The Challenge of Predicting the Immunotoxic Potential of Chemicals

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14.1 What Is Immunotoxicology?

14.1.1 Introduction

Well before the mechanisms were understood, pulmonary immune diseases had been associated with environmental chemicals, i.e., air contaminants. During the second half of the twentieth century, purpose and function of the different

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components of the immune system as well as their interactions with chemicals were extensively investigated and ultimately understood in more detail. In addition to a deeper understanding of immunological interactions and mechanisms, a series of accidents involving immunotoxic compounds pushed the development of immunotoxicological science in the last century.

In Europe in particular, the field of immunotoxicology came to the forefront following the accidental release of 2,3,7,8-TCDD near Seveso in 1976. This accident marked the starting point of public discussion around chemical-induced immune deficiencies which still continue to this day. Broad public attention led to a flood of activity by academics, authorities, and the industry. Immunotoxic effects induced by drugs had been observed and experimentally investigated prior to this incident. However, these studies were – in most cases – not conducted as special immunotoxicity investigations, i.e., based on understanding of immunological processes. Furthermore, they did not obtain wide publicity. Authorities started the process of drafting immunotoxicity guidelines, and several workshops were initiated to discuss possible testing strategies for immunotoxicological screening (e.g., by IPCS) as well as intra- and interlaboratory validation studies (ICICIS, BGA, NTP, etc.). Several proposals for such screening batteries had been published between 1982 and 1998 from various sites.

Development of guidelines for industrial chemicals and agrochemicals reached a milestone as a result of the findings published by M. Luster et al. in 1992 and 1993, in which the authors presented data from studies of 51 substances, 35 of which were declared immunotoxic, in a comprehensive test battery in mice to investigate changes in functional parameters after 28-day administration of the substance. The key finding in this study was that immunotoxic effects (immunosuppression of host resistance) could not be detected by the incorporation of a single immune parameter into the routine toxicological testing. A combination of two or three additional parameters was required. One of these additional parameters was a functional assay, the Plaque-Forming Cell Assay (PFCA; Fig. 14.1). Another test was the analysis of subpopulations of spleen cells by flow cytometry. However, from the very beginning, many discussions about whether advanced histopathology of lymphoid organs alone could be sufficient to pick up all of the immunotoxic effects of chemicals, a discussion which persists to this day.

14.1.2 Definitions

The first international seminar on the immune system as a target for toxic damage was held in Luxembourg in 1984 (UNEP, ILO, WHO: IPCS, CEC; 1987). During the meeting the definition for the term “immunotoxicology” was agreed upon for the first time by all the participants. The definition reads as follows:

> Immunotoxicity is defined as undesired effects as a result of interaction of xenobiotics with the immune system.

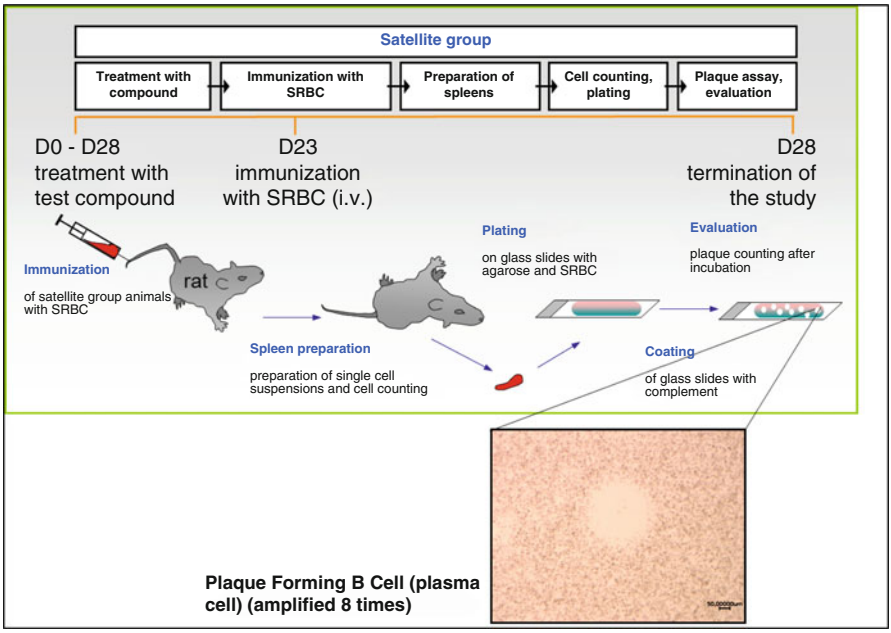

Fig. 14.1 Scheme of the Plaque-Forming Cell Assay (PFCA) as performed in accordance to relevant guidelines (Fischer, A, modified). In most cases, satellite animals are immunized with SRBC for the PFCA instead of animals in the main group to avoid any interference of the toxic effects by a strong immune reaction to SRBC

Such interactions of chemicals with the immune system can induce immunosuppression or enhancement (undesired immunostimulation). While immunosuppression may result in decreased resistance to infections or increased tumor development, overstimulation may increase the risk of autoimmune or allergic reactions. Although several additions and/or alterations have been made to the definition over the years, the end points of immunotoxicity are still valid. The meeting in Luxembourg was the starting point for several workshops, seminars, and symposia focusing on definitions and testing strategies for immunotoxic evaluation.

A workshop held in Hannover, Germany, in 1989 (IPCS 1990) was followed by a workshop in 1992 organized in Bilthoven, Netherlands (Vos and van Loveren 1995). Two special joint workshops of IPCS and the Norwegian National Institute of Public Health were held in Oslo in 1995 and 1996, of which the first was entitled "Environmental Chemicals and Respiratory Hypersensitisation" (Dybing et al. 1996). Finally, two additional scientific symposia were organized in Bilthoven, Netherlands, in 1997 (Van Loveren et al. 1999) and in 1999 in Berlin, Germany.

