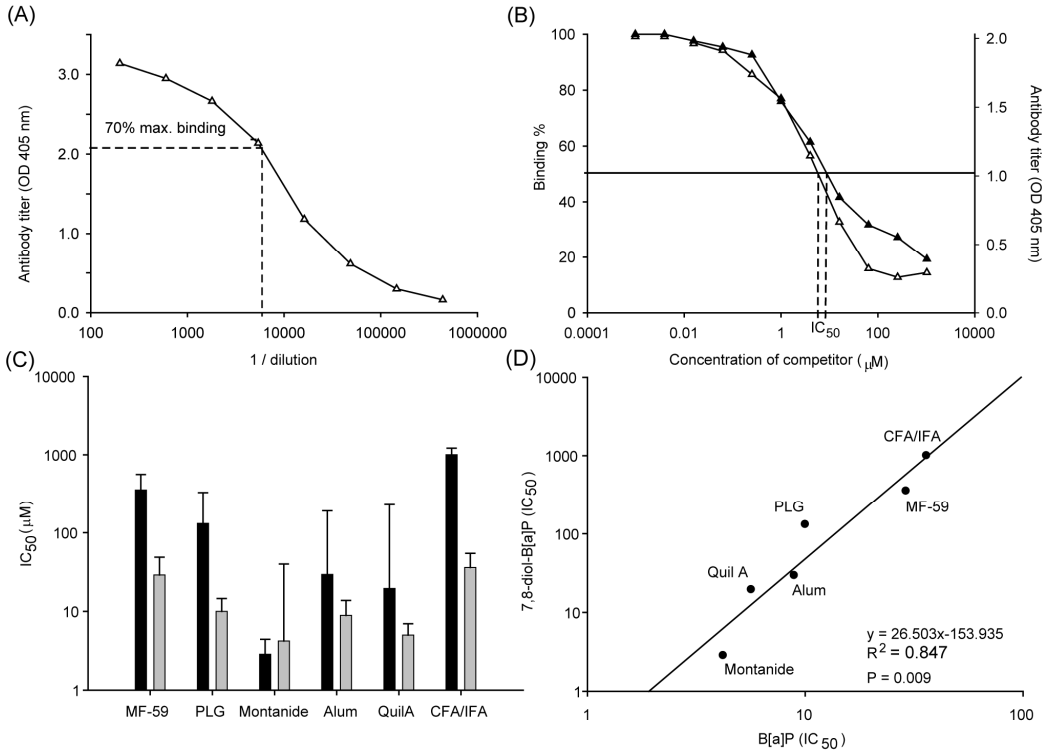

Figure 3: Antibody specificity determined by competition ELISA. (A) Representative example of a three-fold serial titration of a serum of an immunised mouse on B[a]P-OVA coated plate by indirect ELISA, to determine the serum concentration that gives 70% of maximal binding (dashed line), to be used in competition experiment. (B) Example of a titration curve (mouse immunised with MF-59) using B[a]P (Δ) and 7,8-diol-B[a]P (▲). Competition is expressed in percent of maximal binding (left axis) determined by titration of competitor. IC_{50} is determined at 50% of maximal binding (dashed lines). (C) Antibody specificity determined by competition ELISA. B[a]P (grey bar) and 7,8-diol-B[a]P (closed bar) were used as competitors to compete for binding of specific antibodies to B[a]P-OVA as coated antigen. Antibody affinity was determined as IC_{50} value (μM) of each competitor. Values are expressed as median ± S.E. of 6 mice per group. Mice without specific antibodies are set to 1mM. (D) Correlation (linear regression) of the median IC_{50} of B[a]P versus 7,8-diol-B[a]P.

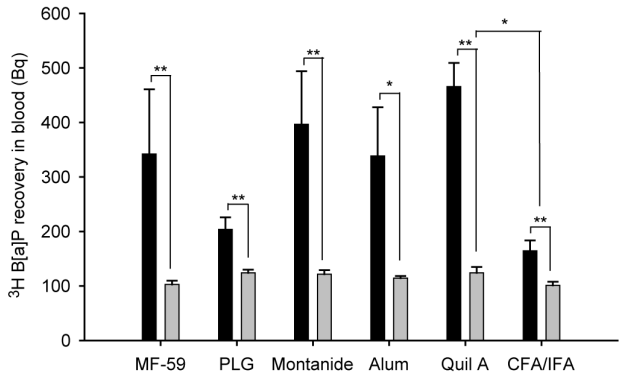

Figure 4: Radioactivity recovered in the blood of mice B[a]P immunised (closed bar) or mock immunised (grey bar) with the different adjuvants, 24 h after ip challenge with [^{3}H]-B[a]P (2 µg/kg, 6.7 10^4 Bq/mouse). Values represent mean ± S.E. of 6 mice per group. One way ANOVA on ranks (Kruskal-Wallis) procedure was applied, * $p<0.05$ statistically significant difference from mice immunised with Freund adjuvant (Dunn's test for post-hoc comparison) and a Mann-Whitney t-test for individual comparison between B[a]P immunised and mock immunised group.

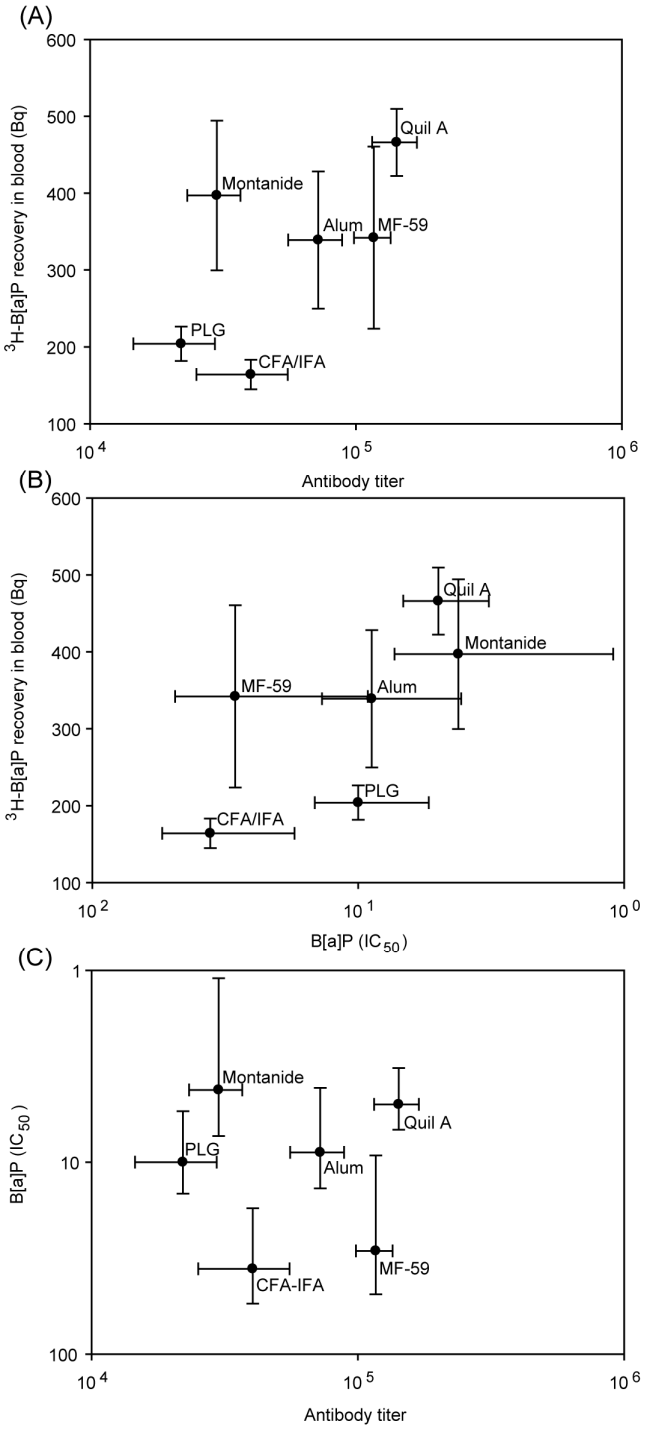

Figure 5: Comparison of antibody titers, [^{3}H]-B[a]P recovery in blood and IC_{50} as a measure of antibody specificity. To compare the immune response with the biological effect for the different adjuvants, [^{3}H]-B[a]P recovery in immunised mice 24 hours after single ip injection is plotted against antibody titers (A) and IC_{50} values for B[a]P (B). In panel C the antibody titer compared with the IC_{50} values for B[a]P. IC_{50} values are expressed as median and [^{3}H]-B[a]P recovery and endpoint titers as mean ± S.E. of 6 mice per group.

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Chapter 5

Immunogenicity of a promiscuous T cell epitope peptide based conjugate vaccine against benzo[a]pyrene: Redirecting antibodies to the hapten

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Abstract

The prototype polycyclic aromatic hydrocarbon benzo[a]pyrene (B[a]P) is an environmental pollutant and food contaminant of epidemiological importance. To protect against adverse effects of this ubiquitous carcinogen, we developed an immunoprophylactic strategy based on a B[a]P-protein conjugate vaccine to induce B[a]P specific antibodies (Grova et al., Vaccine. 2009;27:4142-51). Here, we investigated in mice the efficacy of B[a]P-peptide conjugates based on promiscuous T cell epitopes (TCE) into further improve this approach.

We showed that B[a]P-peptide conjugates induced very different levels of hapten-specific antibodies with variable functional efficacy, depending on the carrier. In some cases peptide carriers induced a more efficient antibody response against B[a]P than tetanus toxoid as a protein carrier, with the capacity to sequester more B[a]P in the blood. Reducing the carrier size to a single TCE can dramatically shift the antibody bias from the carrier to the B[a]P. Conjugates based on the TCE FIGITEL induced the best anti-hapten response and no antibodies against the carrier peptide. Some peptide conjugates increased the selectivity of the antibodies for the activated metabolite 7,8-diol-B[a]P and B[a]P by one or two orders of magnitude. The antibody efficacy was also demonstrated in their ability to sequester B[a]P in the blood and modulate its faecal excretion (15-56%). We further showed that pre-existing immunity to the carrier from which the TCE was derived did not reduce the immunogenicity of the peptide conjugate.

In conclusion, we showed that a vaccination against B[a]P using promiscuous TCEs of tetanus toxin as carriers is feasible even in case of a pre-existing immunity to the toxoid and that some TCE epitopes dramatically redirect the antibody response to the hapten. Further studies to demonstrate a long-term protection of an immunoprophylactic immunisation against B[a]P are warranted.

1. Introduction

Benzo[a]pyrene (B[a]P) is a ubiquitous environmental pollutant and food contaminant belonging to the group of polycyclic aromatic hydrocarbons (PAH). B[a]P is produced during incomplete combustion of organic matter and emanates from natural and anthropogenic sources including industrial processes, cooking, barbequing and tobacco consumption (Bostrom et al., 2002). Uptake in humans is mostly by inhalation of contaminated air, cigarette smoke and ingestion of contaminated food or water. As a consequence exposure to B[a]P by the general public is unavoidable.

