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Re:- Trichloroethylene: Request for Time for a Presentation at the Meeting of the Board of Scientific Counselors Report on Carcinogens Subcommittee

Dear Dr Wolfe:

With this letter I am requesting time to give a presentation during the session on trichloroethylene that is expected to take place on December 13 at the meeting of the Board of Scientific Counselors RoC Subcommittee.

Included with this e-mail as attachments, and as part of the facsimile submission, you will find comments by Dr J. Gnarra and another by Professors Adami and Trichopoulos sponsored by HSIA. None of the authors will be available to speak on December 13 because of prior engagements. I will refer to these documents in my talk and I would be grateful if they can be distributed to the Board. I will cover additional topics and will provide a summary of my talk on the day.

Thank you for your assistance.

Yours sincerely,



Paul H. Dugard, PhD
Director of Scientific Programs

**TRICHLOROETHYLENE and THE VHL TUMOR SUPPRESSOR GENE:
COMMENTS FOR CONSIDERATION BY
THE NTP BOARD OF SCIENTIFIC COUNSELORS REPORT ON
CARCINOGENS SUBCOMMITTEE**

**by JAMES R. GNARRA, PhD
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This commentary is written in response to the Report on Carcinogens Background Document for Trichloroethylene to be discussed at the Meeting of the NTP Board of Scientific Counselors Report on Carcinogens Subcommittee, December 13-14, 2000. I will restrict my comments to aspects of the report involving a possible relationship between Trichloroethylene (TCE) exposure and VHL tumor suppressor gene mutations and animal models, since the role of VHL in renal tumorigenesis is my major area of expertise. I will also point out aspects of the report that I consider to be either inaccurate or misleading. This commentary is submitted in conjunction with the Halogenated Solvents Industry Alliance, Inc. for submission to the subcommittee members. My affiliation and contact information are included at the end of the report.

Conclusions

The report from Brauch et al (1999) had many unique and interesting findings, but also raised some questions:

1. They identified a high frequency of multiple VHL mutations within individual tumors along with an association between multiplicity of VHL mutations and TCE exposure levels.

The lack of a precise classification of TCE exposure levels of the study population, as well as the presence or absence of other potential risk factors, is a weakness. It is difficult mechanistically to account for multiple VHL mutations present in a clonal population of tumor cells. Multiple mutations within the VHL gene would not be likely to contribute a selective advantage to the transformed cells. It will be critical to confirm these findings in independent laboratories and with the same and additional RCC samples from TCE-exposed patients

Background on Human Renal Cancer and the VHL tumor suppressor

The classification of human renal cell carcinoma (RCC) is complicated and is based on histologic and cytologic evaluation.

1. Clear cell RCC account for about 75% to 80% of all cases. The term "clear cell" was derived because these RCC cells store excess lipid and glycogen and appear as nucleated cells with an empty cytoplasm after standard tissue processing with organic solvents for diagnosis.
2. Chromophilic RCC account for about 15% of all cases and often have a papillary growth pattern, characterized by vascularized stalks of connective tissue surrounded by neoplastic cells. These tumors have historically been called papillary RCC in the literature.

The genetics of human RCC correlates well with histology and indicate that clear cell RCC and papillary RCC may have distinct genetic origins. Patients with von Hippel-Lindau disease (an inherited human cancer syndrome) have VHL gene mutations (chromosome 3p25.5) in the germ line and are strongly predisposed to developing clear cell RCC (reviewed in Gnarra 1996). The mechanism of tumorigenesis in VHL patients appears to be through deletion (loss of heterozygosity) of the chromosome arm 3p carrying the inherited wild type VHL allele and

retention of the chromosome arm 3p carrying the inherited mutant VHL allele. There is strong evidence supporting a "gatekeeper" role for the VHL tumor suppressor in renal tumorigenesis.

