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QUESTIONS FROM THE FOURTH SON: A CLINICIAN REFLECTS ON IMMUNOMONITORING, SURROGATE MARKERS AND SYSTEMS BIOLOGY

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Abstract. The fourth son is the one who doesn't even know how to ask a question. Tumor immunology is challenged by the failure to identify reliable surrogate markers in vaccine and other experimental therapies for cancer; perhaps investigators haven't yet asked the right questions. Unlike prophylactic vaccines for infectious disease, where the development of antibody is considered a satisfactory endpoint, no such endpoint exists for human therapeutic vaccines. Why is this? Despite an extensive roster of in vitro assays that correlate immune responses to favorable clinical outcomes, no assay is sufficiently reliable to be usefully predictive for vaccine therapy. The discussion reviews some of the historical developments in tumor immunology and the problem of defining a causal relationship when strong correlations are identified. The development of mathematical models from empirical data may help inform the clinician/scientist about underlying mechanisms and help frame new testable hypotheses.

The field of tumor immunology has made some enormous strides over the last ten to twenty years. It is clear that vaccines can causes tumors to regress, ([36, 38, 27]), and a vaccine for prostate cancer has just been approved by the FDA on the basis of prolongation of survival. Yet a nagging problem persists: a measurable upregulated immune response does not guarantee a clinical response and the necessary and sufficient factors for clinical responses remain poorly understood. Investigators have yet to pose the right questions and receive an answer that can inform improved design of clinical trials. The problem of asking the right questions always reminds me of the traditional Passover dinner, which actually is a teaching event: asking questions is part of the liturgy, and that liturgy describes the different questions asked by four sons. Each of us acts from time to time like each of the sons, so consider the classification: there is the wise son, the rebellious son, the simple son, and the son who knows not what to ask. Might the paradigm of the four sons help break the impasse troubling immunotherapy? Might it be possible that there is a lesson in the data for which we just haven't framed the right question? If all of the facts almost, but not quite, fit a theory, maybe there is something naïve about the theory. That is, there are critical questions that haven't been framed, the characteristic of the fourth son.

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The overarching dilemma of cancer immunotherapy has been the ability to develop quantifiable in vitro evidence of anti-tumor immune responses without achieving clinically beneficial regressions [25, 32] . Inherent in this problem is the need for more sophisticated understanding of the immune response. In practical terms, the evaluation of current studies and the development of new clinical trials suffers from the absence of one or more reliable surrogate markers [33].

Two issues are involved: the first is relating any given assay to actual mechanisms occurring within the tumor, especially if one is studying antibody or cellular immune responses from peripheral blood. Gene expression or other studies on biopsies from regressing tumor or organ allografts partly avoid this problem, and Marincola has worked extensively in this area to identify the "immunological constant" [32] but further investigation is still needed to interpret the actual function of the particular genes identified and the choreography of the various cells involved.

The second issue is to find a test that reliably predicts that the immune response developed will result in tumor regression and, of course, concomitant improvement of survival. For example, Galon et al. studied infiltrating lymphocyte gene expression of primary colon cancer, and developed a classification rivaling, perhaps surpassing, currently used tumor staging [15]. But in general, immune responses are considered prognostic, not predictive; that is, they may inform about disease outcome but do not reliably correlate with response to treatment [20]. No single test is accepted uncritically, and none have yet emerged to find application by the practicing physician.

So the immunologically oriented physician/scientist is left with a bewildering array of antigens, cytokines, cell types, and immune responses. One interesting approach to organize and classify immune responses may be found in the paper by Tieri et al., who did a network analysis of 19 cells involved in immune responses and 90 cytokines, [30]. In the five years since that publication, no doubt the complexity of the network map has increased greatly. Because of the large amount of data, and the complexity of the immune systems, the concept of "data mining", rarely heard of in the 70s, now becomes a common practice and the discipline of "systems biology" has emerged. There is rapid growth in the literature of various mathematical models of the immune response, and such models can serve many purposes.

To be clear, the problem of cancer immunotherapy is quite a bit different from models of immune responses to infectious disease. While there are exceptions, most cancer vaccine programs involve multiple periodic injections- weekly or at least several times a month, and the injections are continued for several months. The need for multiple vaccine inoculations would be required given the accepted model of T-cell immunity, in which T-cell number is down-regulated rapidly (within weeks) after the initial response.

In some ways the therapeutic application of vaccines, providing additional antigen(s) in the form of whole cells, extracts, peptides etc., is paradoxical. Whatever the capabilities or defects of the host immune system might be, an advanced, clinically identifiable metastatic cancer must be releasing very large amounts of tumor antigen. Yet even in this context, therapy-produced regressions certainly can and do occur.

