

To whom it may concern:

The 3M Company has asked me to review the animal studies cited in the draft NTP Monograph on Immunotoxicity Associated with Exposure to Perfluorooctanoic Acid (PFOA) or Perfluorooctane Sulfonate (PFOS). I am a Professor of Immunology with some 28 years working in the field of immunology. I have a PhD in immunology from the Weill Graduate School of Cornell University, with postdoctoral training at The Rockefeller University. I also worked for the drug discovery unit of a major pharmaceutical company, working on developing novel immunosuppressants. Over the past 17 years, I have worked as researcher at 2 major research universities in the field of immunology, investigating pathways that regulate the immune response, including T cells, B cells, mast cells and eosinophils, in animal models of diseases, including infectious disease and allergic models.

Comment on review of Perfluorooctane Sulfonate (PFOS).

Following systematic analysis of the literature on the effects of PFOS on immune parameters, the National Toxicology program concluded that:

“...that PFOS is *presumed to be an immune hazard to humans* based on a high level of evidence that PFOS suppressed the antibody response from animal studies and a moderate level of evidence from studies in humans. Although the strongest evidence for an effect of PFOS on the immune system is for suppression of the antibody response, there is additional, although weaker, evidence that is primarily from studies in experimental animals that PFOS suppresses disease resistance and natural killer (NK) cell activity. The evidence indicating that PFOS suppresses multiple aspects of the immune system supports the overall conclusion that PFOS alters immune function in humans. Although the mechanism(s) of PFOS-associated immunotoxicity is unknown, suppression of the antibody response and NK cell function are both potential mechanisms by which PFOS may reduce disease resistance.”

Effects PFOS on Antibody response:

Overall concerns over the cited studies leading to this decision include the seemingly very high levels of PFOS exposure utilized for these studies, which at the highest doses results in significant weight loss (partially associated with reduced food intake, see e.g. Qazi et al 2012), and elevated levels of corticosteroids that accompany these high dose treatments.

While consistent effects of PFOS are observed on the induction of an IgM response in response to injection of PFOS treated mice with Sheep Red Blood Cells (SRBC), intended to mimic a T-dependent immune response, this is generally observed at high concentrations of PFOS, and seems to be dose dependent. However, no effect is seen in studies with rats, although this may be in part due to altered pharmacokinetics of PFOS in rats. Indeed, in rats, higher doses of PFOS increase serum levels of IgM and IgG, contrary to the findings in mice (Lefebvre et al. 2008). Furthermore, in one study, Qazi et al, 2010 reported no effect on IgM responses in mice exposed to 7 mg/Kg PFOS. This latter study was quite comprehensive and unlike all the other studies, examined numbers

of CD138+ B cells, along with antibody Plaque Forming Cells (PFCs) or antigen specific antibody secreting cells. Qazi et al suggested that the difference between this and other reports may be due to the different methods of delivery (oral gavage in most studies vs dietary delivery in the Qazi et al studies). Zheng et al 2011, reported that PFOS (at 5 and 10 mg/Kg, with the latter dose resulting in severe weight loss) resulted in reduced IgM and no change or increase in IgG depending on the dose. Dong et al, 2011 reported that IgM is suppressed by increasing concentrations of PFOS (up to 50 mg/Kg), but that IgG and IgE is enhanced at the highest tested levels of PFOS.

While these studies are consistent in their finding that PFOS at high doses suppresses the IgM response, suggestive of immunosuppression, the fact that some cited studies report that exposure to PFOS can lead to no change or an increase in IgG suggests that PFOS may not act as an immunosuppressant (since both antibody isotypes would be expected to be reduced if it was acting as an immunosuppressant). As a comparison, treatment of mice with the known immunosuppressant Cyclophosphamide results in suppression of both IgM and IgG responses (Speirs and Speirs, 1979). Rather, the published effects seem to suggest that PFOS at high doses may modulate the immune system, e.g. altering the response from IgM to IgG. It is possible that the consistently observed reduction in IgM in PFOS exposed mice is a result of a number of factors: the significant weight loss observed at high concentrations and accompanying increase in corticosteroids, which could lead to reduced B cell numbers in response to high dose PFOS; or altered B cell class switch such that less IgM is made and more IgG is made. None of the published studies specifically examined these factors.