In course of this period, the most accepted definitions of immunotoxicology evolved significantly:

> Immunotoxicology is defined as the study of adverse effect on the immune system associated with exposure to environmental chemicals, pharmacologic agents, and biological.

> Generally these effects can be categorized as immunomodulation (immune suppression or potentiation), hypersensitivity (i.e., allergy), and autoimmunity.

While some authors added "chronic inflammation" and/or "flu-like reactions" to this enumeration of effects, Jacques Descotes published a further refined view on the possible end points of immunotoxicity. In 2005 he made precise distinctions between specific (immune-mediated) and nonspecific interactions, allergic and pseudoallergic reactions, etc. (Descotes 2005):

> The immunotoxic effects of drugs are divided into immunosuppression, immunostimulation, hypersensitivity and autoimmunity. The major adverse consequences of immunosuppression are infectious complications and virus-induced malignancies. Flu-like reactions, more frequent autoimmune diseases and hypersensitivity reactions to unrelated allergens, and inhibition of drug-metabolising enzymes are the adverse effects related to immunostimulation. Hypersensitivity reactions are the most frequent immunotoxic effects of drugs. They include immune-mediated ("allergic") and non-immune-mediated ("pseudoallergic") reactions. Drug-induced autoimmune reactions, either systemic or organ-specific, are seemingly rare.

Although Descotes' definition referred only to drug interactions with the immune system, the same holds true for environmental and industrial chemicals.

Immunotoxic effects can be a significant cause of morbidity or in some cases even mortality. Early immunotoxicological investigations in a regulatory environment were predominantly based on *in vivo* studies (28/90 days or short-term tests) with rats or mice. There were ongoing discussions regarding what parameters are essential for a sound immunotoxic assessment, and in parallel a set of relatively robust, standardized, and validated assays were established. However, with the exception of type IV investigations (guinea pig assays or LLNA), focus and experiences were mainly based on immunosuppression and not on immunostimulation. For non-clinical immunotoxicity, safety assessments of unexpected immunostimulation, like systemic or local hypersensitivity reactions, types I to III, or autoimmunity, the situation was unsatisfactory, because no validated and widely accepted assays for determining these end points were available.

Great efforts were made during the last few years to generate reliable assays for the prediction of immunogenicity of biologicals, but much less for the detection of immunostimulating properties of small molecules. Authorities and the public increased pressure on immunotoxicologists to develop additional *in vitro* assays and to extend immunotoxicity screening to animal species more relevant to humans (e.g., nonhuman primates (NHP)). While this effort has resulted in an increasing number of new models, protocols, and parameters, these are still a long way from standardization and validation. Few models and assays have so far been validated and used in preclinical safety assessment of undesired immunostimulation. The situation is more dire with respect to the prediction of hypersensitivity and autoimmune reactions. Last but not the least situation, industrial and environmental chemicals remain largely unstudied in relevant species. This is in contrast to therapeutic drug development where most studies are performed on rodents and other species such as dogs or monkey. In addition, side effects of drugs observed during

preclinical development can shortly afterward be compared with findings in clinical investigations, i.e., in humans. This is unfortunately not the case for environmental chemicals. This will be the focus of the following chapters.

14.1.3 International Guidelines on Immunotoxicology

As described below, authorities started to think about immunotoxicity guidelines in the late 1970s and 1980s. In order to support the development of guidelines backed by a firm set of data, several national and international validation studies were initiated, one of which will be subsequently described in more detail.

The first guideline which was adapted to immunotoxicological end points was the OECD 407 (Repeated Dose 28-day Oral Toxicity Study in Rodents, 1981) for chemicals in 1992. Of note is that the immune parameters added to the existing guidelines were not the most sensitive ones as described by Luster et al. in 1992/1993 (Fig. 14.2). The history of the development of immunotoxicity guidelines is described in the following chapter.

14.1.4 Collaborative "BGA" Study

14.1.4.1 Cyclosporin A

In 1989, the German Federal Health Authority (Bundesgesundheitsamt, BGA) initiated a collaborative study in Europe to determine the most sensitive parameters for the detection of immunological side effects induced by chemicals after a 28-day treatment period. All five participating laboratories agreed to incorporate several additional investigations into the toxicological investigation in accordance with the OECD 407 guideline. Advanced histopathology of lymphoid organs was incorporated as well as a functional assay (PFCA), analyses of NK cell activity, subpopulations in the spleen, mitogen stimulation of splenic cells, and antibody titer in the sera of the treated animals. The "chemical" chosen for the first round was a well-known immunosuppressive standard, cyclosporin A. This drug was chosen not only because of its clear immunosuppressive potential but also because Cyclosporin A was used during the second international ring trial of ICICIS Dayan et al. (1998). Thus, the hope was to ultimately compare the two sets of data from these collaborative studies. Unfortunately, this has never been done, although the reason is not quite clear.

