Appendix 5 Guidelines Of The International Programme On Chemical Safety (IPCS) On The Use Of Compound Specific Adjustment Factors (CSAFs)

All the information in this summary was excerpted from a same titled paper (Meek et al. 2001) published in Comments on Toxicology.

Tolerable intakes or concentrations are based on approximation of "subthreshold" (i.e., considered "safe") concentrations through division of a no-observed (adverse) effect level (NOAEL) or benchmark dose/concentration (i.e., effects without measurable or small responses) for critical effects most often in animals by an uncertainty factor. This NOAEL or benchmark dose/concentration is considered to be at or below the threshold in animals; uncertainty factors are applied to estimate the subthreshold in sensitive human populations. The factor is generally the product of two 10-fold factors, one for interspecies differences (i.e., the variation in response between animals and a representative healthy human population) and one for inter-individual variability in humans (variation in response between a representative healthy human population and sensitive subgroups) (IPCS 1994).

Chemical Specific Adjustment Factors (CSAFs) are considered to represent part of a broader continuum of increasingly data-informed approaches to account for inter-species differences and human variability, which range from default ("presumed protective") to more "biologically-based predictive" (Meek, 1999). Depending upon the data available, these approaches range from simple incorporation of default values for inter-species differences and human variability (often 10 x 10) to more refined approaches. Additional compound-related data permit replacement of components of inter-species or inter-individual variation with chemical-specific adjustments including those derived from physiologically-based pharmacokinetic (PBPK) models.

Renwick (1993) analyzed data on inter-species differences and human variability in toxicokinetics and toxicodynamics for a limited number of compounds as a basis for subdivision of these 10 fold default factors to address kinetics and dynamics separately. Subdivision of the 10-fold uncertainty factors was based primarily on data for pharmacokinetic parameters such as clearance (CL) and area under the concentration-time curve (AUC). The dynamic data for humans were based on pharmacokinetic-pharmacodynamic (PK-PD) modeling of a range of pharmacological and therapeutic responses, in which the inter-individual variability in response was corrected for any inter-individual variability in kinetics by the application of the model to the individual data.

Based on this analysis, it was proposed that each of the 10-fold default factors could be subdivided into factors of 4 ($10^{0.6}$) for toxicokinetics and 2.5 ($10^{0.4}$) for toxicodynamics. The limited data analyzed (Renwick, 1993) indicated greater potential variability within humans in kinetics than in dynamics, so that a larger factor was suggested for kinetic variability. It was considered that the database analyzed by Renwick (1993) was insufficient to justify an uneven subdivision of the 10-fold factor for human variability,

and therefore this factor was divided evenly into two sub-factors each of $10^{0.5}$ (3.16 or 3.2).

The CSAF (IPCS 1994) to address kinetics and dynamics separately collapses back to the usual 100-fold factor in the absence of appropriate data, but allows the incorporation of quantitative chemical-specific data, relating to either toxicokinetics or toxicodynamics, to replace part of the usual 100-fold uncertainty factor. The composite chemical specific adjustment/uncertainty factor (CCSAUF) as a data-derived uncertainty factor applied to the NOAEL or benchmark dose/concentration is the composite of the chemical-specific factors and any remaining defaults for which appropriate data were not available. The total factor could be either greater than or less than 100-fold, depending on the quantitative scientific data that has been introduced to replace defaults.

The compound specific adjustment factor for animal to human toxicokinetic differences [AK_{AF}] is a ratio in humans and animals of a measurable metric for internal exposure to the active compound (e.g., AUC, Cmax, clearance). This is generally determined on the basis of comparison of outcome of *in vivo* kinetic studies with the active compound both in animals and a representative sample of the healthy human population. The ratio reflecting the internal dose or target organ is expressed in the form of human/animal, correcting the kinetic parameter as appropriate or when necessary to reflect internal exposure (eg 1/clearance), because AUC and clearance are inversely related (CL = dose/AUC).