Effects of PFOS on Disease Resistance/Infectious Disease Outcomes:

The animal evidence for an effect of PFOS on disease resistance/infectious disease outcome is weak, relying essentially on 1 controlled murine study (Guruge et al, 2009). This study used fairly low (relative to other studies) doses of PFOS (up to 25 µg/Kg/day) for 21 days, followed by infection with a mouse adapted strain of influenza. They found that 5 and 25 µg/Kg/day PFOS led to enhanced weight loss in response to flu infection, however, all mice recovered body weight, apparently clearing the infection, although no viral titers were determined to conclude whether indeed anti-viral activity was affected. In some cases, enhanced weight loss in response to flu infection in mice could be an effect of enhanced anti-viral activity leading to some pathology and weight loss. As a comparison, treatment of mice with the known immunosuppressant Cyclophosphamide has a clear effect influenza virus infection in mice, leading to increased viral replication (Singer et al, 1972, Mastino et al, 1991), and the mice are actually somewhat protected due to the reduced inflammatory response of the immunosuppression. Thus in the absence of viral titers, it is therefore not clear whether anti-viral activity was affected by PFOS.

Effects of PFOS on Natural Killer (NK) Cell Activity:

The animal evidence for an effect of PFOS on NK cell activity is weak. The Qazi et al (Qazi et al 2009) study that examined the effect of PFOS on NK cell numbers actually measured NK and NKT cells combined, not NK cells separately. These are two different cell populations and so the effect on NK cell numbers is unknown. An effect on combined

NK and NKT cell numbers (reduced by 38%) was observed at the highest dose of PFOS, 0.02% dietary PFOS. Function was not determined. Dong et al, 2009 was also cited but in this study, the effect of PFOS on splenic NK activity was not corrected for by number of NK cells in the spleen. Keil et al, 2008, tested effects of 4 and 8-week exposure to PFOS (5 mg/Kg) and reported an effect on splenic NK cell activity at 8 but not 4 weeks. Again, these studies did not correct for number of NK cells in the spleen. Peden-Adams et al 2008 reported that splenic NK cell activity was not affected (up to 5 mg/Kg PFOS). Given that the well described immunosuppressant Cyclophosphamide has clear effects on depressing splenic NK cell activity in similar types of assays (see Mantovani et al, 1978), these studies cannot be conclusively interpreted as effect of PFOS on NK cell activity.

The other studies cited to support an effect of PFOS on NK cells (Dong et al. 2011, Zheng et al. 2011, Dong et al. 2012), did not actually examine NK cells or their function, but extrapolated from the effect of PFOS on cytokines such as IFN γ that are associated with NK cells. However, IFN γ is also made by a number of other cells, including CD4, CD8 and $\gamma\delta$ T cells. Thus this interpretation cannot be made.

The NTP also cited in vitro and in vivo studies where PFOS is reported to alter inflammatory cytokines such as TNF α , IL6, IL4 and IFN γ . These in vitro studies come to opposite conclusions in some cases, and in some cases, the findings are opposite to the in vivo findings. For example, Qazi et al, 2009 reported that dietary PFOS at 0.02% enhanced LPS-induced IL6 and enhanced or suppressed LPS-induced TNF α when cells were cultured in vitro, but serum levels of these cytokines from the PFOS exposed animals challenged with LPS were not affected. Thus the response differed dependent on how the experiments were done, and which site (bone marrow, spleen peritoneal cells, serum) was being analyzed. By contrast, Dong et al 2012, reported that PFOS enhanced basal as well as LPS induced serum levels of TNF α , IL1 β and IL6 at doses starting at 50 mg/Kg. In addition, Zheng et al, 2011 reported that 20 mg/Kg PFOS enhanced IL4 production by splenocytes cultured in vitro, but these cells were not activated. In addition, at this dose there was significant weight loss and corticosteroid increase. Thus the effects of PFOS on cytokine production is inconsistent and clear conclusions cannot be drawn.