Known adverse effects of B[a]P include carcinogenicity, immuno- , neuro- , geno-, reproductive and developmental toxicity (Thyssen et al., 1981; Wolterbeek et al., 1995; Davila et al., 1996; Carlson et al., 2004; Younglai et al., 2007; Grova et al., 2008; Niu et al., 2009; Sipinen et al., 2010). B[a]P is a very effective pulmonary carcinogen in human and experimentally in rodents (Perera, 1981; Likhachev et al., 1992). The total dose experienced by a smoker in a lifetime is remarkably close to the lowest total dose shown to induce tumours in rats (Hecht, 1998). The aryl hydrocarbon receptor (AhR) plays an important role in B[a]P-induced carcinogenesis. Human and animal studies showed a significant correlation between the inducibility of the arylhydrocarbon hydroxylase activity and lung carcinogenesis induced by B[a]P (McLemore et al., 1978; Ross et al., 1995). B[a]P mediated carcinogenicity can also be induced by its genotoxicity. Human lung and liver metabolically activate B[a]P to 7,8-diol-9,10-epoxide-B[a]P (BPDE) by phase one enzymes (Prough et al., 1977) (Figure1). In human lung, DNA adducts of B[a]P have been detected (Hecht, 1996a; Boysen and Hecht, 2003). Metabolic manipulations by isothiocyanates that decrease the formation of DNA adducts, without lowering levels of chemical exposure, have been shown to reduce the number of tumours (Hecht, 1996a; Hecht, 2001). Mechanistic studies have shown that the chemopreventive activity of isothiocyanates, that modify carcinogen metabolism specifically by inhibiting Phase one enzymes and/or by inducing Phase two enzymes, result in increased carcinogen excretion or detoxification and decreased carcinogen DNA interactions (Hecht, 1996b). BPDE adducts have been linked to G:C to T:A transversions in the Tp53 gene at an unusual series of mutational hotspot codons in smoking-associated lung cancer (Hainaut and Pfeifer, 2001). Mutations in critical regions of this tumour suppressor gene or of oncogenes (e.g. Ras, Myc) can result in deregulation of normal cell growth and cancer development (Osada and Takahashi, 2002).

Therefore, we have started to develop strategies based on B[a]P-carrier conjugates to explore the ability of B[a]P specific antibodies to protect against the adverse effects of this carcinogen (De Buck et al., 2005a; De Buck et al., 2005b; De Buck and Muller, 2005; Grova et al., 2009; Schellenberger et al., 2009; Schellenberger et al., 2011). The use of hapten-carrier conjugates using proteins for vaccination have been successful in the case of nicotine and its major metabolite cotinine, or cocaine using various carrier proteins (Carrera et al., 1995; Deng et al., 2002; Hatsukami et al., 2005; Keyler et al., 2008). Some of these conjugates are already tested in clinical trials (Haney et al.; Hatsukami et al., 2005). However, only limited data are available for low molecular weight carcinogens (Silbart et al., 1997; De Buck et al., 2009; Polonelli et al., 2011). Concerns about local carcinogenesis at the site of injection are probably unsubstantiated considering the low doses and the low metabolic activation rates of (conjugated) B[a]P in muscles in contrast to lung and liver tissues.

Our previous in vitro studies showed that monoclonal antibodies against B[a]P may provide some protection against low doses of B[a]P (De Buck et al., 2005a; De Buck et al., 2005b). In a bi-compartment model of polarised Caco-2 cells, we showed that antibodies reduced the transport of B[a]P and its metabolism by sequestration of B[a]P and its metabolites in the cell culture supernatant (De Buck et al., 2005a). In addition, antibodies modulated the kinetic of B[a]P metabolism in HepG2 cells and human peripheral lymphocytes (De Buck et al., 2005b).

Our first in vivo experiments demonstrated that a hapten-protein conjugate vaccine based on tetanus toxoid (TT) and diphtheria toxoid (DT) in combination with various adjuvants licensed in humans was able to induce high levels of antibodies in mice which were specific for B[a]P, its detoxified metabolites and its activated form, the BPDE (Grova et al., 2009; Schellenberger et al., 2009; Schellenberger et al., 2011). Although most experimental models use high concentrations of B[a]P to achieve an experimental read out after short-term exposure, such high concentrations cannot be used to study protection of B[a]P specific antibodies because they cannot be matched by stoichiometric concentrations of specific antibodies. However, environmental relevant concentrations of B[a]P can easily be matched by the antibody levels obtained by immunisation with B[a]P-TT conjugates (Schellenberger et al., 2009) but they do not provide a short-term readout in relation to their carcinogenicity.

Conjugating the B[a]P to a bulky carrier protein (e.g. TT or DT) tends to divert the immune response to the immunogenic carrier protein and away from the hapten (Herzenberg and Tokuhisa, 1980). Carrier proteins have been replaced by small immunogenic peptides to induce prophylactic or therapeutic T cell responses against pathogens, tumours and other diseases but not against low molecular weight compounds such as B[a]P. In this study, we aimed to further investigate our prophylactic immunisation strategy against B[a]P using T cell epitope (TCE) peptides to redirect the antibody response to the hapten. TCEs were selected that were promiscuous with respect to a large variety of human and mouse MHC class II molecules.

2. Materials and Methods

2.1. Peptide synthesis and B[a]P conjugation

Peptides (Table 1) were synthesised by automated solid phase peptide synthesis using standard Fmoc chemistry on Rink resin on a Syro II peptide synthesiser (Multisyntech, Witten, Germany). B[a]P butyric acid (B[a]P-BA, Biochemical Institute of Environmental Carcinogens, Grosshansdorf, Germany, Figure 1B) was coupled N-terminally to the protected peptide and was washed 3 times each with methanol and ether. Peptide-B[a]P conjugates were purified by RP-HPLC on the ÄKTA explorer 10S system (Amersham Biosciences, Uppsala, Sweden) on a C18 column (250 x 8 mm, 120A, 5µm) using a linear gradient of 25-95% water/acetonitrile (ACN), 0.1% TFA (v/v) and monitored at 214 nm (max absorption of peptide bond, (Højrup, 2003)) and 297 nm (max absorption of B[a]P-BA) and lyophilized in an Alpha 24 lyophilisator (Christ, Osterode am Harz, Germany). B[a]P-BA was coupled to ovalbumin (OVA, Sigma-Aldrich, Bornem, Belgium) and purified tetanus toxoid (TT, Serum Institute of India) by adopted two-step zero-length cross-linking procedure using active esters as described in Grova et al. (Grova et al., 2009).

2.2. Mass Spectroscopy

Masses of B[a]P-protein and -peptide conjugates were analysed using a positive ion MALDI-TOF ULTRAFLEX TOF/TOF mass spectrometry (Bruker Daltonics, Bremen, Germany) equipped with a 337nm, 50 Hz N_2 laser of 100µj as described in (Prodhomme *et al.*). For synthetic peptides the sequence was verified with the MALDI mass spectrometer using its post-source decay (PSD) capacity for fragmentation (Sequence Editor of Biotools software, Bruker Daltonics, Bremen, Germany). The N-terminal linkage of B[a]P on peptides was determined by MS/MS using an ion trap mass spectrometer (Agilent 6340 ion trap) equipped with a Chip Cube interface for infusion at nano flow rate. The parameter settings for positive ion ESI-MS were as follows: capillary voltage 2000V; end plate offset 500V; capillary exit 100V and tarp drive 85. For CID, the fragmentation amplitude was set to 1.3V scanned from 30% to 200% of this value.

2.3. Immunisation with B[a]P-peptide conjugates

All animal experiments were done in compliance with the rules of the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the Ministry of Agriculture, Viticulture and Rural Development (22 December 2008). A group of 6 mice Balb/c (10 week olds, female, Harlan, Horst, The Netherlands) were immunised as described previously (Schellenberger et al., 2009). Briefly, mice were primed i.p. with 25 µg of B[a]P-peptide or B[a]P-TT bioconjugates (50 mM ammonium bicarbonate buffer) on day 0, emulsified in 50% complete Freund's adjuvant (CFA, v/v in PBS, Sigma-Aldrich). On days 14, 28 and 42 mice were boosted i.p. with the same antigen emulsified in 50% incomplete Freund's adjuvant (IFA, v/v in PBS, Sigma-Aldrich,). Mock immunised control group (n=5) was primed with 50% CFA and boosted with 50% IFA (v/v in PBS) alone.

2.4. Detection of specific antibodies after immunisation

On day 53, mice were bled retro-orbitally and serum antibody levels were determined in 384-well microtiter plates (Greiner, Wemmel, Belgium) as described previously (Schellenberger et al., 2009). Briefly, microtiter plates were coated overnight at 4 °C with 0.25 µM B[a]P-OVA (100 mM carbonate buffer, pH 9.6) for detection of B[a]P specific antibodies or with homologous non conjugated peptides (VNNESSE-14, -15, FIGITEL-16, -17 or TT) for the quantification of carrier specific antibodies. After washing, free binding sites were saturated with 1% BSA at RT for 2 h. After washing, diluted serum or carcinogen-specific mouse monoclonal antibody (P9E1R4 produced by immunisation with B[a]P-DT (Grova et al., 2009)) was incubated for 90 min at RT. Binding was assessed by alkaline phosphatase-conjugated goat anti-mouse IgG (1/750 dilution, ImTec Diagnostics NV, Antwerpen, Belgium) and 4-nitrophenyl phosphate disodium salt hexahydrate (Sigma-Aldrich). Absorbance was measured at 405 nm (Spectromax Plus, Sopachem, Brussels, Belgium). For relative quantification, endpoint titers (EPT) were defined as serum dilutions corresponding to 5 fold the background. Absolute antibody quantification is described in (Schellenberger et al., 2009) and Figure 2A and B. For sera with no detectable antibodies, endpoint titer was set to 200 (highest serum concentration tested).

2.5. Antibody selectivity by competition ELISA

The selectivity of serum antibodies was determined by competition ELISA as described previously (Grova et al., 2007). Briefly, B[a]P-BA, B[a]P (Sigma-Aldrich) and 7,8-diol-B[a]P (National Cancer Institute Chemical Carcinogen Reference Standard Repository, Midwest Research Institute, Kansas City, MO, USA) were used as competitors to inhibit antibody binding to coated heterologous B[a]P-conjugate (B[a]P-OVA). For these competition experiments, the optimal amount of coating antigen and the serum dilution were determined by indirect ELISA as described above. The minimal amount of antigen required for saturation was coated on 384-well microtiter plates. Sera dilutions were determined to obtain 70% of saturation. Dilution series of the competitors were mixed 1:1 with the diluted serum to final concentrations of 0 to 1024 µM. No competition (highest signal) and 100% competition (background signal) were determined using no competitor. The difference between the two values corresponds to the dynamic range of the assay (ΔOD max). For each competitor concentration, the percent binding of antibody was determined using the following formula:

% binding = [(OD- test – OD+ background)/ Δ OD max *100]

From the resulting inhibition curves, the 50% inhibition concentration (IC_{50}) of each competitor was determined. In mice without selectivity, the IC_{50} were actively set to 1 mM. A low IC_{50} value corresponds to high selectivity.

