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Chemically-Induced Immunopathology and Immune Functional Changes

Joseph G. Vos[1,2] and C. Frieke Kuper[3]

[1]National Institute for Public Health and the Environment, Bilthoven, The Netherlands
[2]Faculty of Veterinary Medicine, Utrecht, The Netherlands
[3]TNO Nutrition and Food Research, Zeist, The Netherlands

Abstract: Toxicological pathology of lymphoid organs plays an important role in the risk assessment process of immunotoxicants. Identification of chemicals that have the potential to cause injury to the immune system is of considerable public health significance because of the role of the immune system in infectious diseases, hypersensitivity reactions and autoimmune diseases. In assessing immunotoxicity, a two-tier testing system is usually employed in rodents in which the first tier is a general screen for (immuno)toxicity including enhanced histopathology of lymphoid organs and the second tier consists of more specific immune function studies including host resistance tests or mechanistic studies. Studies with the potent immunotoxicants TCDD, TBTO, HCB, azathioprine and cyclosporin A are discussed, which provide data correlating histopathology with immune function changes. This is followed by a discussion of the outcomes of enhanced histopathology investigation in the interlaboratory validation studies with azathioprine, cyclosporin A and HCB in the rat, as well as the results of a recent evaluation of enhanced histopathology in the mouse as an indicator of immunotoxicity. From these studies, that have been the basis for a number of regulatory activities, the following conclusions can be drawn: i) the consistency between histopathology and functional tests, ii) the complimentary information of pathology and immunology observations, and iii) the dependence on standardised protocols and trained toxicologic pathologists to accurately identify and grade microscopical changes in lymphoid organs and tissues. (J Toxicol Pathol 2004; 17: 137–146)

Key words: immunotoxicity, immunopathology, immune function, immunosuppression, immunostimulation, TCDD, TBTO, cyclosporin A, HCB

Introduction

Toxicological pathology plays a most important role in the risk assessment process by identifying and defining the health effects following exposure to xenobiotics. This includes the field of immunotoxicology, as identification of chemicals that have the potential to cause injury to the immune system is of considerable public health significance because of the role of the immune system in infectious diseases, hypersensitivity reactions and autoimmune diseases. Examination of lymphoid organs and tissues has increased the database on these compounds[1–5]. A solid examination includes the analysis of various microenvironments in lymphoid tissues and organs with respect to both the stationary and the passenger components. This also entails interpretation of changes with regard to the dynamic nature of lymphoid organs and tissues. A thorough knowledge of the histophysiology of the immune system is therefore required. Several laboratories have developed and validated methods to determine the effects of chemicals on the immune system of rats and mice[6]. In assessing immunotoxicity, a two-tier testing system is usually employed in rodents in which the first tier is a general screen for (immuno)toxicity including enhanced histopathology of lymphoid organs and the second tier consists of more specific immune function studies including host resistance tests or mechanistic studies[7].

Following a discussion of investigations with the potent immunotoxicants TCDD, TBTO, cyclosporin A and HCB that provided data correlating histopathology with induced immune function changes, the outcomes of enhanced histopathology in the interlaboratory validation studies of azathioprine, cyclosporin A and HCB in the rat, as well as the results of a recent evaluation of extended histopathology in the mouse with potent and weak immunotoxicants as an indicator of immunotoxicity are discussed. From these studies conclusions are drawn regarding the role of histopathology and immune function parameters in identifying immunotoxic agents, of investigating immune-

Mailing address: JG Vos, National Institute for Public Health and the Environment, P.O.Box 1, Bilthoven, The Netherlands
TEL: 31-30-2742075 FAX: 31-30-2744437
E-mail: j.vos@rivm.nl

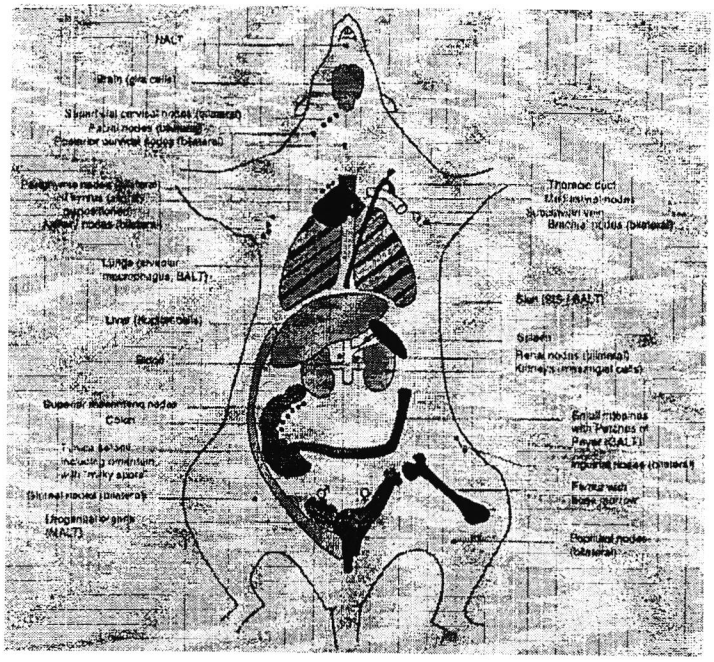


Fig. 1. Primary and secondary lymphoid organs are presented in black, mucosal and skin lymphoid tissues in green and mononuclear phagocyte systems in pink. From: IPCS (6), with permission.

related changes in non-lymphoid target organs, and on training of toxicologic pathologists.