The compound specific adjustment factor for animal to human toxicodynamics differences [AD_{AF}] is a ratio of the dose/concentration which induces the critical toxic effect or a measurable related response in animals and a representative sample of the healthy human population. Data that inform this adjustment factor are those that define relative target site sensitivity directly, without any toxicokinetic influences. They include kinetic dynamic link models but more often, are based on comparative effects of the active compound in animal and human tissues *in vitro*. At its simplest, the replacement to the default factor for inter-species differences in dynamics is the ratio of the effective doses in animal versus human tissues (e.g., ED_{10} values).

The adjustment factor for human toxicokinetic variability [HK_{AF}] could potentially be addressed on the basis of *in vivo* kinetic studies in a sufficiently broad range of subgroups of healthy and potentially susceptible populations to adequately define the population distribution. However, since this may not be practicable or even possible, more often, factors responsible for the clearance mechanisms are identified (eg renal clearance, CYP-specific metabolism, etc). and a chemical-related adjustment factor derived based on known human variability in the relevant physiological and biochemical parameters. Alternatively, this variability including that in potentially susceptible sub-groups, is modeled by a PBPK model based on knowledge of variability in biochemical and physiological parameters relevant to the fate of the chemical.

The population distribution for the relevant metric (e.g., AUC, Cmax, renal clearance) for the active entity is analyzed and the CSAF (HK_{AF}) calculated as the difference between the central values for the main group and given percentiles (such as 95th, 97.5th and 99th)

for the whole population. These differences are analyzed separately for any potentially susceptible sub-group. The ratio reflecting the internal dose or target organ exposure is expressed in the form of sensitive human/average human, correcting the kinetic parameter as appropriate or when necessary to reflect internal exposure (eg 1/clearance).

In the rare cases where there are adequate data to define dose/concentration response in sensitive subgroups of the human population, the relevant effect level or benchmark dose/concentration is used directly without additional need for adjustment for either interspecies differences or human variability (e.g., nitrate) (IRIS 1991). However, where there are adequate *in vivo* data in healthy adult humans only, or where data on dose/concentration response are available only from studies in animals, there is a need for adjustment to allow for potentially susceptible subpopulations. As for AD_{AF}, data that inform the human variability toxicodynamic adjustment factor [HD_{AF}] are those that define relative target site sensitivity directly, without any toxicokinetic influences. They include kinetic dynamic link models, but more often, are based on comparative (either the critical or related) effects of the active compound in human tissues from healthy versus sensitive subgroups *in vitro*. At its simplest, the replacement to the default factor based on *in vitro* data on response in healthy human and susceptible subpopulations is the ratio of the dose/concentration which induces a specified measurable response in average versus sensitive humans (e.g., the ED_{10}).

Composite chemical-specific adjustment/uncertainty factor for interspecies differences and human variability is the product of above mentioned four different factors, each of which could be a chemical-specific adjustment factor or a default, as follows (UF = default uncertainty factor):

$$CCSAUF = [AK_{AF} \text{ or } AK_{UF}] \times [AD_{AF} \text{ or } AD_{UF}] \times [HK_{AF} \text{ or } HK_{UF}] \times [HD_{AF} \text{ or } HD_{UF}]$$

The extent to which CCSAUF differs from the normal default is dependent, therefore, on the amount of adequate and relevant quantitative data available on the chemical. If no such data are available the factor will be the normal default value. CCSAUFS should be developed for several effects which might be considered critical to ensure that resulting tolerable, acceptable or reference intakes/concentrations are sufficiently protective.

If the CCSAUF for a potentially critical effect is less than the normal default, then outcome must be considered in the context of different toxic effects detected at higher doses, for which there are not relevant toxicokinetic or toxicodynamic data. Under such circumstances, application of normal default to the effect levels may result in lower tolerable, acceptable or reference doses/intakes/concentrations. These latter effects are then critical as the basis for protective measures of dose/concentration response.

Appendix 6. Assumptions Used In This Evaluation

Sufficient data are available on the toxicity of perchlorate in order to define a RfD, RfC or MOE based on our understanding of the underlying US EPA methods (Barnes and Dourson, 1988; Dourson, 1994; US EPA, 1994; Jarabek, 1994, 1995). Standard assumptions in our evaluation of the proposed noncancer risk values by US EPA include.

