Nonclinical assessment of immunotoxicity in the EU, United States, and Japan

Wissenschaftliche Prüfungsarbeit

zur Erlangung des Titels

„Master of Drug Regulatory Affairs“

der Mathematisch-Naturwissenschaftlichen Fakultät

der Rheinischen Friedrich-Wilhelms-Universität Bonn

vorgelegt von

Dr. Rainer Paffenholz
aus
Bad Neuenahr

Bonn 2005

Betreuer und 1. Referent: Dr. Gerd Bode
Zweiter Referent: Prof. Dr. Klaus Olejniczak

List of Abbreviations

ACA	active cutaneous anaphylaxis (assay)
ADME	absorption, distribution, metabolism, excretion
ASA	active systemic anaphylaxis (assay)
CDER	Center for Drug Evaluation and Research
CFU	colony forming unit
CHMP	Committee for Medicinal Products for Human Use
CTL	cytotoxic T lymphocyte
DHR	delayed hypersensitivity response (assay)
EFPIA	European Federation of Pharmaceutical Industries and Associations
ELISA	enzyme-linked immunosorbent assay
EMEA	European Medicines Agency
EWG	Expert Working Group
FACS	fluorescence activated cell sorter
FDA	US Food and Drug Administration
GPMT	guinea pig maximization test
HIV	human immunodeficiency virus
ICH	International Conference on Harmonisation
IND	investigational new drug
JPMA	Japan Pharmaceutical Manufacturers Association
KLH	keyhole limpet hemocyanine
LLNA	local lymph node assay
MAA	marketing authorisation application
MEST	mouse ear swelling test
MHLW	Ministry of Health, Labour and Welfare
NfG	note for guidance
NIHS	National Institute of Health Sciences
NK	natural killer (cells)
NOEL	no observed effect level
OECD	Organisation for Economic Co-operation and Development
PCA	passive cutaneous anaphylaxis (assay)
PhRMA	Pharmaceutical Research and Manufacturers of America
PLNA	popliteal lymph node assay
PMDA	Pharmaceuticals and Medical Devices Agency
PND	post natal day
SRBC	sheep red blood cells
STS	standard toxicity studies
TDAR	T-cell dependent antibody response

Table of Contents

1. Introduction

The interference of pharmaceuticals with the immune system can be the intended active principle of a therapeutic, as it is the case with immunosuppressants that are used in organ transplantation treatments. On the other hand, unwanted changes in functionality of the immune system can lead to an adverse drug reaction. In this sense, administration of immunotoxic therapeutics may result in various forms of disturbances of the immune system. Probably the most dangerous forms of immunotoxicity are hypersensitivity and autoimmunity induced by the administration of pharmaceuticals. Indeed, several pharmaceuticals have been withdrawn from the markets due to induction of such immunotoxic effects in patients.
At a first glance, suppression of immune functions appear to be less critical than hypersensitivity and autoimmunity, with the exception of cytotoxic anti cancer drugs and long term immunosuppressive therapy of patients that received organ transplantation (10). Nevertheless, unintended immunosuppression can be a major health risk for patients that are already immunocompromised or are at risk of immunodeficiency for other reasons.
Awareness that pharmaceuticals can cause a variety of immunologically mediated adverse effects in patients has been constantly growing over the past two decades. Some of the early systematic observations of immunotoxic effects of pharmaceuticals refer to the anaphylaxis associated with penicillins, the increased incidence of infections in tumor patients treated with cytotoxic compounds and a higher risk of organ transplant recipients treated with drugs such as azathioprine to develop a tumor (24). It is important to know that all these early observations of adverse effects were made clinically. The obvious lack of adequate preclinical animal studies triggered efforts by several research groups to develop assays for the detection of immunosuppressive effects (7,8).
As a result, strategies were developed to assess immunotoxicity in rodents by incorporating immune parameters in the routine protocols of repeated dose studies. These tiered test strategies were originally aimed at the detection of immunotoxic potentials of industrial and environmental chemicals, but were later adopted by the pharmaceutical industry. The basic issue in the discussion of the tier approach in pharmaceutical safety assessment was about the clinical relevance of findings in some of the tier assays in the absence of more general signs indicative of immunotoxicity in standard toxicology studies (19).
It is the aim of this study to review the requirements for non-clinical immunotoxicity assessment within the regulatory framework of the major pharmaceutical markets (EU, US, Japan) and to analyse the harmonisation efforts of the ICH process that recently reached step 2 with the release of the draft for the “ICH S8 guideline on Immunotoxicity Studies for human Pharmaceuticals”. As the regulations for the assessment of biologicals are defined e.g. by the ICH S6 guideline on “Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals”, they will not be covered herein.

2. Immunotoxicity

For a long time, the term “immunotoxicity” has been used synonymous with “immunosuppression”. With the emerging understanding of the immune system and the mechanisms behind adverse drug effects elicited by immunotoxic pharmaceuticals, most authors nowadays use the term in a broader sense.
Several authors suggested to further divide immunotoxic effects into the two subcategories of direct immunotoxicity and indirect immunotoxicity (45). Direct immunotoxicity can be further divided into immunosuppression and immunostimulation, while indirect immunotoxicity describes the reaction of the immune system in an antigen-specific way and is usually expressed as hypersensitivity and autoimmunity.

2.1. Immunosuppression

Immunosuppressive effects of pharmaceuticals are well known based on the experience following the introduction of immunosuppressive drugs in the early 60s. There are two major types of adverse effects of immunosuppression, namely virus-induced malignancies and infectious complications.
Early observations of patients receiving immunosuppressive treatments (e.g. cyclosporin, azathioprine) following organ transplantations provided evidence of an increased risk of lymphoproliferative disorders and skin cancers. This effect is independent of the immunosuppressive drug used and malignancies are more frequent when immunosuppression is more profound. For example, cardiac transplant patients undergoing a more aggressive immunosuppressive therapy show an increased incidence of lymphomas compared to renal transplant patients (50). Very often malignancies caused by immunosuppressive drugs are associated with latent viral infections (e.g. Ebstein-Barr virus in B lymphomas or HSV 8 in Kaposi´s sarcoma) (49).
Most immunocompromised patients suffer from frequent and relapsing infections. In principle, all types of pathogens like bacteria, viruses, fungi and parasites can be involved in these infections. Nevertheless, some pathogens are more often found in the patients, e.g. mycobacteria, Ebstein-Barr virus and Listeria monocytogenes.

2.2. Immunostimulation

Immunostimulation results in prolonged or excess inflammatory reactions against a pathogen. In addition, dysregulation of the immune system can facilitate the development of allergy or autoimmune reactions that may have other causes than immunostimulation itself. The development of autoimmune diseases in patients receiving treatment with interferons or recombinant IL-2 has been reported in several studies (26). The predominantly immune disease in cancer patients receiving recombinant IL-2 is the development of autoimmune thyroiditis, while hepatitis-C patients treated with interferon-α do not have a preference for a specific type of autoimmune disease. At least in some cases a genetic predisposition of unknown nature appears to be of some importance (12). Hypersensitivity to unrelated

allergens is a rather rare event. One example is the use of IL-2 in cancer treatment, with several studies showing an increased risk for patients to develop hypersensitivity reactions to radiocontrast media (38) or to chemotherapeutic agents (20).
Flu-like reactions like hyperthermia, arthralgias and malaise have also been reported. In serious cases the reaction can be very severe with a body temperature >40°C and cardiovascular and neurological symptoms. (12).
An adverse effect of immunostimulative drugs that is often overlooked is the reduction of activity of certain drug-metabolising enzymes. Experimental data and human studies show a negative impact of several drugs including interferon-α or influenza vaccines on drug metabolism, resulting in clinically significant drug interactions (37,18).

2.3. *Hypersensitivity*

Drug hypersensitivity describes a severe, idiosyncratic multi-system reaction that usually manifests as fever, rash, and the involvement of inner organs. Although the incidence is not known, it is a frequent and dangerous adverse event in drug treatment, with mortality estimated to be around 8 % (52). Allopurinol, anticonvulsants (particularly carbamazepine, phenobarbitone and phenytoin) and sulphonamides are among the most frequent causative agents (43). Since hypersensitivity reactions can be either immune-mediated or non-immune-mediated, the general term hypersensitivity has been recommended instead of allergy.
Although the limitations of the classification system introduced by Gell and Coombs have become evident with increasing knowledge of immune mechanisms (13) most authors still use the scheme to divide drug allergies into four pathophysiological types (see Table 1):
Type I (immediate – type) hypersensitivity reactions are reactions in which antigens combine with specific IgE antibodies bound to membrane receptors on mast cells or basophiles. This binding leads to the rapid release of vasoactive and inflammatory mediators, which trigger vasodilation, increased permeability of capillaries, smooth muscle spasm, and infiltration of inflammatory cells into the tissue.
Type II hypersensitivity reactions are cytotoxic reactions triggered by the binding of an antibody to an antigen of a cell or tissue element or to an antigen or hapten that is coupled to a cell or tissue. The antibody reaction may activate cell-mediated cytotoxicity through killer T-cells or macrophages. This reaction usually includes the activation of the complement system.
Type III reactions usually result from the deposition of soluble circulating antibody-antigen "immune complexes" in vessels or tissues. The immune complexes lead to an activation of complement, which culminates in acute inflammation.

Type I	Anaphylactic hypersensitivity depends upon the reaction of an antigen with specific IgE antibody bound to mast cells
Type II	Antibody dependent cytotoxic hypersensitivity
Type III	Complex mediated hypersensitivity
Type IV	Delayed type hypersensitity (i.e. contact hypersensitivity)

Table 1: Gell and Coombs classification of hypersensitivity reactions (36)

Type IV, or delayed-type, hypersensitivity reactions are caused by sensitised T lymphocytes activated by a specific antigen. The activated T-cells may cause immunologic injury directly or through the release of lymphokines that activate other cells of the immune system.

2.4. Autoimmunity

Autoimmune diseases are pathologic conditions in which immune responses against autoantigens produce structural and/or functional damage. While in the general population autoimmune diseases are quite common, reports of drug – induced autoimmunity are rare (12). Autoimmune diseases are generally distributed within a spectrum of "systemic" and "organ- or tissue specific". In systemic autoimmune diseases, the pathological response is directed against antigens present throughout the body, and consequently the lesions are also widely distributed. Systemic Lupus Erythematosus (SLE) and Rheumatoid arthritis are examples of systemic autoimmune diseases. Among the drug – induced autoimmune diseases, the systemic forms are far more common than organ- or tissue-specific manifestations. In the later, expression of the autoantigen is restricted to certain cells or tissues. Multiple Sclerosis and Inflammatory Bowel Disease are examples of organ-specific autoimmune diseases. Reports of drug induced organ-specific autoimmune reactions are rare and refer to only very few drugs (e.g. penicillamine). Nevertheless, the events like the eosinophilia/fasciitis syndrome associated with L-tryptophan underline that drug induced autoimmunity can affect the general population and can result in significant morbidity and mortality (42).

2.5. Developmental Immunotoxicity

The structural and functional characteristics of many organ systems differ significantly between children and adults as a result of the growth and development that takes place during maturation. One example is the adult level of IgG and IgA antibody response, which is not achieved in humans until about 5 and ten years of age, respectively (32).
The ontogenesis of the immune system in vertebrates is a well characterised process. Major features are multiple switching of haematopoietic compartments, migration of cells into primary and secondary lymphoid organs and the differentiation within these organs under microenvironmental influences. The time course of the development of the immune system is considerably different between humans and rodents. While in humans most features of the immune system are well developed by the end of the first trimester (13 weeks), in rodents development continues throughout gestation and well into early postnatal life. In spite of these differences in time course, the basic principles of the development are conserved between rodents and humans, and the rat is considered the preferred species for the assessment of developmental immunotoxicity (29).
Mainly based on the stages of immune system development, "windows of vulnerability" have been proposed to exist during specific periods of immune ontogeny and are shown in Figure 1 (14).