The Immune System

Components of the immune system are present throughout the body (Fig. 1). The lymphocyte component is lodged in lymphoid organs. Phagocytic cells of the monocyte-macrophage lineage, occur in lymphoid as well as non-lymphoid organs like liver and lungs. The lymphoid organs can be classified in two ways. First, primary or central (antigen-independent) and secondary or peripheral (antigen-dependent) lymphoid tissues are distinguished. Bone marrow and thymus are primary lymphoid organs where lymphocyte proliferation and maturation takes place, independent of exogenous antigen exposure. Antigen-dependent development and proliferation takes place in the secondary lymphoid organs spleen, lymph nodes and mucosa-associated lymphoid tissue (MALT), as well as in the bone marrow. A second classification is based on the location of the lymphoid organs: internal organs such as thymus and most lymph nodes, and external tissue along secretory epithelial surfaces, the MALT, with their draining lymph nodes. Another organ that contributes to the immune system is the skin. It does not contain organized lymphoid tissue, but immune components in skin are interconnected with other lymphoid organs, leading to the concept of the skin immune system or skin-associated lymphoid tissue[8].

Examples of Immunotoxic Changes

After exposure to immunotoxic chemicals, the thymus is often the first lymphoid organ that shows weight changes and morphological alterations[9]. Most thymotoxic compounds induce atrophy of the organ as the result of lymphocyte depletion of the cortex: lymphocytes of the thymic cortex appear especially susceptible to the action of toxic compounds, although the cellular targets for toxicity differ[10]. Examples of such compounds are 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), bis(tri-n-butyltin)oxide (TBTO), cyclophosphamide and 5-fluorouracil. There are a few compounds for which the effect is expansion of a distinct thymic compartment without a major change in thymus weight. An example is cyclosporin A (CSA).

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)

TCDD causes profound effects on the thymus and peripheral lymphoid organs. As a result, cell-mediated and humoral immunity is compromised. Flow cytometry reveals that the thymic atrophy is due to a reduction in cortical-type thymocytes (CD4/CD8 double positive cells). Subsequent to thymic changes, T-cell areas in peripheral lymphoid organs become depleted. The lymphocyte depletion in the thymic cortex caused by TCDD (Fig. 2) may result from changes in the status of thymic epithelial cells, rather than from direct toxicity to the lymphoid compartment[10]. One hypothesis is that TCDD causes terminal differentiation of epithelial cells such that they are no longer able to support the functional

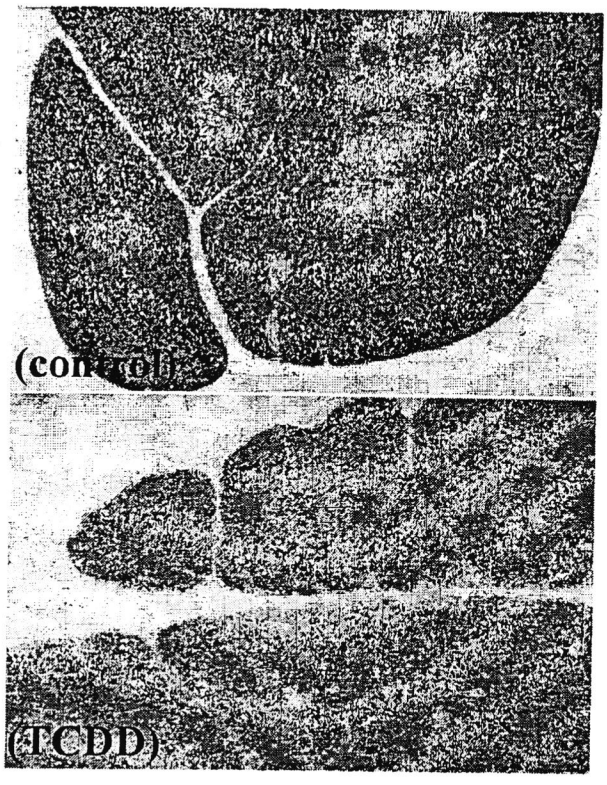


Fig. 2. Thymus of control and TCDD-treated rat showing severe atrophy of the cortex. The cellularity of the remaining cortex is less dense, making a distinction between the cortex and medulla difficult; haematoxylin and eosin stain. From: IPCS[6], with permission.

