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The transferability from rat subacute 4-week oral toxicity study to translational research exemplified by two pharmaceutical immunosuppressants and two environmental pollutants with immunomodulating properties

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ABSTRACT

Exposure to chemicals may have an influence on the immune system. Often, this is an unwanted effect but in some pharmaceuticals, it is the intended mechanism of action. Immune function tests and in depth histopathological investigations of immune organs were integrated in rodent toxicity studies performed according to an extended OECD test guideline 407 protocol. Exemplified by two immunosuppressive drugs, azathioprine and cyclosporine A, and two environmental chemicals, hexachlorobenzene and benzo[a]pyrene, results of subacute rat studies were compared to knowledge in other species particular in humans. Although immune function has a high concordance in mammalian species, regarding the transferability from rodents to humans various factors have to be taken into account. In rats, sensitivity seems to depend on factors such as strain, sex, stress levels as well as metabolism. The two immunosuppressive drugs showed a high similarity of effects in animals and humans as the immune system was the most sensitive target in both. Hexachlorobenzene gave an inconsistent pattern of effects when considering the immune system of different species. In some species pronounced inflammation was observed, whereas in primates liver toxicity seemed more obvious. Generally, the immune system was not the most sensitive target in hexachlorobenzene-treatment. Immune function tests in rats gave evidence of a reaction to systemic inflammation rather than a direct impact on immune cells. Data from humans are likewise equivocal. In the case of benzo[a]pyrene, the immune system was the most sensitive target in rats. In the *in vitro* plaque forming cell assay (Mishell–Dutton culture) a direct comparison of cells from different species including rat and human was possible and showed similar reactions. The doses in the rat study had, however, no realistic relation to human exposure, which occurs exclusively in mixtures and in a much lower range. In summary, a case by case approach is necessary when testing immunotoxicity. Improvements for the translation from animals to humans related to immune cells can be expected from *in vitro* tests which offer direct comparison with reactions of human immune cells. This may lead to a better understanding of results and variations seen in animal studies.

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Abbreviations: AH-receptor, aryl hydrocarbon receptor; AZA, azathioprine; BaP, benzo[a]pyrene; BN, Brown Norway (rat strain); BfR, *Federal Institute for Risk Assessment*; BgVV, *Federal Institute for Health Protection of Consumers and Veterinary Medicine*; BW, body weight; CSA, cyclosporine A; CYP, cytochrome P450; ELISA, enzyme-linked immunosorbent assay; EPA, *Environmental Protection Agency*; F, female; FACScan, fluorescence activated cell sorter; Fig., Figure; HCB, hexachlorobenzene; H&E, hematoxylin and eosin; HEV, high endothelial venules; IARC, *International Agency for Research on Cancer*; ICH, *International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use*; IC_{90}, relative 90% inhibitory concentration; ICISIS, *International Collaborative Immunotoxicity Study*; M, male; MD, Mishell–Dutton (*in vitro* plaque forming cell test); n.d., not determined; NK, natural killer cells; OECD TG, *Organisation for Economic Co-operation and Development Test Guideline*; PAH, polycyclic aromatic hydrocarbons; PALS, periarteriolar lymphatic sheath; PBMC, peripheral blood mononuclear cell; PFCA, plaque forming cell assay; Satell., satellite groups; SRBC, sheep red blood cells; TMPT, thiopurine methyltransferase

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1. Introduction

Starting in the 1990s, increasing awareness of potentially adverse chemically induced immune effects in humans led to the investigation of several model compounds in toxicological animal studies. The OECD TG 407 guideline provided a framework for the conduction of subacute 28-day studies in rodents and still belongs to the most common types of toxicity studies used in preclinical drug development and in the toxicological investigation of chemicals. Though not specifically designed to detect immune effects, efforts were made to improve the study in order to detect changes which could be related to primary immunotoxicity. Due to the recommendation of the very first immunotoxicity guideline published in 1998, it is necessary to differentiate from the context of the study data between primary and secondary immunotoxicity, the latter being an unspecific sequel of toxicity to other organs (U.S. EPA, 1998). Examples for both mechanisms have been reported (Vohr, 1995; Vohr and Rühl-Fehlert, 2001). For primary immunotoxic substances, immunosuppression is markedly more frequent than immunostimulation. On the whole, primary effects occur relatively seldom in toxicological screening. For in depth investigation a tiered approach was developed: *Tier 1* was the routine testing, *tier 2* included specific immune function tests and *tier 3* mechanistic studies.