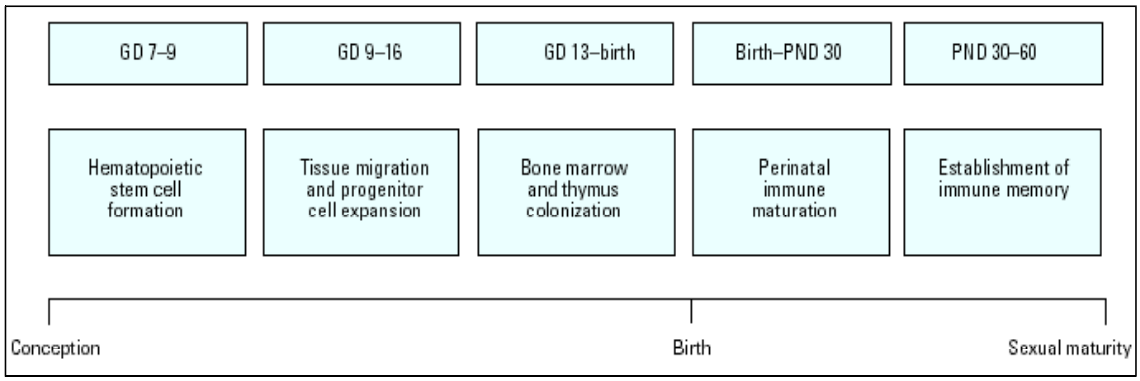

Figure 1: Relative time line of critical windows of exposure for immune system development in mice and rats (29, adapted from [14]). Abbreviations: GD, gestational day; PND, postnatal day.

The immune system of the foetus and neonates is characterized by T_{H2} type of interleukins. In the early postnatal period the immune system matures to provide a balanced T_{H1}/T_{H2} state. It has been shown that infections and vaccinations, that may influence the T_{H1}/T_{H2} balance, have an impact on the maturation of the immune system (48).
Taken into account that many cellular and molecular processes are specific for the developing immune system and are no longer present in the adult vertebrate, it is far from surprising that certain drugs that have no immunotoxic effect in adults can be a serious hazard for the developing immune system. One such example is Acyclovir, which has no immunotoxic effects in adults. In contrast, perinatal exposure to a single dose at day 10 of gestation resulted in abnormal thymus development and reduced thymus weight in the offspring. Host resistance and antibody production was reduced in the *Trichinella spiralis* host resistance model, a highly T-cell dependent defence model (41).

3. Relevant assays

The immune system is one of the most complex systems of the mammalian organism. An effective immune response to a pathogen is based on the close interaction of many different cell types and functional responses. Drugs can interfere at many different points of this sophisticated system and hamper the ability of the organism to establish an effective immune response.
Due to the complexity of the immune system, derangements cannot be detected by a single test. Consequently, tiered approaches combining multiple tests have been developed that address different aspects of the immune system.

3.1. Immune suppression/Immune enhancement

In the last two decades, many methods have become available in animal models to assess aspects of immune disturbance after exposure to chemicals, both *in vitro* and *in vivo*

(7,33). Methods for assessing direct immunotoxicity in experimental animals may be classified into two principal categories: Non-functional tests and functional assays.

3.1.1. Non-functional tests for immunopathology

Most of the non-functional test can be included in the standard repeated dose toxicity tests. The major parameters are:

Determination of body and lymphoid organ (spleen, thymus, kidney, liver) weights.
Changes in body weight may give information on the general health status of the animal. Changes in the weight of lymphoid organs can be indicative for immunotoxic effects, but should always be combined with a thorough histological examination of these organs.

Histopathology
The thymus usually is the first organ that shows morphological changes after exposure to immunotoxic agents. Changes can be a decrease in size or an expansion of a distinct compartment of the organ which does not result in altered total organ weight (27). Since histological changes in lymphoid organs are often the first evidence for altered immune function that are observed in general toxicity testing, the relevant OECD guideline 407 on 28-day repeated dose toxicity test was revised in 1995 to include weight and histological examination of spleen, thymus, Peyer´s patches and draining and distant lymph nodes. Histopathology according to OECD 407 is able to detect the majority of substances with known immunotoxic potential (47). Nevertheless, performance of the methods depends strongly on the use of standardised procedures and terminology (27).

Complete blood and differential counts
Haematological changes, usually seen in peripheral blood samples, can be evidence of myelosuppression. Parameters to be evaluated are total leukocyte counts and absolute differential leukocyte counts.
Serum globulins are a rather insensitive marker of immunotoxicity. However, changes in globulins that occur without a plausible explanation can indicate potential immunotoxicity and provide additional information for the better understanding of the target cell population or the mechanism of action.

Lymphocyte subpopulation assessment
Usually done from peripheral blood, spleen and bone marrow by FACS or immunohisto-chemistry.

3.1.2. Functional tests to evaluate functional competence of immune cells

The functional tests intend to study the consequences of immunotoxic effects on the performance of different components of the immune system (e.g. non-specific responses by macrophages and natural killer cells, humoral and cellular responses). The most relevant

endpoint for immune dysfunction is altered host resistance as it reflects the functionality of the immune system in toto (46).

3.1.2.1. Ex vivo/in vitro cell immune function assays

Ex vivo/in vitro immune function assays are defined as assays in which constituents of the immune system are evaluated for their ability to perform a specific function. In *ex vivo* assays, experimental animals are exposed to the test compound and a challenge with a specific antigen is given. The immune function is assayed by measuring the specific immune response using serum or isolated cells (23).

Primary antibody response to T-cell dependent antigen

Also known as Plaque-forming cell assay, this test measures the ability of isolated spleen cells (B-cells = PFCs) to generate IgM and IgG to a T cell dependent antigen, for example sheep red blood cells (SRBC), keyhole limpet haemocyanin, or tetanus toxoid. The T-cell dependent antibody response is often recommended as a follow-up assay for immune function studies. The T-cell dependent assay demonstrates most components of the classical immune response, including B cell release of antigen specific antibody, macrophage antigen presentation, and T helper cell lymphokine production for B-cell proliferation. Using sheep red blood cells as the T-cell dependent antigen, the assay demonstrates an organized immune response dependent on the functional capacity and cooperation of numerous cell types. Due to the involvement of both cellular components and the antibody response, the assay is sensitive and frequently used as an initial immunotoxicity assay. It should be taken into consideration that the SRBC assay does not describe the mechanism of immunosuppression. Use of T-cell independent antigens such as lipopolysaccharide, which requires only B cells for an antibody response, or dinitrophenyl-ficoll, which requires B cells and macrophages, may be of use in characterizing the cell types subject to immunosuppression. Since SRBC vary considerably in their immunogenic potency and are not available as a standardised reagent, the use of better defined antigens is encouraged by many researchers.
The assay may be modified using ELISA and ELISPOT as the readout to quantify antibody response and antibody-producing cells, respectively (25,44).

Natural killer cell activity assessment

Natural killer (NK) cells constitute the immune system's first line of defense against tumors, virus infected cells, and cells carrying a different type of major histocompatibility (MHC) Class I at the surface. This protective action executed by NK cells is spontaneous and selective in the sense that no pre-activation is needed and that normal cells are not targeted. Assessment of NK activity gained substantial importance in the non-clinical evaluation of immunotoxicity with the EMEA NfG (note for guidance) on repeated dose toxicity (CPMP/SWP/1042/99) that accepts a test of NK cell function in combination with immunophenotyping of lymphocyte subsets as full alternative to the evaluation of primary antibody response to a T-cell dependent antigen.

The cytolytic activity of NK cells (effector cells) is most commonly monitored by quantifying the relative number of tumor cells (target cells) that have been killed following co-incubation with NK cells. Effector cells are isolated from the spleen or peripheral blood.
Studies of NK cell function can be performed either *ex vivo* on subjects treated with the test substance, or *in vitro* after addition of the test substance directly to the cell cultures. Most commonly, NK cell activity is determined by the ^{51}Chromium release assay or by a flow cytometry assay (3).
For the^{51}Chromium release assay effector cells are collected from the spleen or peripheral blood. The spleen is prepared as a single cell suspension and depleted of red blood cells. Effector cells and target cells are mixed after labeling with ^{51}Cr. The cell cultures are incubated at 37°C and 5% CO2 for 4 h. The supernatants are harvested and the radioactivity is measured. The amount of radioactivity is proportional to the number of killed target cells (15).
For the flow cytometry assay, effector cells are isolated as described for the ^{51}Cr-release assay and stained e.g. with carboxy-fluoresceine succcinimidyl ester (CFSE). Cell cultures are incubated for 18 h at 37°C, 5% CO2. Propidium iodide is added and the samples are measured by flow cytometry within 60 min. Two thousand target cells are collected and the results are given as percentage of dead targets gated in a dot-plot. (3). The flow cytometry assay facilitates the incorporation of NK cell assessment into repeated dose studies as fewer cells are needed and it circumvents the need for the use of radioactive markers.
Other functional *ex vivo*/*in vitro* tests include

- Test for macrophage activity
- Antigen specific antibody responses
- Responsiveness to B-cell mitogens (LPS)
- Responsiveness to T-cell mitogens (PHA, ConA)
- Mixed lymphocyte reaction (MRL)
- Cytotoxic T lymphocyte cytolysis.

A detailed description of these assays is beyond the scope of this studies and can be found elsewhere (e.g. [2])
The ability of *ex vivo*/*in vitro* immune functional assays to detect immunotoxic properties of test compounds has been evaluated. Several groups determined the concordance between the results of a battery of *ex vivo*/*in vitro* functional immune assays and a set of host resistance models which are generally regarded as the most relevant test for immune system functionality (30,31). A number of individual tests showed a high concordance (>70%) for the outcome of the host resistance assays, with the test of the primary antibody response to T-cell dependent antigen (e.g. SRBC) being one of the most sensitive assays. Used in combination with either the NK cell activity test or surface marker analysis a pairwise concordance for predictability of more than 90% is achieved.

3.1.2.2. *In vivo* disease models

Since the major function of the immune system is the protection of the organism against infection or neoplastic diseases, most researchers consider assays that directly test the resistance of the host against an infectious or neoplastic agent to be the most relevant to

examine the possible impact of a compound on the immune system, especially as they indicate clinically relevant changes.
In general, experimental animals are challenged with either an infectious agent or a transplantable tumor at a level sufficient to produce disease in a low number of animals. Over the past decades, the endpoints of these host resistance assays have evolved from relatively non-specific (e.g. animals morbidity) to more quantitative (e.g. viral titres or bacterial cell counts) parameter, thus significantly increasing the sensitivity of the assays. An overview of commonly employed host resistance models is given in Table 2. Usually, it is not necessary to perform all of these host resistance assays. The appropriate models should be selected based on experimental consideration and the results obtained in previous experiments.

Challenge	**Endpoints**
Listeria monocytogenes	Liver CFU, spleen CFU, morbidity
Streptococcus pneumoniae	Morbidity
Plasmodium yoelli	Parasitemia
Influenza virus Morbidity	Morbidity, viral titer/tissue burden
Cytomegalovirus	Morbidity, viral titer/tissue burden
Trichinella spiralis	Encysted larvae, adult parasites
PYB6 sarcoma	Tumor incidence (subcutaneous)
B16F10 melanoma	Tumor burden (lung nodules)

Table 2: Commonly employed disease resistance models (16)

3.2. Hypersensitivity

From case to case, hypersensitivity reactions can have different aetiologies, which require various methods for the assessment of the hypersensitivity potential of pharmaceuticals.

3.2.1. Assays for Type I hypersensitivity

The Passive Cutaneous Anaphylaxis (PCA) or the Active Systemic Anaphylaxis (ASA) assay in guinea pigs are the main assays to study type I hypersensitivity (anaphylactic) reactions.
In principle, in the PCA, the IgE-containing serum of guinea pigs sensitised to the test compound is injected intradermally in a naive animal. Subsequently, the animals are intravenously challenged with the compound and a dye. After a rest period the skin of the animals is evaluated. The local vascular permeability resulting in extravasation of the dye is a measure of the amount of antigen-specific IgE present in the original injection.
The ASA assay is used to determine whether a drug can induce anaphylaxis in an animal following immunization with the drug. Guinea pigs are sensitised to the test compound in combination with adjuvant (via various routes of exposure); subsequently, the animals are challenged with the test compound either i.v. (ASA) or dermally (ACA) and the shock reaction or skin reaction is monitored.
A recent analysis of the correlation between immune–based assays (namely ASA/PCA) for hypersensitivity in the guinea pig and reported post-marketing systemic hypersensitivity

reactions suggests that these assays have only limited ability to predict human systemic hypersensitivity potential of pharmaceuticals (51).