- The use of experimental animal data as a surrogate for humans, or the use of data gather in average groups of humans to extrapolate the likely effects (or lack of effects) in sensitive human subgroups,

- The use of T4 serum decrease in dogs, rats or other experimental animal species for extrapolating to human disease,

- The extrapolation of experimental doses in either rats or dogs to humans by division by a 10-fold uncertainty factor, if appropriate, or by the use of data to replace this default uncertainty factor when available,

- The use of a 10-fold default uncertainty factor for extrapolating the results from an average group of humans to that expected in a sensitive subgroup, or by the use of data to replace this default uncertainty factor when available,

- The use of factors based on a logarithmic scale (10, 3 or 1) with the RfD that address additional scientific uncertainties in the overall data base,

- The use of 1 digit of arithmetic precision to the RfD, RfC or MOE (generally) because our understanding of the underlying biology is unlikely to be more precise than this.

The use of these and similar assumptions is common practice in conducting dose response assessments by other environmental and health agencies throughout the world (Meek et al, 1994; IPCS, 1994; Pohl and Abadin, 1995; Rademaker and Linders, 1994).

Appendix 7. EVALUATION OF THE EPA's ASSESSMENT OF THE IMMUNOTOXICITY STUDIES FOR AMMONIUM PERCHLORATE

REVIEWER: Norbert E. Kaminski, Ph.D.
Professor
Department of Pharmacology & Toxicology
College of Human Medicine
Michigan State University
East Lansing, MI 48824

Tel (517) 353-3786
FAX (517) 432-3218
e-mail kamins11@msu.edu

DATE: February 15, 2002

A. GENERAL COMMENTS: The primary sources of immunotoxicity data utilized in the EPA's perchlorate risk assessment report where studies performed in the laboratories of Dr. Deborah Keil and BRT-Burleson Research Technologies, Inc. Collectively, the studies performed by the two laboratories represent a broad-based evaluation of the effects of perchlorate on innate, humoral and cell-mediated immune functions in mice. The assays utilized by the two laboratories are widely accepted and routinely employed by immunotoxicologists for evaluating the immunotoxicity of xenobiotics. Although technical problems and issues were raised primarily with several of the experiments performed in the Keil laboratory, for the most part these issues were corrected through the replication of those experiments and/or by statistical reanalysis of the data sets that were in question. An additional issue, concerns one study performed by BRT-Burleson Research Technologies, Inc in which the positive control in a local lymph node assay (LLNA) produced no effect.

B. SPECIFIC COMMENTS: In this section, comments are provide on the interpretation of the results from the immunotoxicologic evaluation discussed in section 5.6 of the EPA's perchlorate risk assessment report.

1. General Toxicology, Organ Weight and Cellularity Measurements: Collectively, the Keil studies showed no signs of general toxicity or consistent changes in organ weight or cellularity by perchlorate treatment in the 14, 90 or 120-day studies.

2. Immune Function Assays: As discussed in the GENERAL COMMENTS section above, a comprehensive assessment of the effects of perchlorate on innate, humoral and cell-mediated immune functions was performed in mice. No consistent alterations were observed in any of the immune function assays with the exception of *in vitro* macrophage phagocytosis of *L. monocytogenes*, the anti-sRBC IgM PFC assay and LLNA. Therefore

the comments in this section will principally focus on results obtained from studies assessing the effects of ammonium perchlorate on these three immune parameters.

• ***In vitro* macrophage phagocytosis of *L. monocytogenes*:** In the "Final Report", Keil reported that perchlorate decreased phagocytosis of *L. monocytogenes* at 1.0 and 30 mg/kg-day in the 14-day study and at 0.1, 1.0, 3.0 and 30 mg/kg-day in the 90-day study. Moreover, after a 30-day recovery period following 90 days of treatment, phagocytic function was comparable to the controls at all dose levels. I concur with the interpretation that the data suggests that ammonium perchlorate suppressed phagocytic capacity of peritoneal macrophages in the *ex vivo studies* at the doses employed in the above two studies. In addition, the recovery study (120-day) demonstrates that the inhibition of phagocytosis at the treatment doses employed in the study is reversed after 30 days. The conclusion from the recovery study on page 5-100 that "this suppression may be reversed after a 30-day recovery" is curious in light of the fact that complete recovery was observed at all perchlorate dose levels. The interpretation from these studies is modified in the RESULTS SUMMARY on page 5-106 stating that the data show that *in vitro* phagocytic capacity "is" reversed following a 30-day recovery period. I also concur with the criticism raised by Dr. White (page 5-100 line 22) that assessment of macrophage function *in vivo* would have been more appropriate than the *in vitro* assay employed by Keil and coworkers.

