# APPLICATION of FLOW CYTOMETRY to IMMUNOTOXICITY TESTING

## SUMMARY of a WORKSHOP

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**OCTOBER 9–10, 1997**

**INTERNATIONAL LIFE SCIENCES INSTITUTE**

**HEALTH AND ENVIRONMENTAL SCIENCES INSTITUTE**

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ILSI Press
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ISBN: 1-57881-056-6

Printed in the United States of America

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The ILSI Health and Environmental Sciences Institute (HESI) gratefully acknowledges the time and expertise generously given by the members of the Workshop Steering Committee, Editorial Board, and session speakers and rapporteurs toward the development of this summary. HESI additionally acknowledges the outstanding support provided by Ms. Judith Dickson, Ms. Amy Burk, Ms. Suprina Johnson, and Ms. Candice Hamilton in planning and implementing the workshop program and the final editing and production of the report. This workshop was funded by ILSI. The opinions expressed herein are those of the Editorial Board and participants of the workshop and not necessarily the views of either their organizations or ILSI.

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# INTRODUCTION

The toxicology and risk assessment communities have a continuing interest in evaluating chemicals that have the potential to affect the immune system. Despite or perhaps because of this interest, specific regulatory requirements for assessing for immunotoxic effects of chemicals are in various stages of development and implementation. In Europe the Organization for Economic Cooperation and Development (OECD) test guideline #407, which was published in 1981 and amended in 1995, calls for determining the effect of test compounds as rodent hematology and serology, lymphoid organ pathology and histopathology, and lymphocyte subpopulation analysis (OECD 1995). Further changes to OECD test guideline #407 have been proposed. In the United States the Food and Drug Administration (FDA; 1993) has proposed a two-tiered immunotoxicity testing framework where significant changes in tier 1 screening parameters (i.e., comparable with OECD test guideline #407) would necessitate tier 2 testing to establish the functional consequences of such changes. These guidelines have yet to be finalized. Under the harmonized Toxic Substances Control Act (TSCA) and Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) testing guidelines, the U.S. Environmental Protection Agency (US EPA 1998) recommends the use of immune cell phenotyping only when chemical exposure results in a demonstrable suppression of a T cell–dependent primary immune response.

The possible use of flow cytometric analysis to detect chemical-mediated effects on immunity and immune system status has engendered much discussion. Among the issues of concern are whether flow cytometric analysis is appropriate for tier 1 (screening) or tier 2 testing; the applicability of flow cytometric analysis to specific problems in immunotoxicology, for example, hypersensitivity testing (see Gerberick et al. 1997); and whether findings in animals are predictive of human responses. Underlying such concerns are questions about the relationship between changes in the expression of phenotypic and/or activation markers and measures of immune function, the biologic significance of changes detectable by flow cytometric methods, and whether changes and correlations seen in animals are predictive of the human response.

During a 1997 workshop, clinicians, immunotoxicologists, and other scientists examined available data, discussed the implications of those findings, and identified gaps in scientific understanding that could be resolved through specific research programs. The workshop was organized around two themes: clinical and experimental applications of flow cytometry and the relevance of flow cytometry to human health risk assessment. Ten invited presentations provided background on current uses and applications of flow cytometry in both clinical and preclinical testing arenas and its use for hazard identification and risk assessment. These presentations were followed by rapporteur reports and a panel discussion addressing current scientific understanding of the potential role of flow cytometric analysis in preclinical immunotoxicity testing and human health risk assessment. This discussion, which focused on a number of specific topics and questions (Table 1), is summarized herein (see Rapporteur Reports and Roundtable Discussion). Because most of the illustrative data described herein refer to cell phenotypes based on antibody binding characteristics, Table 2 defines these terms.

# INVITED PRESENTATIONS

## Session 1: Use of Flow Cytometry in Assessing Human Immune Status

**Use of Flow Cytometry for the Evaluation of Immune Status in HIV Infection.** *Dr. Janet K. A. Nicholson, Centers for Disease Control and Prevention, Atlanta, GA* , reviewed the phenotypic changes that occur during the course of human immunodeficiency virus (HIV) infection, a disease that is characterized by a depletion of CD4+ T cells. The greater the viral load, the faster the decline in CD4+ T cells. A hallmark of disease progression is a decrease to <200 CD4+ cells/mL with a corresponding loss in immune function, such as sequential loss of responsiveness to recall antigens. In addition, there is a suggestion that the CD45 RO subset of CD4+ cells is a selective target for HIV (Cayota et al. 1990, Schnittman et al. 1990), but further research is needed to verify this observation. In contrast to a gradual decline in CD4+ cells during HIV infection, CD8+ T cells initially increase in number and remain elevated for many years before decreasing during the later stages of the disease. Subset marker analysis has shown an increase in the expression of the activation markers CD38, HLA-DR, and CD57 on cytotoxic T lymphocytes (Vanham et al. 1991, Ho et al. 1993, Giorgi 1996). Metabolic activation of these cells is believed to ultimately result in their death by apoptotic mechanisms.

Over the course of the disease, there is generally a correlation between changes in viral load and changes in lymphocyte subsets, in particular CD4+ and CD8+CD38+ cells (Bouscarat et al. 1996). Antiviral therapies using protease inhibitors usually decrease viral loads with a concomitant increase in CD4+ cells, decrease in CD8+CD38+ cells, and decreased expression of CD38 on CD8+ cells (Kelleher et al. 1996). However, there is no specific value or magnitude of change in these parameters that can be used to assign risk that is due to immunosuppression because multiple patient-related factors must be taken into account. Thus, there is a gap between quantitating phenotypic changes and applying this information to human health risk assessment.

**Primary Immunodeficiency Disorders: Assessment of Cellular Elements.** *Dr. Hans D. Ochs, University of Washington School of Medicine, Seattle, WA* , described how flow cytometry methods are used in the diagnosis of primary immune deficiency disorders to characterize absolute numbers of different blood lymphocyte phenotypes in patients with various immune deficiencies. The absence of T and B lymphocytes is characteristic for some patients with reticular dysgenesis (Heymer et al. 1990), Rag1/Rag2 deficiency (Schwarz et al. 1999), or adenosine deaminase deficiency (Buckley et al. 1997). B cell–positive, T cell–negative, and natural killer (NK) cell–negative deficiencies suggest X-linked severe combined immune deficiency (SCID) resulting from mutations of the interleukin-2 receptor γ (Buckley et al. 1997) chain. A similar phenotype with a defect in intracellular kinase signaling related to Jak-3 deficiency (Buckley et al. 1997) is observed in an autosomal recessive form of SCID. Absence of T cells in the presence of B cells in the peripheral blood is often seen in purine nucleoside phosphorylase deficiency (Markert 1991). In contrast, in SCID resulting from ZAP-70 deficiency, patients have normal absolute lymphocyte counts, normal numbers of nonfunctional CD4+ cells, but no CD8+ cells (Elder et al. 1995). Patients with bare lymphocyte syndrome lack major histocompatibility complex class II molecule expression by B cells or activated T cells. Circulating B cells (CD19+, CD20+) are absent in X-linked agammaglobulinemia (Bruton's tyrosine kinase deficiency) and in autosomal recessive agammaglobulinemia (μ heavy gene or the gene for λ/14.1 chain [Conley 1999]).

Flow cytometry is also useful in assessing the function of activated lymphocytes and phagocytes. The diagnosis of X-linked hyper immunoglobulin (Ig) M (XHIM) syndrome, caused by a mutation in the CD40 ligand gene, is confirmed by demonstrating that activated T lymphocytes from XHIM patients are unable to express functional CD40 ligand (Aruffo et al. 1993). Defects in phagocytic cell function can also be determined by flow cytometry. The absence of a metabolic burst in activated neutrophils, as demonstrated by flow cytometric analysis

of the uptake of 2,7-dichlorofluorescein diacetate or by using dihydrorhodamine for the fluorescent detection of hydrogen peroxide production, is diagnostic for chronic granulomatous disease (Emmendörffer et al. 1994).

Dr. Ochs emphasized the importance of not only characterizing cell phenotypes but also assessing immune cell function in vivo, such as measuring primary and secondary immune responses in humans after immunization with bacteriophage (ϕX174). Because the response to ϕX174 is T cell dependent (Nonoyama et al. 1993), this in vivo challenge will identify patients with a predominant B cell defect (e.g., X-linked agammaglobulinemia) as well as those with abnormal T cell function (e.g., XHIM). Thus, immunization with bacteriophage ϕX174 is a useful way to assess both T and B cell function. This technique has also been used to study how the therapeutic ablation of T lymphocytes affects immune responsiveness in humans (Witherspoon et al. 1984).

**Flow Cytometric Immune Monitoring Following Allogeneic Bone Marrow Transplantation.** *Dr. Lawrence S. Lamb, Richland Memorial Hospital, University of South Carolina School of Medicine, Columbia, SC* , focused on monitoring the recovery of immune parameters by flow cytometry in patients after myeloablative therapy for allogeneic bone marrow transplant. A study was reviewed in which patients with refractory leukemia were transplanted with human leukocyte antigen mismatched bone marrow cells that were depleted of T cells. Lymphocyte reconstitution during the first 2–4 weeks of recovery was characterized by CD 56+ NK cells (85–95%) expressing high-density CD 122 as well as a small percentage of CD3+ TCR-αβ+ T cells (0.2–1.5%) and CD 19+ B cells (0.1–1.5%). During subsequent months for up to 1 year, both T cells and functional NK cells gradually recovered to normal levels (60–70% and 6–29%, respectively). Restoration of normal CD4-CD8 ratios was also delayed for 1 year or more. B cell recovery was negligible until about 6 months after therapy and then gradually returned to normal (Lamb et al. 1998). During the recovery phase the occurrence of fungal infections provided the clearest evidence of depressed immune function.

T lymphocytes were able to respond to mitogen stimulation as early as 1 month after transplantation; responses returned to normal after about 1 year. Acute graft rejection could be characterized by a predictable pattern of host-derived CD3+CD8+ T lymphocyte regeneration during the first 2–8 weeks of recovery (Lamb et al. 1995). Additionally, polymerase chain reaction analysis and Southern blotting indicated that patient rejection episodes were not necessarily mediated by a polyclonal response against the graft but might have been induced by $CD3^{low}$ $CD8^{bright}$ cells of host origin that possessed restricted TCR-Vβ gene expression. Approaches to the treatment of graft rejection include donor leukocyte infusion (Godder et al. 1998) or high doses of granulocyte/macrophage-colony stimulating factor if diagnosed before a rapid decline in white blood cell counts. A subset of patients developed a transient increase in TCR-(γδ+ T cells at 3–6 months posttransplant and had prolonged survival (Lamb et al. 1996). The role that TCR-(γδ+ T cells play in the observed increased survival of these patients is currently under investigation and appears to be related to the number of (γδ+ T cells infused with the graft (Lamb et al. 1999).