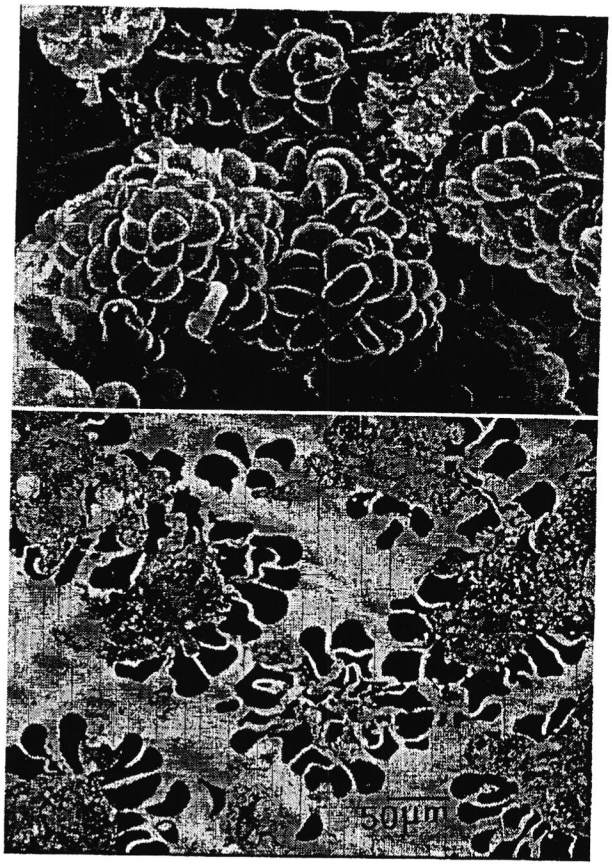


Fig. 3. Scanning and transmission electron microscopy of mesenteric lymph node of TBTO-treated rat showing erythrocyte rosettes in medullary sinus. Note the intimate contact of the macrophages with the erythrocytes without signs of phagocytosis, indicating impaired phagocytic function.

maturation and selection of T-lymphocytes. By electron microscopy a higher incidence of electron-dense epithelial cells has been observed, which indicates an increased differentiation of electron-lucent cells that normally form the major epithelial component[11]. This indicates that the epithelial compartment can be a target for toxicity, which confirms data from *in vitro* experiments. A direct action of TCDD on bone marrow stem cells also has been proposed. TCDD appears to act primarily through a cytosolic aryl hydrocarbon (Ah) or TCDD receptor, which is present in relative high concentration on thymic epithelium.

It has been shown that TCDD suppresses thymus-dependent immune responses. As a result, the resistance to infectious diseases is impaired. From studies in rodents as well as in man it appears that the developing immune system is especially vulnerable. TCDD administered during the perinatal period by maternal dosing causes severe suppression of the thymus-dependent immunity in rats and mice[12,13]. These effects appear long-lasting and at low level exposure: maternal treatment of a dose as low as 0.1 μg TCDD/kg bw on gestational day 14 suppressed the delayed-type hypersensitivity response in male offspring at 14 months of age[14]. The importance of TCDD immunotoxicity in human risk assessment is illustrated by the Tolerable Daily Intake (TDI) set for TCDD by the WHO. Among the most sensitive endpoints (on a body burden basis) were developmental neurobehavioral (cognitive) effects, developmental reproductive effects (sperm counts, female urogenital malformations), hormonal effects (endometriosis) and immunotoxic effects, both adult and developmental[15].

Bis(tri-n-butyltin)oxide (TBTO)

Organotin compounds are used widely as pesticides, as preservatives of wood, paper, textiles and leather, in heat/light protection of PVC plastics and in anti-fouling paints. Various organotin compounds, such as dibutyl- and dioctyltin chloride and TBTO have been shown in rodents to be T-cell immunotoxicants, resulting in suppression of thymus-dependent immune responses. Thymus atrophy is a sensitive parameter of exposure to these compounds. Histologically, there is depletion of large lymphoblasts located in the outer cortex, resulting in an inverse pattern of lymphocyte density in the cortex and medulla. This

Table 1. Summary Immune Function Study Following Long-term Oral TBTO Exposure in the Wistar Rat

		4–6 mo.			15–17 mo.		
		0.5	5	50	0.5	5	50
T. spiralis:	muscle larvae	–	↓	↓↓	–	↓	↓↓
	IgE response	–	↓	↓↓	–	↓	↓
	inflamm. react.				–	–	↓
Listeria monocytogenes		–	–	↓↓	–	↑	↓↓
DTH reaction		–	–	–	–	–	–
T- mitogen:	thymus	–	–	–			
	spleen	–	–	–	–	–	–
NK activity:	PEC	–	–	–			
	spleen	–	–	–	↓	↓	↓

one arrow: slight to moderate suppression.
two arrows: strong suppression. Adapted from Vos *et al.*[18]

depletion is due to an anti-proliferative action on thymocytes in the cortex. After prolonged exposure to organotin compounds, lymphopenia occurs and thymus and lymph node weights are reduced. The reduction in peripheral lymphoid organs affects particularly the T-cell compartment, such as the PALS in spleen and the paracortex in lymph nodes. Another finding was the occurrence of erythrocyte rosettes in the medullary sinuses of mesenteric lymph nodes. In scanning and transmission electron microscopy it appeared that there was intimate contact of the macrophages with the erythrocytes without signs of phagocytosis, indicating impaired phagocytic function (Fig. 3). The occurrence of rosettes increased in a dose-related fashion and appeared to be the most sensitive parameter[16].