The overall interpretation of the *in vitro* phagocytosis studies in the RESULTS SUMMARY section is balanced with the findings being interpreted in the context of the results obtained from the *L. monocytogenes* host resistance studies. Specifically, it is noted that although perchlorate treatment inhibited phagocytosis of *L. monocytogenes in vitro*, perchlorate treatment did not adversely affect host resistance to *L. monocytogenes* challenge. In this reviewers opinion, collectively these data demonstrate that the inhibition of phagocytic activity is not biologically significant since perchlorate treatment did not adversely affect host resistance to *L. monocytogenes* as well as a number of other immune function responses requiring phagocytosis of antigen in the immunotoxicology testing battery.

• **Anti-IgM sRBC PFC assay** - BRT-Burleson Research Technologies, Inc reported that perchlorate treatment in the 14-day study produced no significant differences for control at any of the treatment doses. Conversely, in the 90-day study, perchlorate increased the PFC response in the 2.0 and 50 mg/kg-day groups when expressed as PFC/spleen and increased only in the 50 mg/kg-day group when expressed as PFC/10^6 spleen cells. In contrast, Keil demonstrated no increase in serum anti-sRBC IgM or anti-sRBC IgG due to perchlorate treatment in either the 14-day and 90-day study at doses as high 30 mg/kg. In the RESULTS SUMMARY section (page 5-106) it is stated that the ELISA data "do not corroborate" the enhancement observed in the sRBC PFC assay. It is important to emphasize that although both assays are measurements of humoral immunity, each provides different information. The PFC response enumerates the number of B cells driven by antigen (sRBC) to differentiate into IgM secreting plasma cells. Conversely, the anti-sRBC IgM and IgG ELISA measures the total amount of antigen-specific (anti-sRBC) immunoglobulin that is being produced by the

differentiated plasma cells. Collectively, the results from the two assay systems suggest that although perchlorate treatment enhanced the number of B cells that differentiated into antibody secreting plasma cells, there was no significant increase in the total amount of anti-sRBC IgM or IgG being produced as compared to the controls. Therefore, it is unlikely that the increase in the PFC response is an adverse effect. Moreover, as stated on page 5-107, the studies demonstrate that perchlorate treatment does not suppress the humoral immune response to sRBC. Lastly, I concur with the statement that the studies do suggest that under the dosing conditions utilized in the PFC assay, ammonium perchlorate may have adjuvant-like or immune enhancing activity.

- **Local Lymph Node Assay (LLNA)** - BRT-Burleson Research Technologies, Inc reported an increase in the LLNA response to the contact sensitizer DNCB in the 14-day and 90-day perchlorate study. In the 14-day study, ammonium perchlorate at 0.06, 0.2, and 50.0 mg/kg-day, but not at 2.0 mg/kg-day, increased the LLNA response to DNCB. In the 90-day study, ammonium perchlorate increased the LLNA response to DNCB at 0.6 and 2.0 mg/kg-day and suppressed the response at 50 mg/kg-day. It is notable that in the 90-day study, the cyclophosphamide control did not suppress the LLNA response.