**Measurement of Activation Markers on Lymphocytes Using Flow Cytometry.** *Dr. Paul E. Hurtubise, University of Cincinnati Medical Center, Cincinnati, OH* , reviewed how flow cytometric characterization of activation markers could be incorporated into an evaluation of immune responsiveness after stimulation with drugs classified as biologic response modifiers. This analysis can be done together with functional assays (cytotoxicity, cytokine production, proliferation, chemotaxis, and superoxide generation) and phenotyping. Dr. Hurtubise noted that activation could be defined as the movement of resting cells into an activated state (e.g., macrophage killing and lymphocyte-activated killing), initiation of differentiation and maturation, or manifestation of certain phenotypic changes. B cell maturation, for example, can be monitored by quantifying changes in CD19+CD38- and CD19-CD38+ cell populations (Lanier 1993). For T cells, useful markers of activation include HLA-DR, CD71, and CD25 (Paxton et al. 1996). Activation can be characterized by flow cytometry in a variety of ways, including scoring the percentage of activation marker positive cells, determining the proportion of dimly and brightly staining cells, determining mean relative fluorescence intensity, or measuring antibody-binding capacity (number of molecules per cell).

For example, in hepatic transplantation therapy, flow cytometry is used to monitor HLA-DR+, CD2+, and CD3+ T cells (Herrod et al. 1988, Winkler et al. 1989). These cells are eliminated by treating the patient with murine anti–T lymphocyte antibody (OKT3), which is directed against CD3+ T cells (Goldstein 1987). An increase of CD3+ cells in circulating blood indicates that rejection may be occurring because of the production of host antibody to the OKT3 antibody. T cell–activation markers are also used as indicators of T cell–mediated rejection episodes in heart transplantation (Garner et al. 1989). In this case only CD2 and HLA-DR antigens are useful markers because such patients are treated with cyclosporine and therefore do not express CD25 (interleukin-2 receptor) on their T cells (Almawi et al. 1993).

A novel activation marker on CD8+ cells is the S6F1 (CD11a) antigen. Some studies indicate that this surface marker increases in density on CD8+ lymphocytes as HIV disease progresses (Morimoto et al. 1987, Ferbas et al.

1995). Relative fluorescence intensity measurements from flow cytometry can be used to evaluate the expression of activation markers such as CD38 and HLA-DR. The elevated mean fluorescence intensity of CD38 expression on CD8+ cells is useful for following the progression to acquired immune deficiency syndrome (AIDS); the greater the fluorescence intensity, the greater the risk of developing AIDS (Giorgi 1996). Similarly, mean fluorescence intensity measurements may also be useful in reflecting responses of patients to antiretroviral treatment.

## Session 2: Human and Animal Studies on Drugs and Chemicals

**Application of Flow Cytometry to Immunotoxicity Testing of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD).** *Dr. Nancy I. Kerkvliet, Oregon State University, Corvallis, OR* , examined the immunotoxicity of TCDD, a widespread, persistent environmental contaminant. Dr. Kerkvliet emphasized that immunophenotyping has been applied somewhat differently in human and animal studies. In laboratory animal studies the emphasis has been either on hazard identification (e.g., identifying phenotypic changes and establishing the dose response for specific chemicals) or on mechanistic studies (e.g., assessing phenotypic changes as they relate to altered immune function). In contrast, in human studies immunophenotyping has been used primarily to survey cohorts of individuals possibly exposed to immunotoxic chemicals and to attempt to demonstrate an immunotoxic event on the basis of the presumption that altered phenotypes would be present. These differences in the application of immunophenotyping have affected the types of information generated relative to the characterization of TCDD immunotoxicity in human and laboratory animal studies.

A number of human studies were reviewed, including several that were based in part on an analysis of the effects of TCDD on lymphocyte phenotypes (Hoffman et al. 1986, Webb et al. 1989, Roegner et al. 1991). These studies have mostly been inconclusive, prompting some to suggest that humans are uniquely resistant to the immunotoxic effects of TCDD. Dr. Kerkvliet emphasized that this conclusion is premature because the cohorts and exposure conditions were poorly defined and because phenotypic analysis has not been established as a biomarker of TCDD exposure.

The immunosuppression associated with exposure to TCDD in laboratory animals was reported by a number of laboratories and includes endpoints reflecting the functional status of both humoral (antibody-mediated) and cell-mediated (e.g., cytotoxic T lymphocyte [CTL] activity) immunity. Dr. Kerkvliet and her coworkers demonstrated via analysis of B and T cell subsets that acute exposure to immunosuppressive doses of TCDD did not alter the phenotypic profile of mouse spleen cells (Kerkvliet and Brauner 1990). This profile was not changed when the mice were challenged with sheep red blood cells (SRBCs) even though the antibody response to this antigen was markedly suppressed. In contrast, reductions in B220+ B cells and CD8+ T cells were demonstrated after exposure to TCDD in an allogeneic tumor model (Prell and Kerkvliet 1997). Taken together, these results suggested that there is a difference in the sensitivity of resting cells and antigen-activated cells to suppression by TCDD and that the type of antigen used to activate the immune system can have a profound effect on the ability to detect phenotypic changes.

Dr. Kerkvliet also reviewed several mechanistic studies of the role that activation antigens play in the suppression of the CTL response by TCDD (Prell and Kerkvliet 1997, Oughton et al. 1995). She and her colleagues characterized a novel CD8+ cell phenotype expressing high levels of CD44, CD28, and CD54 that appears to represent an activated CTL precursor. The percentage of cells expressing this novel phenotype was reduced by exposure to TCDD under conditions that suppressed CTL activity, suggesting that an early activation event was the primary target for TCDD. Dr. Kerkvliet's laboratory also has studied the effects of chronic (15 months) exposure to TCDD to determine whether phenotypic changes occur in the spleen and peripheral blood (Oughton et al. 1995). In these studies chronic exposure to TCDD did not alter the frequency of CD4+, CD8+, B220+, or Mac-1+ cells or the expression of I-A on the latter two cell subsets. However, several age-dependent changes in these markers were observed and were highly correlated in the spleen and peripheral blood. Although chronic exposure to TCDD did not alter overall frequency of CD4 + cells, TCCD did affect functionally discrete subpopulations of CD4+ T cells. The number of CD4+ cells expressing the memory phenotype ($CD44^{Hi}$ $CD45RB^{Lo}$) was reduced and the number expressing the naive phenotype ($CD44^{Lo}$, $CD45RB^{Hi}$) was increased in TCDD-exposed mice (Kerkvliet et al. 1996). These results are consistent with both the TCDD immunosuppression observed in animals and the important role played by antigen activation in the sensitivity to TCDD.

In light of these results, Dr. Kerkvliet expressed doubt that non-antigen-challenged laboratory rodents are an appropriate model for studying TCDD, particularly when they are raised in an ultraclean environment where exposure to environmental antigens is decreased by the imposed barriers. She concluded the following: 1) Phenotypic analysis of major cell subsets (i.e., T and B cells and macrophages) is not sensitive to low-level exposure to TCDD and therefore is not a good candidate for biomarker development in humans. In this regard, she challenged

the significance of recent studies in Dutch and Japanese infants that present conflicting results regarding exposure to background levels of polychlorinated biphenyls and corresponding changes in the expression CD8+ T cells. 2) Chronic exposure to TCDD produced a decrease in the number of memory T cells in mice, an effect previously demonstrated in marmosets. However, she emphasized that it may be difficult to ascribe similar changes to humans exposed to TCDD. 3) Phenotypic analysis is best suited for mechanistic investigations and should not be used as an initial screening approach in immunotoxicity testing.

**Immunologic Effects of 2´,3´-Dideoxyinosine (ddI).** *Dr. Dori R. Germolec, National Institute of Environmental Health Sciences, Research Triangle Park, NC* , described the effects of ddI, a purine analog with antiretroviral activity that is currently used in combination therapy for HIV-infected adults and children. She reviewed the status of ddI toxicity in humans on the basis of data from clinical trials in patients with HIV infections and in vitro studies in human cells or cell lines. In general, these studies indicated that ddI has a good-to-excellent therapeutic index when the control of viral load is compared with immunotoxicity (Perry and Balfur 1996). In particular, clinical trials have indicated that treatment with ddI caused a concomitant increase in cell-mediated immunity (i.e., as measured by delayed hypersensitivity reactions) and in the number of CD4+ T cells, the principal cellular target for HIV (Yarchoan et al. 1989, Drusano et al. 1993). She noted that ddI's toxicity has not been evaluated in clinically normal individuals, especially in the context of studies of the functional status of the immune system.

Dr. Germolec's review of the animal studies highlighted two general models: the murine model for AIDS (MAIDS), in which C57BL/6 mice are infected with the LP-BM5 murine leukemia virus, and B6C3F1 mice, used by the National Toxicology Program for most of its immunotoxicity testing. Dr. Germolec noted that peak plasma ddI concentrations were used to compare doses in mice with those used in human studies. These comparisons indicated that a dose of 100 mg/kg in a mouse study was comparable with the effective clinical dose of ddI.

C57BL/6 mice infected with the MAIDS virus exhibit splenomegaly, lymphadenopathy, elevated serum immunoglobulin concentrations, decreased of CD8+ cells (i.e., resulting in a corresponding increase in CD4/CD8 ratios), and suppressed lymphoproliferative responses (Harvie et al. 1996). Thus, MAIDS-infected C57BL/6 constitute a useful model of HIV disease. Treatment of MAIDS-infected mice with ddI reduced the severity of lymphadenopathy and splenomegaly, partially reversed the effects on CD8 cells, but had no effect on either the serum immunoglobulin concentrations or the suppression of the lymphoproliferative responses.

Subchronic treatment (180 days) of B6C3F1 mice with ddI resulted in the suppression of a number of immune parameters (Phillips et al. 1997). ddI was myelotoxic at higher doses (i.e., 500 and 1000 mg/kg) as evidenced by reduced numbers of granulocyte and macrophage progenitor cells. Spleen and thymus weights were significantly decreased and lymphoid depletion was evident in these tissues. Although there were significant alterations in the absolute numbers of Ig+, CD3+, CD4+, CD8+, and CD4+/CD8+ splenocytes, there were no differences in the percentages of the various subpopulations when compared with control animals, suggesting that the reductions in cell numbers were simply due to reduced spleen cellularity and weight associated with exposure to high doses of ddI (i.e., 500 and 1000 mg/kg). In contrast, the antibody response to SRBCs was significantly suppressed at all doses examined, including 100 mg/kg, which corresponded to the standard clinical dose. Selected T cell functions, such as CTL activity and the mixed lymphocyte reaction, were also suppressed by intermediate (≥250 mg/kg) doses of ddI.