Some comments are necessary with regard to the accuracy of identifying clinical response. One additional problem inherent in these studies has been the reliance on imaging studies as an endpoint for evaluating clinical response. It is quite possible significant antitumor responses could occur without notable changes in volume of

tumor lesions. Leung and Patt [19] described a study of aggressive chemotherapy for hepatoma, for which little benefit seemed identifiable by imaging. Nonetheless, several patients subsequently had resection of the the liver tumors and, remarkably, were found to have replacement of tumor by scar tissue and complete pathological remission. This remarkable finding would not have been identified by imaging methods alone (perhaps PET scanning might have helped).

Schlom [26] has extended discussion of this problem further, giving several possible scenarios where there was little obvious response to immunotherapy but clear evidence supporting a changed natural history when patients were treated subsequently with hormones or chemotherapy.

Wolchok has asked for a revision of the RECIST criteria in evaluating vaccine trials, as 1) clinical responses may be slow and require many months to manifest; 2) stable tumor size may represent a response, with tumor shrinkage occurring after weeks or months; 3) some new or established tumors may grow despite the immunotherapy, only to regress with continued inoculations [37]. So the search for mechanism and surrogate markers will also require some attention to which particular end-point is chosen.

But for this discussion, let us consider regression of advanced, measurable, metastatic cancer. Investigators over the years have argued that the immune response will not be very effective in metastatic disease [11]. This notion is clearly falsified by so many examples of other investigators [25, 1] as well as examples shown in this discussion.

Consider a melanoma patient treated initially with an allogeneic whole cells vaccine who subsequently had near complete regression of a visceral metastasis. The patient subsequently relapsed, was treated with an autologous vaccine and again responded in a different visceral metastasis [35].

In another example, a patient with breast cancer was treated with a genetically-engineered allogeneic whole cell vaccine transfected to release sargramostim. The clinical trial employed 3 vaccines at 2 week intervals, followed by monthly maintenance for 3 months. Insofar as this was a Phase I program, 6 inoculations were the maximum allowed by FDA recommendations. Two concerns must be addressed here: tumor regression occurred very rapidly (after 3 vaccines) and relapse occurred promptly with cessation of vaccine. The very rapid response of far-advanced disease is unlike most reports in the literature, and it is not obvious which immune mechanism(s) might be responsible. Secondly, the patient relapsed 3 months after completing the protocol, but again had a response when vaccine inoculations were reinitiated, with demonstrable regression of multiple tumor sites, not only in the breast but also in visceral areas and several in the cerebellum [36].

That the vaccine regimens were causative of the regression, and reinduction of regression on retreatment certainly dispels arguments for “spontaneous remission” or some other intervening agency. Yet a clear understanding of the mechanism is still elusive. This problem has not yielded solutions since the 70s when the state of the art immunomonitoring assays were fluorescence microscope antibody techniques [34], delayed-type hypersensitivity testing, elegantly studied in breast cancer by Black [3], lymphocyte blastogensis, and Chromium release cytotoxicity. In the forty plus years since immunotherapy became common in clinical trials, we have many more sophisticated immunomonitoring techniques but their utility remains a work in progress [6]. State of the art laboratories use gene-expression profiling, flow cytometry assays of simultaneous target apoptosis and cytotoxicity

[40], elispot and tetramer assays, among others. Some of these methods involve high-throughput methods which generate a very large amount of data, and perhaps thats very much needed to extract insights into the immune response.

Undeniably, some very basic principles unknown forty years ago are indeed much better understood: the role of MHC antigen presentation, activity of NK cells and other components of innate immunity, cross-priming, the ineffectiveness of antibody therapy against internal tumor-associated antigens . Tumor immunologists are pleased at the FDA approval of a vaccine for prostate cancer, a new development that has just occurred while preparing this manuscript! Several other vaccines may be close to FDA approval, but the fact remains that vaccine therapy has not been widely applied outside clinical trials. Therefore, characterizing mechanisms has stimulated a burgeoning growth of immunomonitoring programs.

Current thinking suggests the key factor in tumor regression is the activity of CD8+ cytotoxic lymphocytes and the ratio of those cells to FOXp3 regulatory cells [17]. The problem is complicated by issues of function vs. number, of transit from node to blood compartment to tumor, of neutralizing or inhibiting effector cells within the tumor, or relative rates of change between tumor growth and expansion of the effector clone (or clones), and the cross-talk with and participation of innate immune mechanism, eg. NK cells, neutrophils and others. Immunologists are quite aware of many mechanisms of tumor resistance to host immune responses [5]. But the complexity of the immune response makes it very difficult to identify the necessary and sufficient conditions for understanding effective vaccine therapy.