Comment on review of Perfluorooctanoic Acid (PFOA).

Following systematic analysis of the literature on the effects of PFOA on immune parameters, the National Toxicology program concluded that:

“... PFOA is *presumed to be an immune hazard to humans* based on two separate lines of evidence: (1) the high level of evidence that PFOA suppressed the antibody response from animal studies and the moderate level of evidence from studies in humans, and (2) high level of evidence that PFOA increased hypersensitivity-related outcomes from animal studies and low level of evidence from studies in humans. Although the strongest evidence for an effect of PFOA on the immune system is for suppression of the antibody response and increased hypersensitivity, there is additional, although weaker, evidence that is primarily from epidemiological studies that PFOA reduced infectious disease resistance and increased autoimmune disease. The evidence indicating that PFOA

affects multiple aspects of the immune system supports the overall conclusion that PFOA alters immune function in humans. However, the mechanism(s) of PFOA-associated immunotoxicity is unknown and effects on diverse endpoints such as suppression of the antibody response and increased hypersensitivity may be unrelated.”

Overall concerns of these studies include the seemingly very high levels of PFOA exposure utilized for these studies, which at the highest doses results in significant weight loss (partially associated with reduced food intake (see e.g. Qazi et al 2012), and elevated levels of corticosteroids that accompany these high dose treatments. In addition, in vitro experiments may be significantly affected by the finding that PFOA can act as a detergent on cells (albeit tested at concentration greater than 0.9 mM, see Levitt and Liss, 1987).

Effects of PFOA on Antibody response:

Consistent effects of PFOA are observed on the induction of an IgM response in response to injection of PFOA treated mice with Sheep Red Blood Cells (SRBC), intended to mimic a T-dependent immune response, this is generally observed at high concentrations of PFOS, and seems to be dose dependent. No effect is seen in studies with rats, although this may be in part due to altered pharmacokinetics of PFOA in rats. Indeed, in rats, higher doses of PFOA increase serum levels of IgM and IgG, contrary to the findings in mice (Loveless et al. 2008). In some studies, PFOA exposure led to increases in IgG (in response to SRBC, DeWitt et al, 2008) and/or IgE (in response to immunization with Ovalbumin, Fairley et al, 2007) suggesting that the immune response is being altered rather than being suppressed.

While these studies are consistent in their finding that PFOA at high doses suppresses the IgM response, suggestive of immunosuppression, the fact that some cited studies report that exposure to PFOA can lead to an increase in IgG or IgE suggests that PFOS may not act as an immunosuppressant (since the other antibody isotypes would be expected to be reduced if it was acting as an immunosuppressant). As a comparison, treatment of mice with the known immunosuppressant Cyclophosphamide results in suppression of both IgM and IgG responses (Speirs and Speirs, 1979). Rather, the published effects suggest that PFOA at high doses may modulate the immune system, e.g. altering the response from IgM to IgG or IgE. It is possible that the consistently observed reduction in IgM in PFOA exposed mice is a result of a number of factors: the significant weight loss observed at high concentrations and accompanying increase in corticosteroids, which could lead to reduced B cell numbers in response to high dose PFOS; or altered B cell class switch such that less IgM is made and more IgG or IgE is made. None of the published studies specifically examined these factors.

Effects of PFOA on Increased hypersensitivity:

The animal evidence for an effect of PFOA on increased IgE is not as strong as for the reduction in IgM, relying on 1 study (Fairley et al, 2007). Again, however, this was observed at higher doses of PFOA and seemed to be dose dependent. This is further evidence that PFOA does not suppress, but rather alters the nature of the type of immune response that will be generated. However, the evidence for an effect of PFOA on airways hypersensitivity in animals is weak. This conclusion relies on 2 animal studies, Fairley et

al and Ryu et al (Ryu et al, 2014).