2.6. Distribution of [^{3}H]-B[a]P in immunized mice after a single injection

The quantification of [^{3}H]-B[a]P in organs and excretion products were described before (Grova et al., 2009). Briefly, two weeks after a complete immunisation schedule, each animal received a single i.p. injection of [^{3}H]-B[a]P (2 µg/kg, 2.67 104 Bq/mouse GE-Healthcare, Belgium) and were placed in individual metabolic cages (Technilab, Someren, Netherlands). Urine, faeces and organs (Liver, Lung, Brain, Spleen, and Kidney) were collected 24 h later and stored at – 20°C before analysis. EDTA-blood (500 µL) was obtained by retro-orbital bleeding and analysed immediately. To study the pharmacokinetic of B[a]P, mice were challenged with 2

μg/kg [3H]-B[a]P (2.67 104 Bq/mouse) and sacrificed after 15 min to 48 h intervals. Samples were collected and stored as described above.Samples were analysed for [^{3}H]-B[a]P recovery as described previously (Grova et al., 2007). Pre-hydrated faeces (20 mg) or tissues (100 mg) were solubilised in Soluene-350 according to Perkin Elmer procedure and previously described and measured for radioactivity (Grova et al., 2002). All samples were corrected for quenching of radioactivity. Radioactivity was expressed in Bq or Bq/g accounting for the effective counting efficiency.

3. Results

3.1. T cell epitope peptide synthesis and B[a]P conjugation

After solid phase synthesis peptides were purified by liquid chromatography and analysed by positive ion MALDI-TOF ULTRAFLEX TOF/TOF mass spectrometry for their correct masses. All synthesised peptides had the correct molecular mass (Table1). The Figure 3B shows as an example the MS spectrum of peptide VNNESSE-14. After conjugation, the mass spectra showed the correct shift in size of 320 Da corresponding to B[a]P-BA (Figure 3B and E). In addition, a shift in the elution from the HPLC from 61.5% to 86.5% ACN confirmed the successful conjugation of B[a]P-BA to the peptide by its increased hydrophobicity (Figure 3A and D). The peptide sequence and the N-terminal linkage of the B[a]P-BA were also confirmed by MS/MS analysis. The peak of 320.64 Da corresponds to the $[B[a]P\text{-}BA+H]^+$ molecule (Figure 2F). The mass difference of 320.64 Da (corresponding to the $[B[a]P\text{-}BA+H]^+$) between the b2 ions in Figure 3F and C proves the N-terminal linkage.

3.2. Detection of B[a]P- specific antibodies after immunisation with B[a]P-peptides

Four known TCE of different length of TT were conjugated to B[a]P-BA (VNNESSE-3, -14, FIGITEL-6, -16, -29, PNRDIL-8 and SYFPSV-19, Table 1) and tested for their potential to induce B[a]P specific antibodies. These peptides reacted with mouse H2d and were promiscuous for human MHC class II molecules (Demotz et al., 1989; Panina-Bordignon et al., 1989; Ho et al., 1990; James et al., 2007). For instance according to the literature FIGITEL peptides react with most DR and some DQ and DP MHC class II molecules whereas PNDRL and SYFPSV peptides react with a variety of human DR molecules and some mouse class II molecules (Panina-Bordignon et al., 1989; James et al., 2007). In addition, B[a]P was conjugated to several of variants of these TCE peptides. The serum of each mouse was titrated 2 weeks after the fourth injection. Figure 4 shows the variable immunogenicity of a selection of B[a]P-peptide conjugates. From B[a]P conjugates based on VNNESSE-motive only VNNESSE-14 and -15 (EPT 1/120,000 and 1/250,000) induced higher levels of specific antibodies against B[a]P (Figure 4A and E) than the B[a]P-TT protein conjugate (EPT 1/99,500, Figure 4E) but EPT did not significantly differ. All

other VNNESSE-peptides induced significantly lower levels or no antibodies (Figure 4A and E). Peptide conjugates from the FIGITEL group which induced higher levels of B[a]P specific antibodies than B[a]P-TT included FIGITEL-16 and -17 (EPT 1/300,000 and 1/325,000 respectively, Figure 4B and E). Only peptide SYFPSV-20 (EPT 1/19,000, Figure 4C and E) induced specific antibodies in the group of SYFPSV peptides, but less efficient than B[a]P-TT. None of the tested peptides of the PNRDIL-motive induced specific B[a]P antibodies (Figure 4D).

3.3. Detection of carrier specific antibodies after immunisation with B[a]P-peptides

The sera with the highest titers against B[a]P were further tested for antibodies against the homologous carrier peptide. Mean antibody levels were 6.5 times lower for VENNESSE-14 (EPT 1/60,000) and 3 times lower for VENNESSE-15 (EPT 1/125,000) compared to immunisation with B[a]P-TT (EPT 1/390,000 Figure 4F). Interestingly for the FIGITEL-16 and -17 conjugates no antibodies against the carrier were detected (EPT < 1/200).

3.4. Antibody selectivity by competitive ELISA

The selectivity of the anti-B[a]P antibodies was analysed by competitive ELISA in all sera of mice with detectable antibodies against B[a]P. In a competitive ELISA assay IC^{50} values inversely correlate with the selectivity for the competitor. IC^{50} values for B[a]P-BA as competitor ranged from 0.7 µM for B[a]P-peptide conjugate VNNESSE-18 to 1.20 µM for FIGITEL-16 (Figure 5A). Compared to B[a]P-TT, the antibodies induced by VNNESSE-15, -18, FIGITEL-17, -21 and SYFPSV-20 showed a somewhat higher selectivity for B[a]P-BA (although not statistically significant). Competition with B[a]P, gave IC_{50} values that were 5 to 30 times higher than those of B[a]P-BA (except for VNNESSE-16 with similar IC50 values) ranging from 0.14 µM for B[a]P-peptide conjugate VNESSE-15 to 17.00 µM for FIGITEL-16 (Figure 5B). The IC_{50} of 7,8-diol-B[a]P was also higher than those of B[a]P-BA ranging from 0.45 µM (VNESSE-15) to 9.25 µM (VNESSE-14, Figure 5C). All tested peptide conjugates, except the FIGITEL-16, showed also a higher selectivity for B[a]P and 7,8-diol-B[a]P than the TT protein conjugate.

3.5. Modulation of [^{3}H]-B[a]P distribution in immunised mice 24 h after a single injection

[^{3}H]-B[a]P recovery in the blood was on average 3-6 times higher than in mock immunised animals ($p<0.05$, Figure 6A) for all peptide conjugates tested except for FIGITEL-16. VNNESSE-14 and -15 increased [^{3}H]-B[a]P recovery above the level of B[a]P-TT immunised animals but the difference was not statistically significant (Figure 6A). In the different solid tissues tested [^{3}H]-B[a]P recovery was on average 1.1-2.6 fold increased (Figure 6B-F). The difference compared to the mock immunised animals was highest in the liver (Figure 6B) and the spleen (Figure 6D) for VNNESSE-14 and VNNESSE-15 (but this did not reach statistical significance) respectively. No difference was observed in the brain (Figure 6C). Animals immunised with FIGITEL-16 showed no enhanced retention of [^{3}H]-B[a]P in any of the four organs tested. In faeces, a significant decrease of 15-56% of the radioactivity recovered was observed ($p<0.05$) in mice immunised with peptide conjugates (except for FIGITEL-16) while no decrease was observed for B[a]P-TT (Figure 6H). Excretion of radioactive B[a]P in the urine was essentially the same in both immunised and mock control mice (Figure 6G). Figure 5 shows that there is a good and highly significant correlation between increasing B[a]P antibody concentration and levels of [^{3}H]-B[a]P recovered in the blood and in the liver (Figure 2C and D).

3.6. Modulation of the pharmacokinetic of [^{3}H] -B[a]P

The pharmacokinetic of B[a]P was investigated over 48 h in blood, solid tissues and excretion products in mice immunised with peptide VNNESSE-14 (Figure 7). In mock immunised mice a rapid accumulation of [^{3}H]-B[a]P was observed in the blood with peak concentrations 3 h after B[a]P administration (Figure 7A). In B[a]P immunised mice a significantly higher peak was observed 4.5 h after B[a]P injection and was constant for at least 48 h (Figure 7A). In solid tissues, the highest levels of B[a]P were detected in the liver and the kidney, the lowest in the brain 3-4.5 h after injection. In B[a]P immunised mice, the peak concentration was delayed by 1-2 h. In kidney and brain, the concentration of B[a]P was lower in the immunised group during the uptake phase (first 10 h), and was then constant over the observation period, while a decrease was observed for the kidney in the control group (Figure 7C

and E). For the other organs tested (liver, spleen, lung) no difference was observed early after B[a]P administration (Figure 7B, D and F). As a consequence of B[a]P sequestration by antibodies, excretion in the urine and faeces was reduced (Figure 7G and H). In the urine of immunised mice, lower levels of B[a]P were detected during the first 18 h, whereas in the faeces this difference was only observed during the first 3 h (Figure 7G and H).

3.7. Effect of a TT pre-vaccination on the B[a]P vaccination

The influence of a pre-existing immune response to the carrier protein (TT) was tested by immunising mice with TT prior to immunisation with B[a]P-peptides and B[a]P-TT. Antibody levels in mice pre-immunised with TT were higher or similar to those without pre-vaccination, excluding a negative effect of pre-existing antibodies to TT (Figure 8B). Also the antibodies against TT were not influenced by a vaccination with B[a]P-peptide conjugates (Figure 8A). There was also no negative effect of TT pre-exposure on antibody specificity for B[a]P or 7,8-diol-B[a]P (Figure 8C and D). The in vivo recovery experiment also reflected this observation (Figure 9). 24 h after [^{3}H]-B[a]P injection the recovery of radioactivity was similar for FIGITEL-16 and -17 compared to those without pre-vaccination and for VNNESSE-14 and -15 it was even significantly increased.

4. Discussion

In our previous work we showed that the conjugation of B[a]P-BA to TT or diphtheria toxoid (DT) induces high titers of B[a]P-specific antibodies (Grova et al., 2009). Here we demonstrate that similar or even better B[a]P-specific antibody titers can be induced by reducing the carrier size to a single promiscuous TCE.

It was previously shown that vaccination with a co-linear peptide containing a peptide corresponding to an important neutralising epitope of the measles hemaglutinin protein and various TCEs induced antibodies with activities that ranged from simply binding to in vitro neutralisation and in vivo protection against the virus (Bouche et al., 2005). Similarly, we here show that TCE-peptides induced very different levels of hapten specific antibodies with varying functional efficacies, depending on the carrier peptide. In some cases the peptide carrier induced a more efficient immune response than the protein conjugates. Not all known TCE-peptides (Demotz et al., 1989; Panina-Bordignon et al., 1989; Ho et al., 1990) induced hapten specific antibodies (Figure 4A), which may reflect difficulties of the antigen processing and presentation machinery to properly cleave the conjugate.

Peptide conjugates are simple and cost effective to synthesise in large quantities, in high quality and safety (Muller and Putz). In addition, peptide carrier conjugates are stoichiometrically well defined (Figure 3F) which is not the case of protein conjugates. The TT-protein carrier conjugate had on average 8 haptens per molecule, corresponding to 1 hapten for 18 kDa of protein (Grova et al., 2009), while the hapten/carrier ratio was 1 in the case of the peptide conjugate, corresponding to 1 hapten per 3 kDa of carrier. Under the simplest assumption that the molecular weight is a crude estimate of the relative number of B cell epitopes (BCEs), the peptide conjugate would have a six fold better ratio of hapten to carrier BCEs. Indeed, while some peptide conjugates induced similar or even higher anti B[a]P titers, antibody levels against the carrier peptide was up to 6 fold lower for some of the VNESSE conjugates. Interestingly, the conjugate FIGITEL-16 and -17 induced the best anti-hapten responses and no antibodies against the carrier peptide. Although this may be suggestive of a hole in the B cell repertoire despite a strong T cell immunogenicity against the latter peptide, vaccine conjugate based on appropriate small peptide carriers strengthen the B cell response towards the hapten at the detriment of the carrier.