To investigate whether immune function suppression observed in an earlier study after short-term exposure[17] also occurred after long-term treatment, function studies for specific and nonspecific resistance were performed after exposure of weaned male rats to diets containing 0, 0.5, 5, or 50 mg TBTO/kg for 4–6 and 15–17 months[18]. Treatment for 4.5 months had no effect on body weight but reduced thymus weight at 50 mg/kg. As in the short-term study, the resistance to the nematode Trichinella spiralis was dose-relatedly suppressed at the 5 and 50 mg/kg levels, in both experiments (5.5 and 16.5 months exposure), as shown by increased counts of muscle larvae and depressed serum IgE titers. Also the inflammatory reaction around cysts in parasitized musculature was reduced (Table 1). A dose-related shift was observed in T- and B- cell numbers in mesenteric lymph nodes as shown by flow cytometry using monoclonal antibodies: treatment for 6 and 18 months reduced the relative count of T-lymphocytes and consequently increased the percentage of B-lymphocytes. As a result, the T:B ratio was reduced in the 5 and 50 mg/kg groups. Concerning the nonspecific resistance, TBTO exposure for 5 and 17 months reduced macrophage function at 50 mg/kg as shown by impaired splenic clearance of Listeria monocytogenes bacteria. Natural cell-mediated cytotoxicity of spleen and peritoneal cells was investigated in a 51Cr-release assay with YAC-lymphoma target cells. TBTO treatment significantly suppressed natural killer (NK) activity in spleen cells. Significant suppression was noted in all treatment groups following 16 months TBTO exposure; in contrast to treatment for 4.5 months. The TDI for TBT of 0.27 microgram /kg/day set by WHO is based on the NOAEL of 0.5 mg/kg diet observed with T. spiralis host resistance model[19]. The US EPA gives a Bench Mark Dose (BMD10) of 0.03 mg/kg/day based on reduction in IgE responses to T. spiralis; taking a safety factor of 100 this leads to an TDI of 0.30 μg/kg/day[20].

Cyclosporin A (CSA)

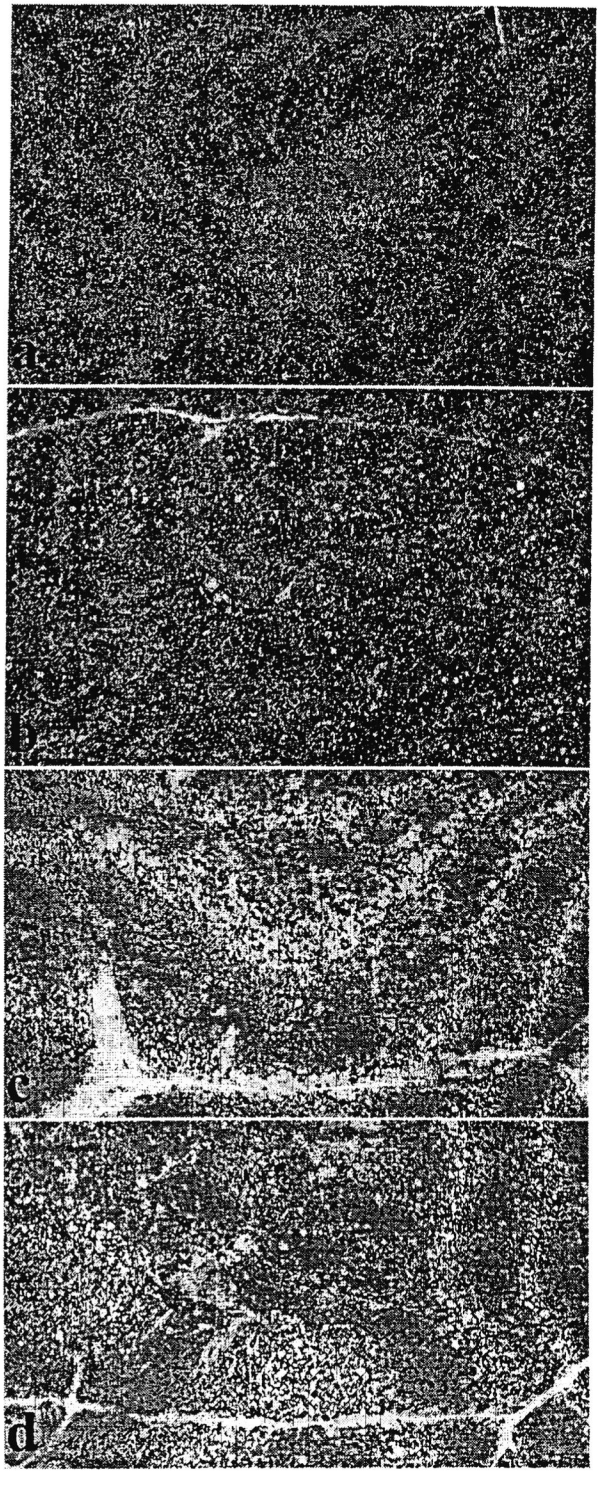
CSA is a drug that is widely used as an immunosuppressant in organ transplantation and in the treatment of certain autoimmune diseases. In contrast to the above-mentioned effects on the thymic cortex, CSA affects the thymic medulla[21]. The thymus of a CSA-treated rat shows an increased cortex:medulla ratio, based upon cell density and morphology. The outer medulla area is densely packed with small-sized cortical thymocytes and in effect there is a 'cortification' of the medulla (Fig. 4). In the medulla, there is a decrease of interdigitating dendritic cells and more mature medium-sized lymphocytes. The disappearance of interdigitating dendritic cells has been related to a disturbance in intrathymic selection of relevant (precursor) T-cells, resulting in the emergence of autoimmune phenomena upon withdrawal of treatment[22].