Fairley et al used a murine model of OVA induced allergic airway inflammation to examine the effect of PFOA. However, while they observed that PFOA led to elevated IgE responses to the antigen, they used a method, whole body plethysmography, which reports a unit less value PenH, to measure airways hyperresponsiveness (AHR). The is method, PenH, has been found to be unreliable for the measure of AHR, and reliable studies use mechanical ventilation of the lung to measure lung resistance (see Glaab et al, 2007). Thus these studies cannot be relied upon to provide a measure of the effect of PFOA on airways hyperresponsiveness and hypersensitivity.

Ryu et al also used a mouse model of OVA induced allergic airway inflammation to examine the effect of PFOA. Ryu et al used mechanical ventilation, an accepted and reliable method to measure AHR. They found that PFOA led to elevated AHR at the basal state (in the absence of any sensitization), accompanied by elevated macrophages in the lung. However, no effect of PFOA was observed on OVA induced AHR. This suggests that PFOA does not affect immune mediated AHR. Ryu et al did not examine IgE levels.

Singh et al, 2012, reported that PFOA enhanced IgE mediated Passive Cutaneous Anaphylaxis (PCA), done by injecting PFOA (1 and 5 mg/kg) into the ear, followed by IgE and antigen. However, the authors did not perform controls with PFOA alone to determine whether this amount of PFOA induced spontaneous histamine release as per suggested detergent properties and effects on cells in vitro (see further discussion of this point below). Furthermore, the in vitro studies cited to support this conclusion also raise concerns as to their interpretation. Singh et al (Singh, et al, 2012) use concentrations of PFOA (up to 400 μ M) on a mast cell line and reported elevated histamine release. However, the concentrations of PFOA used were enough to cause cell death, and potential detergent effects of PFOA at high concentrations could lead to similar effects. In addition, Singh et al incubated the cells with PFOA in vitro for 24 hours prior to analysis of histamine, when histamine is generally released over a much shorter period (less than an hour) when mast cells are stimulated. This suggest that the authors may be seeing a mild detergent effect that increases spontaneous release of histamine from the mast cell line. However, direct injection of PFOA in the peritoneum led to enhanced histamine release after 1 hour, but with the caveat discussed above about potential detergent properties of PFOA. The studies by Yamaki and Yoshino (Yamaki and Yoshino, 2010) cited used PFOA concentrations that cause cell death, as well as a rat basophil cell lines as a model. Such concentrations, as in the Singh et al study, can cause spontaneous release of histamine and are not reliable.

The NTP also cited in vitro and in vivo studies where PFOA is reported to alter inflammatory cytokines such as $TNF\alpha$, IL6, IL4 and $IFN\gamma$. Theses in vitro studies come to opposite conclusions in some cases, and in some cases, the findings are opposite to the in vivo findings. For example, Qazi et al (Qazi et al, 2009) reported that dietary PFOA at 0.02% enhanced LPS induced IL6 and enhanced or suppressed $TNF\alpha$, but this response differed dependent on how the experiments were done, and which site (bone marrow, spleen peritoneal cells, serum) was being analyzed. Furthermore, the study by

Singh et al (Singh et al, 2012) reported that PFOA enhanced mRNA for TNF α , IL1 β and IL6, but the caveats to this study applies here as well.

Finally, the effects of PFOA in vitro on intracellular pathways are not consistent or reliable enough to form any conclusions. Singh et al suggest that PFOA induced p38, caspase activation and NF κ B in a dose dependent manner. However, these doses induce cell death (as indicated by caspase activation), while other reports suggest that PFOA suppress NF κ B activation (e.g. see Corsini et al, 2012). Thus the effects of PFOA on cytokine production is inconsistent and clear conclusions cannot be drawn.

Respectfully,

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