Some peptide conjugates did not only induce similar or higher levels of antibodies against B[a]P, but they also showed an increased specificity and an improved ability to sequester [^{3}H]-B[a]P. In particular peptide VNESSE-14 and -15 showed an improved immune response in terms of antibody quantity, quality and B[a]P sequestration in the blood.

Our TCE were derived from TT, against which 90% of the world population is vaccinated. Thus a large proportion of a population will have T cells specific for the promiscuous TCE selected here. To exclude that a pre-existing T cell immunity against TT interfere with the immune response against the TCE (from TT) undermining the anti-hapten antibody response, we tested the effect of a TT pre-vaccination on the vaccination with B[a]P peptide-conjugates. B[a]P antibody levels were in general higher in pre-immunised animals for all peptide conjugates tested (except for FIGITEL-16, Figure 8). Also antibody specificity and effectiveness did not suffer as a result of pre-vaccination with TT, for most of the peptide conjugates. In fact, the recovery of [3H]-B[a]P was significantly increased in mice pre-immunised with TT and boosted with VNESSE-14 and -15. These results are in agreement with Putz et al. who tested the immunogenicity of B cell epitope peptides conjugated to DT or TT derived TCE in mice after active priming with the toxoids (Putz et al., 2004). Both TT and DT peptide conjugates induced high titers of anti-measles antibodies which cross-reacted with the virus and protected against a lethal challenge with the virus, even after active priming with the homologous toxoid (Putz et al., 2004). Similar observations were found in humans, active priming against the carrier enhanced the response to the antigen conjugated to TT and DT irrespective of whether proteins, or peptides were used (Lise et al., 1987; Barington et al., 1994; Granoff et al., 1994; Kurikka, 1996).

Kinetic experiments showed that B[a]P specific antibodies modulate the pharmacokinetics and slow down its excretion by capturing B[a]P or its metabolites in the blood stream and by reducing its excretion via the faeces (Figure 2H). The higher levels of B[a]P recovered in solid tissues in immunised mice are due to sequestration by antibodies and not by the blood in the organs. The results are in accordance with Johanson and colleagues who showed a higher recovery of dinitrophenol in the liver (20% in spleen and 50% lung) in rats passively immunised with anti-dinitrophenol antibodies (Johansson et al., 1996). B[a]P specific antibodies are able to capture B[a]P or its metabolites in the blood away from sensitive tissues and thereby mitigating P450 enzyme induction and reducing its metabolism especially to its toxic

metabolite 7,8-diol-9,10-epoxide-B[a]P. In general antigen-antibody complexes are depleted mostly by Kupffer cells of the reticuloendothelial system of the liver (Skogh et al., 1985; Skogh et al., 1988; Johansson et al., 1996). Because of the lower sensitivity of the liver to B[a]P carcinogenesis, metabolism of B[a]P in this organ is much less likely to cause damage. In addition, we can speculate that the sequestration by specific antibodies of environmental (i.e. very low) concentrations of B[a]P (mean daily uptake 200 ng (2002)) would be considerably higher resulting in an even better protective effect against its toxicity.

In previous experiments we demonstrated in vitro and in vivo that B[a]P specific antibodies are also able to capture its endpoint metabolites and the 7,8-diol which is the precursor of the ultimate carcinogen 7,8-diol-9,10-epoxide-B[a]P (De Buck et al., 2005b; Schellenberger et al., 2009). In addition, our kinetic data show that B[a]P and its metabolites are captured for a prolonged time in the blood (Figure 7A). This lowers intracellular peak concentrations preventing enzyme induction of Cyp1a1 and 1b1 responsible for the formation of 7,8,-diol-9,10-epoxide-B[a]P (Schellenberger et al., 2009).

In conclusion, we demonstrated that a vaccination against B[a]P using promiscuous T-helper cell epitopes as carriers is feasible. Some peptide conjugates were more immunogenic and induced more and better antibodies against the hapten. We further showed that appropriate small peptide carriers can redirect the antibody response against the hapten at the detriment of the B cell response to the carrier. This effect may partially explain the improved response to these peptide conjugates. Pre-exposure to TT did not negatively affect the immune response against B[a]P-peptide or B[a]P-TT conjugates. This lends further support to the use of TT derived peptides or protein as carriers for an immunoprophylactic conjugate vaccine against low molecular weight carcinogens such as B[a]P. While we demonstrated previously that our vaccination strategy is protective against short term adverse effects of B[a]P, such as immunotoxicity (Schellenberger et al., 2009) and neurotoxicity (unpublished data), further studies to demonstrate a long term protection against carcinogenesis are needed.

Acknowledgements

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Table 2: Summery of peptides and their expected and measured masses (mutations in the peptide sequence are in bold).

ID	Origin/ Antigen/	Position	Sequence	Expected masses $[M+H]^+$	Measured masses $[M+H]^+$	Δ	Purity
VNNESSE-3	TT (wt-short)	916-932	**B[a]P–**PGINGKAIHLVNNESSE	2098.100	2098.278	0.178	70%
VNNESSE-14	TT (wt-long)	909-932	**B[a]P–**PDAQLVPGINGKAIHLVNNESSE	2721.428	2721.513	0.085	95%
VNNESSE-15	TT		**B[a]P–**PDAQLV**V**GINGKAIHLVNNESSE	2723.443	2723.979	0.536	80%
VNNESSE-18	TT		**B[a]P–AENK**PGINGKAIHLVNNESSE	2540.317	2539.942	0.375	40%
VNNESSE-23	TT		**B[a]P–ALAYYVL**PGINGKAIHLVNNESSE	2891.537	2891.286	0.251	85%
VNNESSE-24	TT		**B[a]P–PILFFRLK**GINGKAIHLVNNESSE	3015.685	3015.178	0.507	80%
PNRDIL-8	TT (wt-short)	1273-1284	**B[a]P–**GQIGNDPNRDIL	1630.862	1630.836	0.026	95%
PNRDIL-10	TT		**B[a]P–A**LGLVGTHNGQIGNDPNRDIL	2493.328	2493.416	0.088	80%
FIGITEL-6	TT (wt-short)	830-844	**B[a]P–**QYIKANSKFIGITEL	2044.155	2043.896	0.259	90%
FIGITEL-29	TT (wt-medium)	826-844	**B[a]P–**NILMQYIKANSKFIGITEL	2515.406	2515.220	0.186	40%
FIGITEL-16	TT (wt-long)	823-844	**B[a]P–**QSKNILMQYIKANSKFIGITEL	2858.592	2858.986	0.394	80%
FIGITEL-17	TT		**B[a]P–**QSK**Q**ILM**V**YIKANSKFIGITEL	2843.617	2843.606	0.011	90%
FIGITEL-21	TT		**B[a]P–AENK**QYIKANSKFIGITEL	2486.372	2486.182	0.190	40%
FIGITEL-27	TT		**B[a]P–ALAYYVL**QYIKANSKFIGITEL	2837.952	2837.872	0.080	60%
FIGITEL-28	TT		**B[a]P–PILFFRLK**YIKANSKFIGITEL	2930.734	2930.340	0.394	80%
SYFPSV-19	TT (wt)	580-599	**B[a]P–**NSVDDALINSTKIYSYFPSV	2552.299	2552.572	0.273	90%
SYFPSV-20	TT		**B[a]P–**NSVDDALI**V**STKIYSYFPSV	2537.324	2537.514	0.190	95%
SYFPSV -22	TT		**B[a]P–AENK**ALINSTKIYSYFPSV	2464.319	2464.154	0.165	70%

Tetanus toxoid (TT), wild type (wt)

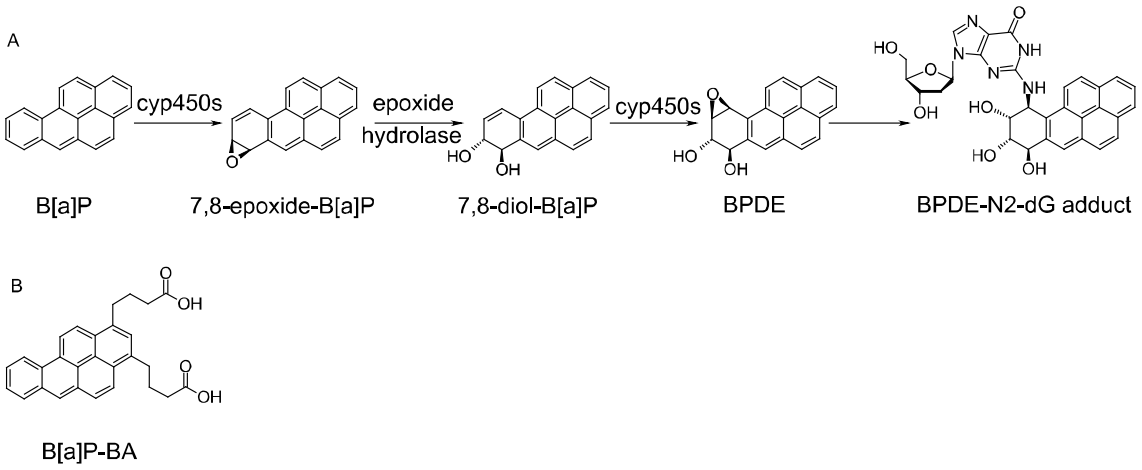

Figure 1: Metabolic activation of B[a]P. (A) During detoxification a small fraction of B[a]P is activated to 7-8-diol-B[a]P which is further converted to the highly reactive 7,8-dihydroxy-9,10-epoxy-B[a]P (BPDE) the ultimate DNA carcinogen. (B) Chemical structure of the Benzo[a]pyrene butyric acid isomeric mixture (B[a]P-BA), the derivative used for the conjugation to T cell epitope peptides.