3.2.2. Assays for Type II and Type III hypersensitivity

Basically, no standard preclinical methods are available to evaluate the potential of a compound to evoke Type II or Type III hypersensitivity reactions. In some cases, suitable biomarkers may be available for assessing the sensitising potential of the drug (Putman et al 2003)

3.2.3. Assays for Type IV hypersensitivity

The guinea pig is the model animal for most methods to study contact sensitisation (Type IV) potential of compounds (51).
The guinea pig maximization test (GPMT) with the use of an adjuvant and the occluded patch test of Buelher without adjuvant are most commonly used assays to identify skin sensitising capacity. In both tests previously sensitised animals are challenged. Sensitisation is usually developed after 2-3 weeks following first exposure. 24 and 48 h after topical or intradermally application of the challenges, the inflammatory reactions are measured as a function of erythema and/or oedema (a qualitative classification). The GPMT is regarded as a more sensitive assay that may also, for certain substances, overestimate the sensitisation hazard of the compound tested. The Buehler test is less sensitive.
Two additional tests for Type IV hypersensitivity in the mouse have become available in recent years:
The ear swelling test (MEST) is based on the evaluation of challenge induced reactions in previously sensitised animals. The sensitising potential is correlated to the degree of oedema (ear swelling/thickness at 24 and 48 h after exposure) and the percentage of animals displaying a reaction.
The local lymph node assay (LLNA) measures the primary T-cell response through incorporation of radiolabelled thymidine in the draining lymph node following topical application on three consecutive days to the mouse ear. In contrast to the guinea pigs assays and the MEST, the LLNA has a quantitative endpoint and measures the initiation phase, rather than the elicitation phase, of the contact sensitivity. Additionally, the LLNA can be performed in a substantially short time frame, i.e. 6 days vs. 5–6 weeks for the guinea pig assay and 12 days for the MEST.

3.3. Autoimmunity

The determination of autoantibody titres is a frequently used method to evaluate autoimmunity. While for organ specific autoimmunity, the titre of a highly specific and homogeneous autoantibody population is a critical parameter, the correlation of autoantibody titres to more ubiquitous antigens like histones or DNA are only poorly correlated to the autoimmune disease (11)

The most important assay to detect the autoimmune inducing potential of pharmaceuticals is the Popliteal Lymph Node Assay (PLNA) and its variations.
The direct (primary) PLNA measures the enlargement of the popliteal lymph node 6–8 days after subcutaneous injection of the test substance in the hindpaw of a naïve mouse or rat (Figure 2).

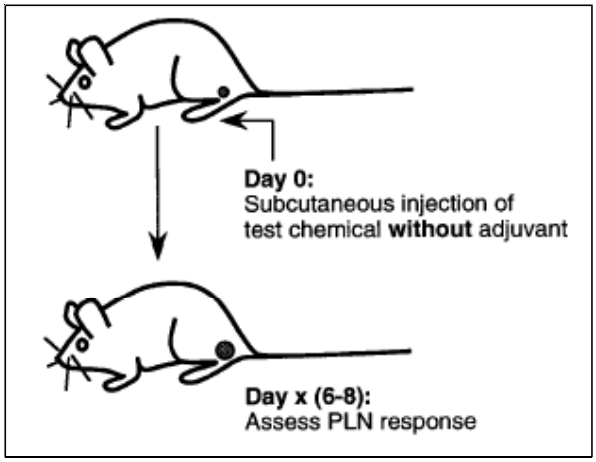

Figure 2: Direct (primary) PLNA (from:[1])

The end point of the assay is either the ratio of cell numbers or weight of the draining lymph node of control vs. treated animals. The primary PLNA is unable to assess the involvement of T cells, i.e. discriminate between mere inflammatory agents and sensitisers.
The secondary or modified PLNA allows to assess the involvement of (memory) T-cells in the immune reaction. It detects challenge reactions in the PLNA to non-sensitising doses of a compound in presensitised animals or in un-sensitised animals that received an adoptive transfer of pre-sensitised syngenic T cells (Figure 3). The modified PLNA uses defined reporter antigens TNP-OVA (T-cell-dependent antigen) and TNP-Ficoll (T-cell-independent) to distinguish sensitising from non-sensitising (IgG1-response or not to TNP-Ficoll) and mere inflammatory from complete innocent (IgG1 response or not to TNP-OVA) chemicals.
However, it should be noted that although the PLNA can discriminate sensitisers from non-sensitisers, it cannot distinguish autoimmunity inducing compounds from sensitisers. This is caused by the overlap between the mechanism involved in hypersensitivity and autoimmunity responses (36).

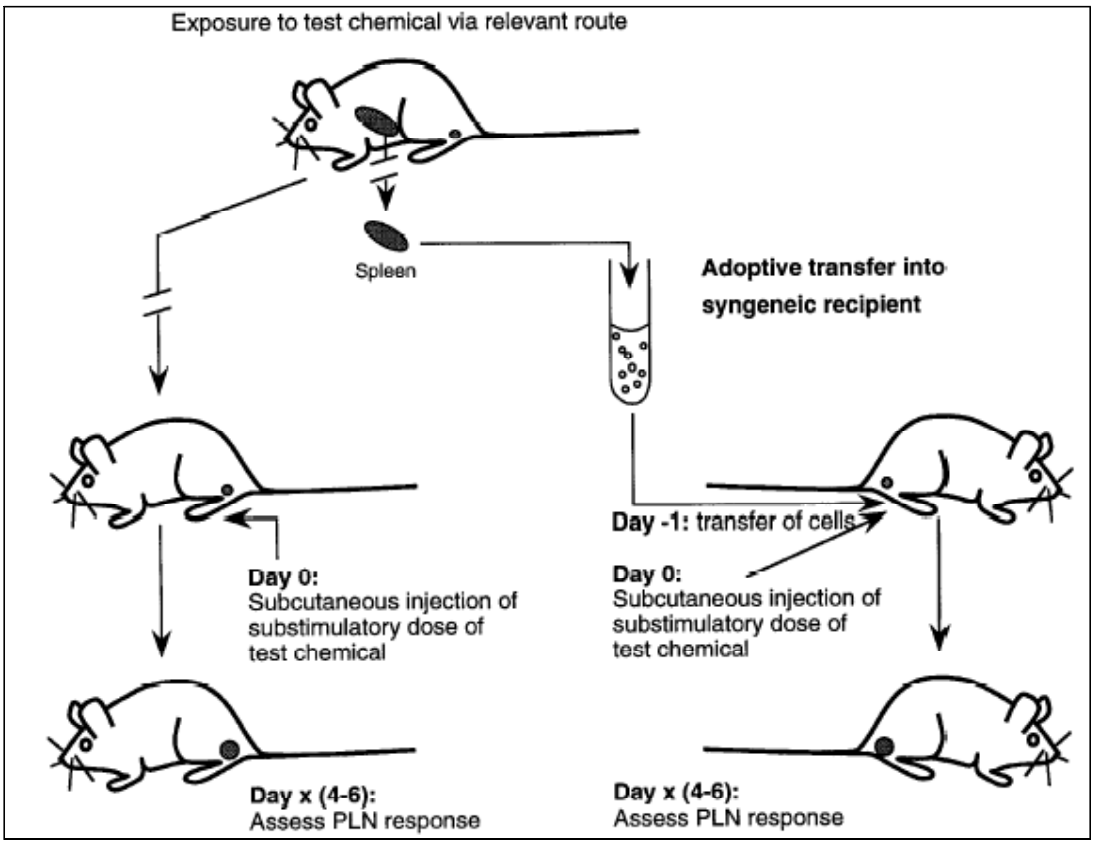

Figure 3: Secondary PLN assay (from: [1])

3.4. Developmental Immunotoxicity

The rat is regarded as the species of choice for the study of developmental immunotoxicity in terms of hazard identification. When it comes to address the mechanism of action, the mouse and genetical mouse models should also be considered (21,5,39).
Basically, the assays used for evaluation of immunotoxicity in adult animals can also be applied for studies on adverse effects on the developing immune system. As standard immunotoxicity studies are usually performed in adult, mostly inbred rodents, evaluation of developmental immunotoxicity requires a different experimental setup. Ideally, such a protocol should

- cover all critical exposure windows in the development of the immune system
- should be incorporated into existing developmental toxicology protocols
- should allow to study long term effects as well as recovery
- should carefully consider the optimal time for measurement since certain immunotoxic insults during prenatal development yield an effect only in the fully mature immune system.

Several experiments have been proposed (22). One procedure published by Chapin et al.(4) proposed the integration into standard obligatory reproductive toxicology studies (Figure 4).

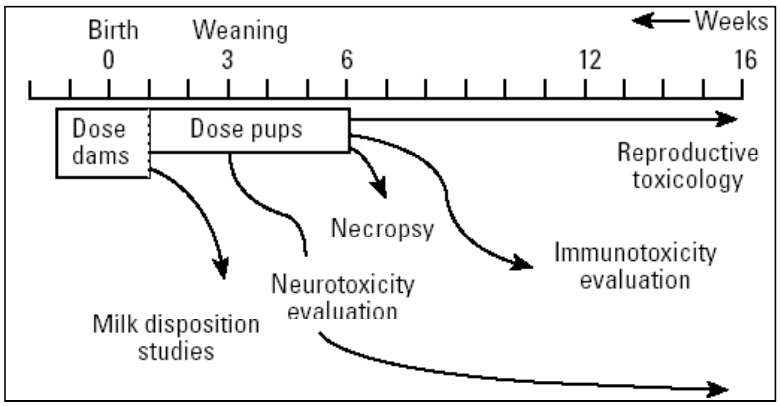

Figure 4 : Schematic diagram of proposed experimental design in rats that include Immunotoxicology testing (29, adapted from [4]).

Pregnant females are dosed from gestational day 12 to post natal day (PND) 7. Starting at PND 8, the pubs receive the same dose as their mothers before until PND 42, which covers the entire period of immunological ontogenesis. After PND 42, some animals are selected for immunotoxicity assays like T-cell dependent antigen response, delayed hypersensitivity response and NK cell activity. Histology and weight of immune relevant organs are also analysed.
Limitation in this type of study is that relatively few animals are examined, limited mechanistic information is provided, and neiter long term effects nor recovery is routinely assessed (29).

4. Regulatory requirements in the ICH regions

4.1. EU

Of the three major global pharmaceutical markets, the European Union was first to implement guidelines for the assessment of direct immunotoxicity in drug development.
EMEA recommendations on nonclinical testing for immunotoxicity are now part of the revised NfG (Note for Guidance) on repeated-dose toxicity (CPMP/SWP/1042/99). In principle, the guidance applies to all conventional medicinal products except biotechnology-derived drugs and vaccines, for which separate ICH/CHMP guidelines exist (see NfG on Preclinical safety evaluation of biotechnology-derived pharmaceuticals, CPMP/ICH/302/95, and NfG on Preclinical pharmacological and toxicological testing of vaccines, CPMP/465/95). The requirements, which came into operation in October 2000, are applicable only at the stage of marketing authorisation application (MAA) and do not comprise a region-specific variation to the ICH M3 guideline on nonclinical studies to support clinical trials.
In the revised version of the “NfG on non-clinical local tolerance testing of medicinal products” (CPMP/SWP/2145/00) guidance is provided for the assessment of the sensitising potential of drugs that are intended to be applied to the skin.

EU regulations are based on a two step approach to evaluate the immunotoxic potential of drugs. The initial screening phase, using a rat or mouse model preferably in a 28-day study (14- or 90-day studies also being acceptable), comprises predominantly of non-functional endpoints including

- haematology (differential cell counting)
- lymphoid organ weights (thymus, spleen, draining and distant lymph nodes, Peyer´s patches)
- microscopy of lymphoid tissues
  and
- bone marrow cellularity.

As a functional assay, distribution of lymphocyte subset and NK-cell activity or alternatively an assay to evaluate the primary antibody response to a T-cell dependent antigen (preferably the sheep red blood cell test, SRBC) may be included in the initial screening phase for all drugs.
Any changes observed in the parameters monitored in the initial screening may trigger additional testing summarized as extended studies.
The goal of the extended studies is to provide information on the type of immunotoxicity observed, on the target cell populations(s) involved, and on the dose-response-relationship in order to facilitate risk assessment. The need and design of the extended studies should be determined on a scientifically motivated case-by-case basis. The extended studies consist of functional assays and include:

- Delayed-type hypersensitivity
- Mitogen- or antigen-stimulated lymphocyte proliferative responses
- Macrophage function
- Primary antibody response to T-cell-dependent antigen (if not already undertaken)
  and
- *In vivo* models of host resistance.

The integration of obligatory functional assays in the initial screening phase has been discussed controversially (e.g. 40, 35). The major considerations leading to the adoption of the EU guidelines have been summarized by Snodin (40) and include

- Histopathological examination is qualitative and subjective and provides a static examination of a dynamic functional system.
- Generally, functional testing is preferred for studying the performance of functional organ systems because some defects are seen only after the organ is challenged.
- Functional testing is generally more sensitive and easier to interpret than histopathological data. The correlation between morphology and function is sometimes difficult to ascertain.
- Significant changes in function may not always be preceded or accompanied by detectable morphological changes in the immune system.
- The availability of biomarkers for evaluating the immune system in clinical trials is limited and, therefore, increased emphasis should be placed on nonclinical data.

- Determination of the potential drug effect in response to a challenge with a T-cell dependent antigen is likely the best stand alone assay.