Hexachlorobenzene (HCB)

HCB is one of the compounds with immunostimulating or immuno-adjuvant properties, HCB is a highly persistent environmental chemical that has been used in the past as a fungicide. Presently, emission in the environment may occur as a result of the use of HCB as a chemical intermediate or as a byproduct in chemical processes. In rats, prominent changes following dietary exposure include elevated IgM levels and an increase in the weights of the spleen and lymph nodes. Histopathologically, the spleen shows hyperplasia of B-lymphocytes in marginal zones and follicles. Lymph nodes are activated as shown by the appearance of secondary follicles and an increase in the number of high endothelial venules in T-dependent areas[23]. High endothelial-like venules are also induced in the lung (Fig. 5), in particular in Lewis strain rats[24]. In the Brown Norway rat, HCB causes eosinophilic and granulomatous lung pathology that appeared associated with in vitro and *in vivo* airways hyperresponsiveness[25,26].

In immune function studies, cell-mediated immunity was enhanced and to an even larger extent an increase in humoral immunity was noted. Functional tests revealed an increase in cell-mediated immunity and humoral immunity. The developing immune system appears to be particularly vulnerable, as observed in two dietary studies investigating the prenatal and postnatal toxicity of HCB. In the first study, Wistar rats received 0, 50 or 150 mg/kg HCB starting at days 1–3 of pregnancy[23]. Clear immunostimulatory effects were noted in the 50 mg/kg group, therefore a second prenatal and

postnatal study was performed in which rats were exposed to 0, 4, 20 or 100 mg/kg diet[27]. Most prominent findings were increased serum IgM, increased primary and secondary IgM and IgG responses to tetanus toxoid and increased DTH reaction to ovalbumin, at a dietary level as low as 4 mg/kg. Liver weight increases and histopathology only occurred in the 100 mg/kg group. In contrast to rat data, HCB appears to be immunosupressive in the mouse as shown by suppressed



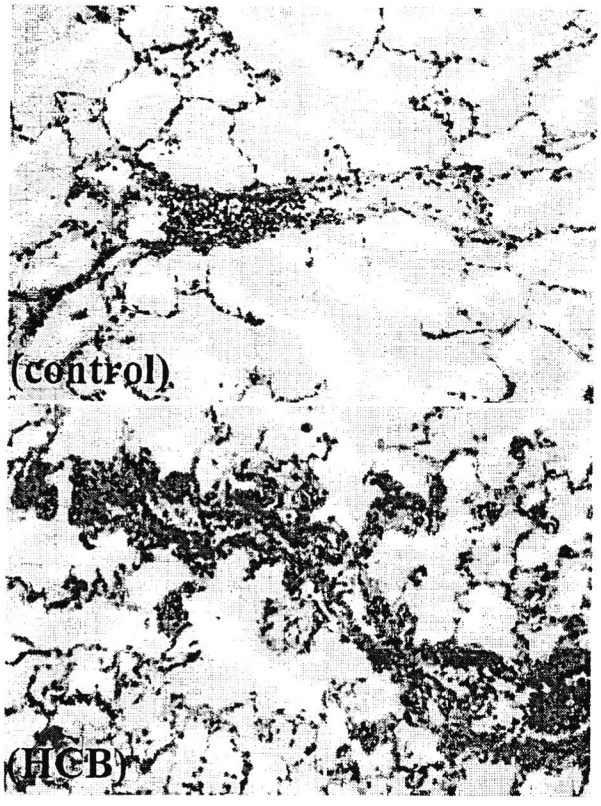


Fig. 5. Lung of control and hexachlorobenzene-treated Wistar rat showing venule with high endothelial cells and perivascular infiltrate of mononuclears; haematoxylin and eosin stain. From: IPCS[6], with permission.