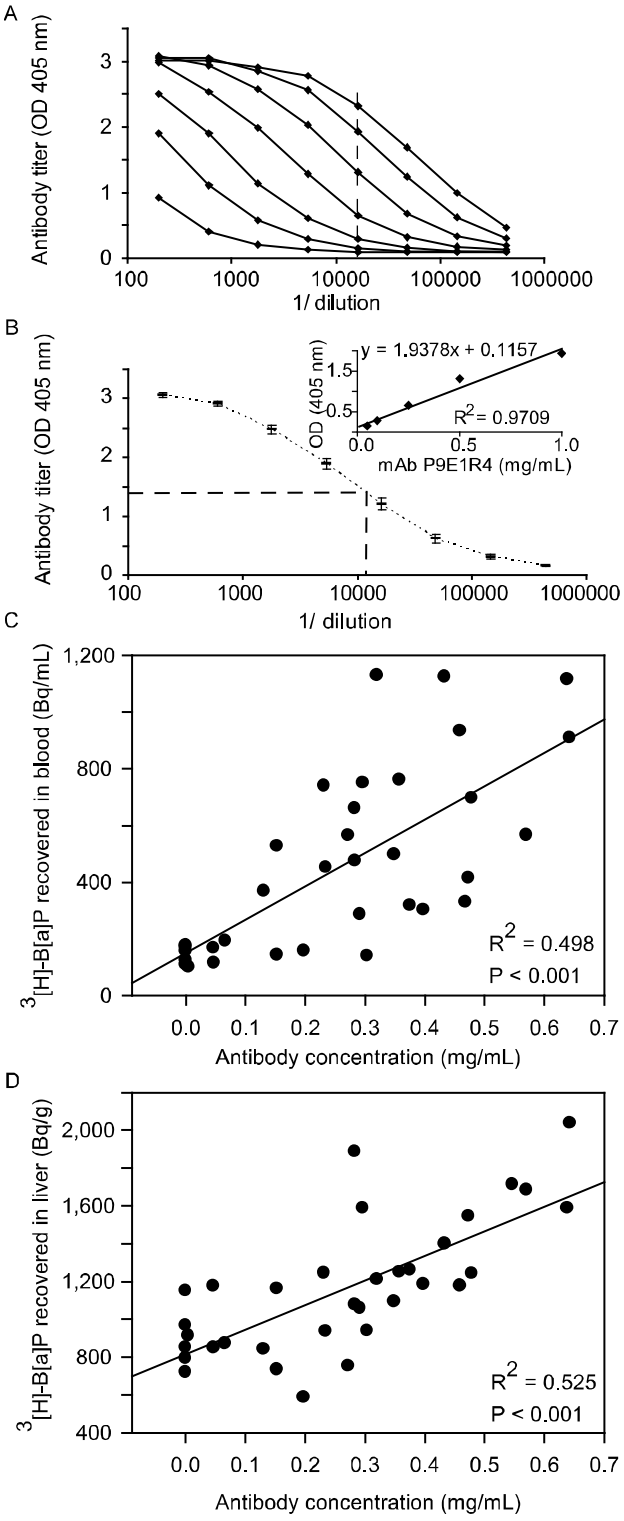

Figure 2: Anti-B[a]P antibody concentration determined by indirect ELISA and correlation with antibody levels. To estimate absolute antibody concentrations a standard curve (insert in panel B) was used, that was based on dilution curves (1/200-1/437,400) of different known concentrations (0.01-1 mg/ml, represented by a line for each concentration in panel A) of purified monoclonal antibody against B[a]P (P9E1R4). 1/16,200 dilution (dashed line) was chosen to plot the standard curve (Insert in panel B, R^2 = 0.9709) and to calculate the antibody concentration. (B) Example of a titration curve of serum antibodies of mice immunised with B[a]P conjugated TT 2 weeks after the fourth injection . Values are presented as mean ± S.E.M of 6 mice per group. (C, D) Correlation between antibody level and [^{3}H]-B[a]P recovery after a single i.p. injection of 2 µg/kg [^{3}H]-B[a]P in individual mice mock immunised and immunised against B[a]P using B[a]P-peptide or B[a]P-TT conjugates. [^{3}H]-B[a]P recovery in the blood (C) and liver (D). The statistical significant relationship between the two variables as shown in C and D was estimated by linear regression.

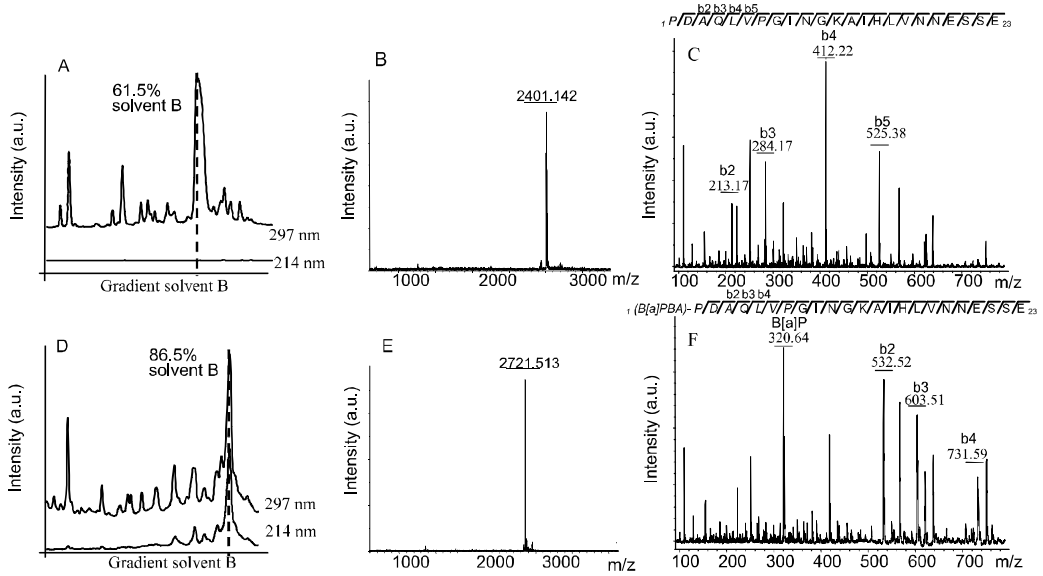

Figure 3: Peptide conjugation. HPLC chromatogram before purification (A, D). MS/MS spectrum (B, E) and the N terminal sequence (C, D) of VNNESSE-14 after purification. Panel A, B, C represent the unconjugated and panel D, E and F the B[a]P-BA conjugated peptide.

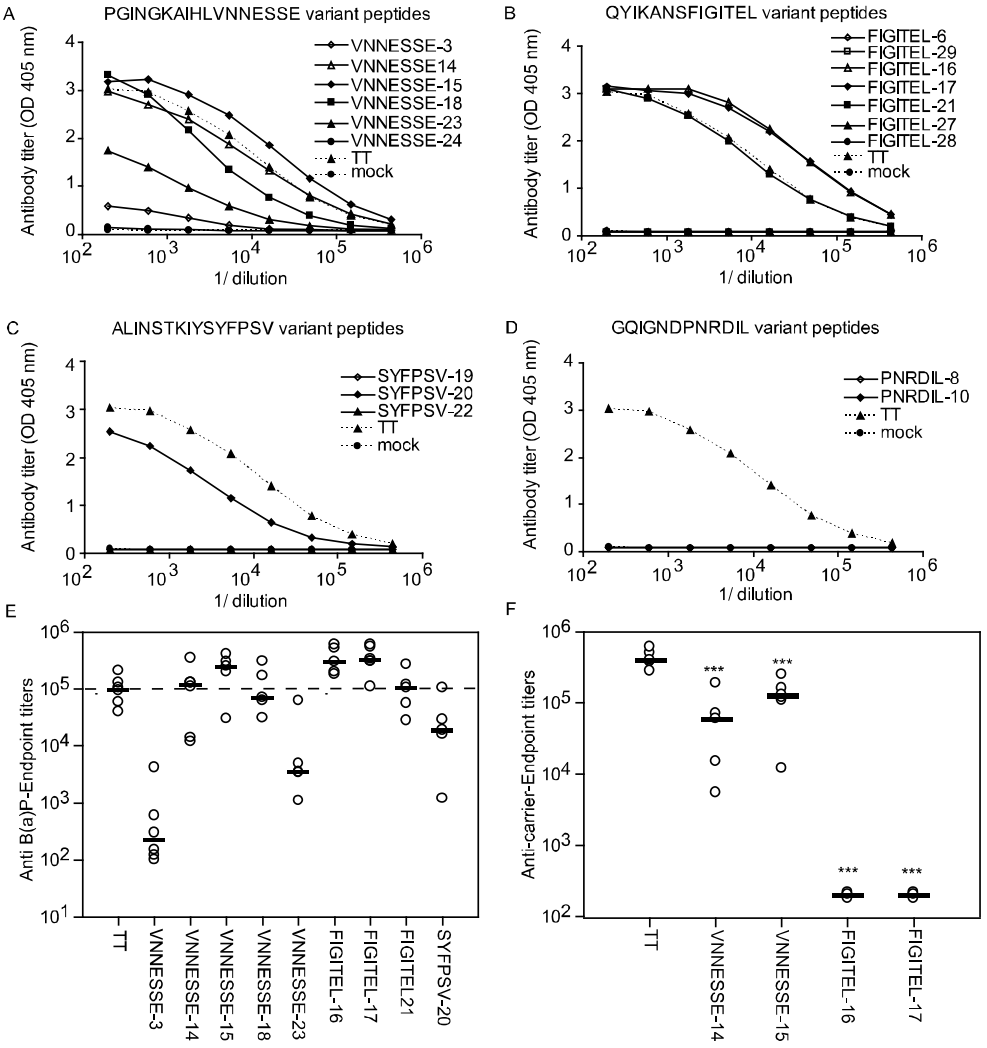

Figure 4: Antibody titration curves of sera from mice immunised with B[a]P-peptide conjugates. (A) VNNESSE-variants, (B) FIGITEL-variants, (C) SYFPSV-variants and (D) PNRDIL-variants. Control mice were immunised with B[a]P conjugated to TT-protein (dashed line). Values are mean of 6 mice per group determined by indirect ELISA using heterologous conjugates (B[a]P-ovalbumin) as coated antigen. (E) Serum endpoint titers (serum dilution reaching 5 times the background) of B[a]P specific IgG antibodies of individual mice (○) and median value (—). Groups correspond to a selection of panel A to D. Dashed line represents the endpoint titer for immunisation with tetanus toxoid. (F) Endpoint titers against homologous carrier peptide. For sera with no detectable antibodies endpoint titers were set to 1/200. Results are presented for each mouse (○) and median value (—). *** $p < 0.001$, statistically significant difference from TT immunised mice (One Way ANOVA test followed by Student-Newman-Keuls t-test for multiple comparison).