Regulatory guidance for drugs applied to the skin is laid down in the revised NfG on local tolerance (CPMP/SWP/2145/00). Two tests, the guinea pig maximization test (GMPT, as described in OECD test guideline 406), and the local lymph node assay (LLNA) are accepted as stand-alone methods for evaluation of sensitising potential.
The EMEA does not request routine testing of photosensitising of drugs but rather advocates a case by case approach (CPMP/SWP/398/01). Conditions have been defined, though, in which a compound may be suspected of photoallergic potential and additional information may be required. These conditions are bioavailability in sun exposed areas and absorbtion of UV/VIS at 270–700 nm. Additionally, information obtained in photostability tests and structure–activity relationships should be taken into consideration (36).
So far, the EMEA has not released guidance in autoimmunity testing.

4.2. US

In October 2002, the Center for Drug Evaluation and Research (CDER) as the responsible body within the FDA released the final version of the guideline on “Immunotoxicology Evaluation of Investigational New Drugs”. This document is the result of more than a decade of discussion between the regulatory authorities and the pharmaceutical industry. It provides recommendations to sponsors of investigational new drugs (INDs) on the parameters that should be routinely used in standard toxicology studies to determine changes in immune function and on the need for additional immunotoxicity studies. The guideline does not apply to biological products that are regulated in ICH guideline S6 (*Preclinical safety evaluation of biotechnology-derived pharmaceuticals*).

4.2.1. Immunosuppression

As a basic principle, the guideline does not recommend a defined set of tests to be conducted for every IND, but rather emphasizes the inclusion of immune relevant parameters into standard toxicology studies and a weight-of evidence approach to decide on the necessity of further studies. According to the guideline, special attention should be given to indicators of immunosuppression than can be observed in standard nonclinical toxicology studies including

- evidence of myelosuppression, such as pancytopenia, leukopenia, lymphopenia, or other blood dyscrasias
- alterations in immune system organ weights and histology (e.g., hypocellularity of immune system tissues such as the thymus, spleen, lymph nodes, or bone marrow)
- decreased serum globulin levels
- increased incidence of infections.

A clear difference should be made between unintended (adverse) immunosuppressive and intended or expected (pharmacodynamic) effects (e.g. of certain anticancer drugs or of drugs for the prevention of transplant rejection).

In general, all investigational new drugs should be evaluated for their potential to produce immunosuppression. In the first line, this can generally be achieved within the standard repeat-dose toxicology studies using standard clinical and anatomic pathology methods, including determination of serum biochemical markers such as globulin levels, haematology (including differential), gross pathology findings, immune system-related organ weights, and histological examination of immune system-related tissues. The histological examination should be focused on immune-relevant organs and tissues (spleen, thymus, lymph nodes, and bone marrow) and the lymphoid tissue that drains or contacts the site of drug administration. A more quantitative histopathological assessment of lymphoid organs as well as the use of immunohistochemical techniques might be useful in some cases. An increase in the serum albumin/globulin ratio may indicate impaired immune function, while decreased serum globulin levels is a relatively insensitive indicator; when observed, the affected protein component should be determined.
Lymphoproliferative type tumors and treatment-related infections can be indicative of impaired immune function and should be monitored carefully in all animal studies performed. It should be mentioned that an increased tumor incidence is most likely related to more frequent tumorigenic mechanisms (e.g. genotoxicity or hormonal effects). However, if the cause of tumor findings is not apparent, the potential role of immunosuppression should be considered. In general, it is the intention of the guideline to create an alertness of sponsors to consider an immunosuppressive cause for abnormal findings and initiate further evaluations where appropriate.

Additional studies on the functionality of the immune system should be considered if warranted by observations in non-clinical or clinical studies or by other consideration like the intended patient population (e.g. patients with impaired immune functions), or known drug class effects. Concerns can also be based on observed pharmacokinetic effects (e.g. accumulation of drug and/or metabolites in immune-relevant tissues).
The most useful test for immune function is the determination of the effect of the drug on the response to a T-cell dependent immunogen, preferably using the sheep red blood cell test and its modifications. Depending on the initial findings, several other tests can be more suitable or should be performed additionally. Particularly the NK-cell assay may also be useful. Host resistance assays can also be used in the assessment of potential immuno-suppression.
Although most methods used to assess drug-induced immunosuppression are conducted using standardised protocols, the dose, duration, and route of administration used in functional assays should be consistent, where possible, with the nonclinical toxicology study in which an adverse immune effect was observed.
Immune cell phenotyping by flow cytometry or immunohistochemical analysis is not considered to be an adequate stand-alone test of impaired immune function, but is regarded to be useful as an adjunct to other immune function assays or as a method for the identification of biomarker(s) that could be used in clinical trials.
In general, the guideline advocates that signs of immunosuppression should be appropriately evaluated using scientifically sound methodology, and the decision for a specific evaluation strategy has to be based on scientific principles.

All indications of immunosuppression in nonclinical toxicology studies should be evaluated with respect to

- statistical significance,
- biological significance,
- likely or demonstrated mechanisms,
- relevance to other adverse drug effects,
- intended use of the drug,
- and the potential role of stress.

Small but statistically significant changes in some parameters might not be a cause for concern as they do not necessarily indicate a biological significant effect. A weight-of-evidence approach is recommended in which all adverse effects observed in nonclinical toxicology studies would be considered in determining if follow-up immune function studies should be conducted. Potential immunosuppressive and/or pharmacodynamic effects should be evaluated using animal/human comparisons in terms of dose (per unit of body surface area) and, data permitting, systemic exposure. The reversibility of any immunosuppressive effects should also be investigated. Immunological changes related to stress and to the pharmacological activity of the test material occur commonly in animal studies and only when these are ruled out the possibility of a direct effect on the immune system should be considered.

4.2.2. Immunogenicity

Immunogenicity (i.e. the ability of a drug to induce an immune response) is a very common phenomenon for polypeptides or protein drugs > 10kDa. Smaller peptides or proteins of a molecular weight between 5 and 10 kDa may also be immunogenic, but the response is usually rather weak. Low molecular weight compounds are only immunogenic if they are covalently bound to proteins in hapten-protein complexes.
Although immunogenicity is an important property of protein allergens, it does not inevitably lead to drug allergy. It is very difficult to predict the allergenic potential of a protein drug in nonclinical toxicology, and validation of known methods is regarded as not being sufficient to justify a recommendation by the guideline.

4.2.3. Hypersensitivity

The guidance documents refers to the classification of Coombs and Gell that identifies four different types of hypersensitivity. The recommendations for the assessment of hypersensitivity should only be used for small molecular weight drugs, which by the time the guidance was published were the major type of products to be reviewed by the CDER.

Type I immediate-type hypersensitivity reactions are mediated by IgE. Several methods are described that intend to tackle the risk of IgE mediated hypersensitivity, including the passive cutaneous anaphylaxis (PCA) assay, the active cutaneous anaphylaxis (ACA) assay, and the active systemic anaphylaxis (ASA) assay. In general, the usefulness of these tests to identify a

Type I sensitising potential of small molecules is regarded as limited, especially when biotransformation would be important for production of potential haptens. In conclusion, none of the tests is recommended for routine safety evaluation in terms of Type I hypersensitivity. Drugs that will be administered via inhalation should be evaluated for their potential to induce Type I hypersensitivity reactions using appropriate methodology. The guideline recognizes that almost all methods need further evaluation in terms of usefulness for drug safety testing. Nevertheless, a modification of the mouse local lymph node assay (LLNA) might be useful for identification of respiratory sensitisers.

Type II and Type III hypersensitivity reactions are mediated by IgG and/or IgM antibody responses and tend to occur simultaneously. Pathological findings in Type II and Type III hypersensitivity reactions include anemia, leukopenia, pneumonitis and are often indistinguishable from autoimmune reactions. Furthermore, most of the pathological signs of type II and Type III reactions are also indicative of direct, nonimmune-mediated toxicity. The guideline states that no standard nonclinical methods are available to predict these effects, and consequently routine testing is not recommended. However, if pathological findings can be interpreted as the result of an immune reaction, follow up studies are recommended on a case by case decision.

Type IV immunopathies are T-cell mediated and most commonly occur as delayed-type hypersensitivity skin reactions (contact dermatitis). For all drugs intended for topical application the sensitising potential should routinely be evaluated as part of the non-clinical safety assessment. The Buehler assay and the guinea pig maximization test (GPMT) are accepted by CDER as reliable and well established methods that have shown a high correlation with known human skin sensitisers. These methods, along with the split adjuvant technique and the Draize test, are currently accepted by CDER for the determination of the sensitising potential of drugs for topical use.
The mouse ear-swelling test (MEST) is mentioned, but no statement is made if it is regarded as appropriate for the evaluation of Type IV reactions.
In contrast, the murine LLNA is accepted as a stand alone assay to detect the induction phase of delayed-type hypersensitivity reaction. LLNA results correlate quite well with results from classical guinea pig test and provides a quantitative endpoint. If the LLNA is used to support the safety of clinical trials, the sensitising potential not only of the drug substance, but also of the clinical excipients and the clinical formulation should be evaluated.
As no reliable animal model could be identified for the assessment of the phototoxic potential of drugs, no routine nonclinical testing of photoallergenic potential is requested by CDER.
Taking into account the possibility of pseudoallergic (anaphylactoid) reactions, the observation of anaphylaxis in animal studies should trigger follow-up studies to determine the nature of the reaction. This analysis might provide valuable information on biochemical markers to be used in clinical trials.

4.2.4. Autoimmunity

Autoimmunity is a pathological process in which the immune system reacts against self-antigens. In rather rare cases, autoimmunity can be triggered by drugs. As no standard methods for predicting the autoimmune potential of a compound are available, CDER does not recommend testing for autoimmune potential routinely. Nevertheless, the popliteal lymph node assay (PLNA), the LLNA, and modifications of these assays could be useful where needed.
Adverse immunostimulation refers to any antigen-nonspecific and inappropriate activation of the immune system. It is recognized as a general problem in drug development, but again no test method exists that CDER would recommend for routine use in clinical development. Nevertheless, special attention should be given to a possible involvement of adverse immunostimulation in the interpretation of findings from other nonclinical studies.

4.2.5. Developmental Immunotoxicity

Additional care has to be taken if a drug is intended to be used in pregnant women or if immunosuppressive effects have been shown for a drug in adults.
In these cases, incorporation of immunotoxicology in the ICH stage C to F reproductive toxicology study should be considered. In this type of study, the pregnant females are exposed from gestation day 6 (time of implantation) through PND 21 (time of weaning). Offspring is reared to sexual maturity. For the detection of immunotoxic effects during development, lymphoid system organ weight, histology, and haematology in the F1 generation should be determined. No recommendation is made for the use of functional assays.

4.2.6. Assessment strategy

The procedure of immunotoxicology nonclinical safety assessment according to current CDER regulations is shown in Figure 5.
For topical administration and for drugs administered via inhalation tests in addition to the standard toxicology studies are necessary to assess dermal or respiratory sensitising potential, respectively. GPMT, Buehler assay, murine LLNA or the guinea pig inhalation induction and challenge assay are regarded as appropriate tests, but alternative tests may also be used when scientifically justified.
For all drugs, the findings from other animal studies should be examined very carefully for signs of potential immunotoxic effects. If evidence for drug–induced immunosuppression is found, appropriate follow-up studies may be conducted. In particular, assays evaluating the effect on T-cell dependent antibody response (e.g. the SRBC) and immune cell phenotyping (e.g. by FACS analysis) should be considered.
If drugs are intended to be used in HIV-infected patients, studies of the potential to induce immunosuppression are obligatory (e.g. effect of the drug on response to a T-cell dependent immunogen.

Apart from special application routes (topical administration or inhalation) and special population of patients (HIV patients, pregnant women) the FDA/CDER does not request routine immunotoxicology/immunosuppression assessment, but rather relies on data from the general toxicological studies.
Instead, a weight-of-evidence approach is advocated where special focus is placed on immune relevant findings in standard toxicology and other nonclinical animal studies.