In this reviewer's opinion, if the objective of the LLNA study was to determine whether ammonium perchlorate is a dermal contact sensitizer, which seems logical based on clinical human data indicating that some patients treated with potassium perchlorate for Grave's disease developed a skin rash, there is a significant flaw with the experimental design as performed by BRT-Burleson Research Technologies, Inc. Specifically, the critical treatment group(s) not included in the study would be mice sensitized and challenged with ammonium perchlorate by dermal treatment in the absence of DNCB. It is important to emphasize that enhancement of the LLNA response to DNCB by ammonium perchlorate does not provide information whether perchlorate is a contact sensitizer. In fact the EPA's Health Effects Test Guidelines, OPPTS 870.2600 Skin Sensitization, state on page 4 that the approved methods for assessing whether an agent is a dermal contact sensitizer requires either "topical administration" or "intradermal injection" of the test agent. In the absence of a treatment group where mice were sensitized and challenged with perchlorate by dermal treatment, a conclusion can not be drawn whether perchlorate is or is not a dermal contact sensitizer. Therefore, the existing study design measuring the ability of ammonium perchlorate to modulate the DNCB-mediated LLNA response, is primarily assessing whether perchlorate exerts an effect on cell mediated immunity. The LLNA results combined with the anti-sRBC IgM PFC response suggest that ammonium perchlorate, under certain dosing regimens, may possess adjuvant-like or immune enhancing activity. Additional evidence supporting the conclusion that perchlorate may enhance cell-mediated immune responses is the observed enhancement of the DTH response to soluble Listeria antigen (SLA) at 30 mg/kg-day in the 14-day (Table 31; Keil Final Report) and 90-day (Table 32; Keil Final Report) studies. It is important to emphasize that based on the studies presented in the EPA's perchlorate risk assessment report, there is no compelling experimental evidence to support the suggestion in the EXECUTIVE SUMMARY (page E-9 line19) that perchlorate is a dermal contact sensitizer.

C. RESPONSE TO SPECIFIC QUESTIONS POSED BY TERA CONCERNING EPA's PERCHLORATE RISK ASSESSMENT REPORT

Evaluate the adequacy of the EPA's assessment of the immunotoxicology studies. As summarized above, the immunotoxicity assessment of perchlorate was comprehensive employing well-established assays. For the most part the studies appear to be of good technical quality and designed appropriately with the exception of the LLNA studies. Specifically, if the intent of the LLNA was to determine whether perchlorate is a dermal contact sensitizer, the experimental design utilized was flawed.

Did the EPA completely characterize the findings of the studies? Yes with the exception of the LLNA studies. As discussed, in my opinion the LLNA studies should be repeated using the appropriate experimental design.

Were there aspects of the EPA's assessment that you agree with or conversely with which you particularly disagree? For the most part I believe the immunotoxicology studies were interpreted in an appropriate and balanced manner with the exception of several issues primarily pertaining to the LLNA discussed above.

Where the battery of immunotoxicology tests complete enough to adequately characterize the potential for perchlorate to cause immunotoxicity? The battery of immunotoxicity tests used to evaluate perchlorate was comprehensive and should have provided a definitive characterization of the potential for perchlorate to cause immunotoxicity. However, due to the inappropriate LLNA study design, the one immunotoxic sequelea potentially produced by perchlorate that was anecdotally suggested by human clinical date, dermal contact sensitization, was not adequately examined. In my view, this is a major shortcoming in the immunotoxicology assessment of perchlorate.

Where any critical tests missing? Yes, the LLNA should be repeated with the appropriate experimental design to assess whether perchlorate is a dermal contact sensitizer.

Were the tests that were done predictive of potential immunotoxicity in people? The battery of assay utilized for evaluating the potential immunotoxicity of perchlorate in mice by the Keil and BRT-Burleson Research Technologies, Inc. laboratories are widely believe to be predictive of immunotoxicity in people.

In your opinion, what was the biological significance of the results of the immunotoxicology tests? With the exception of the *in vitro* macrophage phagocytosis of *L. monocytogenes*, the anti-sRBC IgM PFC assay, enhancement of the DTH response to SLA (30 mg/kg-day only) and LLNA, no consistent alterations attributable to perchlorate treatment were observed in any of the immune function assays as compared to the appropriate control groups. Based on the fact that none of the immune function responses, with the exception of 50 mg/kg-day in the 90 day LLNA study were inhibited and many of the responses are critically dependent on phagocytosis of antigen by