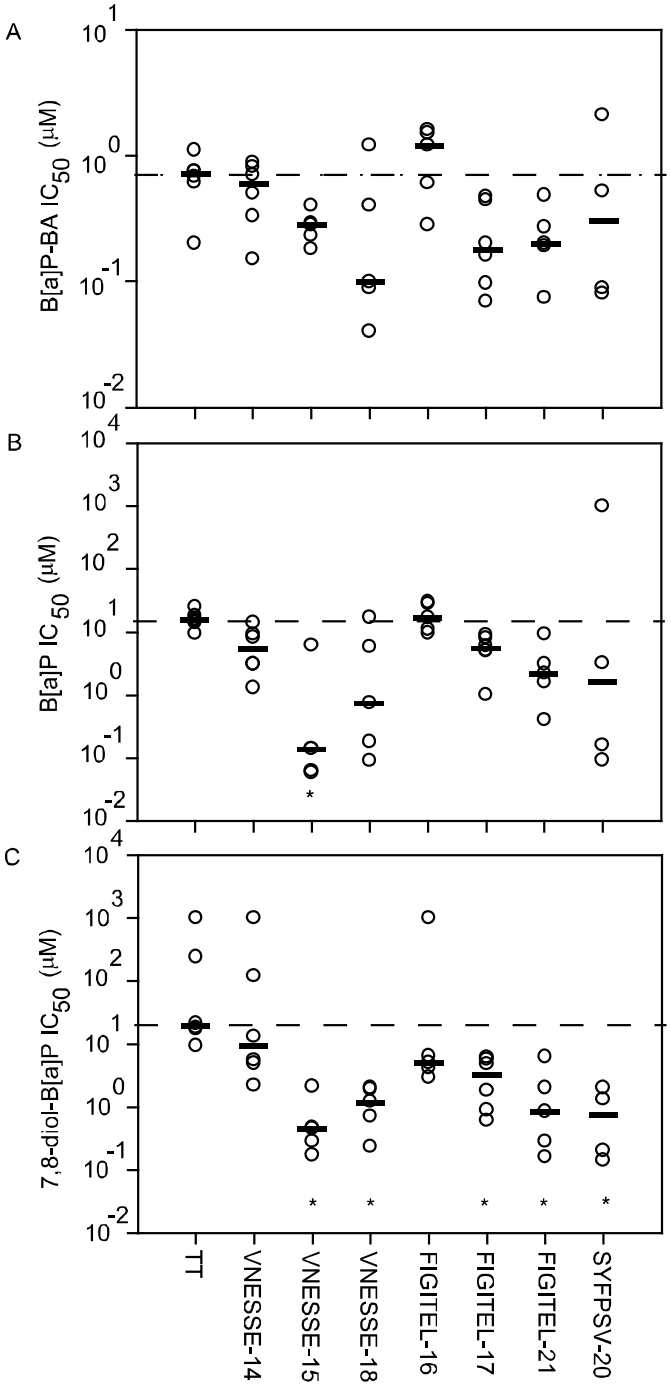

Figure 5: Antibody selectivity determined by competitive ELISA in sera immunised with B[a]P peptide or TT conjugates. (A) B[a]P-BA, (B) B[a]P, and (C) 7,8-diol-B[a]P were used as competitors to compete for binding of specific antibodies to B[a]P-ovalbumin as the coated antigen. The IC_{50} (concentration of competitor for 50% inhibition) was calculated as a measure of antibody selectivity for each tested competitor. The IC_{50} is inversely correlated to the antibody selectivity. Results are represented for each mouse (○) and median value (—). * $p < 0.05$, statistically significant difference from TT immunised mice (One Way ANOVA on Ranks followed by Dunn's method).

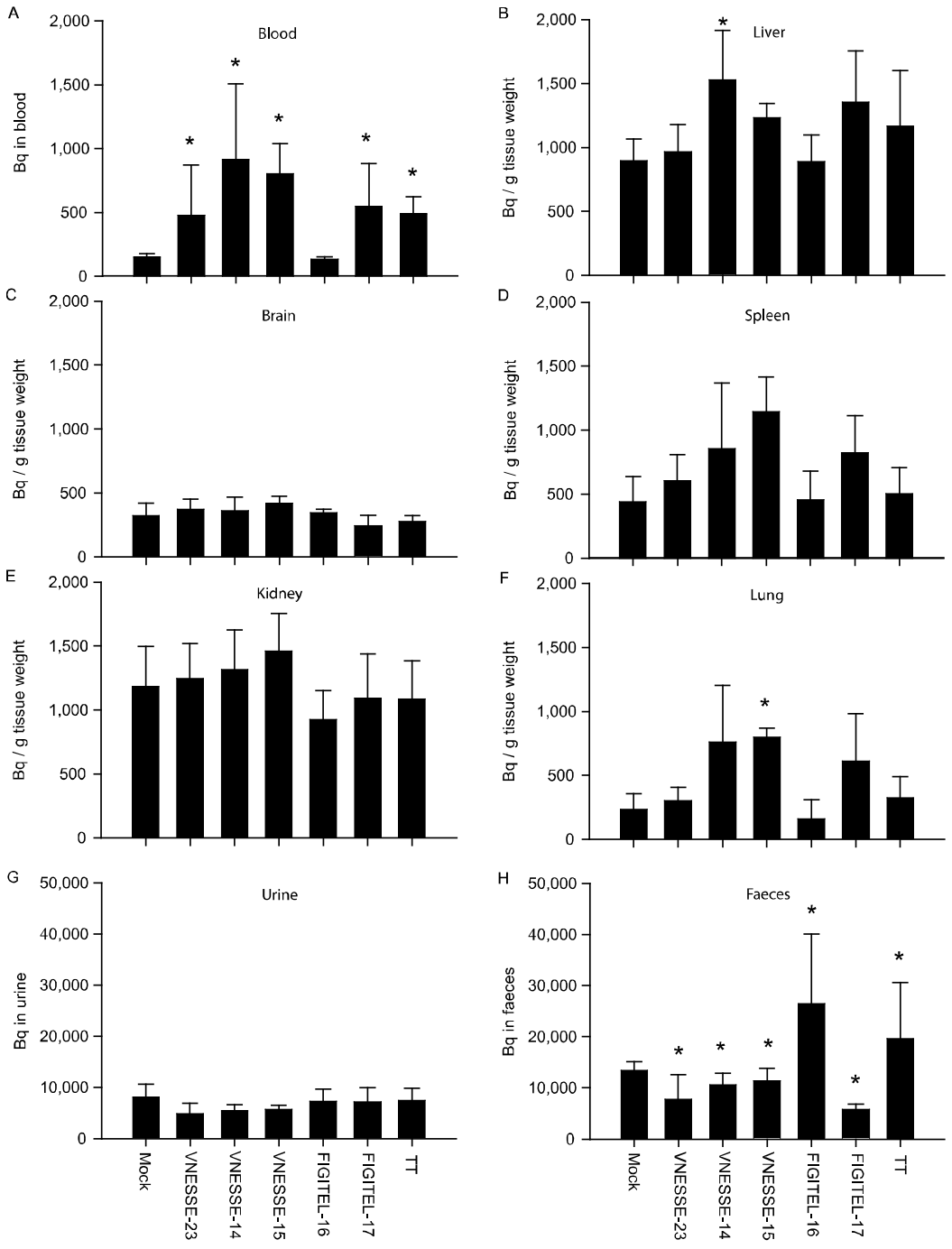

Figure 6: B[a]P recovery in mice immunised against B[a]P. [^{3}H]-B[a]P (in Bq/g tissue weight or Bq) recovered in blood (A), tissues (B-liver, C-brain, D-spleen, E-kidney, F-lung), urine (G) and faeces (H) 24 h after a single i.p. injection of [^{3}H]-B[a]P (2 µg/kg) in B[a]P-peptide, B[a]P-TT or mock immunised mice. Results are expressed as mean ± S.E.M of 5 mice per group. * $p<0.05$; statistical significant difference from control (Mock) (Student-Newman-Keuls-t test for multiple comparisons).

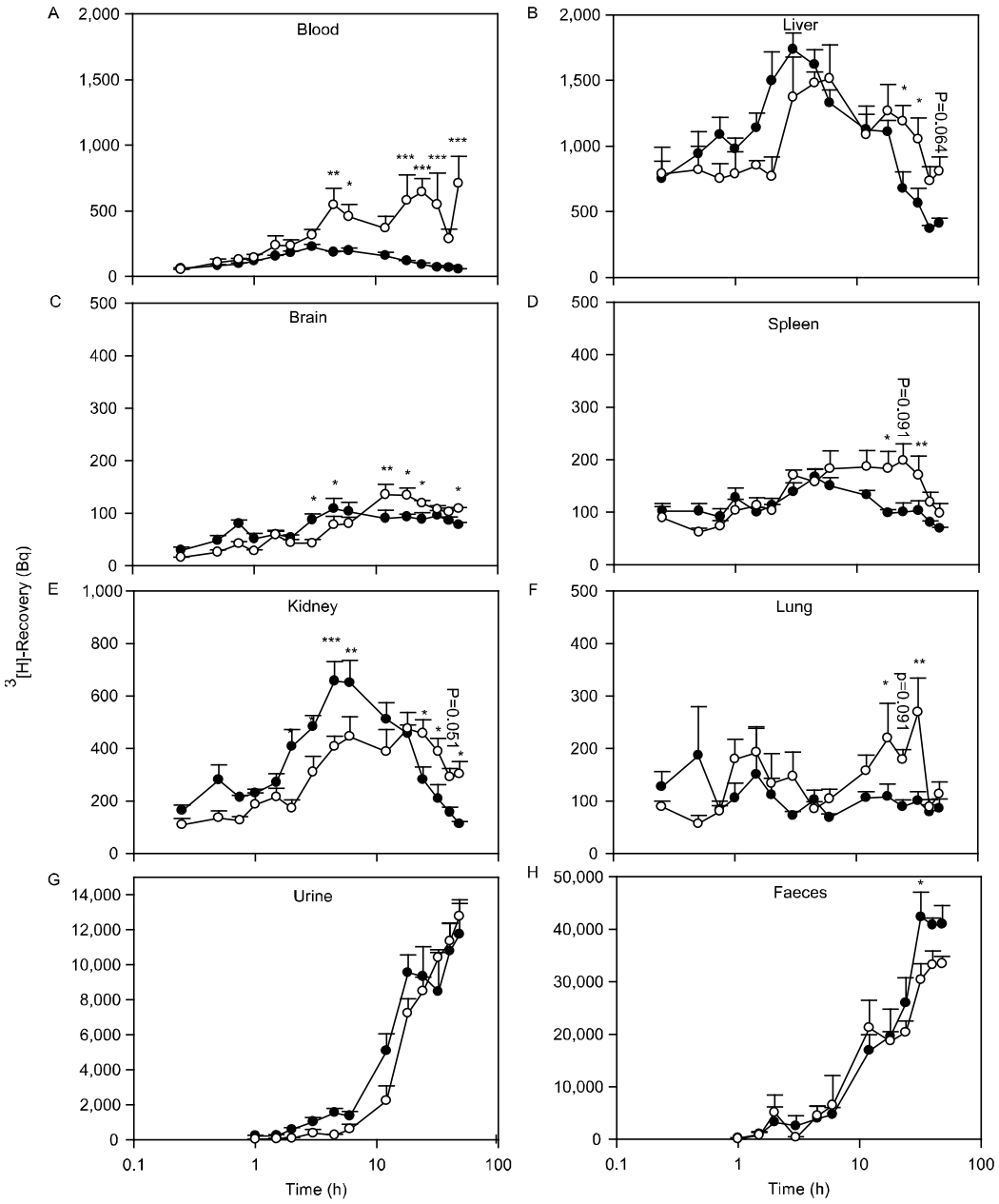

Figure 7: B[a]P pharmacokinetic. Pharmacokinetic of [^{3}H]-B[a]P in mice immunised with VNESSE-14 (○) and mock immunised mice (●) over 48 hours after a single i.p. injection of 2 µg/kg [^{3}H]-B[a]P. (A) blood, (B) liver, (C) brain, (D) spleen, (E) kidney, (F) lung, (G) urine, (H) faeces. Results are expressed as mean ± S.E.M of 5 mice per group. * $p<0.05$; ** $p<0.01$ and *** $p<0.001$, statistical significant difference from controls (Two way ANOVA procedure followed by Student-Newman-Keuls-t test).