Dr. Germolec's overall conclusions were that 1) ddI does not appear to be immunotoxic in humans, 2) exposure of mice to clinically relevant doses of ddI produced immunotoxic effects including changes in certain immune function parameters (suggesting that for the ddI studies reviewed, animal studies are not predictive of human toxicity), and 3) the latter studies also demonstrated that changes in immune function were not correlated with phenotypic changes among lymphoid cell populations.

**Immunotoxic Effects of Lead.** *Dr. Raymond E. Biagini, Centers for Disease Control and Prevention, Cincinnati, OH* , examined the immunotoxicity of lead. He observed that the effects of lead on the immune systems of animals include changes in antibody synthesis, macrophage activity, lymphoproliferative responses, and autoimmune responses (McCabe 1994, McCabe and Lawrence 1994, Zelikoff and Cohen 1996). Such studies often yield conflicting results reflecting, at least in part, interlaboratory variability in study design, sampling, and analysis. He concluded that the most consistent, reproducible effect was decreased host resistance to bacterial, viral, and parasitic challenges. There have been few studies using flow cytometry to determine the effects of lead on lymphoid cell phenotypes other than in the context of examining potential mechanisms of toxicity. For example, lead-associated changes in cell-mediated immunity were correlated with differential effects on CD4+ T cell subsets in the spleen, whereas the lead-induced increase in IgM production was associated with enhanced splenic B cell Ia molecule expression and differentiation (McCabe and Lawrence 1990, 1991). The mechanistic studies in animals

generally relied on cell sources that are not amenable to sampling in humans (e.g., spleen or lymph nodes).

Dr. Biagini concluded that data from the animal studies are not directly comparable with the results of human studies, where immunophenotyping is performed on peripheral blood mononuclear cells from workers exposed to lead in the workplace (Pinkerton et al. 1998). Comparisons between animals and humans are constrained because peripheral blood typically has not been used for immunophenotyping studies in animals. Dr. Biagini reviewed recent epidemiologic studies in lead-exposed workers in which their immunophenotypic profiles were examined (Cohen et al. 1989, Fischbein et al. 1993, Ündeer et al. 1996, Sata et al. 1997). These studies exclusively used peripheral blood and yielded inconsistent results. Depending on the study, exposure to lead resulted in increases, decreases, or no change in the absolute numbers and/or percentages of cells expressing CD3, CD4, CD8, CD16, CD19, CD56, and other surface markers. Although there is little evidence that the results of animal studies correspond to observed human responses to lead exposure, the animal findings are aiding in the design of epidemiologic studies. For example, the observation of consistent changes in host resistance in lead-exposed animals (Lawrence 1981, Kowolenko et al. 1991) has prompted the revision of questionnaires used in epidemiologic studies to include questions about recent illness and infections. However, the limitations of cohort size (usually <200 people) makes it difficult to detect statistical differences in nonspecific illness and infection prevalence.

## Session 3: Flow Cytometry for Hazard Identification and Risk Assessment

**Correlation of Splenic Phenotypic Markers with Functional Immunologic Assays.** *Dr. Kimber L. White, Jr., Medical College of Virginia, Virginia Commonwealth University, Richmond, VA,* described studies conducted by the National Toxicology Program in which changes in splenic lymphocyte populations, as determined by flow cytometry, were observed in mice exposed to various chemicals (Luster et al. 1992). Such changes were highly concordant with the immunotoxicity of the compounds being tested, but only pan T and pan B cell probes were used to assess subpopulations in most of the studies. To expand on these findings, data from these and other chemicals (a total of 59 chemicals) studied by the immunotoxicology program at the Medical College of Virginia were reevaluated to determine whether phenotypic changes measured by flow cytometry correlated with changes in any measured immune function. Functional assays evaluated included the IgM plaque-forming cell response to SRBCs, CTL response, MLR, DHR, NK cell activity, and mitogenic responses to concanavalin A or lipopolysaccharide. Spleen cells were evaluated for expression of surface Ig, CD3, CD4, and CD8; CD4+CD8+ cells were considered to be immature T cells. Data analysis was performed as described by Luster et al. (1992).

Dr. White found that changes in splenic lymphocyte populations were sometimes correlated with changes in functional assays in studies conducted in both rats and mice. Of the 59 compounds reviewed, 14 affected neither immune function nor the distribution of spleen cell populations, 24 had statistically significant effects on both measures of immune function and on the phenotypic profiles of spleen cells, 10 produced statistically significant effects on phenotypic profiles with no effect on the outcomes of the functional assays, and 11 produced significant alterations in function but no changes in spleen cell populations. The sensitivity of the phenotyping (i.e., the ability of changes in phenotypic profiles to correctly predict changes in immune function) was 69%. The specificity of phenotypic profile changes (i.e., the probability of correctly predicting no change in the immune function) was 58%. The concordance, or probability of making a correct decision regarding immune function, was estimated to be 64%, which is considerably lower than the value (81%) reported by Luster et al. (1992). The number of false positive and false negative results observed in this study indicates that additional compounds need to be evaluated to determine the usefulness of changes in phenotypic profiles as predictors of immunotoxic effects.

**Phase I and II of an Interlaboratory Evaluation of the Quantification of Rat Splenic Lymphocyte Subtypes Using Immunofluorescent Staining and Flow Cytometry.** *Dr. Gregory S. Ladics, E.I. du Pont de Nemours and Co., Newark, DE* , described a two-phase interlaboratory study in which the enumeration of rat splenic lymphocyte populations by immunofluorescent staining and flow cytometry was evaluated by using a standard protocol. Six independent laboratories participated in phase 1 (Ladics et al. 1997) and four participated in phase 2 (Ladics et al. 1998). Phase 1 was designed to establish baseline values for rat splenic lymphocyte populations, examine variability in flow cytometry data both within and among laboratories, evaluate single vs. dual labeling of T cell subpopulations, and compare quadrant and histogram analysis procedures. The specific aim of phase 2 was to determine whether each laboratory could detect similar changes in splenic lymphocyte populations of rats exposed to the immunosuppressive agent, cyclophosphamide.

In phase 1, B cells were examined by using a single immunofluorescent label whereas T cells were examined by using both single and dual labeling (Ladics et al. 1997). Enumeration of rat splenic lymphocyte populations by

the six laboratories yielded overall mean baseline values of 39%±2% B (OX12+) cells and 43%±2% T (CD5+) cells; 20%±1% of the T cells were CD4+ and 17%±2% were CD8+. Intralaboratory variability with respect to the relative and absolute numbers of lymphocytes was similar across analysis and labeling procedures. Intralaboratory variability was greater and interlaboratory reference ranges were larger when data were reported as absolute numbers rather than as relative numbers. Statistical performance indexes indicated that no analysis or labeling method was performed consistently among the participating laboratories. Because monoclonal antibodies to CD4 and CD8 antigens may recognize cross-reacting epitopes on other types of cells, Dr. Ladics recommended the dual labeling of T cell subpopulations and the use of quadrant analysis procedures.

In phase 2, rats were dosed intraperitoneally for 4 days with cyclophosphamide at 1, 3, or 10 mg/kg, and the effects on splenic lymphocyte subpopulations were compared. All four laboratories reported a decrease in spleen cell numbers in cyclophosphamide-exposed rats. Interestingly, the percentage of total T cells and CD4+ and CD8+ cells increased whereas the percentage of B cells decreased. However, the absolute numbers of splenic lymphocytes in each of these populations decreased. On the basis of changes in spleen cell numbers, the no-observable-adverse-effect level (NOAEL) was 1 mg/kg for three of the laboratories and 3–10 mg/kg for the fourth. The NOAEL based on spleen weight was 1 mg/kg for two of the three reporting laboratories and 3 mg/kg for the third. Based on relative numbers (i.e., percentages) for each splenic lymphocyte population, the NOAELs for three of the four laboratories were within one dose level of each other. Except for the absolute numbers of OX8+OX19+ (CD8+CD5+) cells, the NOAELs based on absolute numbers of lymphocyte population were within one dose level for three of the laboratories. At the fourth laboratory, the NOAEL based on each cell population expressed in absolute numbers was >10 mg/kg. Overall, each of the laboratories was able to use flow cytometric analyses to demonstrate that cyclophosphamide is an immunotoxic agent. However, Dr. Ladics noted that further standardization of sample preparation and analysis procedures (i.e., red blood cell lysis and cell gating) is needed to facilitate the use of flow cytometry in immunotoxicology studies.

**Assessing Human Populations for Immunotoxic Effects: Epidemiology from a Laboratory Perspective.** *Dr. Robert F. Vogt, Jr., Centers for Disease Control and Prevention, Atlanta, GA* , noted that epidemiologic studies of immunotoxicity frequently involve laboratory analysis of peripheral blood leukocytes and lymphoid cell subpopulations defined by the expression of various biomarkers. Analysis of these data is critical and requires knowledge of both laboratory and epidemiologic methods and their respective limitations (see van Loveren et al. 1998). The application of epidemiology in risk assessment presents special challenges because the ultimate goal is to prove beyond a reasonable doubt the absence of an association between exposure and disease. Conventional surveillance for overt disease cannot provide that kind of information because proving with 95% confidence that exposure to a chemical contributed less than 1 case of a disease per 100,000 (with an overall prevalence of 1 case per 10,000) would require control and exposure populations greater than that of the entire United States. However, markers of intermediate biologic effects that lie in the exposure-disease continuum may be sufficiently prevalent to make epidemiologic studies of exposure effects feasible.

Dr. Vogt suggested that peripheral blood B lymphocytes may be appropriate sentinels for human exposure to certain immunotoxicants in the environment. He noted that peripheral blood is readily available and contains long-lived memory cells that recirculate through most tissues. Such cells exhibit inherent genomic instability and are characterized by well-defined surface markers for various cell maturation stages as well as for various proliferative disorders.

Dr. Vogt described an Agency for Toxic Substances and Disease Registry health investigation that used the Basic Immune Test Battery to evaluate the immune status of approximately 6500 individuals in cross-sectional studies at 12 sites (Marti et al. 1997). In this investigation, 14 cases of B cell chronic lymphocytic leukemia were identified by examining various markers for detecting B cell disorders (i.e., CD20 vs. CD3 and CD5 vs. CD19). In 11 patients with chronic lymphocytic leukemia–like disorders, the peripheral blood had greater numbers of both CD20+CD3- and CD5+CD19+ lymphocytes. Other parameters such as white blood cell, total lymphocyte, and total B cell counts were used to characterize these individuals. Assessment of monoclonal gammopathies by serum electrophoresis was used as an indicator of malignant lymphocyte disorders. These studies and others suggested the following with respect to B cell lymphoproliferative disorders: overall, the rate of non-Hodgkins lymphoma increased; the risk of a B cell lymphoproliferative disorder increased with immunosuppression; smoking and radiation were not risk factors; family history of these disorders correlated with an increased risk; and the reported association of these diseases with exposure to volatile organic compounds, pesticides, and nitrates as well as possible synergies with infectious agents requires confirmation by well-designed studies with objective exposure measurements. Additional disease-based studies in humans are needed to identify and characterize relevant biomarkers of immunotoxicity.