Although the additional immune parameters, especially flow cytometry and PFCA, turned out to be most sensitive for picking up significant effects on the immune system, the histopathology of the thymus was also more affected than other organs. This fact led to a discussion on whether the "new" additional immune parameters are valuable or not or if advanced histopathology would suffice. A final agreement about the necessity of additional immune parameters was also not reached by the participating labs of the BGA study (Richter-Reichhelm et al. 1995; Vohr 1995), and the discussion about this topic remains undecided to the present day. This is especially astonishing considering that Germolec et al. (2004), Lappin

	Plaque Forming Cells	NK Cell Activity	T Cell Mitogens	MLR	DHR	CTL	Surface Markers	Leukocyte Counts	Thymus/BW Ratio	Spleen/BW Ratio	Spleen Cellularity	LPS Response
Plaque Forming Cells	**78 (45)**	P<.0001										
NK Cell Activity	94 (34)	**69 (36)**	P=.0014									
T Cell Mitogens	85 (40)	79 (34)	**67 (46)**	P=.0003								
MLR	82 (34)	74 (31)	73 (37)	**56 (39)**	P=.0458							
DHR	89 (27)	84 (19)	82 (28)	74 (23)	**57 (30)**	P=.0348						
CTL	100 (8)	78 (9)	71 (7)	75 (8)	- (0)	**67 (9)**	P=.2380					
Surface Markers	91 (23)	90 (21)	92 (24)	87 (23)	93 (14)	100 (5)	**83 (24)**	P=.0017				
Leukocyte Counts	86 (28)	71 (24)	62 (29)	59 (27)	67 (18)	67 (6)	80 (20)	**43 (30)**	P=.4490			
Thymus/BW Ratio	92 (38)	81 (31)	83 (36)	77 (30)	75 (24)	71 (7)	90 (21)	72 (29)	**68 (40)**	P=.0009		
Spleen/BW Ratio	85 (39)	75 (32)	76 (37)	65 (31)	71 (24)	75 (8)	86 (22)	62 (29)	73 (40)	**61 (41)**	P=.0395	
Spleen Cellularity	80 (35)	72 (29)	72 (32)	63 (30)	67 (21)	71 (7)	76 (21)	60 (25)	75 (32)	63 (32)	**56 (36)**	P=.0694
LPS Response	81 (37)	73 (30)	69 (39)	65 (31)	58 (24)	83 (6)	90 (20)	56 (27)	74 (34)	71 (35)	63 (27)	**50 (40)** P=0.2260

Fig. 14.2 Individual and pairwise concordance to establish predictability using the immune panel. Values are presented as percentage concordance which is the sum of specificity (–/–) and sensitivity (+/+). Individual concordance values are shown in boldface on the diagonal of the matrix and combinations, using two tests on the off-diagonal element. Values in parenthesis are the number of chemicals tested for the assay. Since the individual tests were also used to establish the "immunotoxic classification," the frequency of concordance will obviously increase as the number of tests included for the analysis is increased (–). No overlapping studies were performed. P values are given for individual concordance only (Slightly modified after Luster et al. (1992))

and Black (2003), as well as Vohr and Rühl-Fehlert (2001) showed that only the combination of both advanced histopathology of lymphoid organs and determination of additional immune parameters are sufficient to pick up all immunotoxic effects of chemicals.

14.1.4.2 Hexachlorobenzene (HCB)

For the second round of the BGA ring study with nine participating labs, an "immunostimulating" chemical had to be chosen to investigate immunotoxic end points, immunosuppression, and immunostimulation. HCB had been selected for the second round on the basis of some reports, e.g., Vos et al. (1979), on the

immunomodulating properties of this compound. However, during the evaluation of the histopathology, it became clear that an irritant property of the compound was primarily responsible for the observed changes. In accordance with this finding, the additional immunological parameters verified a secondary immunotoxic effect of HCB due to nonspecific activation of the immune system via irritation, i.e., inflammation. While a faint immunostimulating effect had been picked up for HCB, the additional immunological parameters clearly demonstrated the indirect effect of this reaction. As a result, there was no final agreement about the favorable additional immunological parameters to be included in the normal routine toxicology package to flag immunotoxicological changes.

Mike Luster from the National Toxicology Program (NTP) of the USEPA investigated a series of 52 chemicals, 39 of which were known immunosuppressives.

While some information on potential immunotoxic effects may be obtained from hematology, lymphoid organ weights, and histopathology, the data published by Mike Luster demonstrate that these end points alone are not sufficient to predict immunotoxicity (Luster et al. 1992, 1993).

14.1.5 Guidelines

The US Environmental Protection Agency (USEPA) was the first public authority to really push the development of guidelines on immunotoxicity. After a longlasting discussion about the optimal test battery for immunotoxicological screening, the USEPA developed its guideline, OPPTS 780.8700 (1998), which is exclusively based on the findings of Luster et al. mentioned above.

The USEPA further agreed and defined on the following definition: *"Immunotoxicity refers to the ability of a test substance to suppress immune responses that could enhance the risk of infectious or neoplastic disease, or to induce inappropriate stimulation of the immune system, thus contributing to allergic or autoimmune disease. This guideline only addresses potential immune suppression."* Nevertheless, the USEPA excluded all aspects of immunostimulation as this was also not part of Luster's analyses, and there were no validated widely accepted test methods available to pick up an allergic or autoimmune potential of chemicals. This is still true today for the prediction of allergic reactions of types I, II, and III and autoimmunity, but not for type IV reactions (contact allergy), which have been investigated for decades using a guinea pig assay (OECD TG 406) or the mouse local lymph node assay (OECD TG 429, 442A and 442B).

In principle most of the other guidelines on immunotoxicity published thereafter pursued similar concepts. Only the harmonized tripartite guideline ICH S6 "Preclinical Safety Evaluation of Biotechnologically Derived Pharmaceuticals" (1997; revision 2009) included immunostimulation as one of the end points to be determined. This is understandable because immunogenicity of biologicals is an issue during preclinical and clinical development of pharmaceuticals.