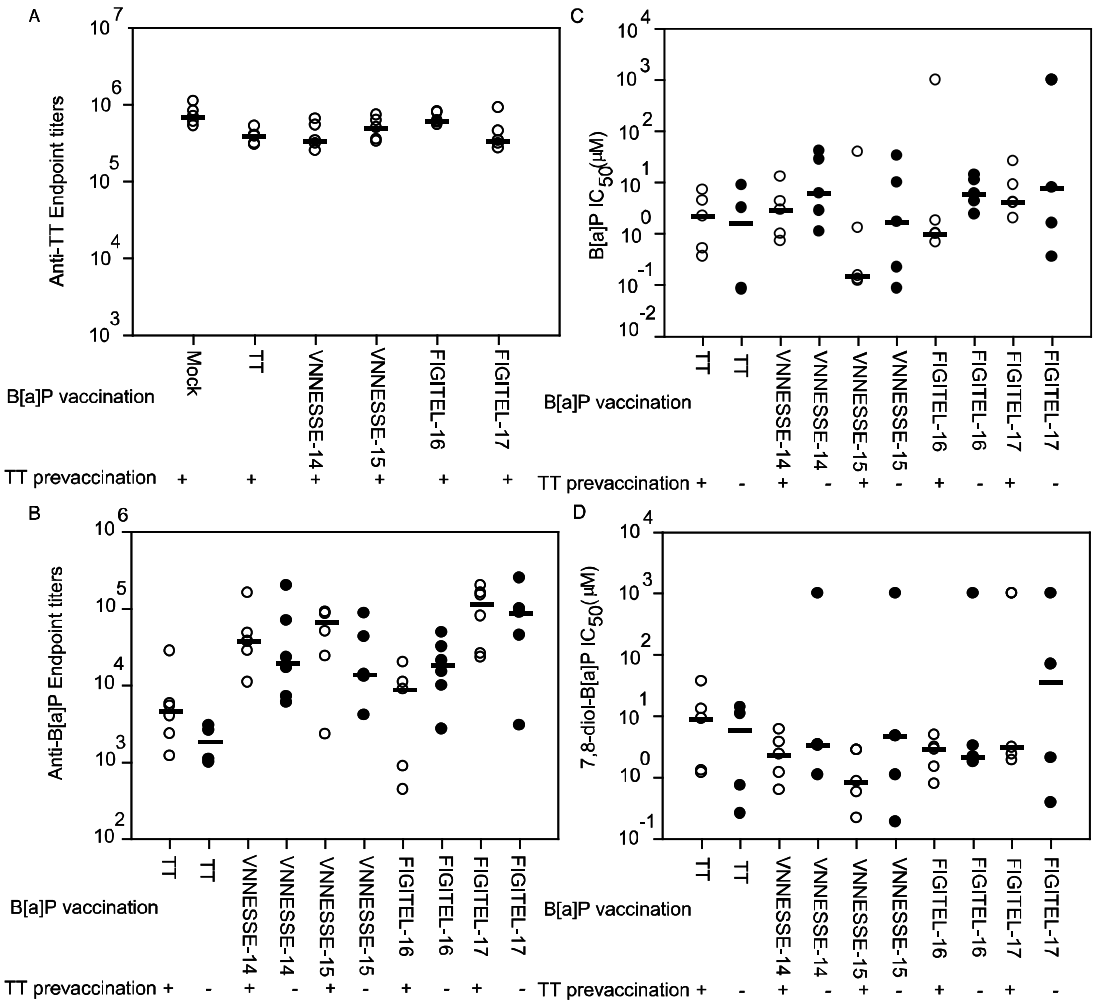

Figure 8: Immunogenicity of B[a]P-peptide conjugates after tetanus toxoid pre-vaccination. (A) Endpoint titers (serum dilution reaching 5 times the background) for TT specific IgG antibodies determined by indirect ELISA for sera pre-immunised with tetanus toxoid (TT) followed by B[a]P-peptide or B[a]P-TT conjugate vaccination. (B) B[a]P specific IgG antibodies with and without pre-vaccination. Results are presented for each mouse (○) and median value (—). There was no statistical significant difference between animal with and without pre-vaccination (One way ANOVA procedure followed by Student-Newman-Keuls-t test). (C, D) Antibody selectivity determined by competitive ELISA in sera immunised with B[a]P peptide or B[a]P-TT conjugates with (○) and without (●) tetanus toxoid (TT) pre-vaccination. B[a]P (C) and 7,8-diol-B[a]P (D) were used as competitors to compete for binding of specific antibodies to B[a]P-ovalbumin as the coated antigen. The IC50 (concentration of competitor for 50% inhibition) was calculated to determine the antibody specificity for each tested competitor. The IC50 is inverse correlated to the antibody affinity. Results are presented for each mouse (circle) and median value (—).

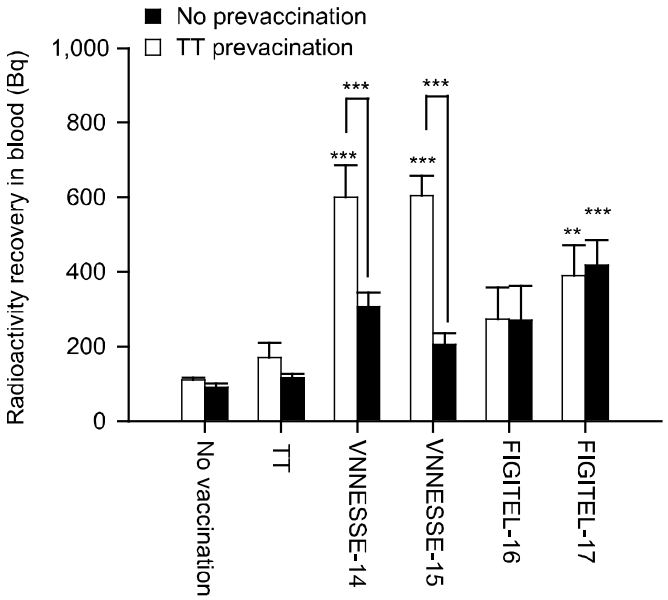

Figure 9: B[a]P recovery in mice vaccinated against B[a]P and tetanus toxoid. [^{3}H]-B[a]P recovered in blood 24 h after a single i.p. injection of [^{3}H]-B[a]P (2 µg/kg) in mice immunised with B[a]P-peptide or B[a]P-TT conjugates, with (open bars) or without (closed bars) tetanus toxoid (TT) pre-vaccination. Results are expressed as mean ± S.E.M of 5 mice per group.** $p<0.01$, ***$p<0.001$ significant difference from controls (Two Way ANOVA followed by Bonferoni). Control groups are No pre-vaccination (closed bars) or animals without B[a]P vaccination (No vaccination).

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Summary

The main objective of the present thesis was to investigate whether antibody effects observed in earlier *in vitro* studies can translate into the protection against chemical carcinogenesis *in vivo* as the basis of an immunoprophylactic approach against carcinogens.

As model for chemical carcinogenesis, we selected B[a]P the prototype polycyclic aromatic hydrocarbon (PAH), an environmental pollutant emanating from both natural and anthropogenic sources. Many *in vivo* models conveniently use high doses of carcinogens mostly given as single bolus, which provides simple surrogate readouts, but poorly reflects chronic exposure to the low concentrations found in the environment. In addition, these concentrations cannot be matched with equimolar antibody concentrations obtained by immunisation. However, low B[a]P concentrations do not permit to directly measure chemical carcinogenesis. Therefore, in the present thesis, the pharmacokinetic, metabolism and B[a]P mediated immunotoxicity were chosen as experimental read-outs.

B[a]P conjugate vaccines based on ovalbumin, tetanus toxoid and diphtheria toxoid (DT) as carrier proteins were developed to actively immunise mice against B[a]P. B[a]P-DT conjugate induced the most robust immune response. The antibodies reacted not only with B[a]P but also with the proximate carcinogen 7,8-diol-B[a]P. Antibodies modulated the bioavailability of B[a]P and its metabolic activation in a dose-dependent manner by sequestration in the blood.

In order to further improve the vaccination, we replaced the protein carrier by promiscuous T-helper cell epitopes to induce higher antibody titer with increased specificity for the B[a]P hapten. We hypothesised that a reduction of B cell binding sites on the carrier, compared to whole protein carrier, should favour the activation of B cells recognising the hapten instead of the carrier protein. An internal processing of the carrier and cleavage of the B[a]P-BA and subsequent presentation of the carrier peptide by MHC II molecules to T cell receptor should induce a B cell dependent immune response by activating B cells capable to recognise B[a]P. We demonstrated that a vaccination against B[a]P using promiscuous T-helper cell epitopes as a carrier is feasible and some tested peptide conjugates were more immunogenic as whole protein conjugates with increased specificity.

We showed that vaccination against B[a]P reduces immunotoxicity. B[a]P suppressed the proliferative response of both T and B cells after a sub-acute administration, an effect that was completely reversed by vaccination. In immunized mice the immunotoxic effect of B[a]P on IFN-γ, Il-12, TNF-α production and B cell activation was restored. In addition, specific antibodies inhibited the induction of Cyp1a1 by B[a]P in lymphocytes and Cyp1b1 in the liver, enzymes that are known to convert the procarcinogen B[a]P to the ultimate DNA-adduct forming metabolite, a major risk factor of chemical carcinogenesis.

In order to replace Freund adjuvant and to improve the immunisation strategy in terms of antibody quantity and quality, several adjuvants that are potentially compatible with their use in humans were tested. In combination with Freund adjuvant, the conjugate-vaccine induced high levels of B[a]P-specific antibodies. We showed that all adjuvants tested induced specific antibodies against B[a]P and 7,8-diol-B[a]P, its carcinogenic metabolite. The highest antibody levels were obtained with Quil A, MF-59 and Alum. Biological activity in terms of enhanced retention of B[a]P was confirmed in mice immunised with Quil A, Montanide, Alum and MF-59. Our findings demonstrate that a vaccination against B[a]P is feasible in combination with adjuvants licensed in humans.

Based on these results and with the current understanding of the mechanisms of chemical carcinogenesis of the ubiquitous carcinogen B[a]P and of the effects of specific antibodies, an immunoprophylactic approach against chemical carcinogenesis is absolutely warranted. Nevertheless, the direct effects of B[a]P-specific antibodies on the different stages of carcinogenesis (e.g. adduct formation) and whether these effects may translate into long-term protective effect against tumourigenesis needs to be proven in further experiments.

Zusammenfassung

Das Ziel der vorliegenden Thesis war es, zu untersuchen, ob die schützenden Effekte von Benzo[a]pyrene (B[a]P) spezifischen Antikörpern aus früheren *in vitro* Experimenten auf *in vivo* Versuche übertragen werden können und dieses auf der Basis einer immunprophylaktischen Anwendung gegen Karzinogene angewendet werden kann.

Als Modell für die chemische Karzinogenese wählten wir B[a]P, den Prototyp der polyzyklischen aromatischen Kohlenwasserstoffe (PAH), ein Umweltschadstoff der natürlich und durch menschliche Verursachung entsteht. Zahlreiche *in vivo* Modelle verwenden hohe Dosen an Karzinogenen, meistens als Einzeldosis verabreicht, welche einfache experimentelle Auslesungen ermöglichen, aber kaum die chronische Exposition zu umweltbedingten Konzentrationen reflektieren. Zusätzlich können diese Konzentrationen nicht von Antikörperkonzentrationen gebunden werden, die durch Immunisierungen erreicht werden können. Geringe B[a]P Konzentrationen erlauben jedoch keine direkte Untersuchung der chemischen Karzinogenese. Deshalb wurde in der vorliegenden These, die Pharmakokinetik, der B[a]P Metabolismus und die Immunotoxizität als experimentelle Auslesung für geringe B[a]P Konzentrationen *in vivo* untersucht.