# RAPPORTEUR REPORTS AND ROUNDTABLE DISCUSSION

The second day of the workshop featured a structured discussion about the role of flow cytometry in detecting and understanding the effects of chemicals on the immune system. Rapporteurs from academia, government, and industry explored the implications of this information relative to assuring the safety of chemicals and protecting public health. The rapporteurs and speakers (Table 3) participated in a roundtable discussion that addressed a number of previously identified questions (Table 2) critical to understanding the potential role of flow cytometry in assessing the immunotoxic potential of chemicals. The remainder of this report summarizes those discussions.

## Terminology

Although the term flow cytometry is sometimes used to refer almost exclusively to immunophenotyping, flow cytometry encompasses much more than phenotyping alone. Here the terms phenotyping and immunophenotyping are used synonymously and refer to differentiating and enumerating cells on the basis of their expression of specific surface or intracellular molecular markers.

Flow cytometry was considered to be a powerful tool for measuring the specific position of a cell in the cell cycle or in cascades of biochemical events on the basis of the expression of a number of intracellular and/or surface markers. However, the significance of such information was unclear. Some panelists considered these types of markers as additional indicators of cell phenotype; others ascribed a functional quality to these markers (i.e., activation markers). If the measurement of activation status by flow cytometry is considered to be synonymous with immune function, then there is no question about the biologic significance of such measurements. However, if activation status is considered to be an indicator of phenotype, then the biologic significance will depend on whether there is an association between a change in such a marker and a change in function. Such considerations are further confounded because there are applications of flow cytometry that can measure specific cell functions, for example, superoxide generation by granulocytes; various cytotoxicity endpoints (i.e., NK cells and CTLs); and antigen-specific IgM, IgG, and IgA production during an antibody response. Thus, flow cytometry can be used to assess a variety of parameters reflecting the functional competence of immune cells; such approaches are distinct from immunophenotyping.

## Preclinical and Clinical Applications of Flow Cytometry

Clinical applications of flow cytometry are based largely on disease conditions in which changes in phenotypic distribution, activation status, or both are unequivocal and not subtle. In contrast, most preclinical studies in animals focus on much more subtle changes in immunologic parameters. The extent to which many of the clinicians on the panel failed to appreciate this distinction was reflected in the discussion of establishing the biologic significance of changes in parameters evaluated by flow cytometry. In the absence of a clear and unambiguous marker or outcome against which to make such an evaluation, gauging biologic significance of an altered assay parameter is difficult if not impossible. Most immunotoxicology studies (except those designed to assess for changes in host resistance) do not incorporate outcomes such as lethality or morbidity and thus offer no independent outcome for assessing the relevance of a phenotypic or activational change. For example, although exposures to TCDD (Dr. Kerkvliet), ddI (Dr. Germolec), and lead (Dr. Biagini) were associated with immunotoxic effects in animals, there was no clear evidence that they were associated with immunosuppression in humans.

Clinical investigators have invested considerable time and resources in the characterization of parameters that can be assessed by flow cytometry that serve as surrogates for measures of immune function. Conversely, preclinical investigators have focused on characterizing sensitive models of immune function (i.e., primary immune responses) and have sought to apply flow cytometry to

animal studies, either as a tier 2 assay to more fully understand the mechanisms of immunotoxicity or to explore its potential ability to predict results likely to occur in humans.

## Immunophenotyping

Lymphocyte immunophenotyping is used in diagnosing AIDS and in monitoring the responses of patients to anti-HIV therapies (Dr. Nicholson); in characterizing immunodeficiencies, leukemias, and lymphomas (Drs. Ochs and Vogt); for monitoring responses of patients to organ and tissue transplantation (Dr. Lamb); and in identifying stem cells (Dr. Hurtubise). There are no known parameters that are measurable by flow cytometry that are used by clinicians as biomarkers of unintentional or undocumented human exposure to chemicals. Immunophenotyping is helpful in diagnosing diseases for which changes in the distribution of certain phenotypic markers have been extensively studied (e.g., changes in numbers of CD4+ lymphocytes in HIV-positive patients). However, the clinicians were skeptical of using changes in the distribution of various phenotypic markers to predict the risk of infection in patients. Even flow cytometric monitoring of the recovery of the immune system in transplantation patients provides little insight into their risk for developing infections.

Most clinical experience with immunophenotyping focuses on lymphocyte populations involved in acquired immunity whereas phagocytes and other types of cells associated with the innate immune response are less well characterized. Panelists agreed that a person's response to antigen challenge or immunization would be more indicative of their risk of infection than information currently attainable by flow cytometry. Determining the phenotype of blood lymphocytes in patients is probably more useful for understanding mechanisms associated with immune competence. For example, monitoring patients with repeated episodes of infection for changes in lymphocyte subset populations could include examination of activation markers, intracellular cytokine concentrations, or CD40 ligand expression. The evaluation and interpretation of subtle changes in any or all of these parameters would need to be considered in the context of the entire clinical picture before medical decisions could be made.

The panel acknowledged that there are limited data available that offer insight into how much change in blood lymphocyte subsets is sufficient to cause clinical concern. For example, in AIDS, reductions in numbers of CD4+ cells to <200 cells/mL is generally linked to an increased risk of opportunistic infection. Absence of B cells or granulocytes in certain inherited immunodeficiencies is also associated with an increased risk of infection. Although phenotypic data are useful on a case-by-case basis, the panel considered changes in leukocyte populations as determined by phenotypic analysis alone to be insufficient for making judgments about risk of infection.

Some panelists considered immunophenotyping to be of limited value for predicting the immunotoxic potential of chemicals in animals because it is not a measure of immune function. Rather, it provides a picture of the cellular composition of the spleen at the time of sampling. Flow cytometric analyses generally are not applied to animals that have been challenged with an antigen and, therefore, observed changes may not reflect the ability of the immune system to respond to a biologically relevant insult.

The appropriate tissue to use for immunophenotyping of laboratory animals may need to be determined on a case-by-case basis. Panelists generally agreed that immunophenotyping of the spleen rather than the peripheral blood should be done when the goal is hazard identification and the prediction of immunotoxic effects. Information on the effects of immunotoxicants on peripheral blood leukocytes is limited and more studies in various species are needed to determine whether data from peripheral blood correlates with data from the spleen. It was suggested that investigators conducting immunophenotyping studies in animals should evaluate both peripheral blood and spleen cells to generate the requisite data. If a response in the peripheral blood of mice could be related to a response in the mouse spleen, then changes in human peripheral blood might be considered indicative of changes occurring in the human spleen.

When immunophenotyping is to be performed, the implications of changes detected by flow cytometry need to be evaluated in the context of the study design. For example, such findings may be most relevant when the investigation focuses on the mechanisms underlying an observed effect (e.g., a diminished SRBC response). Although panelists suggested that immunophenotyping might best be performed as an adjunct to immune function tests, there remains much uncertainty as to the significance of a change in a functional parameter. Further characterization of such a change by flow cytometry may better enable investigators to gauge its biologic significance. The panelists considered how small or large a quantitative change in a parameter assessed by flow cytometry is necessary to predict biologically significant consequences. This question is fundamental to the continued evolution of immunotoxicology as a scientific discipline and could well be posed for a variety of other assays used by immunotoxicologists. This question had not been satisfactorily answered during previous workshops on immunotoxicology and remained unanswered at this workshop.

Some investigators suggested that the types of results obtained by immunophenotyping may complement

those obtained by histopathologic and immunohistochemical evaluation. Indeed, the pairing of flow cytometry and hematopathologic (e.g., cytospin preparations) examination is a routine part of clinical diagnosis. Part of the value of a histologic approach is that it examines immune cells in a relevant context, unlike the finding of activated cells in a lung lavage that reveals nothing about the tissue context of their activation. Some panelists noted the potential value of using immunohistochemistry to examine lymphoid cell distribution in tissues as part of an immunotoxicity testing strategy.

Even when flow cytometric analysis is used as a tier 2 test, it is important to determine the significance to ascribe to the results in the context of regulatory and risk assessment decisions. Often, any dose-related, statistically significant change in a measured parameter is considered to be relevant for regulatory purposes. From an immunotoxicologic perspective, this overlooks the issue of whether a change in a parameter is biologically relevant and thus is inconsistent with the understanding of the panel.

## Sources of Cells Used for Flow Cytometry

Panelists emphasized the advantages of using peripheral blood in clinical studies, noting that human blood is generally available and readily accessible and can be obtained through a minimally invasive procedure and that multiple samples can be collected over time (Dr. Vogt). However, peripheral blood only provides information on the types, activity, and/or numbers of cells in the periphery and thus reflects the trafficking of immune cells at the time of sampling. It was noted that patterns of cellular traffic in the periphery can be affected by glucocorticoids, epinephrine, and other stress factors, which may confound the distinction between treatment-induced changes and changes resulting from stress (e.g., blood sampling).

Perhaps the greatest concern about the use of peripheral blood was that it may reveal little about the immune competence of the patient. The immune response occurs principally in the primary and secondary lymphoid organs, where immunoresponsive cells are positioned to engage antigens. Panelists noted that the types, numbers, and other characteristics of peripheral blood lymphocytes are unlikely to reflect lymphocyte populations in the lymphoid tissues. For example, in the pathogenesis of HIV disease, many of the cellular changes are first observed in the lymphoid organs and not in the blood. Bone marrow hematology is of greater relevance for the diagnosis of leukemia than is determining the phenotype of blood cells, and the early detection and diagnosis of lymphomas is more likely to be based on lymphoid tissue biopsy specimens than on flow cytometric analysis of peripheral blood.

In contrast to clinical studies, flow cytometry has been most commonly used in preclinical immunotoxicity testing to determine the phenotype of immune cells in the spleens of mice and, to a lesser extent, rats. This reflects the greater number and availability of reagents for immunophenotyping mouse cells. The panelists discussed whether flow cytometric analysis can be used with equal confidence in studies with rodents or other laboratory animals. In the case of rodents, the available data suggest that such procedures can be used with confidence. For most other species, lack of knowledge, experience, and suitable reagents have limited the use of flow cytometry for immunophenotyping.

Because most studies in rodents use spleen cells for determining lymphoid cell phenotype, there is limited information available on the effects of immunotoxicants on peripheral blood leukocytes of laboratory rodents. Hence, there is considerable uncertainty about the relationship between phenotypic changes in peripheral blood and changes in spleen cell populations. This uncertainty is exacerbated when the effects of pharmaceutical compounds that are immunotoxic or immunomodulatory by design are assessed. Spleen cell sampling involves a fatal surgical procedure, and the potential for repeated blood sampling is limited by the small blood volume of mice. The cellular composition of lymphoid organs varies from tissue to tissue and from species to species. For example, lymphocytes constitute 90% of the circulating leukocytes found in mice but 33% or less of the total in humans. Similarly, the spleen is the major hematopoietic organ in mice but not in humans.