Fundamentally, immunology in general, and tumor immunology in particular, deals with multiple specificities. The immune system is characterized by multiple effector mechanisms and multiple specificities within each mechanism. The concept of immunodominance follows directly from this problem: given a variety of potential epitopes to target cytotoxic immune cells or antibodies there will be a spectrum of responses and none will have the exact same characteristics, avidities etc. Furthermore there is a likelihood that the response will be epigenetic, one specificity followed by another. Disis points out that vaccines which develop immune reactivities to defined peptide epitopes of HER2/neu also develop specificities to epitopes entirely unrelated to that which the immunization processes addressed, a desireable effect called “epitope spreading”, [11]. Fulton argues for a division of labor among immune mechanisms. Different roles are assumed by NK cells and innate immunity, cytotoxic CD8+ immunity, and antibody, [14].

In this context, the application of mathematical models may ultimately be pivotal resource. A mathematical model describing one tumor system did seem to validate animal and human responses . Using data from a paper by Diefenbach et al, DePillis et al constructed a mathematical model of a mouse tumor, describing only 3 variables- tumor growth, NK activity, and CD8+ kinetics [9]. See Figure 1. What was especially notable however was that the model was also descriptive of and consistent with published data from human studies, [12]. Specifically, the model demonstrated the need for both innate and adaptive immune responses.

Whatever the specificities, an expanded clone of CD8+ cytotoxic lymphocytes is in some ways analogous to an infectious disease, with effector cells as the etiologic parasite targeting the tumor as the host. To pursue this notion, it is worthwhile to review some of the earlier thinking about pathogens, disease, and causality. Koch’s postulates, Table 1, which produced some degree of clarity in their time, were rapidly recognized as inadequate for much of human infectious disease, even in that

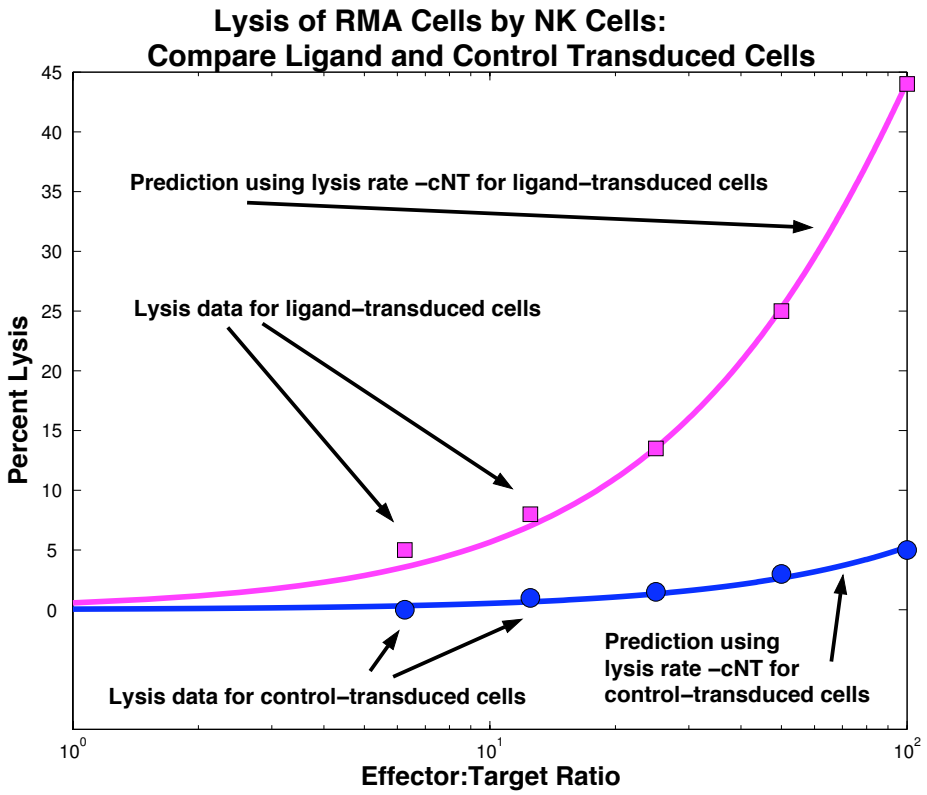

FIGURE 1. Percentage of tumor cell lysis by NK cells. The mathematical predictions from the model (smooth curves) are plotted along with actual experimental data (plotted with squares and circles) from [10] on RMA cells. The shallow curve predicts lysis percentages for the control cells, while the steep curve predicts lysis percentages for the ligand-transduced cells. The conventional product form for NK-tumor competition is used.