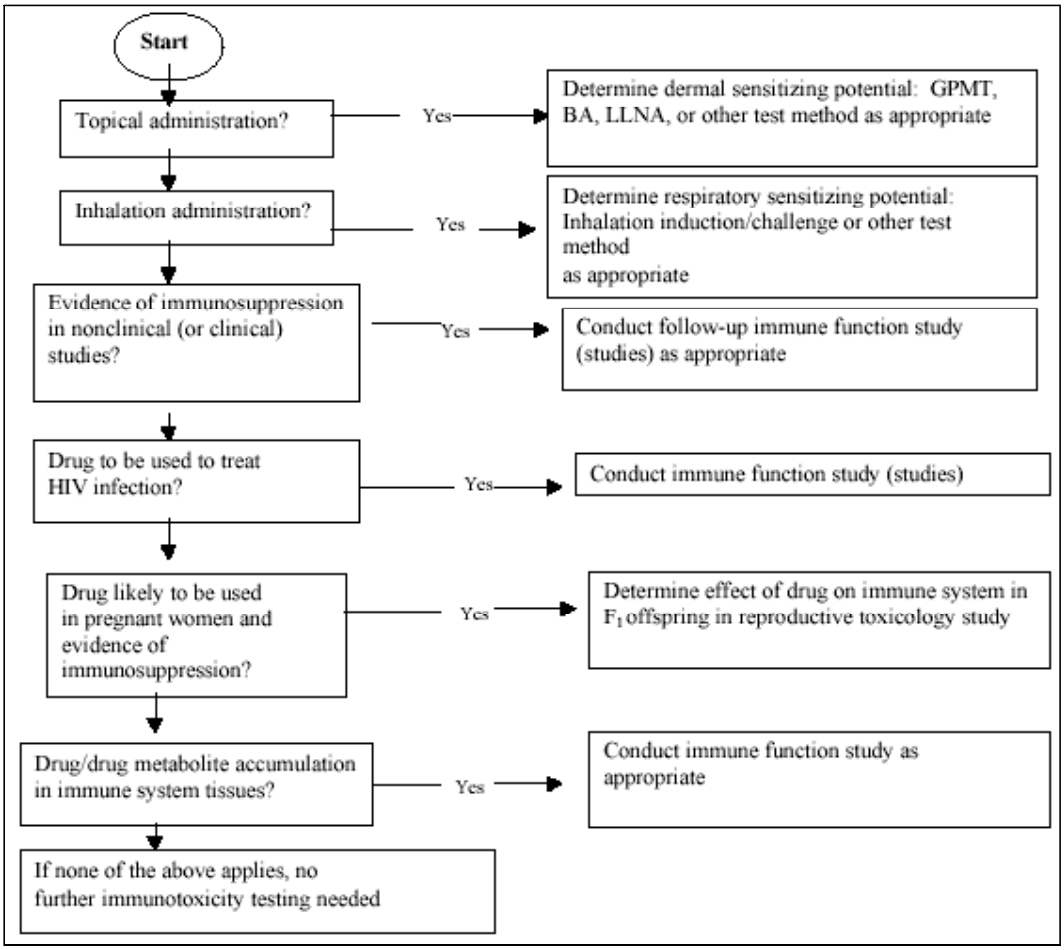

Figure 5: Guidance for Industry: Immunotoxicology evaluation of investigational new drugs, October 2002 (modified from: FDA/CDER)

4.3. Japan

Like both EMEA and FDA, the Japanese Ministry of Health, Labour and Welfare (MHLW) basically adopts the general approach of the OECD for assessment of immunotoxicity. The routine screening for all drugs (exceptions for biologicals, orphan drugs and drugs against allergy) as part of the repeated dose toxicity studies focuses on determination of organ weights (spleen, thymus, adrenals), observation of general appearance and body weight, and histopathology of spleen, thymus, bone marrow smear, unspecified lymph node and Peyer´s patches if draining the site of application. Spleen immunohistochemistry or lymphocyte subset phenotyping is also suggested.

In the case of findings in the routine immunotoxicity studies or in other studies, namely the repeated dose toxicology study, the evaluation of the primary antibody response and - optional - the evaluation of NK-cell activity is required before commencing phase I clinical trials. This second level of evaluation is – somewhat misleading – termed “Tier I”. When effects are observed in Tier I studies, a more in depth analysis of the immunotoxic potential of a drug may follow (Tier II).
To address the potential of a drug to induce hypersensitivity reactions, the MHLW requires testing of all dermatological preparations in at least one skin sensitisation study. There are several tests regarded as acceptable stand alone assays, namely Draize test, adjuvant and patch test, Buehler test, Freund´s complete adjuvant test, maximisation test, open cutaneous test, optimisation test and the split adjuvant test. Beside the broad panel of tests, in most cases the maximation test, the patch test or the Buehler test are used (36).
The testing of photosensitisation is required for dermatological preparations where concerns exist that the product has a photosensitising potential (e.g. a chemical structure similar to known sensitisers). Acceptable methods include adjuvant strip method, Harber method, Horio method, Kochever method, Maurer method, Morikawa method and the Vinson method.

4.4. Summary of regulatory requirements for testing of immunotoxicity

The status of regulatory guidance for the assessment of immunotoxicity differs between the ICH regions. While the EMEA and FDA have published guidelines on direct immunotoxicity, the MHLW are still in the process of finalizing draft guidelines.
Most primary predictive immunotoxicity testing follows the recommendations of the OECD 407 guideline requiring histopathology of lymphoid tissues and haematology including differential cell counting.
All three regions use a concept of tiered testing strategies, although there are differences in the sequence of tests recommended (see Table 3)
The major difference between EMEA and the other regulatory authorities is that the EMEA requires functional testing in routine screening, i.e. test of primary antibody response to T-cell dependent antigen, or NK activity and FACS analysis of lymphocyte subset population. In contrast, the FDA proposes a case-by-case approach on the need for functional assays. The Japanese authorities require the evaluation of the primary antibody response only in case of immunotoxicological findings in the repeated dose toxicity studies. NK-cell activity tests are optional.
In all three regions, hypersensitivity testing focuses on type IV hypersensitivity of locally applied compounds. The EMEA refers to the OECD 406 guideline that recommends the GMPT, the Buehler test, the LLNA and the MEST assays for the evaluation of skin sensitisation potential. In opposite to the OECD, both EMEA and FDA regard the LLNA as a stand-alone assay. The MHLW requires all dermatological preparations to be tested in at least one of several acceptable tests. An overview of the requirements is given in Table 4.

		EMEA	FDA	MHLW
Basic studies	Appearance and body weight	All drugs	All drugs	All drugs
	White cell count and differential	All drugs	All drugs	All drugs
	Clinical chemistry	All drugs	All drugs	All drugs
Organ weights	Spleen	All drugs	All drugs	All drugs
	Thymus	All drugs	All drugs	All drugs
	Adrenal	All drugs	Suggested/All	All drugs
	Lymph node, draining	All drugs	–	–
	Lymph node, distant	All drugs	–	–
Histopathology	Spleen	All drugs	All drugs	All drugs
Tissues	Thymus	All drugs	All drugs	All drugs
	Bone marrow smear	All drugs	All drugs	All drugs
	Lymph node, mesenteric	All drugs	If draining	–
	Lymph node, unspecified	All drugs	All drugs	All drugs
	Lymph node, draining	All drugs	All drugs	–
	Peyer's patch	All drugs	If draining	All drugs
	XALT	–	If draining	–
Other studies	Lymphocyte subsets (A)	A or B/All	Tier I	Suggested/all
	NK cell function (A)	A or B/All	Tier I	Tier I
	T-cell function (SRBC *et al.*) (B)	A or B/All	Tier I	Tier I

All drugs: test required for all drugs. If draining: analysis required if LN is draining site of administration. Suggested/all: test suggested for all drugs, required for Tier I. Tier I: test required if signs of immunotoxicity are observed. XALT: tissue X Associated Lymphoid Tissue (GALT, BALT, NALT).

Table 3: Test requirements for direct immunotoxicity in the three ICH regions (from: [36]).

Assays	EMEA	FDA	MHLW
GMPT	All drugs[b]	All drugs[b]	All drugs[b]
BA	All drugs[b]	All drugs[b]	All drugs[b]
LLNA	All drugs[b]	All drugs[b]	All drugs[b]
MEST	All drugs[a]	All drugs[a]	All drugs[b]
Adjuvant and patch test	–	–	All drugs[b]
Draize test	–	–	All drugs[b]
FCA	–	–	All drugs[b]
Open epicutaneous test	–	–	All drugs[b]
Optimization test	–	–	All drugs[b]
Spilt adjuvant test	–	–	All drugs[b]
MIGET	–	Inhalation drugs	–

[a]All *topically* applied drugs.

[b]Test regarded as stand alone assay.

Table 4: Test requirements for topically applied drugs in the three ICH regions (from: [36])

Neither FDA nor EMEA require routine testing for the photosensitising potential of compounds, taking into account that no sufficiently evaluated test is available (CPMP Note for guidance on photosafety testing, CPMP/SWP/398/01). The need for tests has to be specified in case-by-case decisions. The Japanese authorities require testing of dermatological preparations if the chemical structure of the compound or other information indicate a photosensitising potential.
Although induction of autoimmunity is a significant risk in pharmacological therapy, no sufficiently evaluated assays exist. Consequently, no specific guidance is given by the regulatory authorities.

5. The draft guideline ICH S8

5.1. The International Conference on Harmonisation (ICH)

Starting in the US in the 1930s and followed later by Japan and many European countries, manufacturing and distribution of medicinal products became constantly more regulated by increasing numbers of laws, regulations and guidelines for evaluating and reporting data on quality, safety and efficacy. In parallel, the pharmaceutical industry was becoming more and more international and oriented towards globalised markets. Nevertheless, although a single product was intended to be marketed worldwide, it had to meet the regulatory requirements for every market on a national basis. Facing this situation, an urgent need arose for harmonisation and rationalisation of regulatory requirements to

- avoid duplication of time-consuming and expensive tests
- reduce health care costs for patients
- reduce expenses in research and development
- reduce animal testing
- and reduce time for new drugs to reach the global markets.

To solve this problem, “The International Conference on Harmonisation of Technical Requirements for the Registrations of Pharmaceuticals for Human Use” (ICH) was established in 1990 as a joint institution of industry and regulatory authorities from Europe, United States and Japan to improve the efficiency of drug development and registration through harmonisation of the registration procedure.
All six parties act as equal partners in the scientific and technical discussions of the testing procedures. The parties are
for Europe:

- The European Commission, represented by the European Medicines Agency (EMEA), and the
- European Federation of Pharmaceutical Industries and Associations (EFPIA)

for Japan:

- The Ministry of Health, Labour and Welfare (MHLW), represented by the Pharmaceuticals and Medical Devices Agency (PMDA) and the National Institute of Health Sciences (NIHS),
  and the
- Japan Pharmaceutical Manufacturers Association (JPMA)

and for the United States:

- The US Food and Drug Administration (FDA),
  and the
- Pharmaceutical Research and Manufacturers of America (PhRMA).

In the first phase of ICH activities the focus was almost exclusively on the technical requirements for developing and registering products containing new drug substances in the European Union, Japan and the US. The Expert Working Groups which worked on over 40 guidelines for new drug products were made up of scientists from the six ICH parties. Early in the process a 5-step standard procedure was established for the handling of major harmonisation topics.
After a topic has been accepted by the ICH steering committee, an Expert Working Group (EWG) is formed by Topic Leaders, one for each of the six ICH parties. A rapporteur (usually from industry organisations) is designated that guides the topic through the first steps of the process.

Step 1: Consensus building
It is the task of the rapporteur to prepare an initial draft of a guideline or recommendation based on the initial concept paper by the Steering committee, and in repeated consultations with the experts designated to the EWG.
When a consensus is reached on the technical issues, the document is signed-off by all parties represented in the EWG.

Step 2: Start of Regulatory Action
Step 2 is reached when all six parties of the Steering Committee agree that, based on the report submitted by the EWG, sufficient scientific consensus is reached on the draft guideline or recommendation to proceed to the next stage of regulatory consultation.

Step 3: Regulatory Consultation
At step 3, the guideline or recommendation leaves the ICH process and is included in the normal regulatory consultation procedures in the three regions. In the EU, the document is published as a draft CHMP guideline, in Japan it is translated and published by the MHLW, and in the US it becomes a draft guidance published in the Federal Register.
Comments on the draft document are accepted from industry associations and regulatory authorities both from within and from outside the ICH regions. The input is considered by a new rapporteur (now a representative of regulator authorities) who draws a final document that has to be signed off by the experts from all three regulatory authorities within the ICH regions.

Step 4: Adoption of a Tripartite Harmonized Text
At this step, the report of the regulatory rapporteur is returned to the ICH Steering committee and adopted if all parties are satisfied with the result. The document is than signed off by all three regulatory parties and proceeds to step 5. In case of strong objections against the consensus report, the regulatory parties may agree that the revised text should be submitted to further consultation.

Step 5: Implementation
In this final step the tripartite harmonized text goes back to the national or regional regulatory authorities and is implemented by the same procedure that apply to other regulatory requirements and guidelines not harmonized by the ICH.