## Effects of Chemicals on Rodents and Humans

Previous studies have shown that changes in immune cell phenotypes as determined by flow cytometry are 64% (Dr. White) to 83% (Luster et al. 1992) concordant with the classification of immunotoxic. Moreover, relatively large numbers of compounds yield false positive (17%) or false negative (19%) results (Dr. White). Similar disparities between changes in phenotype and altered immune function were described by other panelists (e.g., Burchiel et al. 1997, Davis et al. 1994). Such results have been frequently cited, often in conjunction with regulatory guidelines, as evidence that flow cytometric analysis can identify immunotoxicants. As noted by the panelists, this conclusion is based on findings for a small number (≤59) of chemicals that are often studied at high doses and with a limited number of phenotypic markers. Indeed, in some of these studies phenotypic changes were observed in the absence of changes in assays of immune function. The utility of immunophenotyping in immunotoxicity testing will remain equivocal until additional immunotoxic compounds are

systematically evaluated.

Another issue related to the application of flow cytometry to immunotoxicity testing is its ability to detect dose-dependent changes in immunophenotypic markers. Clinically, dose-dependent changes in the expression of the CD134 lymphocyte activation marker are observed in patients treated with immunosuppressants (Dr. Lamb) and dose-dependent changes in the number of CD4+ T cells are noted in AIDS patients treated with immunomodulators. Mice treated with 2′,3′-dideoxyadenosine exhibited a dose-dependent decrease in B cells (Dr. White) whereas rats treated with cyclosporin A exhibited dose-dependent changes in T and B cell numbers (Dr. Ladics). Panelists noted that for immunotoxicity testing, doses need to be sufficiently high to detect such changes. This generally requires using a dose at or near the maximum tolerated dose and at least three lower doses. Dose selection is important because many immunotoxicants stimulate the immune system at low doses and suppress it at higher doses (e.g., cyclophosphamide and cyclosporin A). The examples of compounds eliciting dose-dependent effects on lymphoid cell phenotypes presented during the workshop were all immunotoxicants for which there is some understanding of their mechanisms of action. Clearly, establishment of dose-dependent effects will be critical in the evaluation of new chemicals for which only limited mechanistic information is likely to be available.

In the context of immunotoxicity testing, the panelists agreed that evidence is not sufficient for concluding that changes observed through the evaluation of cell surface markers are predictive of disease- or xenobiotic-induced changes in immune function. Thus, such information would not be appropriate for establishing threshold limits or for estimating a no observable adverse effect level, although several exceptions were noted. One such exception is the preclinical testing of therapeutic drugs intended to target the immune system where the principal effect is on a specific lymphocyte subpopulation (e.g., CD4 or NK cells). In such cases the doses used in the preclinical studies may be relevant to the anticipated clinical doses and therefore the NOAEL dose could be used to set initial doses for dose-escalation studies in humans. Phenotypic changes in peripheral blood cells may correlate with clinically significant immunodeficiency states, but in the case of therapeutics, where subtle changes in phenotypes are likely to be seen, it is doubtful that a NOAEL could be established on the basis of phenotypic data alone.

In terms of preclinical testing of therapeutic drugs, there was agreement among the panelists that dose-related changes observed in parameters assessed by flow cytometry indicate the need for further studies to determine whether the tested compound adversely affects immune function. The panel recognized that the definition of adverse would reflect the risks and benefits associated with the compound. Small changes that could, perhaps, be tolerated in short-term therapy may be less acceptable in the context of longer-term therapy.

## Relevance of Findings in Animals for Humans

One of the major goals of the workshop was to examine the relationships between chemical-associated changes in immunologic parameters in laboratory animals and phenotypic changes as measured by flow cytometry and to assess the relevance of this information for human health. Panelists engaged principally in clinical research and diagnosis or in preclinical studies of animals had distinct perceptions about the utility and applicability of flow cytometry. Indeed, the relatively long history of clinical use of flow cytometry led some panelists to wonder why some of the questions and issues raised by the preclinical investigators had not been previously resolved.

The possibility that flow cytometry could be used to detect a xenobiotic-induced effect on a marker of human health risk was discussed. There was considerable skepticism expressed about using data on changes in lymphoid cell phenotypes as markers for environmental exposure to chemicals. Immunophenotyping alone was not considered useful for assessing a population of people unintentionally exposed to poorly defined amounts of a xenobiotic. Although changes in phenotypic markers in humans can be measured, there is no basis for establishing how much of a change is important or even how to interpret observations of statistically significant changes. For example, there was no evidence of increased infection for up to 3 months in patients treated with anti-CTLA-4 antibody to eliminate CD4 cells (G.L. Warner, personal communication). Some patients with idiopathically low levels of CD4 cells are clinically normal and unaware of this abnormality until undergoing lymphoid cell phenotyping for unrelated problems. Conversely, in some organ-transplant patients on long-term immunosuppressive therapy, immune responsiveness or function is decreased relative to immunizations but no significant changes in lymphocyte phenotypes are observed. The panel observed that if phenotypic changes are not evident in patients who are intentionally immunosuppressed, then there should be little expectation of observing any changes associated with exposure to low doses of an environmental contaminant.

Discussion of the use of data obtained by flow cytometry for human health risk assessment revealed a number of uncertainties and data gaps. One area of uncertainty was the relationship between aging and immune function in humans, because age-related changes in lymphoid cell phenotypes are well-known. Do age-related

changes in immune function reflect an increased risk for developing cancer or autoimmune disease? Does long-term exposure to chemicals in the environment or to drugs that affect the immune system increase the risk of autoimmune disease? Several panelists noted a lack of knowledge about the significance of changes in specific subsets of cells within certain human populations, for example, increased percentages of CD5+ B cells in certain individuals living in or near a Superfund site or changes in (γδ T cell populations after exposure to immunotoxicants. It is unclear whether such surface markers could serve as sentinel markers of human exposure.

Another area of uncertainty is whether the test species is an appropriate surrogate for humans. Some panelists found monkeys to be acceptable study subjects whereas others did not consider them to be suitable surrogates for humans. Of particular concern was the observation that the cellular composition of peripheral blood reflects cellular trafficking that can be dramatically altered by stress and is particularly pronounced in some primates and in pigs.

The panel noted the equivocal results of studies of the effects of TCDD on human immune function. Although the immune system is a sensitive target for TCDD in rodents, there is no conclusive evidence that TCDD causes long-lasting immunologic effects in humans. Such concerns reiterate the need to consider phenotyping data on a case-by-case basis, particularly when extrapolating the results of animal studies to humans. A multicenter, multidisciplinary research program combining animal studies and human epidemiologic investigations of past TCDD exposures could be the key to resolving this uncertainty.

The panelists concluded that immunophenotyping has not been sufficiently validated for routine use in immunotoxicity hazard identification and is unlikely to be used by itself to predict the immunotoxic potential of a previously uncharacterized chemical. There was no evidence presented during the workshop to suggest that immunophenotypic analysis was as specific or sensitive as the SRBC assay for identifying immunotoxic effects. Because of the limited data available about the use of lymphocyte activation markers (e.g., intracellular cytokines) for detecting the effects of immunotoxicants, the value of such markers for immunotoxicity testing remains to be determined. Panelists emphasized that flow cytometry should not be applied to immunotoxicity testing without an understanding of the biologic significance of the parameter(s) measured (i.e., phenotypic or activation markers) and without sufficient validation of the protocol. Although recognizing the power of flow cytometry for understanding activation status, panel members acknowledged that most of these types of applications have not been validated and cited the recent controversy about the relationship between CD 69 expression and lymphoproliferative responses in humans as an example.

## Regulatory Considerations

In preclinical immunotoxicity testing, the most informative work has focused on the effects of chemicals on the responses of laboratory animals to antigen challenge (i.e., either a primary antibody response or a delayed hypersensitivity response). Animal researchers agreed that the primary antibody response to a T cell–dependent antigen (e.g., SRBCs) is the most sensitive indicator of changes in immune status. Consequently, such assays are increasingly important in supporting the registration of therapeutic agents and other chemicals.

The clinicians endorsed the rationale for this type of approach for animal studies but were hesitant to suggest that a parallel approach might be used to better understand the relationships between changing immune cell phenotypes and immune competence in humans. Panelists noted the technical and ethical issues associated with proposals to inject a foreign protein into humans and the effect of such concerns on vaccine development. However, in the absence of an overt clinical outcome, the response to antigen challenge is likely the best measure of human immune competence. Measurement of blastogenic or lymphoproliferative responses in humans were considered to be of limited value as stand-alone measures for detecting immunotoxic effects but nonetheless may yield important mechanistic insights (Davila et al. 1996). Animal studies indicate that these responses lack the necessary sensitivity to predict the immunotoxic potential of a chemical.

The use of flow cytometry for immunotoxicity testing was discussed by rapporteurs representing the OECD, FDA, and EPA. OECD (1995) test guideline #407 for 28-day oral toxicity studies calls for the evaluation of certain blood parameters and histologic examination of certain lymphoid tissues to provide for an assessment of the immunotoxic potential of chemicals. Test guideline #407 is under review because of concerns that the current testing requirements may be insufficient to detect all potentially immunotoxic chemicals. Proposals have been made to include the SRBC antibody response assay as a tier 1 study and to use flow cytometric phenotyping of spleen cells to enhance the power of guideline #407 to detect potentially immunotoxic chemicals. It is unclear whether either or both proposals will be adopted because of concerns about increased animal use relative to the SRBC assay and to continuing uncertainty about the relationship between phenotypic changes and altered immune function.

In the context of the draft Redbook II (FDA 1993), immune cell phenotyping is considered a tier 2 immunotoxicity test intended to provide information rela-

tive to mechanisms and targets of the immunotoxic effects of chemicals. Flow cytometric analysis may be used at the discretion of the investigator. Although Redbook II specifically addresses the testing of food additives and color additives used in foods, it will likely have broader applications throughout FDA when completed. The FDA Center for Drug Evaluation and Research is currently developing immunotoxicity testing guidelines that are unlikely to require the use of immunophenotyping in a manner consistent with Redbook II.

Under the harmonized TSCA and FIFRA testing guidelines, the EPA (U.S. EPA 1998) recommends immunophenotyping as an optional assay if changes in immune function are observed. Immune function would be assessed by determining the antibody response to a T cell–dependent antigen. The EPA would request that immune cell phenotypes be determined on a case-by-case basis.