era. For example, they did not recognize the carrier state, instead insisting on a direct relation between the microbe and the disease. The valuable contribution of the postulates is the attempt to define causative association between pathogen and pathology. Besides association, Koch required the replicability of this association to produce the disease again in unaffected susceptible hosts. [1]

But we have a problem with characterizing the "necessary and sufficient" factors to associate causality. What is the cause of pneumococcal pneumonia? In this meeting [22], Ran-Kedar [24] has described a system of neutrophil homeostasis with bimodal stability, that is, the neutrophil population could have either a high or low set point. While the pneumococcal organism must be present, so also there must be a deficit of neutrophils for the mouse to develop infection. So what then is the cause of pneumonia, the microbe or the impaired immune response?

[1]Parenthetically, M. tuberculosis produces a peritonitis in guinea pigs, not a pulmonary disease and the animal infection really doesn't compare very well with the human.

1. An alien structure (the microorganism) must always be found with the disease.
2. The alien structure must be shown by isolation and culture to be a living organism and distinct from any others that might be found with the disease.
3. The organism must be distributed in accord with the lesions and clinical phenomena of the disease, and, hence, must be capable of explaining the manifestations of the disease.
4. The organism , cultured through many generations, must produce the disease in [susceptible] experimental animals.

TABLE 1. Kochs Postulates (Carter K.C., from [29]).

The problem of defining causality in science is a serious philosophical issue. We may look to other disciplines for a treatment of this problem. Attorneys employ a concept of enablement. A classic legal paradigm is the "eggshell" skull case. A very minor auto accident nonetheless resulted in tragic injury to the plaintiff who had been afflicted with osteogenesis imperfecta (Judge Eric Younger, ret. personal communication). Is the cause of the head injury the collision or the underlying disease?

Yet another resource might come from epidemiologists, since we are trying to understand and identify a dominant agent among a multiplicity of candidates. One such precedent is provided by the Surgeon General in establishing the role of smoking and lung cancer. Table 2, lists the criteria involved. Such a model may be needed in teasing out the relevant actors in successful vaccine therapy.

Statistical methods cannot establish proof of a causal relationship in an association. The causal significance of an association is a matter of judgment which goes beyond any statement of statistical probability. To judge or evaluate the causal significance of the association between the attribute or agent and the disease, or effect upon health, a number of criteria must be utilized, no one of which is an all-sufficient basis for judgment. These criteria include:
a) The consistency of the association
b) The strength of the association
c) The specificity of the association
d) The temporal relationship of the association
e) The coherence of the association

TABLE 2. Smoking and Health [28]

The success of adoptive immunotherapies and the emerging hopes of a constant of immunological rejection [31] represent major advances, with the data indicating the importance of genes involved with intereferon and T-cell effector processes. However, identifying a panel of genes upregulated and overexpressed as a correlate of immune rejection raises other difficult problems. Bernstein et al describe such a panel as very informative of early indication of cardiac allograft rejection [2]. While there is strong correlation, understanding how these various genes apply to the process of developing recognition and consequent anti-allograft effector cells is not yet clarified.

So the problems still remains: what are the mechanisms of tumor rejection, and what quantifiable changes are involved? The success of vaccines in prophylaxis of

infectious disease is largely facilitated by the acceptance of newly developed titers of anti-microbial antibodies as an endpoint for successful vaccination. A similar, reliable signal still eludes investigators studying tumor rejection. One possible explanation may be inherent in complex dynamics, the sensitivity to initial conditions. There is data to suggest that a very few cells are involved in the initiation of the relevant immune responses in animal models, [23]. Chao suggests that therefore the activity of single cells may influence the outcome, a reframing of the almost universally recognized "butterfly effect", [7]. One must also take into consideration the possibility that there are immune mechanisms not known or whose importance is not fully appreciated. The discovery of mice that resist inoculations of virtually any kind of cancer identified the relevant cells involved as neutrophils, macrophages and NK cells [16]. The role of the innate immune system is of course receiving greater attention, but anti-tumor neutrophil functions are rarely a topic found in the current literature. The reader, and the author, await the development of deeper understanding of tumor immunology, which will likely involve the bench immunologist and the clinician in dialog with the systems biology mathematician.

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