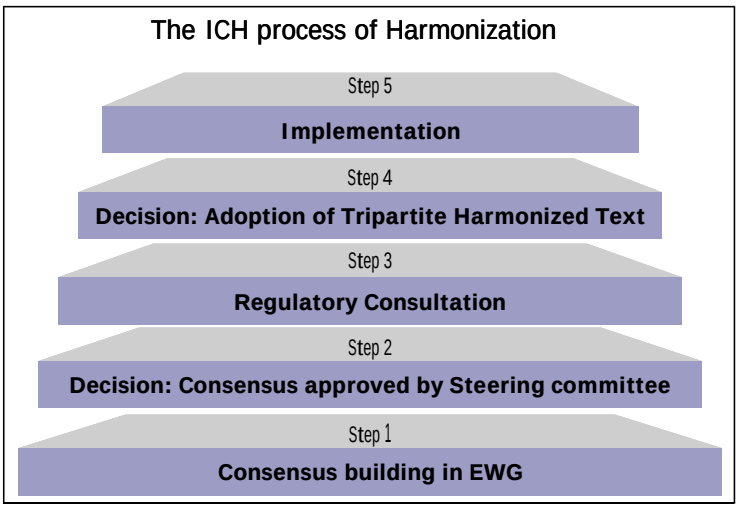

Figure 6: The ICH Process for major harmonisation topics

5.2. The ICH S8 draft guideline for immunotoxicity studies

After years of discussion, at the “Sixth International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use” (ICH 6), which was held November 2003 in Osaka, Japan, the decision was made to accept Immunotoxicity Testing as a topic.
Four key issues should be resolved by a new harmonized guideline:

- the necessity for immune function testing on a routine-basis versus a cause for concern basis
- definitions for “cause of concern” should be provided
- the appropriate conduct of immune function assays
- the timing of conduct of the immune function assays with respect to clinical trials.

It was decided not to include issues as drug hypersensitivity or immunogenicity into the process, either because there was no need perceived for harmonisation, or because no adequately evaluated animal models are available.

This Osaka decision triggered the installation of an Expert Working Group to write a draft guidance. This draft guidance ("ICH S8 Immunotoxicity studies for Human Pharmaceuticals") reached step 2 of the ICH process when it was approved by the ICH Steering Committee in November 2004 and proceeded to step 3 in which comments from industry and regulators from inside and outside the ICH process are collected and considered for the final harmonized proposal.

5.2.1. Objectives and Scope of the S8 draft guideline

Potential adverse effects of pharmaceuticals on the human immune system should be evaluated during standard drug development. The guideline aims at providing

- recommendations on nonclinical testing strategies to identify immunosuppressive properties of compounds

and

- guidance on a weight-of evidence decision making approach to test immunotoxicity.

The guideline gives recommendations on nonclinical testing for immunosuppression only. Other manifestations of immunotoxicity (e.g. immunogenicity or drug hypersensitivity) are not addressed.
The guideline is only applicable for low molecular weight drugs, but not for biologicals (for which immunotoxicity is already addressed by the ICH S6 guideline).
The guideline applies to

- all new pharmaceuticals intended for human use
- marketed drugs proposed for a different indication or other variations in the product labelling that could result in relevant immunotoxic reactions
- drugs in which clinical signs of immunosuppression are observed during clinical trials and following market authorization.

5.2.2. Recommendations for immunotoxicity assessment

In principle, the guideline recommends the evaluation of all new investigational drugs for the potential to generate immunosuppression using standard toxicity studies (STS) and additional immunotoxicity studies where appropriate. Guidance is provided for a weight-of-evidence review of cause of concern when additional studies are considered.
A flow diagram (Figure 7) depicts the decision process when conducting immunotoxicity studies.
On the initial evaluation level, which is obligatory for all new investigational drugs, information on immunotoxicity is gathered both from the STS and from considerations on other putative causes of concern.

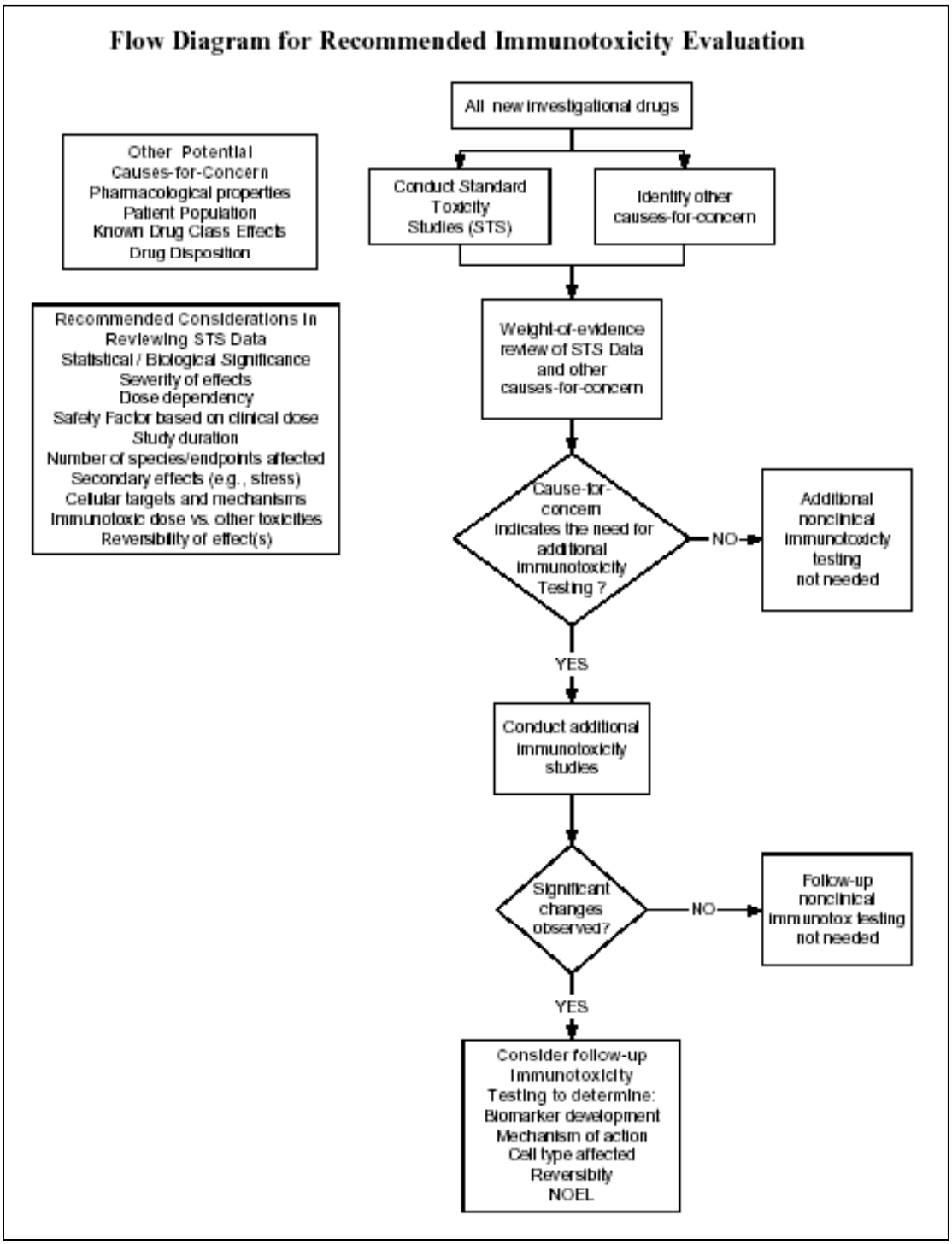

Figure 7: Flow chart ICH S8 immunotoxicity testing (from ICH S8 "Immunotoxicity studies for Human Pharmaceuticals").

Data from both rodent and non-rodent studies and from early short term to repeat-dose studies should be taken into consideration when evaluated for signs of immunotoxic potential with special focus on:

- Haematological changes - Evidence of myelosuppression, usually seen in peripheral blood changes (e.g. pancytopenia, leukopenia, lymphopenia, or other blood dyscrasias). Parameters to be evaluated are total leukocyte counts and absolute differential leukocyte counts.
- Alterations in immune system organ weights and histology (e.g. changes in thymus, spleen, lymph nodes, and/or bone marrow). Weight of thymus, spleen and lymph nodes (optional) should be determined after thorough bleeding of the animals. All lymphoid tissues should be evaluated for gross changes at necropsy. Histopathological changes of the spleen, thymus, bone marrow and Peyer´s patches should be evaluated as potential indicators of systemic immunosuppression. The lymphoid tissue that drains the site of drug application and one additional lymph node should also be examined histologically.
- Serum globulins are a rather insensitive marker of immunotoxicity due to the long half life of immunoglobulins. However, changes in globulins that occur without a plausible explanation can indicate potential immunotoxicity and provide additional information for the better understanding of the target cell population or the mechanism of action.
- Increased incidence of infections.
- Evidence of carcinogenicity, especially in the absence of genotoxicity.

It is very important to distinguish stress related changes of the immune system from real direct immunotoxic effects of a compound. Stress related immunosupressive effects are often observed in toxicity studies with doses near or at the maximum tolerated dose. The data from the STS should be assessed based on criteria that are also used in other toxicity studies, e.g.

- statistical and biological significance of the changes,
- severity of the effects,
- dose dependency,
- safety factor above the expected clinical dose,
- study duration,
- number of species and endpoints affected,
- changes that may occur secondarily to other factors,
- possible cellular targets and/or mechanism of action,
- doses which produce these changes in relation to doses which produce other toxicities, and
- reversibility of effect(s).

Besides the STS, general considerations have to be made for every new investigational drug to identify other causes-of-concern with respect to an immunosuppressive potential. Additional immunotoxicity testing should be considered

- if the targeted patient population is known to already have impaired immune functions (e.g. HIV patients),
- if the compound under investigation is structurally similar to other compounds with known immunosuppressive properties
- if the compound and/or its metabolites are known to accumulate in cells and tissues of the immune system

- if the pharmacological properties of a compound indicate a potential to produce immunosuppression (e.g. if the compounds targets a receptor that is also found to be expressed on immune relevant cells).

In a weight-of evidence review of the STS data and other causes-of-concern a decision is made whether additional immunotoxicity studies have to be conducted.

Additional immunotoxicity studies

If the weight-of-evidence approach leads to the conclusion that additional immunotoxicity studies are needed, several animal models are available. The selection of the appropriate models should be based on the nature of the immunological changes observed and the concerns raised by the class of compounds. At this point, a functional test should be conducted. Preferably, if a specific target is not identified, a T-cell dependent antibody response (TDAR) is recommended, using either SRBC or KLH as test antigen. Adjuvans should not be used without justification.

Immunophenotyping of leukocyte populations, a non-functional assay, can be conducted to identify the specific cell populations affected and to find useful clinical biomarkers. Immunophenotyping is usually conducted by immunohistochemistry or by flow cytometry. Data obtained from peripheral blood by flow cytometry can be useful to develop markers for clinical studies where peripheral blood leukocytes are also evaluated.

Assessment of natural killer (NK) cell can be conducted if indications for a decrease in NK cell number exist or if increased viral infection rates are observed.

Host resistance models may also be conducted as additional immunotoxicity studies. In general, they play an important role in identifying the cell type that is affected by a test compound. Furthermore, host resistance assays are able to detect alteration in innate immune mechanism.

Test of macrophage and neutrophil function can also be conducted *in vitro* and/or *in vivo*, while assays for cell-mediated immunity are not sufficiently validated.

Study design for additional immunotoxicity studies

In general, the studies should employ a daily oral administration regime over 28 consecutive days in rats or mice. The experimental setup should be consistent with the design of the nonclinical toxicology study in which an adverse immune effect was observed. The high dose should be above the no observed adverse effect level, but below a level where unspecific stress can lead to secondary changes in functions of the immune system.

After conducting the additional immunotoxicity studies, all data should be evaluated to decide if information is sufficient to determine the risk of immunotoxicity. If the overall risk-benefit analysis identifies the risk of immunotoxicity to be acceptable, no follow up studies might be necessary.

Follow-up immunotoxicity studies

If changes in immunotoxicity assays are observed, follow-up studies should be considered. A major goal of these studies is to broaden the understanding of the biological mechanism behind the observed disturbances and the identification of the cell types involved. These

information can be helpful for further evaluation of the risk and may lead to the development of biomarkers to be used in clinical studies.
Information of the reversibility and the no observed effect level (NOEL) should also be provided by the follow-up studies.

Timing of the studies

In cases where the weight-of-evidence review concludes the need for additional immunotoxicity studies, these studies should be completed before large number of patients are exposed to the drug. For patient populations that are already immunocompromised, immunotoxicity testing can be initiated at an earlier time point in the development of the drug.