Hereditary papillary RCC (HPRC) has been described (Zbar et al 1995) and these patients have germline activating mutations of the MET proto-oncogene on chromosome 7 (Schmidt et al 1998). HPRC patients show no involvement of the VHL tumor suppressor. Mechanisms of tumorigenesis in HPRC patients include amplification of the copy of chromosome 7 that carries the activated MET allele. Thus, HPRC is one of the few hereditary cancers (MEN2 with the RET proto-oncogene being another) involving proto-oncogene activation rather than tumor suppressor gene inactivation.

In addition to playing a role in inherited disease the VHL tumor suppressor is somatically inactivated in probably about 70% to 80% of sporadic clear cell RCC cases. Mechanisms of somatic VHL inactivation include mutation (microdeletions or insertions leading to frameshift mutations or non-conservative amino acid substitutions) of one VHL allele and loss of heterozygosity of the other VHL allele. Somatic VHL mutations are seen in about 50-60% of clear cell RCC cases. Different studies that analyzed VHL mutations in RCC cases have reported varying percentages of tumors with VHL mutations, ranging from 33% to 57% (Foster et al 1994; Gnarr et al 1994; Shuin et al 1994; Whaley et al 1994; Brauch et al 2000). There are at least two reasons for this variance among studies. First is the issue of diagnosis. Studies that employed multiple pathologists to review tumor histology in a blinded manner tended to show greater VHL mutation rates, probably because they segregated cases into clear cell or papillary histologies more accurately. The second source for variance involves methods of DNA extraction from tissues and mutational analyses. RCC tumors are highly vascular and a tissue sample may contain a greater number of lymphocytes than tumor cells. Therefore, studies in which DNA was extracted from whole pieces of tissue tended to show lower VHL mutation rates because of the large number of contaminating normal cells. On the other hand studies employing clear cell RCC-derived cell lines or tissue microdissection tended to show higher VHL mutation rates. In addition, direct sequencing of the entire VHL gene has proven to be more accurate than analyses using single strand conformation polymorphism (SSCP) gels, which was used as a primary screen in very early studies. In up to about 20% of clear cell RCC cases the VHL gene is hypermethylated with consequent transcription silencing (Herman et al 1995; Brauch et al 2000). The conclusion is that probably up to 80% of all clear cell RCC cases involve VHL tumor suppressor gene activation (mutation or methylation-induced gene silencing). The remaining ~20% of clear cell RCC cases do not yet have a clear genetic basis.

It has also been shown that familial clear cell RCC (FCRC) occurs and is independent of the VHL tumor suppressor. The et al (1997) and Clifford et al (1998) analyzed several kindreds and failed to demonstrate linkage of FCRC families with the VHL locus. To date the genetic basis for these tumors is unknown (Woodward et al 2000). It is likely that when the FCRC gene is described we will also gain an understanding for the genetics on non-VHL related sporadic clear cell RCC.

While VHL clearly plays a "gatekeeper" role in the majority of clear cell RCC, it has been suggested that additional genes on chromosome arm 3p may also play a role in tumorigenesis. The fact that we commonly see loss of heterozygosity of loci on 3p12-21 in renal cancer, as well as many other malignancies such as lung cancer, indicates that additional tumor suppressors may map to these loci. Martinez et al (2000) studied a number of clear cell RCC with or without inactivation of VHL. They showed that both VHL-negative and -positive clear cell RCC showed a similar high frequency of 3p12-21 loss of heterozygosity, but VHL-positive

clear cell RCC showed less frequent loss of heterozygosity at 3p25. Their data support the possibility that loss of VHL alone may be insufficient for renal tumorigenesis and that loss of additional tumor suppressor(s) more centromeric on chromosome arm 3p may be important for RCC development. This is supported by the observation that loss of chromosome arm 3p heterozygosity uniformly occurs in tumors from VHL patients. A situation in which a second mutation occurring in the inherited wild type VHL allele, without chromosome arm 3p loss of heterozygosity, has not been reported.

In summary, RCC in humans is a complicated disease with varying histologies and the probable involvement of multiple genes. Loss of VHL tumor suppressor activity is clearly important for the development of clear cell RCC, but additional genes perhaps also on chromosome arm 3p may also play a significant role in tumorigenesis.