14.1.6 Summary

Based on the EPA guideline recommendations, it is vital to differentiate between primary and secondary immunotoxicities, the latter being a nonspecific sequela of toxicity to other organs. In our studies, we found examples for both mechanisms, where primary immunotoxic substances tend to be markedly more immunosuppressive, although primary effects on the whole occurred relatively seldom during toxicological screening of SMOL, i.e., in less than 10 % of the studies. In both cases, there is a strong correlation between cell analysis and functional parameters on the one hand and pathology on the other, thus ensuring that overt immunotoxicity would not remain undetected in routine studies with high dose levels. However, the higher predictivity of functional parameters and the analysis of special subpopulations are necessary for setting a correct no observed adverse effect level (NOAEL) and for fine differentiation during the screening of comparable immunotoxic compounds. As verified by the collaboration studies, an advanced histopathology of lymphoid organs, combined with flow cytometry of immune competent cells and a functional assay, is able to discriminate between primary and secondary effects as well as immunosuppression and immunostimulation and thus to identify an immunotoxic hazard.

14.1.7 Experiences in Screening Chemicals (Immunosuppression)

The development or selection of suitable tests for immunotoxicological screening and incorporation into guidelines presents a considerable problem. In the beginning, most of the tests which had been proposed for immunotoxicological investigations and the knowledge and experience in immunology were based on mouse models or on a few collaborative studies in rats (cf. above). Adaptation of suitable tests to rats was not always easy, partly because of lack of suitable reagents. The next problem was to find which tests could suitably be used for reliable identification of interactions with the immune system. As mentioned above, publications about collaborative studies as well as the investigations of Luster et al. were of major importance in this regard. Another question which still has not been answered yet was that of the dosages, kinetics, and changes in immunological parameters which are still tolerable over time, i.e., after short-term (28-day) or long-term (90-day) treatment. With respect to ideal time points to screen for immunotoxic effects, the vast majority of experts agree that short-term treatments (14 or 28 days) are of optimal length.

In order to put the discussion on a somewhat more sound footing, it was important to test the various models/parameters for the detection of immunotoxicological potential in practice. For this reason, Bayer AG (Bayer HealthCare AG) has not only been a collaborator in the trial mentioned above, but has also introduced a set of functional immunological tests into its routine toxicological testing of agrochemicals in rats to determine the informative value of these parameters in daily practice. Hence, we started to incorporate additional immunotoxicological parameters according to those used for the collaborative studies into each routine subacute study of agrochemicals as early as 1992. During the first few years, investigations

were performed under GLP-like conditions before being subsequently changed to fully GLP compliant.

14.1.8 Results

During the screening of agrochemicals, we found that pesticides with primary immunotoxic effects were relatively rare (Vohr and Rühl-Fehlert 2001). To gain more experience with positive (immunotoxic) chemicals, we also screened closely related immunosuppressive drugs which showed an impact on the bone marrow. Histopathology revealed reduced hematopoiesis, with the affected dose level varying depending on the compound. The additional immune parameters showed higher sensitivity with respect to the affected dose level and confirmed primary immunotoxicity. Thus, the combination of histopathology of lymphoid organs and measurement of additional immune end points were of especial value in screening assays that can be adjusted according to the class of compound being tested.

It became evident that immunotoxic effects were detectable after only a few days of treatment by screening additional immune parameters. Histopathological changes of the lymphoid organs may only occur with a delay of some days to weeks. On the other hand, the immune system seemed to tolerate the test substance after longer exposure times (>90 days), and overall toxicity became most prominent with time. To check the correctness of these observations, we included an advanced screening battery at different time points of treatment during the development of an immunostimulating drug. As expected, the immunostimulating property of the compound was confirmed histopathologically by the increased number and size of germinal centers in the spleen in the high-dose group. There was no evidence of other organ toxicity that might have been causally related to this finding. As in the cyclosporin A collaborative study, the additional immune parameters were highly sensitive (mid-dose). However, these parameters detected the immunostimulating effect after only 2 weeks of exposure. At that time point, increased splenic germinal center formation was not yet detectable in histopathology. After 1 year of dosing with the test compound, the toxicological effects shifted from immunotoxicity to other organ toxicity.

14.2 What About Immunostimulation?

As mentioned at the end of the last chapter, determination of immunostimulating properties of chemicals is not as simple as it is for immunosuppression. The majority of clear cases of immunostimulation based on scientific evidence are due to pharmaceuticals developed for this purpose like vaccines or per se immunogenic pharmaceuticals like biologics. Especially the immunogenicity of biologics is one of the main concerns during preclinical and clinical development of such therapeutics. Immunogenic epitopes (e.g., OKT 3), directly T-cell-activating therapeutics (e.g., Tegenero antibody), and administration of high amounts of monoclonal antibodies can induce different kinds of inflammatory cytokines and chemokines. As a

consequence patients suffer from flu-like reactions and, in severe cases, of "cytokine storm," i.e., the so-called cytokine release syndrome (CRS).

For environmental chemicals or small molecules (SMOL), such tremendous or comparable effects have not been described so far. However, after binding to carrier molecules, SMOL can act as haptens, i.e., they can elicit an immune response to "altered self" molecules. This immunological principle of anti-hapten antibody response was first described by Mitchison in 1971. The consequence of these hapten-carrier formations can be allergic responses of type I to type IV or autoimmune reactions depending on several additional factors like genetic background, duration of contact, route of exposure, preexisting condition, etc. Unfortunately, no validated and widely acknowledged prediction tools for these undesired health effects exist with the exception for type IV reactions. For the determination of cell-mediated hypersensitivity (type IV reactions), which results clinically in allergic contact dermatitis, there are well-established animal models available: guinea pig assays (Buehler or maximization assay) or a mouse assay (local lymph node assay, LLNA). Other hypersensitivities mediated by antibodies (types I to III) are difficult to predict, and most of our knowledge in this area comes from retrospective human data, i.e., from humans whose history of induction of sensitization remains unknown. It is therefore possible to investigate the specificity of reacting antibodies by different methods like the human prick test, but such investigations will not clarify the underlying mechanisms or intrinsic potential of the chemical to induce these reactions. Therefore, the risk assessment for these end points is often controversial.