macrophages, it is unlikely that the impairment of phagocytic activity observed in the *in vitro* phagocytic assay is biologically significant. The results from the anti-sRBC IgM PFC assay, LLNA and DTH response to SLA suggest that perchlorate under certain exposure regimens – most likely extended exposure (i.e., 90 days) – possesses adjuvant-like or immune enhancing activity. Although immune enhancement as immune suppression can be an adverse effect, in light of the fact that neither enhancement nor immune suppression was observed in virtually any of the other immune function assays, the biological significance of the immune enhancement in the sRBC PFC assay, LLNA and DTH response to SLA is questionable. Moreover, there was neither a significant increase in serum anti-sRBC IgM or IgG detected due to perchlorate treatment nor an increase in spleen cellularity, which further bring into question whether the enhanced anti-sRBC PFC response is biologically significant. Enhancement of the DTH response to SLA was only observed at the 30 mg/kg-day dose. Lastly, it is notable that the experimental design employed for the LLNA can not ascertain whether perchlorate is a dermal contact sensitizer. Based on the available data sets, there is no experimental evidence to suggest that perchlorate is a dermal contact sensitizer.

Were any of the findings adverse? As stated above both immune suppression and immune enhancement can be adverse effects. Immune suppression can increase the host's susceptibility to microbial pathogens and cancer. Likewise, immune enhancement can, under certain instances, lead to hypersensitivity and autoimmunity. In light of the absence of immune inhibition observed throughout the battery of immune function assays, many of which are critically dependent on phagocytosis of antigen by macrophages, I do not believe that the inhibition observed in the *in vitro* phagocytosis assay is an adverse effect. Likewise, based on the absence of an increase by perchlorate treatment in serum anti-sRBC IgM or IgG, I do not believe the increase in the sRBC PFC assay alone constitutes an adverse effect. However, as a follow-up to the increase in plasma cell formation by perchlorate treatment observed in the anti-sRBC PFC response, additional measurements of serum IgE would be appropriate to further assess the potential for an adverse effect. Lastly, the enhancement of the LLNA response, as performed, in my opinion does not constitute an adverse effect and should be performed again utilizing an appropriate experimental design.

EPA added an uncertainty factor of 3 to account for the incomplete characterization of immunotoxicity. Essentially this means that the EPA felt that some key immunotoxicity data was not collected, and if these studies were done, would affect the decision of what a dose would be. Do you agree with this finding? From my reading of the EPA's perchlorate risk assessment report, I believe that the EPA recognizes that the LLNA, as performed by BRT-Burleson Research Technologies, Inc. utilized a flawed experimental design that precluded a definitive conclusion on whether perchlorate is a dermal contact sensitizer. I believe this is evident from the statement in the Executive Summary on page E-10 lines 9-10 - "Finally, a three fold factor was applied for inaccurate characterization of immunotoxicity since recent studies reinforce concern for this endpoint". This point is similarly expressed on page 7-31 lines 28-29 – "Accurate characterization of the immunotoxicity of perchlorate, notably its potential to cause contact hypersensitivity, either secondarily to these hormone effects or possibly via a

direct effect of the anion itself, remains". To resolve this concern, I recommend repeating the LLNA, using an experimental design that would genuinely evaluate whether perchlorate is a skin sensitizer. Collectively and in the absence of additional data, there is no evidence to suggest that perchlorate is a dermal contact sensitizer, especially at doses of 0.01 mg/kg-day. In light of the absence of any observable effects on the immune system in any of the other immune function assays at doses of 0.01 mg/kg-day, addition of a 3-fold uncertainty factor due to an "incomplete characterization" of immunotoxicity in this reviewer's opinion is excessive.

D. CONCLUDING COMMENTS CONCERNING THE IMMUNOLOGICAL EFFECTS OF PERCHLORATE: In this reviewer's opinion, based on the immunotoxicity data presented in the EPA's perchlorate risk assessment report, perchlorate, under certain treatment conditions produces modest adjuvant-like or immune enhancing activity in mice as suggested by an increase in the anti-sRBC PFC assay, DNCB-induced LLNA response and DTH response to SLA. Moreover, based on the profile of activity produced by perchlorate, the immune enhancing activity is likely mediated through direct or secondary effects on T cells.