Animal Models

The development of animal models for RCC has not been straight-forward. The Eker rat model has proven to be very useful in the study of the etiology of RCC. Eker rats have a germline mutation in the tuberous sclerosis 2 (TSC2) tumor suppressor gene (reviewed in Walker 1998). These animals develop spontaneous multiple, bilateral renal tumors at high frequency and exhibit heightened sensitivity to treatment with a variety of carcinogens (Walker 1998). Chemical induction of renal tumors in other rat strains has also been extensively analyzed. Rat RCC tumors (Eker or other strains) are not typically of the clear cell type, but rather tend to be chromophobic. Chromophobic RCC are seen in about 5% or fewer of human cases. Several groups have analyzed a large number of rat renal tumors for VHL and TSC2 mutations. The TSC2 tumor suppressor is a common target for inactivation in rat RCC, while VHL mutations have not commonly been observed (Walker 1998). The situation is probably similar for the mouse, since TSC2 knockouts show RCC susceptibilities and histologies similar to the Eker rat. One group identified 8 rat RCC with a clear cell histology from a large group of tumors induced by N-nitrosodimethylamine (NMDA) (Shiao et al 1998). They classified clear cell RCC in rats as "rare" and did not indicate exactly how many tumors were analyzed to find these 8 samples. Three of the 8 rat clear cell RCC tumors showed VHL mutation, while 40 other NMDA-induced rat tumors of other histologies did not have VHL mutations. This supports the involvement of VHL in formation of clear cell RCC. However, it is clear that conclusions regarding susceptibility to developing carcinogen-induced renal cancer between laboratory animals and humans must be made with a great deal of caution. The fact that rats (and probably mice) and humans appear to have different target genes for RCC tumorigenesis (TSC2 versus VHL) and different RCC phenotypes (chromophobic versus clear cell) complicates the translation of carcinogenesis data. It is not yet clear whether a carcinogen that targets TSC2 and induces renal tumors in rats will similarly target VHL and induce renal tumors in humans. Therefore, any animal data relating TCE and renal tumors must be cautiously interpreted pending evaluation of tumor histology and genetic mutations in the TSC2 and VHL genes.

Specific Comments on the Draft Report

There are several statements in the report that I found to be misleading or inaccurate. These are specifically in reference to the reports by Bruning et al (1997) and Brauch et al (1999), which are cited to support that TCE induces genetic changes in the VHL tumor suppressor. I will discuss them in order of appearance:

Page vi, "*Renal-cell carcinomas from workers occupationally exposed to high levels of TCE exhibited somatic mutations of the von Hippel-Lindau (VHL) tumor suppressor gene, a gene that has been associated with renal cell carcinomas.*" This statement is deceiving, since it implies cause and effect—TCE exposure causes VHL gene mutations, which leads to RCC. These patients were selected for study because they had RCC. As I described above, the fact that the majority of these patients had clear cell RCC indicated a high likelihood that the majority of tumors would have VHL mutations. Neither the Bruning report nor the Brauch report discussed their data in the context of risk factors other than TCE, such as smoking history, diet, family history for kidney disease, or occupational exposure to other potential carcinogens.

Page 58, in reference to the Bruning et al report: "*All 23 patients had aberrations of the von Hippel-Lindau (VHL) tumor suppressor compared to mutation frequencies of 33% to 55% in patients with renal-cell carcinoma but without known occupational exposure to TCE.*" There are two inaccuracies here. First, the authors analyzed 23 tumors by SSCP and found that each had aberrantly migrating bands. However, only 4 samples were actually sequenced and mutations identified in that report. Twenty of the 23 tumors from the Bruning et al study were included in the subsequent report by Brauch et al, where 33 of 44 tumors had VHL mutations. Second, the statement of 33-55% VHL mutation frequencies in RCC was derived from the literature and did not actually come from the Bruning et al report.