Um Mäuse aktiv gegen B[a]P zu immunisieren, wurden B[a]P Impfstoffkonjugate basierend auf Ovalbumin, Tetanus Toxoid und Diphtherie Toxoid als Träger Proteine entwickelt. Die induzierten Antikörper reagierten nicht nur mit B[a]P sondern auch mit dem 7,8-diol-B[a]P, dem ultimativen Karzinogen. Die Antikörper beeinflussten die Bioverfügbarkeit des B[a]P und dessen metabolische Aktivierung in einer dosisabhängigen Weise durch Antikörperbindung im Blut.

Um die Impfwirkung noch zu verbessern, wurde das Trägerprotein durch ein promiskutives T-Helfer Epitop ersetzt. Wir nehmen an, dass die Reduzierung von B Zell Bindungsstellen am Peptid-Träger-Molekül, verglichen mit dem Protein, die Aktivierung von B Zellen die das Hapten erkennen anstelle des Trägers bevorzugen. Die intrazelluläre Verarbeitung des Trägers, die Abspaltung des B[a]P-BA, und die anschließende Präsentation des Träger-Peptides durch MHC II-Moleküle auf T-Zell-Rezeptoren sollte eine B-Zell-abhängige Immunantwort durch die Aktivierung von B-Zellen induzieren. Wir zeigten, dass eine Impfung gegen B[a]P mit T-Helfer-Zell

Epitop Konjugaten möglich ist und einige getestete Peptid-Konjugate immunogener waren als Protein-Träger-Moleküle.

Wir zeigten, dass eine Impfung gegen B[a]P die Immunotoxizität reduziert. B[a]P supprimierte die Proliferation von T- und B-Zellen nach subakuter Injektion, und dieser Effekt wurde durch die Impfung verhindert. In immunisierten Mäusen wurde auch der Effekt von B[a]P auf die IFN-γ, IL-12 und TNF-α Produktion und die B-Zell Aktivierung wiederhergestellt. Weiterhin wurde die Induzierung von Cyp1A1 durch B[a]P in Lymphozyten und Cyp1b1 in der Leber inhibiert. Beide Enzyme, die bekannt sind das Prokarzinogen B[a]P in das ultimative DNA-Addukt-formende Metabolit zu katalysieren, ein bedeutender Faktor für die chemische Karzinogenese.

Um Freund Adjuvant im experimentellen Impfstoff zu ersetzen, testeten wir 5 verschiedene Adjuvantien (Quil A, MF-59, Alum, PLG-Partikel, Montanide) die möglicherweise kompatibel mit der Verwendung im Menschen sind. In Verbindung mit Freund Adjuvant induzierte der konjugierte B[a]P Impfstoff hohe Antikörpermengen. Wir zeigten dass alle getesteten Adjuvantien Antikörper induzierten die mit B[a]P und 7,8-diol-B[a]P reagierten. Die höchsten Antikörpermengen wurden mit Quil A, MF-59 und Alum erzielt. Die Biologische Aktivität *in vivo* in Bezug auf erhöhte Retention des B[a]P wurde in immunisierten Mäusen mit Quil A, Montanide, Alum und MF-59 bestätigt. Diese Ergebnisse bestätigen dass eine Impfung gegen B[a]P mit für Menschen lizenzierten Adjuvantien möglich ist.

Auf Grundlage dieser Ergebnisse und dem gegenwärtigen Verständnis des Mechanismus der chemischen Karzinogenese induziert durch B[a]P und dem Effekt B[a]P spezifischer Antikörper, ist eine immunoprophylaktische Anwendung gegen Karzinogenese absolut berechtigt. Trotzdem sind weitere Experimente notwendig um den direkten Effekt von B[a]P spezifischen Antikörpern auf die verschiedenen Stadien der Karzinogense (z.B. Adduktbildung) zu untersuchen. Ferner ist zu analysieren ob eine Induzierung von B[a]P spezifischen Antikörpern einen langfristigen Schutz gegen Tumorbildung zur folge hat.

List of Abbreviations

1-OH-B[a]P	2-hydroxybento[a]pyrene
2-AAF	2-acetylaminofluorene
3-OH-B[a]P	3-hydroxybento[a]pyrene
7,8-diol-B[a]P	7,8 -benzo[a]pyrene-dihydroldiol
9-OH-B[a]P	9-hydroxybento[a]pyrene
ACN	acetonitrile
AhR	aryl hydrocarbon receptor
ANOVA	analysis of variance
B[a]P	Benzo[a]pyrene
B[a]P-BA	B[a]P butyric acid
BSA	bovine serum albumin
CDNB	1-chloro-2,4-dinitrobenzene
CFA	Complete Freund's adjuvant
DMBA	dimethylbenzanthracene
DMSO	dimethyl sulfoxide
DSS	dioctyl sulfosuccinate sodium salt
DT	diphtheria toxoid
DTT	dithiothreitol
EEC	European Communities Council
GSH	glutathione
GST	glutathione-S-transferase
IFA	Incomplete Freund's adjuvant
ip	intraperitoneally
KLH	keyhole limpet hemocyanin
LPS	lipopolysaccharide
mab	monoclonal antibody
n.a.	not analysed
n.d.	not detectable
NNK	4-(methylnitrosamino)- 1-(3-pyridyl)-1-butanone
OD	optical density
OEHHA	Office of Environmental Health Hazard-Assessment
OVA	ovalbumin
PAH	polycyclic aromatic hydrocarbons
PBDE	7,8-dihydroxy-9,10-epoxide-7,8,9,10-tetrahydro-B[a]P

PBMC	peripheral blood mononuclear cell
PHA	phytohemagglutinin
PLG	poly lactide co-glycosides
RE	reticuloendothelial
RT	room temperature
SE	standard error
TFA	trifluoroacetic acid
TT	tetanus toxoid
WHO	World Health Organisation

Publication and conference participations

Publications

1. *Immunogenicity of a promiscuous T cell epitope peptide based conjugate vaccine against benzo[a]pyrene: Redirecting antibodies to the hapten.* MT Schellenberger, N Grova, S Farninelle, S Willième, D Revets, CP Muller, PLoS One.2012;7(5):e38329. http://www.ncbi.nlm.nih.gov/pubmed/22666501
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3. *Clinical Variability and Novel Mutations in the NHEJ1 Gene in Patients with a Nijmegen Breakage Syndrome-like Phenotype*, V Dutrannoy, U Baumann, D Schindler, K Konrat, H Neitzel, G Kaesbach, J Radszewski, S Rothe, MT Schellenberger, P Nürnberg, K Wee Teik, R Nallusamy, A Reis, K Sperling, M Digweed, R Varon, Human Mutation, 2010 Jul 1;31(9):206-68. http://www.ncbi.nlm.nih.gov/pubmed/20597108
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6. *Modulation of carcinogen bioavailability by immunisation with benzo[a]pyrene-conjugate vaccines,* N Grova, EJ Prodhomme, MT Schellenberger, S Farinelle, CP Muller. Vaccine, 2009 Jun 24;27(31):4142-51, http://www.ncbi.nlm.nih.gov/pubmed/19406187

Conference Participations

1. Evaluation of an Immunoprophylactic Strategy against Benzo[a]pyrene - a Major Lung Carcinogen, MT Schellenberger, Lung Cancer Conference - Lung Cancer Treatment in the Era of personalized Medicine, 11-12.02.2011, Luxembourg. ABSTRACT and TALK
2. *Immune strategy against an ubiquitous carcinogen in food*, MT Schellenberger, N Grova, EJP Prodhomme, S Willième, S Farinelle, CP Muller, "La sécurité dans mon assiette, EFSA and OSQCA", 16.06.10, Luxembourg. ABSTRACT and TALK.
3. *Evaluation of an immunoprophylactic strategy against low molecular weight carcinogens based on benzo[a]pyrene*, MT Schellenberger, S Willième, S Farinelle, CP Muller, "Société des Sciences Médicales Luxembourg", 02.06.2010, Luxembourg. ABSTRACT and TALK.
4. *Evaluation of alternative adjuvants to Freund Adjuvant for a new generation vaccine strategy against benzo[a]pyrene,* MT Schellenberger, S Farinelle, S Willième, CP Muller, "7th world congress on vaccines, immunization and immunotherapie", 26.05.10, Berlin, Germany. ABSTRACT and TALK.
5. *Evaluation of alternative adjuvants to Freund Adjuvant for a new generation vaccine strategy against Benzo[a]pyrene*, MT Schellenberger, S Farinelle, S Willième, CP Muller, "AK Vakzine", 21.05.10, Göttingen, Germany. ABSTRACT and TALK.
6. *Immunoprophylactic strategy against chemical carcinogenesis on the example of Benzo[a]pyrene*, MT Schellenberger, N Grova, S Farinelle, CP Muller, "Symposium", Hochschule Senftenberg, Germany. ABSTRACT and TALK. 26.11.2009
7. *Immunogenicity of a t cell epitope peptide vaccine against Benzo[a]pyrene*, MT Schellenberger, Grova, S Farinelle, CP Muller, "AK Vakzine", 26.06.09, Freiburg, Germany. ABSTRACT and TALK.
8. *Attenuation of benzo(a)pyrene induced immunotoxicity by specific antibodies generated by active immunisation with B(a)P-carrier conjugates*, MT

Schellenberger, N Grova, EJP Prodhomme, CP Muller, "4th EMII-Mugen Immunology Summer School 2009", 17.-24.5.09, Sardinia, Italy. ABSTRACT and POSTER.

9. *Immunoprophylactic strategy against chemical carcinogenesis on the example of Benzo(a)pyrene*, MT Schellenberger, N Grova, EJP Prodhomme, S Willième, S Farinelle, CP Muller, "La sécurité dans mon assiette, EFSA and OSQCA", 9.12.08, Luxembourg. ABSTRACT and TALK.
10. *Modulation of the bioavailability and immunotoxicity of Benzo[a]pyrene in mice actively immunized with B[a]P-carrier conjugates*, MT Schellenberger, N Grova, EJP Prodhomme, CP Muller, "La sécurité dans mon assiette, EFSA and OSQCA", 9.12.08, Luxembourg. ABSTRACT and POSTER.
11. *Modulation of benzo[a]pyrene-induced immunotoxicity in mice actively immunised with B[a]P-carrier conjugates*, MT Schellenberger, N Grova, EJP Prodhomme, S Willième, Farinelle, CP Muller, "Health Aspects of Indoor and Outdoor Air Pollution", 12.11.08, CRP Gabriel Lippmann, Luxembourg. ABSTRACT and POSTER.
12. *Immunoprophylactic approach against environmental carcinogens,* MT Schellenberger, CP Muller, "28^{ième} Journée Nationale de la Biologie Clinique", 19.-20.9.2008, Luxembourg. ABSTRACT and TALK.
13. *Immunoprophylactic approach against environmental carcinogens,* MT Schellenberger; N Grova, S Willième, S Farinelle, Prof. Dr. CP Muller, "AK Vakzine", 12.6.08 Borstel, Germany. ABSTRACT and TALK.
14. *Modulation of benzo[a]pyrene-induced immunotoxicity in mice actively immunized with B[a]P-carrier conjugates,* N Grova, PhD, MT Schellenberger, S Willième, S Farinelle, CP Muller, "Cancer Immunotherapy CIMT 2008", 15.-16.5.2008, Mainz, Germany. ABSTRACT and POSTER.

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