The same holds true and is even worse for the prediction or determination of chemical-induced autoimmune reactions. Development of autoimmune responses is a longlasting process that it is in many cases impossible to narrow down and link to contact with a single chemical or class of chemicals.

Another hurdle of predicting chemical-specific immunostimulation is the fact that several environmental chemicals or SMOL exhibit different kinds of undesired properties to varying degrees. For example, cytotoxicity, severe irritation/corrosion, skin sensitization, photo irritation, photoallergy, or combinations of these belong to such properties. Therefore, it is hard to discriminate the nonspecific from chemical-specific immune reactions because nonspecific inflammatory reactions can show comparable characteristics as severe specific immune reactions (Hölzle et al. 1991).

Ketoprofen illustrates how numerous distinct properties can be expressed by a single chemical compound. Ketoprofen is not only irritating and sensitizing to the skin but is also known to induce photo irritation as well as photoallergy in humans. Although ketoprofen and other nonsteroidal anti-inflammatory drugs (NSAIDs) are not "classical" environmental chemicals due to their prevalence in plants, they are also part of our environment. This illuminates another aspect of the problem in assessing immunotoxicity of environmental chemicals. While drugs and most agrochemicals are intensively screened for immunotoxicological side effects, this is not the case for industrial or other environmental chemicals. With respect to risk assessment for environmental chemicals, we rely therefore mainly on experiences and data from non-environmental chemicals. There is still an enormous gap in the knowledge and understanding of undesired immunological side effects induced by molecules of natural origin or chemicals found ubiquitously in the environment.

14.3 REACH and Its Influence on Immunotoxicological Screening of Chemicals in Europe

Assessment of immunotoxic effects such as immunosuppression and undesired immunostimulation rely at present on several animal-based assays. The use of animals, however, faces a number of issues, e.g., ethical concerns and relevance to human risk assessment. There is a growing belief that non-animal approaches can eliminate these issues without impairing human safety, provided that biological markers are available to identify the immunotoxic potentials of chemicals to which humans may be exposed. As mentioned before, the growing knowledge that the immune system can be the target of many chemicals, resulting in a range of several adverse effects, has raised serious concerns from the public and within the regulatory agencies. In combination with the European REACH legislation (Regulation (EC) No. 1907/2006), immunotoxicological side effects such as skin hypersensitivity must be studied for preregistration. This new EU policy on chemicals has a strong impact on manufacturers, importers, distributors, and downstream users due to the underpinning principle: "no data, no market." Driven by the 7th Amendment to the EU Cosmetics Directive as well as the REACH act, animal-based testing for chemicals is to be reduced to an unavoidable minimum (REACH) or even prohibited (Cosmetics Directive). Hence, there is an enormous pressure on the industry to develop and establish batteries of *in vitro* methods for predicting general toxicity and immunotoxicological side effects. Such *in vitro* methods have to focus on immunosuppressive as well as on immunostimulative properties of chemicals. At present we are far away from predicting the toxicity of chemicals toward the immune system by simple, fast, and reliable cell-based immunotoxicity assays. Some new methods which may lead the way are described in more detail in the last chapter.

This dilemma between advanced animal welfare and the need to (re)evaluate chemicals for their toxic (and immunotoxic) properties has given rise to a series of new *in vitro* and *in silico* methods, many of which lack validation and general acceptance. These methods are nevertheless widely used due to the lack of alternatives. While it is relatively easy to establish and validate *in vitro* assays for simple end points like cytotoxicity (irritation/corrosion), it is much more difficult to develop assays for more complex end points, where metabolism, cell interaction, and induction of factors are all playing a role.

An example of the difference between simple and more complex end points is the *in vitro* testing of skin effects caused by chemicals. For years, skin irritation and corrosion are tested by *in vitro* methods on 3D human skin equivalents. These methods have been established, pre-validated, validated, and finally accepted by regulators over a period of more than a decade. All these efforts resulted in two OECD Guidelines, which came into force in 2004 (OECD TG 431; skin corrosion; update 2013) and 2008 (OECD TG 439; skin irritation; update 2013), respectively.

In contrast *in vitro* methods for predicting skin sensitization are still far away from proposal for an OECD Guideline. One assay, KeratinoSens™ Assay developed by Givaudan, which was published as a draft version in 2014, is the furthest along. However, skin sensitization is a multilayered process which includes penetration of the chemical through the *stratum corneum;* metabolism in the skin; cell

interaction of keratinocytes, dendritic cells, and T cells; chemokine and cytokine induction; cell migration; etc. An interesting review about the complexity of skin sensitization testing has been published previously by Van der Veen et al. 2014.

The implications of the complex interactions mean that for *in vitro* skin sensitization analysis, a battery of tests for several end points has to be established, mimicking all the different steps necessary for interactions to develop contact dermatitis. Indeed, there are *in vitro* methods that are underdevelopment measuring skin penetration, hapten-carrier binding, dendritic cell activation, signal transduction, T-cell activation, or cytokine/chemokine release caused by chemicals. The validation status of these different methods is heterogeneous. While some are already almost accepted by regulators (e.g., KeratinoSens or Direct Peptide Reactivity Assay (DPRA)), others are still far away from any international validation or global acceptance (e.g., hCLAT or signal transduction, IL-18 production by 3D skin models, etc.).