Comments on EPA Reviw of AP Immunotoxicity Studies Conducted by Burleson Research Technologies (BRT) and Keil *et al.*, 1999

FEBRUARY 18, 2002

ATTN: Joan Dollarhide, TERA.

FROM:Deborah Keil, Ph.D., MT (ASCP)
Associate Professor
Department of Health Professions, College of Health Professions
77 President Street, Suite 324, P.O. Box 250702
Medical University of South Carolina, Charleston, SC 29425-5712
Phone (843) 792-3169; Fax (843) 792-3383

Based on the battery of immunological tests performed between these two studies, minimal effects on overall immunity were observed as concluded in studies by BRT and the final report by Keil *et al.*, 1999. Of the notable effects observed in both studies, macrophages, natural killer activity (NK), humoral immune responses (PFC), and contact sensitization were significantly altered. I do not have expertise in the area of contact hypersensitivity and could not adequately address this parameter. However, I have reviewed the comments by EPA regarding the immunosuppression data.

To begin with, the EPA's review of the immunotoxicity data includes a discussion of the interim report that was submitted in 1998 by Keil *et al.* In fact, EPA comments more on the interim report than the final report submitted from this immunotoxicological study. The final report is the project in its entirety and should be the primary source consulted for the review of AP immunotoxicity by Keil *et al.* At the very least, the final report is certainly less confusing, than the interim report. Furthermore, the authors of this interim report requested that it would not be cited, nor quoted. In fact, the label of, "Do not cite or quote", appears on each page of this report. As demonstrated in the current EPA report of January 16, 2002, this request was not respected.

The interim report was indeed interim, and incomplete. Whereas, the final report was complete and accounted for all experiments performed and any technical issues associated with the study. Keil* et al., *1999 should be used as the primary source for the evaluation of immunotoxicity caused by exposure to AP. The final report is a true and factual reflection of the study in its entirety, and I would personally meet any requests to verify this.

Regarding the statistical analysis of these immunotoxicity data in the final report, the EPA describes this correctly. During the compilation of the final report, all data were reviewed from the entire study. Where possible, experiments were combined to provide more power for statistical analysis. As a result, this process may have contributed to some variations between the interim report and the final report.

In regards to the immunotoxicity endpoints, macrophage phagocytic function was consistently and dose-responsively suppressed after exposure to AP. The EPA affirmed this in their review. I agree with the EPA in that this deficit seems to have limited impact on overall immunity, as the response in the listeria challenge model did not reflect any deficit in disease resistance.

On a less important matter, I do not agree with the EPA's use of " *in vitro* " to describe the macrophage phagocytosis assay. The use of this term does not accurately describe this parameter. It is true that the testing for phagocytic function was performed in a 16-well chamber slide; however, the peritoneal macrophages were obtained from AP-exposed mice. Thus, the exposure to AP was *in vivo* and correspondingly, the deficits observed in this functional assay were due to alterations that occurred *in vivo*. Perhaps the use of the term a "*ex vivo*" may offer an improved description for this macrophage evaluation.

In the table listed on page 5-10 of the executive summary, the study by Keil *et al.*, 1999 is described to have used male mice. This is not correct as female B6C3F1 mice were used throughout the entire study.

The EPA mentions that the data from the listeria 'high dose' challenge model (5360 CFU) is not useable. It is true that spleen counts were not available, however, usable information was gathered from liver counts that indicated that immunosuppression was not likely, even with such an increased bacterial challenge.

In mice exposed to 30 mg/kg/day for 90 days, NK cytolytic activity was elevated and sustained 30 days post AP exposure. The EPA mentions this effect briefly in their review and do not include it as a major finding. I would agree that enhanced NK function is not likely to be a considerable finding, and certainly does not reflect immunosuppression. This is corroborated by the fact that resistance to both B16F10 tumor and listeria challenge were not significantly altered, as NK activity is integral to both challenge models.

Based on the interim report by Keil *et al.*, 1998, EPA concluded that there was variability in the control values of the NK assay. This is true for the interim report. However, the final report by Keil *et al.*, 1999 is more thorough and includes repeated experiments assessing NK activity. In the final report, NK control values from experiments performed on different days are of acceptable variability and range from 17 to 30 lytic units per 10^7 splenocytes. I am confident to report that NK data is reliable and the lack of suppression in this endpoint was corroborated in additional studies performed in my laboratory (Fig. 1).