Fig. 4. Rat thymus 14 days after daily administration of cyclosporin A (b and a) and recovery period of four weeks (a and c). Histological appearance in H&E stain (a and b) and immunohistochemistry with MHC Class H antibody that labels epithelium in the cortex in a dendritic pattern and interdigitating cells in the medulla in a more confluent pattern, whereas the epithelium in the medulla is not stained (c and d). The thymus of the cyclosporin A-treated rat has a completely altered cortex:medulla ratio, based upon cell density and morphology. Actually, almost the entire medulla has taken the appearance of cortex (increased cellularity of the medulla or 'cortification of the medulla' (b). Most of the section is filled with cortical-type epithelium in an adendritic staining pattern, due to the collaps of the medullary microenvironment (d). From: IPCS[6], with permission.

antibody responses and reduced host resistance in infection and tumor models. As in the rat, the developing immune system of the mouse is particularly sensitive to HCB. BALB/c mice were exposed to 0, 0.5 or 5.0 mg/kg maternal body weight throughout gestation by daily per os dosing of the females[28]. At 45 days of age, the DTH response to oxazolone was severely depressed in the offspring in both dose groups. Studies indicate that HCB might cause autoimmune-like effects in the rat[29]. Wistar rats treated with HCB produce antibodies to autoantigens: IgM, but not IgG, levels against single-stranded DNA, native DNA, rat IgG (representing rheumatoid factor), and bromelain-treated mouse erythrocytes (that expose phosphatidylcholine as a major autoantigen) were elevated. It is suggested that HCB activates a B cell subset committed to the production of these autoantibodies and associated with various systemic autoimmune diseases[30]. Recent studies in the BN rat indicate that besides T-cells also macrophages and granulocytes play an important role in the immunotoxicity of HCB as shown by the significant reduction of the immuopathology following co-treatment with CSA[31] and the gene expression profiles in different organs of rats exposed to HCB for 4 weeks[32].

Tiered Approach of Immunotoxicity Testing and Interlaboratory Validation Studies

Several laboratories have developed and validated a variety of methods to determine the effects of chemicals on the immune system of rats and mice (IPCS, 1996). Most employ a tier testing system, while some investigators have advocated multiple testing in a single animal. At present, most information regarding tiered testing for immunotoxicity comes from the model developed at the National Institute for Public Health and the Environment (RIVM) in Bilthoven, The Netherlands system[33–35], and the model developed at the US National Institute of Environmental Health Sciences National Toxicology Program (NIEHS-NTP)[36–38]. Other important initiatives in this field are the update of the FDA Center for Drug Evaluation and Research guidance document for immunotoxicity testing of drugs[39], and the FDA Center for Food Safety and Applied Nutrition update of immunotoxicity guidelines in the REDBOOK II.

The RIVM tiered system is based on the guideline 407 of the Organization for Economic Co-operation and Development (OECD), and performed in the rat using at least 3 dose levels, i.e. one resulting in overt toxicity (Maximum Tolerated Dose), one aimed at producing no toxicity, and one intermediate level. The first tier comprises general parameters including conventional hematology, serum immunoglobulin concentrations, bone marrow cellularity, weight and histology of lymphoid organs (thymus, spleen, lymph nodes, mucosa-associated lymphoid tissue-MALT), flow cytometric analysis of spleen cells, and possibly immunophenotyping of tissue sections. This approach has been used for the immunotoxic evaluation of pesticides[40] and pharmaceuticals[41].

In the OECD guideline 407 of 1981 (28-day oral toxicity study), the only parameters for the immune system were hematology including differential cell counting, in addition to histopathology of the spleen. In an evaluation that we made it appeared that this protocol was insufficient for identification direct toxicity for the immune system[42]. Also, results of two international collaborative immunotoxicity studies carried out in the rat by IPCS with the support of CEC showed that basic pathology investigations in the rat specified in OECD guideline 407 did not reveal the immune effects of AZA and CSA[43] and of CSA and HCB[44]. The immunotoxic actions of these compounds could be detected provided the test was extended to include additional pathology parameters. Results of a recent NTP study that addressed the interlaboratory reproducibility of extended histopathology clearly showed the importance of training of the toxicologic pathologist in examining lymphoid tissues[45].

The ICICIS study

The most important aim of the ICICIS study was to investigate whether the limited investigations on haematology, lymphoid organ weight and histopathology of old OECD guideline 407 or more detailed (enhanced) histopathological examination of the lymphoid organs and tissues of the rat in a conventional 28-day subacute toxicity test could serve as the "flagging" system[43]. The approach developed by the pathologists in the ICICIS study emphasized two aspects. First, the lymphoid organs were examined per compartment, as distinct compartments within a lymphoid organ each feature one or more specific functions, and each houses lymphoid and non-lymphoid cells of different lineages and in different ratios. Immunotoxic compounds may have an effect on one compartiment and leave others unaffected. Second, the terminology to describe morphological reflections of functional disturbances had to be quantitative and descriptive rather than interpretative. Because the immune system is highly dynamic, the cellularity and the size of the compartments as well as the development of germinal centres were considered important aspects to be considered in the histopathological examination of H&E-stained sections. Changes in the number of cells were described as decreased or increased cellularity rather than as atrophy, involution, degeneration, or hyperplasia and proliferation. The two model compounds examined were the potent immunosuppressive drugs azathioprine (AZA) and cyclosporin A (CSA)[43]. Results of the AZA study showed overall reduced cellularity in the bone marrow, thymus, spleen, lymph nodes and Peyer's patches. The most consistent indication of the effect of CSA on the immune system was an increase in the thymic cortex-medulla ratio and reduced cellularity of the cortex and medulla (Table 2). The variability of the results of the CSA study was much smaller than in the AZA study, probably as a result of standardisation of protocols, including histopathological