6. Discussion and Outlook

Over the past ten to fifteen years, immunotoxicity has gained increasing attention by the regulatory authorities as a serious issue in drug development. By the beginning of this decade, the EU, US and Japan were working on guidance documents for the preclinical assessment of immunotoxicity. Although these efforts ran in parallel, the requirements differ between the three regulatory regions. A major step to reach a standardisation in regulatory requirement was reached in 2004 when an ICH draft guideline for nonclinical immunotoxicity testing was published. To some extend, the differences between the regulatory regions reflect a critical deficit in standardised and validated models and assays with an established predictive value for the extrapolation to human patients.

Concordance of immunotoxicity test

Almost all of the tests currently available for the assessment of immunotoxicity focus only on a subset of functions and do not address the complex immune system in its entirety. This raises the question of the ability of each assay to detect an immunotoxic potential of a test compound.

Several studies have been performed to tackle this problem. Luster et al. (31) analysed the results for more that 50 compounds that were tested in different assays in mice for their immunotoxic potential. Briefly, the authors found that a combination of two or three immune tests are sufficient to identify immunotoxic compounds with a concordance > 90 % in rodents. The highest association was observed for the T-cell dependent antigen assay and the enumeration of lymphocyte populations and quantitation via cell surface marker analysis. Other frequently employed methods like general leukocyte count or determination of lymphoid organ weights proved to be fairly insensitive.

Lebrec et al. (28) followed a similar approach in the mouse using two groups of pharmaceuticals: non-immunosuppressive and immunosuppressive based on clinical data. The highest concordances were observed for test of primary antibody response, NK cell activity, and CTL activity. Statistical significant effects were also observed for compounds that show no immunotoxicity in clinical use. These effects, however, were often isolated, not dose-depended, and unlikely to be biologically relevant.

Host resistance assays are widely regarded as the most valuable assays as in many experiments pathogens with human relevance are used and the resistance of the organism to a pathogen requires the interplay of multiple combinations of the immune system. Luster et al. (30) used the results from the testing of 50 compounds to analyze the relation between the outcome of immunotoxicity test and host resistance. According to this study, a good correlation exists between findings in the immunotoxicity tests and the altered host resistance in the sense that in all cases in which host resistance was altered at least one test gave positive results. However, in several cases the positive findings in the tests did not correlate with detectable changes in host resistance. The highest concordance values were reached with the delayed hypersensitivity response assay (DHR), and concordance could be further elevated when DHR, T-cell dependent antigen assay or determination of lymphocyte subpopulation were combined with a second assay.

Relevance of findings in nonclinical immunotoxicity studies for the treatment of humans

Experimental immunotoxicology studies in animals have identified many immunotoxic compounds and contributed to a considerable amount of quantitative data. On the other hand, the available data on immunosuppressive compounds in humans is much smaller, particularly regarding quantitative comparisons of dose response in humans and experimental animals. This poses a major problem for the validation of animal models and their predictability for humans.

Most of the general problems animal testing has to face in terms of relevance in humans are also an issue for the assessment of immunotoxicity: “A mouse is not an rat is not a human” (6). Consequently, the regulatory requirements imply the use of different species, and while in many cases information concerning the structure and function of the immune system can be readily translated across species, there are numerous and significant species differences that need to be considered. In some cases, the generation of meaningful immunotoxicology data can be adversely affected by the choice of a species that does not adequately share the immune function of concern with man. Likewise, immunotoxicology testing may produce negative data in one species but positive data in another. Knowing the mechanistic basis through an understanding of species differences in the structure and function of the immune system and the peculiarities of toxicity/ADME profiles is critical for success (see [17] for review of species differences). These differences need to be addressed in the design of experiments with the goal of extrapolating the data to humans. Some of the major differences between test animals and human patients are listed in Table 5.

Some Differences between Animals and Humans Critical to Prediction of Toxicity

	Animals	Man
Subjects		
Number	Large groups	Individuals
Age	Young adult	All ages
State of health	Healthy	Usually sick
Genetic background	Homogeneous	Heterogeneous
Doses		
Magnitude	Therapeutic to toxic	Therapeutic
Schedule	Usually once daily	Therapeutic optimum
Circumstances		
Housing	Uniform, optimal	Variable
Nutrition	Uniform, optimal	Variable
Concomitant therapy	Never	Frequent
Diagnostic procedures		
Verbal contact	None	Intensive
Physical exam	Limited	Extensive
Clinical lab	Limited, standardized	Individualized
Timing	Predetermined	Individualized
Autopsy	Always	Exceptional
Histopathology	Extensive	Exceptional

Table 5: Differences between animals and humans with relevance for immunotoxicity testing (from [34]).

On the other hand, species/strain differences can be advantageous when well-defined differences are used to delineate immunologic mechanisms. For example, mouse strains which are high responders for specific kinds of immunologic disease models, i.e. experimental allergic encephalitis in A/J mice or experimental allergic thyroiditis in C3H/He mice, have been useful as *in vivo* models to evaluate therapeutic efficacy. In particular, the availability of large numbers of mouse and rat strains with well characterized, specific immunologic deficits can be used to determine if the specific immune function plays a role in the toxicity being investigated (17). Besides the inherent differences of the immune system between species and strains other factors may also affected the extrapolation of animal data on the human situation.

Lack of adequate methods for evaluation of hypersensitivity/autoimmunity

Immunotoxicity can best divided into four categories, namely immunosuppression, immunostimulation, hypersensitivity, and autoimmunity. Each of these categories is associated with distinct potential adverse effects in humans. Nevertheless, most animal models and assays address immunosuppression only. In fact, the term “immunotoxicity” is often used synonymous to “immunosuppression”.
Although rare events, hypersensitivity and autoimmune reactions can be life threatening and have led to withdrawals to drugs from the market in the past. No validated models are available to predict this most serious type of immunotoxicity, and there is an urgent need to develop reliable models for drug induced hypersensitivity and autoimmune reactions.
The paucity of adequate assays is reflected in the finding from evaluation of data from preclinical and clinical trials that immune-related problems are the largest single area of adverse events that are not detected by preclinical testing (34).

Regulatory requirements

At the moment, EU, US and Japan have their specific regulatory requirements for preclinical assessment of immunotoxicity. While the EMEA has included recommendations for direct immunotoxicity in the guideline on repeated dose toxicity (CPMP/SWP/1042/99), the FDA has released the final version of a guideline on the “Immunotoxicology evaluations of Investigational New Drugs” in 2002. Japan is still in the process of finalization of a draft guideline.
Currently, predictive immunotoxicity testing is mainly embedded in the context of general toxicity testing. All three guidance documents are based on the concept of a tiered approach, also some differences exist in the sequence of the tests and the acceptance of some assays. Routine testing in all regions consists of haematology (including differential cell counting), histopathology, and weight determination of lymphoid organs.
It is still a matter of discussion whether functional testing should be included into routine assessment of pharmaceuticals. Currently, only the European authorities require the inclusion of functional testing in routine immunotoxicity screening in addition to enhanced histopathology examination. Suitable tests are primary antibody response to T-cell dependent antigen or NK activity and FACS analysis of lymphocyte subset populations. The European requirements are based on the following considerations (36):

- Histopathology is a qualitative and subjective method, and it provides a static view of a dynamic system
- in contrast, functional testing evaluates the performance of the functional immune system (or at least certain functional aspects of it)
- some functional defects of the immune system can only be detected when challenged
- functional testing is generally more sensitive and easier to interpret that histopathology data
- in many cases, functional defects occur well before histopathological changes can be detected.

This position of the European authorities has been repeatedly criticised mainly for the lack of evidence showing the superiority of immune function test to detect immunotoxic properties of pharmaceuticals (40). Critics emphasise that unintended drug-induced immunosuppression is relatively rare phenomenon, and the actual risk for human health is much lower than for drug-induced hypersensitivity and autoimmunity, which have in the past led to significant patient morbidity.
In contrast to the EU, FDA and MHLW advocate case-by-case approach to decide on the need for functional assays.

Regulatory guidance for Type IV hypersensitivity of locally applied compounds basically follows the requirements of the OECD 406 guideline. The guinea pig maximization test (GPMT), the patch test of Buehler, the local lymphnode assay (LLNA) and to some extend the mouse ear swelling test (MEST) are accepted by all three agencies as stand alone assay for the assessment of hypersentitiviy potential of topically applied drugs. In addition, the MHLW requests testing of all topically applied preparations in at least one skin sensitisation study.
No guidance is provided for preclinical examination of the potential to induce Type I-III hypersensitivity reaction. This reflects the lack of generally accept animal models or assays.

Drug induced autoimmunity can be a very serious threat for the patients. Since none of the assays available at the moment is sufficiently evaluated to become the standard model, authorities refrain from giving recommendations for the assessment of autoimmunity. The FDA document discusses the popliteal lymph node assay (PLNA), but states that more research is needed and does not give an explicit recommendation

<u>Timing of immunotoxicity studies</u>
The current guidance documents are somewhat imprecise in the timing of the immunotoxicity studies. The general interpretation is that these studies should be performed prior or parallel to phase II clinical trials, i.e. before larger numbers of humans are exposed to the drug. Only in cases of specific concerns (e.g. indication, chemical class, inclusion of immunocompromised persons already in phase I), evaluation should be done before the first human exposure (35). Nevertheless, this recommendation has recently been challenged based on concerns over exposing volunteers or patients to potentially immunotoxic drugs. Rather, incorporation of immunological endpoints in safety pharmacological studies and the inclusion of similar endpoints, as appropriate, in the clinical trails is advocated (9).

Outlook

Over the last years, immunotoxicity has attracted much attention by clinical and non-clinical research. This research has triggered substantial regulatory activities by all major authorities to develop guidance for the appropriate assessment of immunotoxic risk in drug development. A recent major step was the publishing of a draft guideline on immunotoxicity assessment in all three regions of the International Conference on Harmonisation. Some of the differences between the regulatory regions will be eliminated with the ICH S8 guideline for immunotoxicity studies, which is currently in the regulatory consultation phase. This guideline will be a major step in standardization of regulatory requirements for immunotoxicity and streamline the development of new drugs for global markets. The draft guideline is focused on recommendations for the assessment of immunosuppression only. Basically, it confirms the tiered approach that is being used in immunotoxicity testing for a long time. Functional assays, which are currently required by the EU as part of the routine screen, are no longer requested for all drugs. Instead, the guideline provides detailed advice for information to be considered for a decision on additional studies or follow-up immunotoxicity testing.

In spite of the substantial progress the regulatory framework has made, certain aspects are not yet addressed satisfactorily. Most of the regulatory advice is given for the assessment of immunosuppression or the assessment of local tolerance for topically applied drugs. Only little or no regulatory advice is given for the proper nonclinical assessment of drug induced hypersensitivity and autoimmunity. Also a rather rare event, these manifestations of immunotoxicity can be life threatening and lead to withdrawals of drugs from the market. The underlying reason for this situation is a fundamental lack in understanding the biological mechanisms of drug induced Hypersensitivity/Autoimmunity, and hence the development of reliable test systems. More research is needed to close this gap.

Another field that will receive more regulatory attention is the assessment of developmental immunotoxicity. Embedded in a general tendency to address the special requirements of drug development for children and adolescents, regulatory guidance is now developed for the assessment of adverse drug effects on the developing immune system. Non-clinical evaluation of developmental immunotoxicity is addressed in the FDA guideline on general immunotoxicology evaluation, and the other regulatory authorities are soon to follow.

Generally, there is still a need for reliable and well-validated endpoints in preclinical studies with proven relevance for human patients. In the clinic, usually only little attention is given to the development and assessment of endpoints indicative of immunotoxic effects. This discrepancy is the rationale of many critics of the current immunotoxicity testing that question the relevance of non-clinical data for the situation in humans. It is necessary to produce human data for endpoints used in preclinical studies to bridge the gap between the clinical findings and the results from the preclinical studies.