In collaborative or ring studies which were conducted principally in accordance with the OECD TG 407 prototypical immunomodulating compounds were tested in a standardized setting in rats in order to prove the sensitivity and reproducibility of primary immunotoxic effects. For this purpose, the studies included additional functional testing, analysis of cell populations, lymphoid organ weights and detailed histopathological investigations of immune organs. To reproduce different types of effects cyclosporine A (CSA), azathioprine (AZA), hexachlorobenzene (HCB) (The ICICIS Group Investigators, 1998; Schulte et al., 2002) and benzo[a]pyrene (BaP) were investigated. The CSA and HCB study were part of an effort taken by the former German BgVV (*German Institute for Consumer Health Protection and Veterinary Medicine*, today party reorganized as *Federal Institute for Risk Assessment*, BfR), which initiated a ring study with the participation of 5 laboratories (Richter-Reichhelm et al., 1995; BgVV Hefte, 1996, 2000; Richter-Reichhelm and Schulte, 1998; Schulte et al., 2002). AZA was tested among the first collaborative studies by ICISIS. BaP was tested as a single validation study with a similar study design.

With respect to the further development of guidelines for chemicals, one of the milestones were the findings published by Luster et al. (1992, 1993) in which the authors presented data from studies of 51 substances of which 35 were declared immunotoxic. The most significant result of these investigations was that immunosuppressive effects (immunosuppression of host resistance) could not be detected by the incorporation of one single immune parameter into the routine toxicological testing. Instead, a combination of two or three additional parameters was required. One of these additional investigations was a functional assay, the Plaque Forming Cell Assay (PFCA). Another test was the analysis of subpopulations of spleen cells by flow cytometry (FACScan). Beyond the guideline requirements, investigations of immune cell subpopulations and functional immune tests were thus integrated into the validation studies. The outcome confirmed the overall reproducibility of changes in the immune system in different labs and gave insights into the sensitivity of the investigated parameters. Importantly, the value of the standard investigations for screening purposes was confirmed.

Although the collaborative or ring studies were conducted for validation purposes, they can be seen from a quite different viewpoint when comparing their outcomes with the knowledge in humans with respect to these compounds. This is of interest since there is a perceived gap between the knowledge on animals *versus* humans. Considering the plethora of information present in prototypical immunomodulators, a comparison of data from animal studies and humans for these compounds may further add to the understanding of the relationship of findings in animals and human diseases. In this respect, a differentiation between pharmaceuticals and environmental chemicals is an important issue.

2. Materials and methods

This part gives a short overview on the OECD 407 subacute 28 day oral toxicity study in rodents (OECD, 2008). In addition, it provides information on frequently used methods for investigation of the immune system and specifically describes the design of studies under consideration.

2.1. OECD 407 and similar guidelines

The OECD 407 subacute 28 day oral toxicity study in rodents was first adopted in 1981 and since then was updated in 1995 and 2008. The method is intended to examine effects on a broad variety of potential targets of toxicity, including effects on the immune system (OECD, 2008). The very first guideline for testing immunotoxicity of agrochemicals had been published in 1998 by the U.S. EPA and served as basis for other guidelines. The U.S. EPA requires regularly in rodents functional testing (PFCA or ELISA), and immunotoxicity screening is recommended in combination with subacute (28 day study) or longer study durations, such as 90 days investigations. In addition the proportions of immune cells are required for registration of pesticides according to Health Effects Test Guideline OPPTS 870.7800 Immunotoxicity issued in 1998 (Gehen et al., 2014).

Drug entities belonging to the “small molecules” require a so-called tiered approach as outlined by the ICH S8 guideline (2005). This includes basic testing similar to the OECD TG 407 for direct immunosuppression or immunostimulation in repeated dose studies. Additional testing is needed, if in standard toxicity studies an immunogenic potential is detected. Other conditions demanding in depth testing are defined by the S8 guideline and include the pharmacological properties of the compound, structural similarity with known immunomodulators, accumulation of the compound in immune organs, the intended patient population or observations of immunotoxicity in patients.

For biotechnology-derived drugs or medical devices no standardized procedures, but case by case decisions are required by the respective guidelines ICH S6(R1) – Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals (1997, 2011) and Center for Devices and Radiological Health of the US FDA Immunotoxicity Testing Guidance (1999). According to the S6 guideline beside immunogenicity determination possible immunotoxic effects have also to be evaluated for each biopharmaceutical.

2.2. Overview of examined studies

The following studies were taken into account:

- published BgVV ring studies with CSA and HCB (Richter-Reichhelm et al., 1995; BgVV, 1996, 2000; Richter-Reichhelm and Schulte, 1998; Schulte, 2002);
- studies conducted in the setting of BgVV ring studies at Bayer HealthCare AG:CSA1, HCB;
- published ICISIS collaborative studies with AZA and CSA (The ICICIS Group Investigators, 1998);
- studies conducted as validation studies at Bayer HealthCare AG: AZA1 and BaP;
- studies conducted as validation studies at TNO in the setting of the ICISIS studies: AZA2, CSA2.

The studies were based on different versions of the OECD 407 due to the different times of study initiation.

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