Table 2. ICICIS Cyclosporin A Study: Summary of Histopathology Results[a]

Dose levels	Low	Mid	High
Thymus: increased cortex: medulla ratio	1/9[b]	8/9	8/9
Spleen red pulp: increased cellularity			2/9
PALS: decreased cellularity		1/9	4/9
Marginal zone: altered cellularity		1/9	5/9
Follicles: decreased cellularity		1/8	2/8
Popliteal node follicles: altered cellularity			3/9
Paracortex: altered cellularity			5/9
Interfollicular area: altered cellularity	1/9	1/9	4/9
Mesenteric node follicles: decreased cellularity	1/9	1/9	4/9
Paracortex: decreased cellularity		1/9	3/9
Interfollicular area: altered cellularity		2/9	3/9
Peyer's patch interfoll.area: decreased cellularity			5/7

a: Data adapted from the ICICIS Group Investigators[43]. b: No. of labs with statistically significant differences compared to controls/no. of labs performing the parameter.

Table 3. BGVV Hexachlorobenzene Study: Summary of Histopathology Results[a]

Dose levels	Mid		High	
Sex of rats	M	F	M	F
Bone marrow: increased cellularity			5/8[b]	4/8
Spleen marginal zone: increased cellularity			7/9	4/9
Peyer's patch: hyperplasia of HEV	5/9	4/9	4/9	5/9
Mesenteric lymph node: hyperplasia of HEV	7/9	8/9	8/9	8/9
Popliteal lymph node: hyperplasia of HEV	9/9	9/9	9/9	9/9
BALT: hyperplasia of HEV			2/4	2/4
Lung: hyperplasia of small vessel endothelium		6/7	7/7	7/7
alveolar histiocytosis	7/7	5/7	7/7	5/7

a: Data adapted from Schulte *et al.*[44]. b: No. of labs with statistically significant differences compared to controls/no. of labs performing the parameter. Significant findings reported in at least 50% of the laboratories.

techniques, careful control of procedures, and comprehensive training of investigators.

The BGVV study

Two international ring studies were organized by the Bundesinstitut fur Gesundheitlichen Verbraucherschutz und Veterinarmedizin (BGVV) in Berlin[44]. These studies followed OECD TG 407 and included a number of additional examinations. CSA was selected as model for its immunosuppressive and HCB as model for its immunostimulating effects. Reproducibility of data was defined by significant findings in at least 50% of participating laboratories. Results of the CSA study showed only minor differences with the ICICIS study. HCB enhanced spleen and popliteal lymph node weights. Elevated IgM titers indicated increased antibody formation in HCB-exposed rats. HCB induced cellular proliferation in spleen marginal zones and endothelial activation in HEV of mesenteric and popliteal LN and GALT and in small pulmonary venules (Table 3). Data obtained by specific immune parameters indicated immune effects; however, statistical inference was limited to low numbers of participating laboratories. In order to predict immunomodulatory effects of CSA or HCB, histomorphologic examination of lymphoid tissues resulted in the most reliable and sensitive data to distinguish immunosuppression and -stimulation.

The NTP study

This study addressed the interlaboratory reproducibility of extended histopathology from studies of ten test chemicals and both negative and positive controls from the National Toxicology Program's immunotoxicology testing program[37]. Examined was the consistency between four experienced toxicologic pathologists with varied expertise in immunohistopathology in identifying lesions in thymus, spleen and mesenteric lymph node of the mice used in the immune function studies carried out in the past. Diagnostic terms were based on previously established criteria[4,46]. Agreement between pathologists was highest in the thymus, in particular when evaluating thymus cortical cellularity, good in spleen follicular cellularity and in spleen and lymph node germinal center development, and poorest in spleen red pulp changes. The study concluded that the ability to identify histopathological change in lymphoid tissues was dependent upon i) the severity of the specific lesion; ii) the specific tissue component being investigated; iii) the experience/training of the pathologist in examining lymphoid tissues[45].