To replace the *in vivo* evaluation of skin sensitization, a battery of at least three assays is necessary. It is clear that each of these models has a certain level of sensitivity and specificity and a certain percentage of false-negative and false-positive results. This is the primary disadvantage of such a composite of assays as the overall accuracy and/or variance will increase with the number of test models included. In addition, each *in vivo* or *in vitro* assay has a specific applicability domain (AD), i.e., the classes of chemicals which can be tested by the model are restricted to the intrinsic properties of the compounds like solubility in aqueous vehicles, cytotoxicity, etc. These ADs can be very specific for single test models and so will not always overlap 100 % in a test battery and thus will decrease the classes of chemicals which can be tested by such a battery. Therefore, the aim should be to develop test models with a broader applicability domain, e.g., gene expression analyses or use of organoid models like reconstructed human epidermis. However, these assays must be validated before they can be used in an *in vitro* test battery, which must itself also be validated.

A validated and widely accepted *in vitro* battery for skin sensitization will not be available in anytime soon. On the other hand, the cosmetic industry in Europe has to test new components exclusively in *in vitro* systems, and the same holds true for many chemicals to be re-evaluated under the REACH legislation. A relatively simple end point such as skin sensitization has taken decades to be developed; therefore, an *in vitro* alternative to testing the more complex toxicity effects of small molecules or environmental chemicals will likely require an enormous effort. Other ways to overcome this dilemma could be via biomonitoring and epidemiological investigations which is the topic of the next chapter.

14.4 Risk Assessment for the Immunotoxic Potential of (Environmental) Chemicals

14.4.1 Accidents and Biomonitoring

The history of immunotoxicity is closely related to accidents and pollution with small molecules showing immunomodulating properties like dioxin, heavy metals,

polychlorinated biphenyls (PCBs), asbestos, latex, essential oils, pesticides, isocyanates, diesel engine emissions, etc. All of these molecules are ubiquitously available in the environment or are released into the environment by very different mechanisms. Some of these chemicals were released by accidental spillage where large amounts were released. It was during these accidents that the immunomodulating properties of these compounds became a focal point.

There are several examples of such accidents, be it the methylmercury contamination of fish and shellfish in Japanese Minamata Bay in the 1950s, the contamination of special feed supplement for lactating cows with polybrominated biphenyls in Michigan 1973 and 1974, or the accidental release of 2,3,7,8-TCDD near Seveso in 1976. All such cases of accidental or ignorantly released chemicals led to decades of investigations on the toxic mechanisms observed and raised the general public and regulatory awareness of immunotoxic screening.

Another approach for obtaining information about the possible immunotoxic side effects of (environmental) chemicals is through biomonitoring. The first investigation of workplace-related poisoning was that of chimney sweepers. As early as 1775, the dependency between intensive contact with soot and the development of testicular cancer was reported by Percivall Pott. This report is considered the starting point for the history of biomonitoring of workplace-specific body burden with chemicals. Today biomonitoring in the workplace spans the measurement of concentrations of relevant chemicals, the routes of possible application, as well as the detection of chemicals and their metabolites in a variety of biological fluids and tissues. The result is a sound risk assessment and determination of so-called maximum allowable concentrations (MACs). Many immunotoxic chemicals have been assessed by this manner, although in many cases, the discrimination between, e.g., cancerogenous and immunotoxic properties, was not made clear. Ultimately, whether a worker was protected from cancer or immunotoxicity was irrelevant.

While this is one way of providing a reasonable risk assessment for chemical exposures at work, biomonitoring is not well suited for a similar risk assessment in the overall population. This is the goal of epidemiological investigations as described in the next chapter.

14.4.2 Epidemiological Evaluation

Epidemiology is a scientific discipline dealing with prevalence, mechanisms, and consequences of health conditions and events of the entire population. One part of this discipline is epidemiological evaluation of possible toxic effects of environmental chemicals. Universities, private, and governmental institutions all over the world maintain several positions for this special part of epidemiology. One of the main and most efficient tools used in this discipline is surveys and networking. It is beyond the scope of this chapter to go into too much detail about different surveys. The most important surveys will be mentioned, and a closer look at one example will be undertaken, i.e., skin sensitization.

14.4.3 Surveys

As early as 1976, a comprehensive survey was initiated in the United States by the CDC (Centers for Disease Control and Prevention). The National Health and Nutrition Examination Survey (NHANES) program tested samples from the general population for lead and certain pesticides over a period of several years (Annest et al. 1983). The NHANES program had considerably been expanded over the years, not only to measure pollution in the general population with chemicals by biomonitoring but also to investigate the impact of this pollution on the general health status (Stokstad 2004). The aim in 2004 was to monitor nearly 1000 chemicals in persons from all over the United States. It is clear that this number of chemicals and all their varying interactions, metabolisms, distributions, and kinetics in the body would not simplify the interpretation and evaluation of risk assessment. In 2006 Dennis Paustenbach and David Galbraith critically discussed this aspect of biomonitoring in a much-noticed review.

Similar surveys were initiated in Germany, of which the German Environmental Survey (GerES) is one of the most important. It was started in 1985 with determination of chemicals in the urine of the general population grouped by age, gender, residential area, etc. This program developed stepwise to a more complex determination of different classes of environmental chemicals, in specific groups (ages and gender) or living areas (Angerer et al. 2007; Schulz et al. 2007a, b). For example, GerES IV survey was the first one in Germany to investigate body burden of pollutants in children and the exposure to pollutants in their homes at about 150 different locations in Germany. Due to the experience gained with a large amount of data, human biomonitoring has been subdivided into biological monitoring of exposure and biological effect monitoring. Accordingly the biomarker, organs, or body fluids tested became increasingly comprehensive. To further harmonize these, in order to maximize the value of the ever-increasing data set, the "Human Biomonitoring Commission of the German Federal Environment Agency" was established in 1992 (Schulz et al. 2007a, b).