Page 58, in reference to the Brauch et al report: "*DNA was isolated from tumor tissue of both the TCE-exposed and control patients by microdissection.*" This implies that all samples were isolated in a similar manner and is not true. Brauch et al (1999) report in the "Subjects and Methods" section, "*DNA for VHL mutation screening was obtained from whole blood, fresh and frozen tumor tissues, and formalin-fixed, paraffin-embedded tissues. Tissue sections from paraffin blocks were microdissected before DNA extraction.*" The authors do not in fact report which patient samples were derived by microdissection or from fresh or frozen tissue.

Page 59, in comparing the TCE exposure levels of patients studied it is stated that, "*....TCE exposure was not as severe*," in the Schraml report as compared to the Brauch report. This must be taken with great caution, since exposure levels were self-reported by the patients, using different questionnaires and interviewers.

2. A VHL mutational hot-spot was identified in clear cell RCC from TCE-exposed patients.

This is the first study to report such a hot-spot, and similar results have not been reported previously in other VHL gene mutation studies. To confirm the significance of this hot-spot, it will be necessary to identify and evaluate other RCC patients with similar chemical exposure to determine whether these findings can be reproduced.

In summary, the report by Brauch et al (1999) indicating that the VHL gene may be a target of TCE is potentially very significant. The fact that these patients with clear cell RCC have VHL tumor suppressor gene mutations is expected, given the well-described involvement of VHL in renal tumorigenesis. Issues regarding exposure levels of the study population, the presence of co-existing multiple VHL mutations, and the potentially conflicting data presented by Schraml et al indicate that caution should be used in interpreting these findings. Additional

studies on other TCE-exposed human populations are warranted to confirm these data as well as mechanistic studies to determine if TCE is a renal carcinogen at relevant exposure levels.

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November 30, 2000

NTP Board of Scientific Counselors RoC
Subcommittee
C/o Dr. Mary Wolfe, Executive Secretary**Comments on Epidemiologic Studies of Trichloroethylene and Kidney Cancer
for submission to NTP**

As cancer epidemiologists, we have been asked by HSIA to provide our comments on the literature concerning Trichloroethylene in relation to cancer. Our comments are based on the study of the relevant original literature and also on a careful scrutiny of the following documents:

1. Comment on Additional Substances Proposed for Listing in the Report on Carcinogens, Tenth Edition from Paul Dugard to Dr. Jameson, June 2, 2000.
2. Trichloroethylene and Cancer: Epidemiologic Evidence. Wartenberg, D et al. Environmental Health Perspectives. Volume 108, Supplement 2, May 2000
3. Draft: Report on Carcinogens Background Document for Trichloroethylene. Prepared by: Technology Planning and Management Corporation
4. Comments on the National Toxicology Program (NTP) Proposed Listing for Trichloroethylene (TCE): A critical Review of Epidemiologic Research and Selected Toxicological Issues on Cancer Risks due to TCE Exposure. Mandel, J. et al. June 5, 2000.
5. A Review of the Epidemiologic Studies of Trichloroethylene and Kidney Cancer. J. Mandel. November 29, 2000-12-01

We noticed the explicit criteria defined by 65 Fed.Reg. at 17889 for a compound to be classified as a known human carcinogen, namely that "there is sufficient evidence of carcinogenicity from studies in humans which indicates a causal relationship between exposure to the agent, substance or mixture and human cancer". Moreover, after reading the documents referenced above it was obvious to us that the individual studies have all been carefully reviewed and summarized by previous authors, notably in documents 2, 3, 4 and 5. Hence, it would add little

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if anything to provide a further summary of the original studies. Finally, it was obvious to us that in humans, the only tentative evidence for an association between trichloroethylene and cancer is confined to kidney cancer. Although other malignancies, notably cancer of the liver, lung and haematopoietic system, have been considered, the evidence is indeed reassuring. As summarized in the table of document 4 by Mandel et al, results from seven cohorts of highly exposed individuals reveal that for these three malignancies one out of 21 risk estimates is marginally statistically significantly elevated at the $p \sim 0.05$ level, which is exactly what one would expect to occur by chance alone.

In our review of kidney cancer we focused on the occupational cohort studies which presumably include the most heavily exposed individuals. These studies have been referenced in previous reviews, and we enclose here, to facilitate reading, only a copy of the summary in Table 1 from document 5.