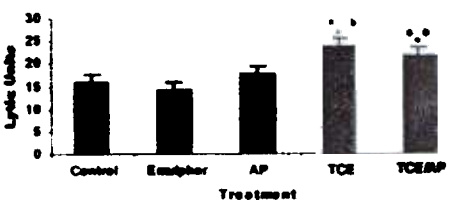

Figure 1. Preliminary and unpublished data recently collected corroborates that AP only at 1mg/kg/day for 90 days does not significantly alter NK activity. However, when drinking water contains both 1.0 mg/kg of AP with 1000 ppb of TCE,

significant enhancement of function is noted. Control is the water only control. Emulphor is the vehicle control used to dissolve TCE in water.

In the matter of EPA's review of the CTL responses, it is simply described in the final report submitted by Keil *et al.*, 1999 that indicates no significant differences in this parameter. This is also corroborated by the fact that resistance to B16F10 tumor challenge was not significantly altered.

I disagree with the EPA, in that the study by Keil *et al.*, 1999, did indeed measure humoral immunity. Specific IgM and IgG responses to SRBC antigen were detected with an ELISA procedure. Generally, this assay agrees with the PFC assay, however, differences can be attributed to the sensitivity of the procedures and the SRBC immunizing dose. Recently in my laboratory, the PFC assay was performed on mice exposed to 1.0 mg/kg/day of AP for 90 days. These results were consistent with those reported by BRT and Keil *et al.*, 1999 in that no significant alterations were detected in the PFC response at this dose level range.

In the BRT study, humoral immune responses were significantly elevated in the treatment groups of 2.0 and 50 mg/kg/day after 90 days of AP exposure. It was also noted that BRT used twice the SRBC immunizing dose (2×10^8) than used in the study by Keil *et al.*, 1999 (1×10^8). As demonstrated frequently in host challenge models, different levels of immunization doses can often impact the immunological response. This was also apparent by the dose response data for SRBC immunization included in the BRT report, as immune response was altered depending on the challenge level. This factor may account in part, for the disagreement between Keil *et al.*, 1999 that reports no effect in specific IgM and IgG antibody levels at 3.0 and 30 mg/kg/day of AP.

Collectively, it is clear that these studies demonstrate that AP does not suppress humoral immunity. In the matter that elevated immunoglobulin production might contribute to an autoimmune state, I would add that the Keil *et al.*, 1999 reports that anti-nuclear antibody screening was negative in mice exposed for 14 or 90 days to AP. This parameter was not included in the EPA's review of these data.

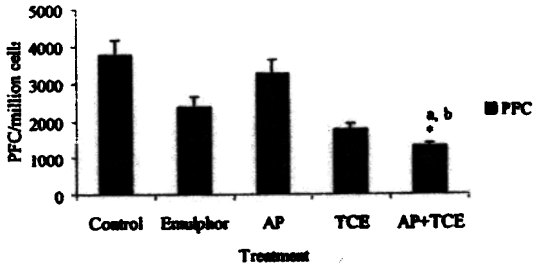

Figure 1. Preliminary and unpublished data recently collected corroborates that AP only at 1mg/kg/day for 90 days does not significantly alter PFC humoral responses to SRBC. This is consistent with the reported ELISA results in the final report of Keil *et al.*, 1999. However, when drinking water contains both 1.0 mg/kg of AP with 1000 ppb of TCE, significant suppression of function is noted. Control is the water only control. Emulphor is the vehicle control used to dissolve TCE in water.

Based on the AP immunotoxicity data submitted in the final report of Keil *et al.*, 1999 and that of BRT, I would reason that AP is generally not immunotoxic. This is based on the overview of all data from the Keil and BRT studies, with the exception of the contact hypersensitivity data. I do not have adequate expertise in the study of contact hypersensitivity to adequately address this parameter.

A future consideration in the risk assessment of AP may include interactions with other chemicals in drinking water. EPA has not considered this in their review. However, preliminary data from this laboratory suggest that this should be a future consideration.