Panel members agreed that in the absence of changes in other measures of immune status or function, phenotypic changes observed in animal studies alone were not sufficiently validated for use in human health risk assessment. Because of the scientific and technical uncertainties associated with its application to immunotoxicity testing, flow cytometry is unsuitable for use as a tier 1 testing protocol. The consensus of the panel was that flow cytometry is most appropriate as a tier 2 test to provide a better understanding of the responsible biologic and molecular mechanisms and processes underlying observed changes in immune function.

## Laboratory Considerations

There is an urgent need for improved characterization of exposure during immunotoxicity testing, particularly in terms of the test compound's dosimetry and toxicokinetics. It is difficult to use immunotoxicology data from animal studies for dose extrapolation because of the general lack of absorption, distribution, metabolism, and excretion (ADME) data. Test compound bioavailability and metabolism can vary tremendously among chemicals and across species. In the absence of relevant ADME data from animal studies, attempting to extrapolate those findings to humans is fraught with uncertainty.

Panelists agreed that it is critical to incorporate appropriate positive (immunotoxic) and negative (nonimmunotoxic) treatment controls when conducting immunotoxicology studies. These controls facilitate comparison of data between laboratories as well as demonstrating that assays are performing satisfactorily. The choice of appropriate controls depends on the class of compound being evaluated. Commonly used positive control compounds such as cyclosporin A and cyclophosphamide may not be appropriate for all classes of chemicals. For example, the immunosuppressive effects of cyclophophamide are due to hepatocyte-generated metabolites of the parent compound (Yang et al. 1986, Kawabata et al. 1990), thus the ability to detect possible immunotoxic effects of chemicals that do not undergo hepatic activation may be compromised.

The lack of standardization of flow cytometry techniques and reagents used in animal studies and the limited availability of phenotyping reagents for laboratory animals, especially rats, dogs, and monkeys, have limited the application of this investigative tool. The importance of such factors was well-documented by Dr. Ladics and further underscored by the rigorous standardization and validation programs described by the participating clinical flow cytometrists. To accomplish this, investigators will need to understand the biologic significance of the various phenotypic, activation, and other markers that can be assessed by flow cytometry and the health and immunologic consequences of changes in those parameters. Such information will need to be established for each species of laboratory animal.

Panel members noted that standardizing the flow cytometer setup was critical to quantitating fluorescence intensity and ensuring the consistency and reproducibility of data among flow cytometers (e.g., Schwartz and Fernandez-Repollet 1993) as well as for single instruments over the course of a long study. The reliability and variability of the instrument should be determined by assaying a number of samples; randomization of the samples enhances the value of such analyses. It is important that investigators understand the flow cytometer being used and the distinction between linear and log fluorescence scales. Light scatter should be appropriate for the identification of the subpopulation of cells bearing the marker of interest. For example, backgating of CD45+ cells is commonly used to identify human lymphocytes by their cell-staining characteristics rather than their physical light-scattering properties; this provides for more objective and specific gating (Loken et al. 1990).

Procedures used to lyse RBCs when preparing mouse splenic lymphoid cells need to be improved. In the multilaboratory study described by Dr. Ladics, lymphoid cell recovery varied from 27% to 90% even though each laboratory used ammonium chloride as the lysing agent. Empirically determined changes in the lysing protocol (e.g., reducing the concentration of the ammonium chloride solution, stopping the reaction with larger volumes of ice-cold buffer, processing fewer samples simultaneously to speed the process, and removing the ammonium chloride as quickly as possible) can result in splenic lymphoid cell recovery rates consistently around 90%. Morris and Komocsar (1997) found that one of two RBC lysis procedures used to recover rat mononuclear cells was more

effective for peripheral blood whereas the other was effective for spleen cells. Bossuyt et al. (1997) compared six methods for lysing human RBCs and found that some affected the outcome of the immunophenotypic analysis. In another study, lysed and unlysed human peripheral blood cells yielded similar results; however, the unlysed cell suspensions were messy and best sorted by backgating. Moreover, different lysing reagents and/or protocols may affect surface marker expression and/or differentially deplete or destroy subpopulations of leukocytes.

## Research Needs

In addition to the specific research needs identified in the preceding sections, there is an urgent need for more information relating chemical- and drug-induced changes in immune cell phenotypes in animals to health effects in humans. The panel emphasized that confidence in flow cytometric assays and their results will be increased through rigorous examination of available data, increasing the size of the available database, and performing multilaboratory studies that use meaningful positive and negative controls. Currently available data on immunophenotyping in animals are limited and those data, as well as the markers used, need to be evaluated and validated to determine what changes, if any, are significant to humans. It was suggested during the discussion that a consortium of regulatory agencies, companies, and academic institutions be formed to address very specific questions relevant to the development and application of immunotoxicity testing guidelines.

# CONCLUSIONS AND RECOMMENDATIONS

The invited presentations and panel discussion were intended to address the focus questions identified in Table 1. The conclusions and recommendations emerging from the workshop relative to those questions are summarized below.

**Can flow cytometric methods be used with equal confidence in studies with mice, rats, or other laboratory animals?**

Currently, phenotypic analysis for immunotoxicity testing is done with spleen cells from mice and to a lesser extent from rats. Although there are a number of reagents available for studies of mice, for most other species the knowledge of and experience with reagents, surface markers, and procedures is limited. **Recommendation:** This lack of information limits the use of flow cytometric analysis in species other than the mouse. Evaluation of surface markers requires an understanding of their biologic significance, the availability of marker-specific antibodies, and the development of appropriate protocols, including instrument setup and data analysis. It is also important to recognize that the expression and relevance of a marker may differ among species and tissues. Additional studies are needed to standardize the methodology and validate its reproducibility from laboratory to laboratory.

**Are primates acceptable surrogates for humans in studies using flow cytometric methods?**

Some investigators found monkeys to be acceptable for conducting phenotypic analyses whereas others did not. Factors potentially limiting the use of primates for immunotoxicity testing include the use of peripheral blood as the source of cells for flow cytometric analysis (discussed below), limited availability of reagents, limited information on most surface markers, susceptibility of monkeys to stress, and limited availability of monkeys. Stress in monkeys results in altered hormone concentrations in the blood that may affect trafficking of lymphocytes or alter phenotypic distribution; this confounds determination of whether immunophenotypic changes are due to the treatment or to stress. **Recommendation:** There is a lack of information on the use of flow cytometry for evaluating lymphoid cells in monkeys and how to extrapolate those results to humans. The acceptability of monkeys as surrogates for humans will need to be established for each marker on a case-by-case basis.

**What is the relationship between the results of flow cytometric evaluation of peripheralblood leukocytes and similar data obtained from the spleen?**

To date, most phenotypic evaluations in preclinical immunotoxicity testing have used the mouse spleen. Only limited data are available on the effects of immunotoxicants on peripheral blood leukocytes in the mouse, and in cases where data from both peripheral blood and the spleen are available, changes in peripheral blood phenotypes do not always correspond to effects on spleen cell populations. Similarly, HIV pathogenesis is associated with certain changes in the lymphoid organs that are not reflected in the blood. Sampling of peripheral blood offers a number of advantages: blood can be obtained by a relatively noninvasive method, multiple samples can be collected over time, and findings in the peripheral blood of animals may facilitate extrapolation to humans because human peripheral blood is generally available for evaluation. However, peripheral blood can only provide information on the types and numbers of leukocytes present at the time of sampling and may simply reflect trafficking of immune cells. Moreover, the volume of blood in mice is limited and the types and numbers of leukocytes in the blood may not reflect those found in lymphoid tissue. This is important in the context of immune function because the primary and secondary immune organs are where immune responses are initiated through cell-cell interactions. **Recommendation:** Immunophenotyping as part of an immunotoxicity assessment should be done using spleen cells rather than peripheral blood cells when the goal is preclinical hazard identification. Because information on the effects of immunotoxicants on peripheral blood leukocytes is limited, more studies (in various species) are

needed to determine whether findings in the periphery correlate with spleen cell data.

**Can flow cytometric methods detect dose-dependent changes in cell population numbers and activation status in preclinical and clinical applications?**

Dose dependence is an important feature of a true toxicologic effect, and for many immunotoxicants, lower doses stimulate the immune system whereas higher doses are suppressive. Some known immunosuppressive chemicals have dose-dependent effects on specific types of murine spleen cells: dideoxyadenosine produces a dose-dependent decrease in B cells, and cyclosporin A produces dose-dependent alterations in T and B cells. However, dose-response data are limited to a small number of well-studied immunotoxicants. **Recommendation:** When assessing for dose-dependent effects on immune cell phenotypes, it is important to use a graded series of concentrations of the test compound, starting with a dose that is at or near the maximum tolerated dose. More information is needed to determine whether dose-dependent changes in phenotypic profiles are a common effect of immunotoxic chemicals.

**Are changes in the distribution of cell phenotypes or activation status indicative of changes in immune competence or of other biologic significance?**

A key area for further investigation is the relationship between changes in phenotype or activation status and changes in immune function as an indicator of immune competence. For a limited number of immunotoxicants, dose-dependent changes in the profiles of T cells, B cells, or both have been reported, but the relationship between these changes and immune function have not been confirmed. In other cases no significant correlations have been observed between changes in phenotypic profile and immune function. This limited information suggests that alterations in cell phenotypes or activation status observed during preclinical testing are unlikely to indicate changes in immune function. However, in clinical testing such changes (e.g., alterations in CD4+ T cells) can indicate altered immune function.

Workshop participants considered the primary antibody response to T cell–dependent antigens (e.g., SRBCs) to be the most sensitive indicator of change in the immune status of laboratory animals. This assay measures the function of the immune system whereas determination of phenotype as currently performed provides little information about immune function; it generally reveals only the types and numbers of cells present. **Recommendation:** Immunophenotyping has not been sufficiently validated for routine use in preclinical immunotoxicity hazard identification studies and may not be useful for predicting the immunotoxicity of an unknown chemical. Immunophenotyping is appropriate for use in tier 2 immunotoxicity testing when the goal is to characterize an immunosuppressive effect and to determine the responsible mechanism(s). This is consistent with respect to testing guidelines in the United States, where neither FDA nor EPA recommend the use of flow cytometry for lymphoid cell phenotyping in tier 1 studies.

**How small or large of a change in parameters assessed by flow cytometry is necessary to predict biologically significant consequences?**

Alterations in parameters assessed by flow cytometry cannot be considered biologically significant in any study design without sufficient validation of those parameters. Clinically, a change in a measured parameter is considered significant only if it can be shown to be correlated with disease outcome, progression, or improvement. Some clinicians and preclinical toxicologists would ascribe biologic significance to phenotypic changes when they can be correlated with changes in parameters reflective of immune function. It will be important to analyze each marker to determine the biologic relevance of an observed change.

Understanding the biologic relevance of changes in measured immunologic parameters is one of the most important challenges to the continued evolution of immunotoxicology as a scientific discipline. Workshop participants recognized that the scientific community may never be able to answer the question of whether a 20% change in a given immune parameter should be considered indicative of significant adverse consequences. **Recommendation:** For each parameter assessed by flow cytometry, it will be important to determine what the observed change portends, when it is biologically significant, and what changes are associated with altered health status. Subtle or minimal changes in parameters measured by flow cytometry, as may occur during preclinical or clinical testing of pharmaceuticals, were not considered representative of relevant changes in immune status.