Table 1 – Summary of Occupational Cohort Studies of TCE Exposed Workers

Authors, Yr	Study Group	No. of Workers	Kidney Cancer
Antilla et al., 1995	Finnish workers monitored for TCE and other solvents	3,974	SIR=0.87 (0.32 - 1.89)
Axelsson et al., 1994	Swedish workers monitored for TCE	1,670	SIR= 1.16 (0.42 - 2.52)
Blair et al., 1998	Aircraft workers, Utah airforce base	14,457	SMR=1.6 (0.5 - 5.1)
Boice et al., 1999	Aircraft manufacturing workers, Burbank, CA	77,965	SMR=0.99 (0.40 - 2.04)
Garabrant et al., 1988	Aircraft manufacturing workers, San Diego CA	14,067	SMR=0.93 (0.48 - 1.64)
Henschler et al., 1995	Cardboard factory workers, Germany	169	SIR=7.97 (2.59 - 8.59)
Morgan et al., 1998	Aircraft manufacturing workers, Tuscon, AZ	20,508	SMR=1.32 (0.57 - 2.60)
Ritz, 1999	Uranium processing plant workers	3,814	SMR=0.65 (0.21 - 1.51)

SIR = Standardized Incidence Ratio, SMR = Standardized Mortality Ratio. 95% confidence intervals in parentheses

We considered first all cohort studies except that of Henschler because this study was an outlier both in terms of size, methodology and results. All seven remaining cohort studies provided risk estimates centered around 1. Of these point estimates, four were below 1 and three were higher than 1, but all confidence intervals included – indeed with wide margins – 1.0. This is exactly the pattern of results one would expect if no causal association existed between exposure to Trichloroethylene and risk for kidney cancer. Therefore, according to our interpretation, these results would lead to the conclusion that evidence from epidemiologic studies in highly exposed humans does not provide any evidence to support an association between Trichloroethylene and kidney cancer. The concern that Trichloroethylene might indeed increase cancer risk in human appears to be driven to a large extent by the cohort study by Henschler et al. and by the related case-control study by Vamvakas et al. (1998). The evidence that these studies had

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a serious impact on the discussion surprised us and we would like to briefly outline the reason for this. While it is widely recognized that bio medical research has undergone a revolution during the last several decades, it may be less widely recognized that a major methodologic development has also taken place in epidemiology over the last half century. Indeed, beginning around 1950 epidemiologic theory has developed dramatically into a sophisticated understanding of the particular features of different study designs, notably cohort studies and case-control studies. This development is to a large extent based on the concept of the study base, of sources of bias and confounding and of different approaches to improve validity in epidemiologic studies.

It appears inconceivable to us that an investigator would pay serious attention to experimental work carried out in the 1990s, but rely on study principles and methodologies that were developed during the first half of the 20th century and disregard subsequent development. Similarly, in the light of current understanding of epidemiologic principles, the methodologic standards of the papers by Henschler et al. (1995) and Vamvakas et al. (1998) appear unacceptable, almost a chaotic. In our view, these studies disregard the methodologic development briefly referred to above. Indeed, in terms of study design and methodologic rigour, these studies do not even meet the standards of epidemiologic studies published in the 1950s. We can only agree with the detailed criticisms of these studies provided by other critical reviewers.

In conclusion, epidemiologic studies of individuals highly exposed to Trichloroethylene show limited if any evidence of a causal relationship with kidney cancer. Indeed none of the Hill criteria for causality appears to be convincingly met. Studies with designs meeting modern criteria for a valid epidemiologic investigation show a pattern that would be expected in a situation in which causality does not exist. Two studies, one in a small cohort (Henschler et al. 1995) and one with a case-control design (Vamvakas et al. 1998) are extraordinary outliers by showing an approximately tenfold excess risk based on small numbers. These studies are, however, so methodologically flawed that they do not even meet basic quality criteria for a modern epidemiologic investigation. Hence, we suggest that they be disregarded in the current evaluation process – at least until the original data have been scrutinized in detail by external reviewers.

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