There are a growing number of surveys across the globe, and due to more harmonized strategies, the focus of different initiatives is more and more refined. Also the existing methods were validated and/or harmonized with time to accurately measure biologically relevant concentrations of the chemicals investigated. However, due to the large amount of data produced and the enormous variety of interactions of the different parameters analyzed, controversial discussions about the reasonable selection of chemicals evaluated took place (Paustenbach and Galbraith 2006a, b) and objections about the reasonableness of such investigations were put forth (Boccia et al. 2007; Baker and Gibson 2014; Chang et al. 2014).

In spite of or maybe because of large amounts of data were generated by different groups, clear-cut and reliable effects of environmental chemicals on the immune system are rare and in most cases controversially discussed. This is often due to external or basic parameters of a survey which were not addressed or over-interpreted depending on the intention of an investigation. In many publications, the mere presence of a toxic compound around the detection limit in the environment or tissue is

discussed as a major health problem. Realistic exposure scenarios or risk assessments based on relevant NOELs are simply not done. Even for experts in the field of immunology/immunotoxicology, it is sometimes hard to eliminate the noise. Therefore, data presented are often looked at with skepticism.

So it is really not clear at all whether huge amounts of date as would, for example, be generated by the American "National Children's Study" which should follow 100,000 children across the United States from birth until age 21, to address the effects of social, economic, and environmental factors on a child's health, would at the end help to understand all the factors influencing the development and the health status of children. Because of the expected extreme costs and the abovementioned shortcomings of such a study, the planning has lasted over 14 years (for details, http://www.nap.edu/catalog.php?record_id=18826).

14.4.4 German Survey on Skin Sensitization

The Information Network of Departments of Dermatology for recording and scientific analysis of contact allergies (IVDK) founded in 1988 cooperates with 55 dermatological hospitals in Germany, Austria, and Switzerland. As a multicentric project, the IVDK collects data about allergens and publishes lists at regular intervals on the prevalence of allergies in different regions (Schnuch et al. 2004; Uter et al. 2007, 2010). At present (2014) data of about 12,000 prick-tested patients are collected and analyzed each year.

The data is extrapolated into incidences of allergic contact dermatitis (ACD) in the general population between 3 (medium case scenario) and 7 (worst case scenario) cases per 1,000 persons a year. Thus far, no comprehensive studies exist to determine the actual incidences of such ACDs in the general populations. The data is based on extrapolation prick test data or interviews of patients by dermatologists (Hermann-Kunz 1999).

Beyond calculation of incidences and prevalence of allergic diseases, the IVDK also publishes ranking lists of the most frequent allergens. For years, nickel has always topped the charts, although it is a weak sensitizer (Geier et al. 2011). Thus, in contrast to the low potency of nickel to induce ACDs, the prevalence of nickel allergies is high due to the extreme frequency of contact in the general population. This discrepancy is one of the reasons for frequent discussions about potency of chemicals relative to their frequency of exposure. In addition, the different assay protocols used in dermatology are not yet fully standardized, and it is not always clear if the data from different sources can be pooled for evaluation of potency (Thyssen et al. 2012a, b, c).

This example illustrates the difficulty in evaluating or predicting the risk of an environmental chemical to induce contact allergy across the population, particularly as the majority of data analyzed is exclusively based on patients with a longer history of disease. If evaluation of a relatively simple side effect such as induction of contact dermatitis is already complex, the difficulty of measuring a more complex side effect of environment chemicals can be envisioned.

In conclusion, prediction of risk assessment of immunotoxic effects of environmental chemicals based solely on epidemiological data should be regarded with skepticism.

14.5 Future Developments

14.5.1 *In Vitro* Screenings

Although there are still several problems to be solved for "classical" immunotoxicity screening, such as autoimmunity or systemic hypersensitivity, the field of immunotoxicity is already expanding into new areas. Such new directions are *in vitro/in silico* immunotoxicology and developmental immunotoxicity. While the roots of the first are more common in Europe, those of the second field of interest are more prevalent in America.

As already mentioned before, there is increasing pressure in the European Union to develop *in vitro* screening methods and thus reduce the number of animals used in toxicological studies, including immunotoxicity screenings. However, a number of questions need to be addressed prior to embarking on validation studies: Which cell source should be used for these *in vitro* studies – human or mouse/rat cell lines or primary cells from lymphoid organs? How can we discriminate between overall cytotoxicity and immunotoxicity to cells? Which end points are to be measured – induction/inhibition of surface marker expression, and/or proliferation, and/or cytokine expression? Which activation stimuli should be used – T-cell mitogens, antigens, B cell, or macrophage stimuli? It is clear that a simple *in vitro* determination of the cytotoxicity of chemicals against cells of lymphoid organs like lymph nodes, bone marrow, thymus, blood, or spleen will not be sufficient to replace *in vivo* screening in immunotoxicology. The fact that the basic principle of immune cell activation is cell–cell interaction will make it absolutely necessary to develop an *in vitro* functional assay which includes coculture of various relevant cell types.

14.5.2 Mishell–Dutton Cultures (*In Vitro* PFCA)

An increasing aim in safety assessment of chemicals and drugs is to reduce, refine, and replace animal testing. Therefore, alternative methods for this purpose are highly desirable. Furthermore, importance of immunotoxicological studies for determining potential adverse effects of pesticides and pharmaceuticals is growing. However, *in vitro* alternatives for immunosuppression are available only at a research level, and up to now, no *in vitro* test for the prediction of immunotoxicity is fully validated or accepted by regulatory authorities.