**Can investigators distinguish between statistically significant changes and biologically relevant changes?**

This question was never specifically addressed during the workshop. However, on the basis of the general discussion, changes in either phenotypic markers or activation status of immune cells as determined by flow cytometry are only considered to be biologically relevant if they are either associated with changes in disease outcome or are correlated with changes in functional endpoints in laboratory investigations. **Recommendation:** Biologically relevant changes need to be established for each marker and correlated with changes in immune function or health.

**Can investigators distinguish between treatment- or disease-associated changes in phenotypic distribution or activation status and stress-induced changes?**

Treatment- and disease-associated changes in human peripheral blood are well-documented, but this question was not specifically addressed in terms of studies of the primary and secondary immune organs of laboratory animals. Peripheral blood immunophenotypic profiles were considered by some participants to primarily reflect cellular trafficking. Stress is known to result in immunophenotypic changes in the peripheral blood of certain primates and in swine and may alter lymphocyte homing patterns. **Recommendation:** The lack of information on stress- vs. treatment-induced changes in phenotypic profile underscores the importance of evaluating the significance of such findings. This is particularly relevant in the context of peripheral blood sampling.

**Can activation markers be used as phenotypic markers of immune function?**

Although not one of the original focus questions, this complex issue was central to the panel discussion. If the measurement of activation status by flow cytometry is considered to be synonymous with immune function, then there is no question about the biologic significance of such measurements. However, if activation status is considered to be another aspect of phenotype, then its biologic significance will depend on whether there is an association between a change in activation and a change in function. **Recommendation:** Flow cytometry was considered to be a very powerful tool for measuring the specific position of a cell in the cell cycle or in another cascade of biologic events based on the expression of various intracellular or surface markers. However, the relationship between the expression of activation markers on immune cells and the function of those cells remains to be determined.

**Are the implications of changes in parameters measured by flow cytometry viewed with the same confidence as are those associated with observed changes in a functional assay (e.g., the primary antibody response to SRBCs)?**

As noted above, immunotoxicologists consider the primary antibody response to T cell–dependent antigens to be the most sensitive indicator of changes in immune status in animal studies. Similarly, physicians consider a patient's response to antigen (e.g., keyhole limpet hemocyanin or bacteriophage) challenge to be a reliable indicator of a patient's immune status. In contrast, immunophenotyping of a patient's blood, though informative, may not necessarily indicate immune status. Moreover, chemical-induced changes in immune function have rarely been shown to be associated with changes in phenotype or activation status, even when these parameters have been measured. **Recommendation:** Because of the limitations associated with peripheral blood immunophenotyping, evaluations of immune status must be done in the context of functional parameters and in consideration of the overall clinical status of an individual.

**Do flow cytometric methods yield information that cannot be obtained by other methods or measures?**

Determining the phenotype of blood lymphocytes from patients was generally considered to be more useful for examining immunologic mechanisms than as a diagnostic tool. For example, in patients who have repeated episodes of infection, changes in lymphocyte subset populations, activation markers, and intracellular cytokine levels or CD40 ligand expression would provide insight into the underlying immunologic mechanisms contributing to the observed infections. **Recommendation:** Subtle changes in phenotypic parameters require informed interpretation relative to the entire clinical picture before clinical decisions can be made.

**Can flow cytometric methods be applied in parallel with other procedures (e.g., lymphoid tissue histopathology or functional assays) to provide a more robust measure of immune system status?**

Several panelists considered immunophenotyping to be more analogous to histopathology than to the measurement of a functional parameter. Some investigators suggested that results from flow cytometric analysis may complement those obtained by histopathology and could be used as an initial approach to assaying for immunotoxic effects. The pairing of flow cytometric analysis with hematopathologic examination is a routine part of clinical diagnosis. There was agreement that phenotyping alone in animal studies (i.e., independent of any assessment of immune function) is not sufficiently validated for predicting immunotoxicity. **Recommendation:** Phenotypic applications of flow cytometry should not be viewed as a standalone approach and should not be considered appropriate for a tier 1 assessment of immunotoxicity; rather it is more appropriate as a tier 2 assay.

**What is the status of immunophenotyping in guidelines for immunotoxicity testing?**

Workshop participants were interested in the application of immunophenotyping and other flow cytometric methods to regulatory testing. At the time of the workshop, the OECD was considering updating guideline #407 to provide for immunotoxicity testing but was not expected to add flow cytometric analyses for determining phenotype. Similarly, FDA has no guidelines recommending immunophenotyping for immunotoxicity testing. Only the

then-pending (and recently released [U.S. EPA 1998]) EPA TSCA and FIFRA regulatory guidelines recommended immunophenotyping in immunotoxicology assessments and only as an optional assay if changes in immune function were observed. The TSCA/FIFRA guideline considers immunophenotyping to be a tier 1 method that would provide information germane to the selection of appropriate tier 2 tests. **Recommendation:** Immune cell phenotyping alone in animal studies is not sufficiently validated for use in risk assessment.

**What limitations are associated with the application of clinical flow cytometry data to medical diagnosis, prognosis, and treatment decisions?**

Human lymphocyte phenotyping by flow cytometry is used in characterizing leukemia and lymphoma, monitoring transplantation therapy, and identifying stem cells. In HIV disease, flow cytometry is used to follow changes in the CD4+ cell population that characterize disease progression, determine when to initiate therapies for opportunistic infections, and monitor the outcome of antiretroviral therapy. Using phenotype data alone to make a prediction about infection risk in patients was considered problematic. For example, immunity to infection involves the innate (phagocytic cells) immune response, yet clinical studies primarily focus on determining the phenotype of lymphocyte populations involved in the acquired immune response. In transplant patients although monitoring the immunologic recovery of patients is informative, flow cytometry data alone reveal little about the risk of infection. **Recommendation:** Determining the phenotype white blood cells alone is not sufficient for making clinical judgments about infection risk; some measure of immune function, such as antigen challenge or immunization, would be much more informative.

**How might flow cytometry be used to assess human health risks associated with xenobiotics such as human and animal drugs, food contaminants and additives, consumer products, and agrochemicals and other commodity chemicals?**

The possibility that flow cytometry could be used to demonstrate a biomarker of effect for a xenobiotic-induced human health risk was discussed by the panel. In general, there was skepticism expressed about using phenotypic data as biomarkers for environmental exposure to chemicals. Patients with idiopathic low levels of T cells may be clinically normal and unaware of their disease. Conversely, in some organ-transplant patients on long-term immunosuppressive therapy, immune responsiveness or function is decreased in regard to immunizations but no significant changes in lymphocyte phenotypic profiles are observed. Such observations raise the issue of whether significant changes should be expected with exposure to very small doses of environmental contaminants if phenotypic changes are not evident in patients who are intentionally immunosuppressed. **Recommendation:** Immunophenotyping alone was not considered useful in assessing the immune status of human populations unintentionally exposed to unknown amounts of xenobiotics. Although changes in human immune cell phenotypic markers can be measured, there is no basis at present for establishing how much change is important or even what it means when there is a statistically significant change.

**Can or should data obtained by flow cytometry be used to define NOAELs or other parameters used in human health risk assessment? Can such data be used to establish threshold levels?**

Changes in the phenotypic profiles of peripheral blood cells may correlate clinically with health in situations where there is severe immunodeficiency. In the case of therapeutics, where subtle changes in phenotypes are likely to be observed, it is doubtful that a NOAEL could be established based on phenotypic data alone. There is no convincing evidence that surface markers are sufficiently predictive of changes in disease status or of xenobiotic-induced changes in immune function. **Recommendation:** Phenotypic data alone cannot be used to define NOAELs either for therapeutics or for environmental exposure to chemicals. Exceptions occur in cases where in preclinical testing of therapeutic drugs intended to target the immune system, the principal effect is on specific immune cell populations such CD4 or NK cells.

**What uncertainties (e.g., false positives and negatives, validation criteria) and data gaps are associated with the potential collection, presentation, and use of flow cytometric data for human health risk assessment?**

There is a lack of knowledge of the significance of changes in certain lymphocyte subsets observed in certain human populations. For example, an increase in the percentage of CD5+ B cells in individuals living in or near a Superfund site is of unknown health significance as is the presence of (* T cells in patients treated with immunosuppressive agents. Phenotypic profiles are known to change with age, yet there is limited understanding of immune dysfunction associated with aging and the distinction between dysfunction related to environmental exposure and age-related changes. **Recommendation:** There is a need for research to evaluate and establish the biologic relevance of such observations and to determine whether there is sufficient evidence to select or target certain surface markers as sentinel markers for immunotoxicity.

Workshop participants noted in particular the inconsistent and equivocal findings from studies of the effects of TCDD on human immune function. Although the immune system is clearly a sensitive target for TCDD in rodents, no evidence has yet been produced that unequivocally implicates TCDD as a cause of long-lasting immunologic effects in humans. **Recommendation:** A multicenter study involving both animal research and human epidemiologic studies would be valuable for improving understanding of the relationship between this common environmental contaminant and immunity.

Better measures of compound exposure in immunotoxicology studies are urgently needed. It is difficult, if not impossible, to perform dose extrapolations of immunotoxicity data from animal studies because ADME data are usually not available. In addition, compound bioavailability and metabolism can vary tremendously across species. Attempting to extrapolate a NOAEL for humans from animal immunotoxicity studies without such data is unsatisfactory. **Recommendation:** It is imperative to have toxicokinetic or dosimetric information from immunotoxicology studies in order to make dose extrapolations for risk assessment.

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**Table 1.** Focus issues for the rapporteur reports and roundtable discussion during the Application of Flow Cytometry to Immunotoxicity Testing workshop, October 9–10, 1997

Applications of flow cytometry to immunotoxicity testing

1. Can flow cytometric methods be used with equal confidence in studies with mice, rats, or other laboratory animals? Are primates accepted as surrogates for humans in studies using flow cytometric methods?
2. What is the relationship between the results of flow cytometric evaluation of peripheral blood leukocytes and similar data obtained from the spleen?
3. Can flow cytometric methods detect dose-dependent changes in cell population numbers and activation status in preclinical and clinical applications?

Statistical and biologic relevance of changes in immune status as determined by flow cytometric analysis

1. Are changes in the distribution of cell phenotypes or activation status indicative of changes in immune competence or of other biologic significance?
2. How small or large a change in parameters assessed by flow cytometry is necessary to predict biologically significant consequences? Can investigators distinguish between statistically significant changes and biologically relevant changes?
3. Can investigators distinguish between treatment- or disease-associated changes in phenotypic distribution or activation status and stress-induced changes?