7. Summary

The exposition of human body to pharmaceuticals can lead to unintended and in some cases even life-threatening changes in the functionality of the immune system. These effects are summarized as immunotoxicity and can best be divided into four categories, namely immunosuppression, immunostimulation, hypersensitivity, and autoimmunity.
Over the past ten to fifteen years, immunotoxicity has gained increasing attention by the regulatory authorities as a serious issue in drug development. By the beginning of this decade, the EU, US, and Japan were working on guidance documents for the preclinical assessment of immunotoxicity. Although these efforts ran in parallel, the requirements differ between the three regulatory regions. While the EMEA has included recommendations for direct immunotoxicity in the guideline on repeated dose toxicity, the FDA has released the final version of a guideline on the “Immunotoxicology evaluations of Investigational New Drugs” in 2002. Japan is still in the process of finalization of a draft guideline.
Currently, predictive immunotoxicity testing is mainly embedded in the context of general toxicity assessment. All three guidance documents are based on the concept of a tiered approach, also some differences exist in the sequence of the tests and the acceptance of some assays.
Routine testing in all regions consists of haematology, histopathology, and weight determination of lymphoid organs. It is still a matter of discussion whether functional testing should be included into routine testing. Currently, only the European authorities require the inclusion of functional tests in first line immunotoxicity screening in addition to enhanced histopathology examination, while FDA and MHLW advocate case-by-case approaches to decide on the need for functional assays.
A major step to reach a standardisation in regulatory requirement was reached in 2004 when an ICH draft guideline for nonclinical immunotoxicity testing was published. This guideline will be a major step in standardization of regulatory requirements for immunotoxicity and streamline the development of new drugs for global markets. It is focused on recommendations for the assessment of immunosuppression only. Basically, it confirms the tiered approach that is being used in immunotoxicity testing for a long time. Functional assays are not requested for all drugs. Instead, the guideline provides detailed advice for information to be considered for a decision on additional studies or follow-up immunotoxicity testing.
In all three regulatory regions, little or no regulatory advice is given for the proper nonclinical assessment of drug induced hypersensitivity and autoimmunity, most likely the most serious manifestations of immunotoxicity. This reflects a fundamental lack in understanding the biological mechanisms of drug induced Hypersensitivity/Autoimmunity, and hence the lack of reliable test systems. More research is needed to close this gap.
Another field that will receive more regulatory attention is the assessment of developmental immunotoxicity. Embedded in a general tendency to address the special requirements of drug development for children and adolescents, regulatory guidance is now developed for the assessment of adverse drug effects on the developing immune system.

8. Reference list

1. Albers, R., W.Seinen, and R.Pieters. 1997. Suitability of the popliteal lymph node assay as a predictive immunotoxicological test. Drug Inf J.31: 1337-1340
2. Burleson G.H., J.H. Dean JH, And A.E. Munson (eds). 1995. *Methods in Immunotoxicology* Wiley-Liss, New York
3. Cederbrant,K., M.Marcusson-Stahl, F.Condevaux, and J.Descotes. 2003. NK-cell activity in immunotoxicity drug evaluation. Toxicology 185: 241-250.
4. Chapin R.E., M.W.Harris, B.J.Davis, S.M.Ward, and et al.. 1997. The effects of perinatal/juvenile methoxychlor exposure on adult rat nervous, immune, and reproductive system function. Fundam Appl Toxicol.;40(1):138-57
5. Chapin,R..E.. 2002. The use of the rat in developmental immunotoxicology studies. Hum.Exp Toxicol. 21: 521-523.
6. Cunningham,M.L. 2002. A mouse is not a rat is not a human: species differences exist. Toxicol.Sci 70: 157-158.
7. Dean,J.H., M.I.Luster, G.A.Boorman, and L.D.Lauer. 1982. Procedures available to examine the immunotoxicity of chemicals and drugs. Pharmacol.Rev 34: 137-148.
8. Dean,J.H., M.L.Padarathsingh, and T.R.Jerrells. 1979. Assessment of immunobiological effects induced by chemicals, drugs or food additives. I. Tier testing and screening approach. Drug Chem.Toxicol. 2: 5-17.
9. Descotes,J. 2004. Importance of immunotoxicity in safety assessment: a medical toxicologist's perspective. Toxicol Lett.149(1-3):103-8.
10. Descotes,J. 1996. Immunotoxicity of pharmaceuticals:An introduction. Drug Inf J 30: 271-274
11. Descotes,J. 2000. Autoimmunity and toxicity testing. Toxicol.Lett. 112-113: 461-465.
12. Descotes,J. 2005. Immunotoxicology: role in the safety assessment of drugs. Drug Saf 28: 127-136.
13. Descotes,J. and G.Choquet-Kastylevsky. 2001. Gell and Coombs's classification: is it still valid? Toxicology 158: 43-49.
14. Dietert,R.R., R.A.Etzel, D.Chen, M.Halonen, S.D.Holladay, A.M.Jarabek, K.Landreth, D.B.Peden, K.Pinkerton, R.J.Smialowicz, and T.Zoetis. 2000. Workshop to identify critical windows of exposure for children's health: immune and respiratory systems work group summary. Environ.Health Perspect. 108 Suppl 3: 483-490
15. Friberg,D.D. J. L.Bryant, and T. L.Whiteside. 1996. Measurements of Natural Killer (NK) Activity and NK-Cell Quantification. Methods Enzymol 9, 316-326.
16. Germolec,D.R. 2004. Sensitivity and predictivity in immunotoxicity testing: immune endpoints and disease resistance. Toxicol.Lett. 149: 109-114.
17. Haley,P.J. 2003. Species differences in the structure and function of the immune system. Toxicology 188: 49-71.
18. Hassan M, C.Nilsson, H.Olsson, J.Lundin, and A.Osterborg. 1999. The influence of interferon-alpha on the pharmacokinetics of cyclophosphamide and its 4-hydroxy metabolite in patients with multiple myeloma. Eur J Haematol. 63:163-170
19. Hastings,K.L. 2002. Implications of the new FDA/CDER immunotoxicology guidance for drugs. Int.Immunopharmacol. 2: 1613-1618.

20. Heywood,G.R., S.A.Rosenberg, and J.S.Weber. 1995. Hypersensitivity reactions to chemotherapy agents in patients receiving chemoimmunotherapy with high-dose interleukin 2. J Natl Cancer Inst. 87 : 915-922.

21. Holladay,S.D. and B.L.Blaylock. 2002. The mouse as a model for developmental immunotoxicology. Hum.Exp Toxicol. 21: 525-531.

22. Holsapple MP.2003. Developmental immunotoxicity testing: a review. Toxicology.;185(3):193-203

23. International Life Sciences Institute (ILSI). 1995. Immunotoxicity testing and risk assessment: summary of a 1994 workshop. Food Chem.Toxicol. 33, 887-894.

24. Irey NS. 1978. Drug adverse reaction reports related to immunotoxicity. In: *Inadvertent modification of the immune response – the effects of foods, drugs, and environmental contaminants*. *Proceedings of the Fourth FDA Science Symposium*.(ed. Asher IM), pp. 140-143. Printing Office; Washington, DC.

25. Kawabata,T.T. 1995. Enumeration of antigen-specific antibody-forming cells by the enzyme-linked immunospot (ELISPOT) assay. In*: Methods in Immunotoxicology* (eds. Burleson GH, Dean JH, Munson AE), pp. 125-135. Wiley-Liss, New York.

26. Krause,I., G.Valesini, R.Scrivo, and Y.Shoenfeld. 2003. Autoimmune aspects of cytokine and anticytokine therapies. Am J Med 115: 390-397.

27. Kuper CF, J.H.Harleman, H.B.Richter-Reichelm, and J.G.Vos. 2000. Histopathologic approaches to detect changes indicative of immunotoxicity. Toxicol Pathol. 28(3):454-466

28. Lebrec,H., R.Roger, C.Blot, G.R.Burleson, C.Bohuon, and M.Pallardy. 1995. Immunotoxicological investigation using pharmaceutical drugs. In vitro evaluation of immune effects using rodent or human immune cells. Toxicology 96: 147-156.

29. Luster MI, J.H.Dean JH, and D.R.Germolec. 2003. Consensus workshop on methods to evaluate developmental immunotoxicity. Environ Health Perspect 111(4):579-583.

30. Luster,M.I., C.Portier, D.G.Pait, G.J.Rosenthal, D.R.Germolec, E.Corsini, B.L.Blaylock, P.Pollock, Y.Kouchi, and W.Craig. 1993. Risk assessment in immunotoxicology. II. Relationships between immune and host resistance tests. Fundam.Appl.Toxicol. 21: 71-82.

31. Luster,M.I., C.Portier, D.G.Pait, K.L.White, Jr., C.Gennings, A.E.Munson, and G.J.Rosenthal. 1992. Risk assessment in immunotoxicology. I. Sensitivity and predictability of immune tests. Fundam.Appl.Toxicol. 18: 200-210.

32. Miyawaki T., N.Moriya, T.Nagaoki, and N.Taniguchi. 1981. Maturation of B-cell differentiation ability and T-cell regulatory function in infancy and childhood. Immunol Rev 57: 61-87

33. Norbury K.C.. 1982. Methods currently used in the pharmaceutical industry for evaluating immunotoxic effects. Pharmacol Rev.:34(1):131-136

34. Olson,H., G.Betton, D.Robinson, K.Thomas, A.Monro, G.Kolaja, P.Lilly, J.Sanders, G.Sipes, W.Bracken, M.Dorato, K.Van Deun, P.Smith, B.Berger, and A.Heller. 2000. Concordance of the toxicity of pharmaceuticals in humans and in animals. Regul.Toxicol.Pharmacol. 32: 56-67.

35. Putman,E., H.van Loveren, G.Bode, J.Dean, K.Hastings, K.Nakamura, F.Verdier, and J-W.van der Laan. 2002. Assessment of the immunotoxic potential of human pharmaceuticals: A workshop report. Drug Inf J 36: 417-427.

36. Putman,E., J.W.van der Laan, and H.van Loveren. 2003. Assessing immunotoxicity: guidelines. Fundam.Clin.Pharmacol. 17: 615-626.

37. Renton,K.W. 2000. Hepatic drug metabolism and immunostimulation. Toxicology 142: 173-178.

38. Shulman,K.L., J.A.Thompson, M.C.Benyunes, T.C.Winter, and A.Fefer. 1993. Adverse reactions to intravenous contrast media in patients treated with interleukin-2. J Immunother. 13: 208-212.

39. Smialowicz,R.J. 2002. The rat as a model in developmental immunotoxicology. Hum.Exp Toxicol. 21: 513-519.

40. Snodin,D.J. 2004. Regulatory immunotoxicology: does the published evidence support mandatory nonclinical immune function screening in drug development? Regul.Toxicol.Pharmacol. 40: 336-355.

41. Stahlmann R, M.Korte, H.Van Loveren, J.G.Vos, R.Thiel, and D.Neubert. 1992. Abnormal thymus development and impaired function of the immune system in rats after prenatal exposure to aciclovir. Arch Toxicol. 66(8):551-559.

42. Sternberg, E.M.. 1996. Pathogenesis of L-tryptophan eosinophilia myalgia syndrome. Adv Exp Med Biol. 398: 325-330

43. Sullivan,J.R. and N.H.Shear. 2001. The drug hypersensitivity syndrome: what is the pathogenesis? Arch.Dermatol. 137: 357-364.

44. Temple.L., L.Butterworth, T.T.Kawabata, et al.. 1995. ELISA to measure SRBC specific IgM: method and data evaluation. *In: Methods in Immunotoxicology* (eds. Burleson GH, Dean JH, Munson AE), pp. 137-157. Wiley-Liss, New York.

45. Van der Laan J.W, Van Loveren,H, J.G.Vos, J.H.Dean, and K.L Hastings. 1997. Immunotoxicity of pharmaceuticals: Current knowledge, testing strategies, risk evaluation and consequences for human health. Drug Inf J, 31: 1301-1305.

46. Van Loveren H.. 1995. Host resistance models. Hum Exp Toxicol;14(1):137-40

47. Van Loveren H., J.G.Vos, and E.DeWaal 1996.Testing lmmunotoxicity of Chemicals as a Guide for Testing Approaches for Pharmaceuticals. Drug Inf J, 30(1): 275-280.

48. van Loveren,H., J.Vos, E.Putman, and A.Piersma. 2003. Immunotoxicological consequences of perinatal chemical exposures: a plea for inclusion of immune parameters in reproduction studies. Toxicology 185: 185-191.

49. Verma,S.C. and E.S.Robertson. 2003. Molecular biology and pathogenesis of Kaposi sarcoma-associated herpesvirus. FEMS Microbiol.Lett. 222: 155-163.

50. Vial,T. and J.Descotes. 2003. Immunosuppressive drugs and cancer. Toxicology 185: 229-240.

51. Weaver,J.L., D.Staten, J.Swann, G.Armstrong, M.Bates, and K.L.Hastings. 2003. Detection of systemic hypersensitivity to drugs using standard guinea pig assays. Toxicology 193: 203-217.

52. Wolkenstein,P. and J.Revuz. 1995. Drug-induced severe skin reactions. Incidence, management and prevention. Drug Saf 13: 56-68.

Danksagung

Mein besonderer Dank gilt Herrn Dr. Bode für die spontane Bereitschaft zur Betreuung dieser Arbeit und Herrn Prof Dr. Olejniczak für die Übernahme des zweiten Referats.