Martin *et al.* reply:

C. Cerdan *et al.*[1] revisit our finding of contamination of human embryonic stem cells (HESCs) by the nonhuman sialic acid Neu5Gc (ref. 2). Studying 18 random human sera and two HESC lines, they find "no correlation" between HESC Neu5Gc content (studied only in a narrow range of ~7.5–8.5% Neu5Gc) and *in vitro* cell killing. (As in our study, there was high background death without serum.) The authors do cite our subsequent publication[3] showing a clear correlation between Neu5Gc content of some other cultured human cell types and complement killing by human sera containing known antibodies to Neu5Gc (anti-Gc-Ig). There are many technical concerns about their study, only some of which can be addressed in the limited space available here. For example, they do not ever measure in their sera anti-Gc-Ig, which we have shown to be highly variable among individuals[3]. They also do not manipulate Neu5Gc levels in HESCs over a wide range, as we did, to see if this affects the results. Nor do they address whether human immunoglobulin is deposited on the cells in a Neu5Gc-dependent manner.

The use of mouse embryonic fibroblast (MEF) cells as a 'positive control' is flawed because there are likely to be many other antigenic differences inducing cell killing. The same is true of using a pure mouse monoclonal antibody against a single major surface epitope as a positive control. Moreover, they use Matrigel, an animal product containing Neu5Gc, which could confound results by acting as a sink for adsorption of anti-Gc-Ig. Notably, they trypsinize their HESC (destroying cell surface glycoprotein–bound Neu5Gc) before examining serum killing. (We gently released our cells with EDTA to avoid this problem and to reduce MEF contamination.) Finally, they ignore our main conclusion that even if significant lysis above background does not occur *in vitro*, deposition of small amounts of antibody or complement could trigger immune attack *in vivo* by various inflammatory cells having Fc receptors, complement receptors or both.

Regardless of these technical concerns, we are disturbed by their concluding statement that "incorporation of Neu5Gc is unlikely to be of any consideration for future basic or clinical practice." Incorporation of Neu5Gc into HESCs is indeed likely not to be of concern for basic research *in vitro*, as long as the HESCs are not exposed to human sera containing anti-Gc-Ig (ref. 2). However, we are mystified as to how the authors could come to the strong conclusion that these issues are of no concern in clinical practice. This conclusion seems unjustified with regard to the realities of clinical medicine. As we emphasized, deposition of even small amounts of antibody or complement could result in problems *in vivo*, as a result of recognition of such molecules by the innate immune system. Moreover, unlike cells *in vitro*, which are exposed to small amounts of serum, an *in vivo* graft would be exposed to a large fraction of the total amount of anti-Gc-Ig in the 2–3-liter volume of circulating blood plasma. We have also recently found that the human polyclonal immune response to Neu5Gc actually targets multiple Neu5Gc-containing epitopes, in a manner that is quite variable among individual sera (unpublished results). In view of all this, it is dangerous to use *in vitro* killing data with small numbers of human sera to predict whether or not this phenomenon will be a problem *in vivo*. In the final analysis, what matters is whether this potential risk is of concern to the clinician and the patient. If any of us were unfortunate enough to require treatment with HESC derivatives, we would strongly prefer that the nonhuman Neu5Gc molecule not be present on grafted cells. We suspect that practicing clinicians would feel the same way, especially given the recent disaster in which even *in vivo* studies in monkeys did not predict serious side effects of monoclonal antibody therapy experienced in human volunteers[4]. Meanwhile, scientists at the US Food and Drug Administration are also expressing increasing concern about the potential immunogenicity of biological therapeutics in humans[5], and the European Medicines Agency (EMEA) has withheld approval of recombinant human antithrombin produced from transgenic goats, citing concerning over the potential immunogenicity of Neu5Gc in this product[6]. Also, others have recently confirmed the problem of Neu5Gc contamination in human ES cells and extended this finding to other cell types used for human therapies, including mesenchymal stem cells (J. Laine, Finnish Red Cross Blood Service, Helsinki, Finland, personal communication), as well as autologous-donor T cells and allogeneic breast cancer cell vaccines (A. Rosenberg, US Food and Drug Administration, Bethesda, Maryland, USA, personal communication). As such cells also incorporated or expressed Neu5Gc when cultured in FCS, they could possibly elicit immune responses. Similar concerns over potential problems during use in humans are mentioned by these researchers. We suggest that the focus of attention be on eliminating this potential problem, rather than on second-guessing whether it has any *in vivo* significance on the basis of limited *in vitro* experiments. In this regard, we have produced MEFs free of Neu5Gc (unpublished results) and are working to define Neu5Gc-free medium components. It also appears that some HESC differentiation conditions can lower Neu5Gc levels by unknown mechanisms[7]—possibly, competitive overproduction of the human sialic acid Neu5Ac.

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