Relationship between changes in cell distribution or activation status and immune function

1. Are changes in parameters measured by flow cytometry indicative/predictive of changes in immune function? What uncertainties are associated with such predictions?
2. Are the implications of changes in parameters measured by flow cytometry viewed with the same confidence as are those associated with observed changes in a functional assay (e.g., the primary antibody response to SRBCs)?
3. Should flow cytometry be viewed more as a tool for segregating cells into defined populations that can be specifically evaluated in a functional context?
4. Do flow cytometric methods yield information that cannot be obtained by other methods/measures? Can flow cytometric methods be applied in parallel with other procedures (e.g., lymphoid tissue histopathology or functional assays) to provide a more robust measure of immune system status?

Relevance of flow cytometric methods for human health risk assessment

1. What limitations are associated with the application of clinical flow cytometry data to medical diagnosis, prognosis, and treatment decisions?
2. How might flow cytometry be used to assess human health risks associated with xenobiotics such as human and animal drugs, food contaminants and additives, consumer products, and agrochemicals and other commodity chemicals?
3. Can or should data obtained by flow cytometry be used to define NOAELs or other parameters used in human health risk assessment? Can such data be used to establish threshold levels?
4. What uncertainties (e.g., false positives and negatives, validation criteria) and data gaps are associated with the potential collection, presentation, and use of flow cytometric data for human health risk assessment?

**Table 2.** Lymphoid cell phenotypic markers

| Designation | Synonyms | Cellular Expression | Putative Function |
|---|---|---|---|
| CD2 | LFA-2, SRBCR | T cells, NK cells[a] | Adhesion molecule |
| CD3 | T3, OKT3 | T cells | Signal transduction |
| CD4 | Leu-4, L3T4 (in mice) | T helper cells | Binds MHC Class II molecules |
| CD5 | OX8 in rats | T cells, some B cells | Adhesion molecule(?) |
| CD8 | OX19 in rats | T cytotoxic/suppressor cells | Binds MHC Class I molecules |
| CD11a | S6F1 | Leukocytes | Adhesion molecule |
| CD11b | MAC-1 | NK cells, granulocytes, monocytes | iC3B receptor |
| CD16 | FcγR III | NK cells, granulocytes, macrophages | IgG Fc receptor |
| CD19 | B4 | Most B cells | Cell activation/ regulation(?) |
| CD20 | B1 | Most/all B cells | Cell activation/ regulation(?) |
| CD25 | IL-2 receptor α-chain | Activated T and B cells and macrophages | IL-2 receptor |
| CD28 | Tp 44 | Most CD4+ cells, some CD8+ cells | B7-1/B7-2 receptor |
| CD38 | T 10 | Plasma cells, activated T cells | Unknown |
| CD40 | gp39 receptor | B cells | T cell induced B cell activation |
| CD40L | CD40 ligand | T cells | Ligand for CD40 |
| CD44 | Pgp-1, Hermes, Lg-24 | Leukocytes | Homing receptor(?), receptor for matrix molecules; T cell activation |
| CD45R | B220 | B cells | |
| CD45RA | | Naive T cells | Signal transduction |
| CD45RB | | B cells, some T cells | Signal transduction |
| CD45RO | | T memory cells | Signal transduction |
| CD54 | ICAM-1 | Activated cells | Adhesion molecule |
| CD56 | Leu-19 | NK cells | Adhesion molecule |
| CD57 | HNK-1, Leu-7 | NK cells, some T cells | Unknown |
| CD71 | Transferrin receptor | Activate T and B cells, macrophages | Transferrin receptor |
| CD122 | IL-2 receptor β-chain | T cells, B cells, NK cells | IL-2 receptor |
| CD134 | OX40, Ly-70 | T cells | T cell/B cell interaction<br>T cell co-stimulation |
| TCR αβ | | T cells | T cell antigen receptor composed of α and β polypeptide chains |
| TCR γδ | | T cells | T cell antigen receptor composed of γ and δ polypeptide chains |
| TCR $V_\beta$ | | T cells | T cell antigen receptor β polypeptide chain, variable region |

[a] NK cells, natural killer cells; MHC, major histocompatibility complex; IL, interleukin.

**Table 3.** Roundtable discussion participants during the Application of Flow Cytometry to Immunotoxicity Testing workshop, October 9–10, 1997

| |
|---|
| Raymond E. Biagini, Centers for Disease Control and Prevention, USA |
| Scott W. Burchiel, The University of New Mexico, USA |
| Jack H. Dean, Sanofi Pharmaceuticals, Inc., USA, Roundtable Moderator |
| Dennis K. Flaherty, Monsanto Company, USA |
| Dr. Thomas A. Fleisher, Rapporteur, National Institutes of Health Clinical Center |
| Dori R. Germolec, National Institute of Environmental Health Sciences, USA |
| Paul E. Hurtubise, University of Cincinnati, USA |
| Nancy I. Kerkvliet, Oregon State University, USA |
| Gregory S. Ladics, E.I. du Pont De Nemours & Company, USA |
| Lawrence S. Lamb, University of South Carolina, USA |
| Gerald E. Marti, FDA Center for Biologics Evaluation and Research, USA |
| Janet K. Nicholson, Centers for Disease Control and Prevention, USA |
| Hans D. Ochs, University of Washington School of Medicine, USA |
| Sheryl Reilly, Environmental Protection Agency, USA |
| Henk Van Loveren, National Institute of Public Health and Environmental Protection, The Netherlands |
| Robert Vogt, Centers for Disease Control and Prevention, USA |
| James L. Weaver, FDA Center for Drug Evaluation and Research, USA |
| Kimber L. White, Jr., Medical College of Virginia, USA |

# APPENDIX 1. WORKSHOP PROGRAM

## Thursday, October 9, 1997
## Clinical and Experimental Applications of Flow Cytometry

| | |
|---|---|
| 7:30 - 8:30 | **Registration and Coffee**<br>New Hampshire Foyer |
| 8:30 - 8:50 | ***Welcome and Introductions***<br>Dr. Jack H. Dean, Sanofi Pharmaceuticals, Inc. |

### SESSION 1: USE OF FLOW CYTOMETRY IN ASSESSING HUMAN IMMUNE STATUS

| | |
|---|---|
| 8:50 - 9:00 | ***Opening Remarks***<br>Dr. Jack H. Dean, Sanofi Pharmaceuticals, Inc. |
| 9:00 - 9:30 | ***HIV/AIDS***<br>Dr. Janet K. Nicholson, Centers for Disease Control and Prevention |
| 9:30 - 10:00 | ***Primary Immunodeficiencies***<br>Dr. Hans D. Ochs, University of Washington School of Medicine |
| 10:00 - 10:30 | ***Transplantation***<br>Dr. Lawrence S. Lamb, Richland Memorial Hospital |
| 10:30 - 11:00 | **Break**<br>New Hampshire Foyer |
| 11:00 - 11:30 | ***Activation Markers***<br>Dr. Paul E. Hurtubise, University of Cincinnati |

### SESSION 2: HUMAN AND ANIMAL STUDIES ON DRUGS/CHEMICALS

| | |
|---|---|
| 11:30 - 11:50 | ***Opening Remarks***<br>Dr. Jack H. Dean, Sanofi Pharmaceuticals, Inc. |
| 11:40 - 12:10 | ***Dioxin***<br>Dr. Nancy I. Kerkvliet, Oregon State University |
| 12:10 - 1:30 | **Lunch**<br>City Centre I & II |
| 1:30 - 2:00 | ***Dideoxyinosine***<br>Dr. Dori R. Germolec, National Institute of Environmental Health Sciences |
| 2:00 - 2:30 | ***Lead***<br>Dr. Raymond E. Biagini, National Institute of Occupational Health and Safety |

### SESSION 3: FLOW CYTOMETRY FOR HAZARD IDENTIFICATION AND RISK ASSESSMENT

| | |
|---|---|
| 2:30 - 2:40 | ***Opening Remarks***<br>Dr. Jack H. Dean, Sanofi Pharmaceuticals, Inc. |
| 2:40 - 3:10 | ***Correlates with Functional Changes***<br>Dr. Kimber L. White, Jr., Medical College of Virginia |

| | |
|---|---|
| 3:10 - 3:40 | **Break** |
| 3:40 - 4:10 | ***Evaluation of Methods for Preclinical Studies***<br>Dr. Gregory Scott Ladics, E.I. du Pont de Nemours & Co. |
| 4:10 - 4:40 | ***Applications for Epidemiologic Studies***<br>Dr. Robert Vogt, Centers for Disease Control and Prevention |
| 4:40 - 5:00 | ***Facilitator's Overview and "Thought Questions" for Roundtable Discussions***<br>Dr. Jack H. Dean, Sanofi Pharmaceuticals, Inc. |
| 5:15 - 6:45 | **Reception**<br>City Centre I & II |

## Friday, October 10, 1997
## Relevance of Flow Cytometry to Human Risk Assessment

| | |
|---|---|
| 7:30 - 8:30 | **Registration and Coffee**<br>New Hampshire Foyer |
| 8:30 - 8:50 | ***Opening Remarks***<br>Dr. Jack H. Dean, Sanofi Pharmaceuticals, Inc. |
| 8:50 - 10:00 | ***Rapporteur Discussion***<br>Dr. Scott W. Burchiel - The University of New Mexico<br>Dr. Dennis K. Flaherty - Monsanto Company<br>Dr. Thomas A. Fleisher - National Institutes of Health<br>Dr. Sheryl Reilly - U.S. Environmental Protection Agency<br>Dr. Gerald E. Marti - U.S. FDA, Center for Biologics Evaluation and Research<br>Dr. Henk Van Loveren - National Institute of Public Health and Environmental Protection, The Netherlands<br>Dr. James L. Weaver - U.S. FDA, Center for Drugs Evaluation and Research |
| 10:00 - 10:30 | **Break**<br>New Hampshire Ballroom Foyer |
| 10:30 - 12:00 | ***Roundtable Discussion with Presenters and Rapporteurs***<br>*Topics of Discussion May Include:*<br>Use of Flow Cytometry as a Predictive Tool<br>Statistical vs. Biologic Significance of Flow Cytometry<br>Changes in Cell Populations<br>Correlations with Functional Changes<br>Predictive Value of Flow Cytometry for Human Risk Assessment<br>Data Gaps and Prioritization of Research Needs<br>Clinical vs. Experimental<br>Phenotypic vs. Activation Marker |
| 12:00 - 1:30 | **Lunch**<br>City Centre I & II |
| 1:30 - 3:30 | ***Roundtable Discussion with Presenters and Rapporteurs Continued*** |
| 3:30 - 4:00 | ***Facilitator's Summary of Consensus Statements and Remaining Issues***<br>Dr. Jack H. Dean, Sanofi Pharmaceuticals, Inc. |
| 4:00 | **Adjournment** |

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