The OECD guideline has been updated and now includes: weight of spleen and thymus, and histopathology of these organs, in addition to lymph nodes, Peyer's patches, and bone marrow[47]. This update appears to be certainly an improvement over the earlier guideline, although even with this updated version, some immunotoxic compounds may not be identified as such[48]. It should be born in mind, that in the updated OECD guideline 407, the immune system is not evaluated functionally. Within OECD a debate as to functional testing, i.e. measurement of antibody responses to sheep red blood cells (SRBC) is going on, in addition to discussions as to the inclusion of measurement of NK activity and inclusion of FACS analysis of lymphocyte subpopulations. One can question whether the inclusion of an in vivo antigen challenge test, for example with SRBC or another thymus-dependent antigen such as tetanus toxoid, to improve the sensitivity would really interfere with the toxicity test. Results of a study by Ladics *et al.*[49] indicate that intravenous injection with SRBC during a 30 and 90-day toxicity study did not alter hematological and clinical chemistry parameters. With the expected exception of the spleen, administration of SRBC did not significantly alter the weights or morphology of routine protocol tissues. The sensitivity of the SRBC assay for identification of potential immunotoxicity was demonstrated by Luster *et al.*[38] using 51

different chemicals. For the quantification of antibodies to SRBC, the enzyme-linked immunosorbent assay (ELISA) is a practical test method[50], which method also appears more sensitive than the plaque forming cell assay[51]. Although not yet included in the OECD 407 guideline, for the testing of pesticides and toxic substances the EPA has developed a guideline for subacute toxicity testing that includes a functional assay in which antibodies to SRBC are quantified by either the plaque forming cell assay or ELISA[52].

Recommendations

It should be noted that the array of tests proposed to be included in general testing of chemicals is aimed at flagging potential immunotoxicity. Further function testing will likely provide information on no-adverse effect levels, and are therefore valuable for the process of risk assessment. Functional tests that are especially valuable in this respect are host resistance assays, in which the animals are also exposed to a pathogen. The fate of the pathogen, and the pathology in the host associated with it, may serve as an indicator of the health implication of the immunotoxicity found for the test chemical. Pathogens used in these host resistance models are chosen in such a way that they are good models for human disease, e.g. the T. spiralis and L. monocytogenes infection models that were used in investigating the immunotoxicity of TBTO (discussed in the previous section).

Repeated dose testing according to the OECD 407 guideline has been very instrumental in identifying immunotoxicants, however, as these guidelines indicate the use of young- adult rodents, the more vulnerable periods regarding immunotoxicity are not addressed. Hence, certain immunotoxicants may not be detected, whereas for those that will be detected, the doses that produce effects in adults may be different from those that may produce effects during pregnancy or early in life[53,54]. For these reasons it is recommended that current OECD guidelines for reproductive toxicity testing should be amended so that also the developing immune system is considered a potential target of immunotoxicity[55,56].

The classical lymphoid organs are not the only sites where cells of the immune system are housed and where immune events take place[46]. For instance, subsets of macrophages reside in almost every organ (Fig. 1). Moreover, immunological processes like autoimmunity and hypersensitivity/allergy lead to tissue damage, protein (immune) complex deposits and/or inflammatory cell infiltrates predominantly in non-lymphoid organs. Well-known non-lymphoid target sites are vasculature, kidneys, synovial membranes, thyroid, skin, liver and lungs[57]. Thus, histopathological changes in non-lymphoid organs may be indicative of immunological reactions induced by a compound.

It remains to be established whether histopathological changes in non-lymphoid organs per se can be indicative of immunological reactions induced by a compound. The morphology of lymph nodes that drain the sites were allergenic compounds or compounds with adjuvant properties have been applied[58], and the morphology of lymphoid organs in animals exposed to immunostimulating compounds suggest that, at least at given time points, increased sizes of compartments, increased germinal centre development, and HEV development may be indicative features[59].

Conclusions

Tiered immunotoxicity testing strategies have been developed and validated in both rats and mice:

- the ICICIS study showed that all participating institutes flagged the immunotoxicity of the potent immunosuppressive drugs AZA and CSA using the 1995 update of the guideline 407.
- the BGVV study of CSA and HCB showed the sensitivity and reliability of histopathology and the further improvement of the enhanced OECD 407 by the inclusion of lymph node weights and histopathology of GALT.

The NTP interlaboratory reproducibility study of extended histopathology showed that the ability to identify histopathological change in lymphoid tissues was dependent upon:

- the severity of the specific lesion;
- the specific tissue component being investigated; and
- the experience/training of the pathologist in examining lymphoid tissues.

From these interlaboratory validation studies, that have been the basis for a number of regulatory activities, in general the following conclusions can be drawn:

- the consistency between histopathology and functional tests;
- the complimentary information of pathology and immunology observations; and
- the dependence on standardised protocols and trained toxicologic pathologists to accurately identify and grade microscopical changes in lymphoid organs and tissues.

The identification of immunotoxic agents may be further improved by:

- the inclusion of an *in vivo* antigen challenge test in the OECD 407 guideline; and
- the inclusion of immune parameters in current OECD guidelines for reproductive toxicity testing (in view of the sensitivity of the developing immune system).

The classical lymphoid organs are not the only sites where cells of the immune system are housed and where immune events take place:

- immunological processes like autoimmunity and hypersensitivity/allergy lead to tissue damage, protein (immune) complex deposits and/or inflammatory cell infiltrates predominantly in non-lymphoid organs;
- thus, histopathological changes in non-lymphoid organs may be indicative of immunological reactions induced by a compound.

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