The production of antigen-specific antibodies represents a major defense mechanism of humoral immune responses, and TDAR, like the PFCA, has been identified in a regulatory context as a main functional test for immunotoxicological investigations. The PFCA *in vitro* equivalent, also known as MD culture or MD test, represents a comprehensive evaluation of immune function based on the interaction of

antigen-presenting cells, T cells, and B cells involved in the antigen-specific antibody response. Using MD cultures of mouse spleen cells treated with 11 different test items, we were able to both demonstrate immunosuppressive effects and clearly discriminate between specific immunosuppression and nonspecific cytotoxicity (Koeper and Vohr 2009). To compare the *in vitro* antibody responses of rats and mice, we performed a study with three standards using spleen cells as well as peripheral blood mononuclear cells (PBMC) from both species. Preliminary data showed an excellent concordance between species (including dog, monkey, and humans) as well as different cell sources (Fischer et al. 2011) (Fig. 14.3).

14.5.3 Developmental Immunotoxicity

Another challenge in the near future will be to screen for the impact of a chemical on the developing immune system, i.e., developmental immunotoxicity (DIT). There are different views and opinions with respect to a reasonable test strategy, but at present we are far away from a validated and fully accepted protocol. There are considerable differences in the time required for full development of the immune system across species and in the placental structure and transport mechanisms. Thus, caution is required both in planning the treatment period and dose in the species selected for developmental immunotoxicity studies and in translating the data from animals to humans (Holladay and Smialowicz 2000). Nevertheless, testing developmental immunotoxicity in rats instead of mice would have the advantage that such studies could easily be incorporated into existing test protocols (Luster et al. 2003; Barnett 2005). On the other hand, there is an ongoing discussion about the feasibility and value of incorporation of a DIT module in complex and longlasting rat studies like the extended one-generation reproductive toxicity study (EOGRTS) (Boverhof et al. 2014).

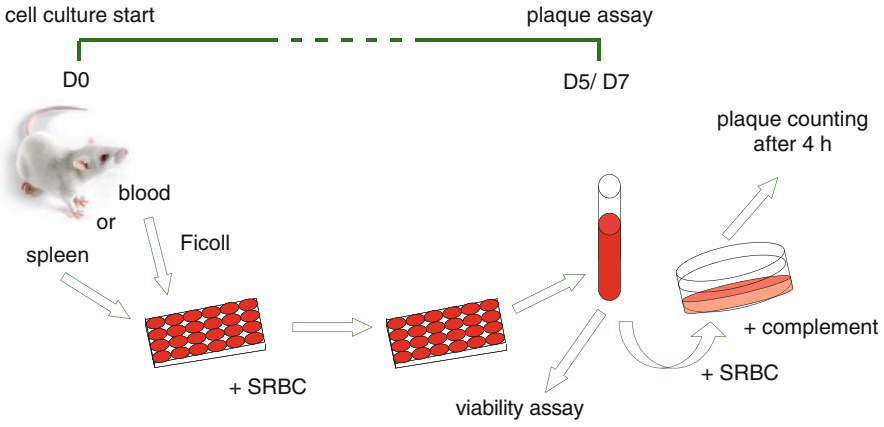

Fig. 14.3 Principle of the in vitro Plaque-Forming Cell Assay, the so-called Mishell–Dutton culture (MD test) as described previously by Koeper and Vohr 2009 and Fischer et al. 2011. The MD test can be performed with splenic or blood cells of rodents as well as other species like dog, monkey, or humans

14.6 Overall Summary

Current immunotoxicology testing approaches for pharmaceuticals, agrochemicals, or animal health products differ significantly from the testing of new and environmental chemicals. Whether comparable immunotoxicological screenings should also routinely be done for industrial, and to some extent also for environmental chemicals, has been discussed controversially for years. Such testing would subject these chemicals to the USEPA OPPTS 870.7800 guideline or the ICH S8 guidance by including a functional test (TDAR) as well as a flow cytometric analysis of blood or splenic cell populations. Especially controversial is whether environmental chemicals should also be subjected to such tests; a trigger-based approach would be necessary.

A trigger-based approach for environmental chemicals could be followed in accordance with the ICH S8 guidance, i.e., evaluation would start with a weight-of-evidence (WoE) assessment. This assessment should include all data available for the relevant chemical, i.e., data from standard toxicity studies, structural similarities to known toxicants, toxicokinetics data, intrinsic properties of a chemical class, and possible routes of exposure. This initial WoE assessment would then trigger an immunotoxicological screening as described in the abovementioned guidelines. Although such an approach sounds reasonable, it raises a number of key questions: Who will collect the data and prepare a WoE assessment? Are there sufficient and reliable data available for environmental chemicals as basis for such an approach? Who will sponsor a full-blown immunotoxicological screening if required? What will be the consequences of positive outcomes of such a study?

Routine toxicological investigations of agrochemicals have produced considerable amounts of data with respect to special immune parameters. These data show that a combination of advanced histopathology and some additional immunological investigations such as PFCA (TDAR) and/or flow cytometric analyses of subpopulations can be used not only to flag immunomodulating chemicals but also to discriminate whether such effects are due to the primary or secondary impact on the immune system. Although direct immunosuppressive as well as immunostimulating effects can be determined by such *in vivo*/*ex vivo* test batteries, there are as yet no robust and validated tests for the determination of other end points such as autoimmunity or type I allergy. The development of widely accepted models for such end points will necessitate much effort in the near future.

Nonetheless, there are already demands for additional new fields of immunotoxicology, i.e., *in vitro* immunotoxicity screenings or developmental immunotoxicity. This will likewise represent a significant challenge for the future.

We are still far from applying these investigations to environmental chemicals as described in this chapter. Our knowledge about the impact of environmental chemicals on the immune system is still fragmented and requires further study. For progress in the field, it will be critical to obtain consensus between the scientific and administrative communities about the path forward. A consolidated expert panel discussion and consultation to move the discussion forward is already being conducted by various associations like ILSI-HESI, ECVAM, and others who have recognized the importance of this alignment. Importantly, these efforts should go beyond publication by providing results in well-defined research projects.

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