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Biomarkers and assessment of vaccine responses

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Abstract
Vaccines for infectious diseases have in the past, and will into the future, relied on a variety of surrogate markers to monitor vaccine efficacy. The primary surrogate markers have been either the antibody titer to vaccine antigens or the measurement of antibody function such as anti-viral neutralizing activity. In recent years, the measurement of T-cell function in conjunction with or independent of antibody measurements have been used to assess vaccine efficacy. ELISPOT, flow cytometry and intra-cellular staining methods are used to determine the impact of vaccines on immune mediators such as interleukins, interferons, MHC expression and pro-inflammatory mediators. The relevant B-cell and T-cell surrogate markers for vaccine efficacy is dependent on the vaccine being used, so that no universal set of surrogate markers can be applied to all vaccines. The use of T-cell surrogate markers can be complicated by the lack of sensitivity to accurately measure intra-cellular mediators. Although typically this is not a problem for infectious disease vaccines, it is a major problem for cancer vaccines.

Keywords: *Vaccine response, ELISPOT, tetramer*

Introduction

The term 'biomarkers' covers a multitude of chemical and biological molecules in a wide range of pathologic conditions including infectious disease, cardiovascular disease, neurological diseases, inflammatory diseases and many more. Recently, the term biomarker has been defined as biomolecules such as mRNA, proteins, peptides and small molecules, that can be quantitated, predict disease or drug activity and are 'novel' (Diller 2004). 'Novel' in this definition means the biomarker is neither an established diagnostic marker nor a surrogate marker used to predict clinical endpoints. With such a strict definition, no markers used in infectious disease and cancer vaccine development would qualify as a 'biomarker', since the only vaccine markers available are surrogate markers measuring various components of the immune system. Therefore, this review is limited to surrogate markers of the immune response to both infectious disease and cancer vaccines.

Biomarkers are utilized at two stages of vaccine development. The first stage is to identify appropriate immunogens where the measurement of end-stage immune function such as antibody levels is utilized. The second stage is to establish vaccine efficacy as well as assess protection of the proposed vaccine (Krause 1998). Both humoral and cell-mediated immunity biomarkers are used in this stage. Immune response biomarkers that correlate with protection are used to determine efficacy

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issues such as the proper vaccine formulation, inoculation schedules, dosage and target populations (Siber et al. 1998). When measuring protection of potential new vaccines, it must be kept in mind that cancer and infectious disease vaccines have different roles in patient protection and, thus, different markers will be used to assess protection. For infectious disease vaccines, the primary goal is the protection of the general population from acquiring disease, while for cancer vaccines protection of the individual patient from residual disease is the primary goal (Käyhty 1998, Lyerly et al. 2001, Weiner & Kim 2002). Due to these different primary roles and the different immune-mediated protection that is required, infectious disease vaccine protection is measured primarily with humoral markers with cellular immune markers being secondary. In cancer vaccines, the opposite has occurred where cell-mediated immune biomarkers are the primary measures used (Lyerly et al. 2001).

Traditional surrogate markers for vaccine evaluation

Surrogate markers for immunity have been in existence for nearly 150 years. In the 19th century, Robert Koch described the use of the skin test (injection of inactivated virus intra-dermally) to indicate the host's delayed-type hypersensitivity to poxviruses. The skin test became the first surrogate marker to indicate the presence of immunity to poxviruses and, thus, an indicator of protection. Since the 19th century, a number of surrogate markers have been used to assess immunity and, in particular, to assess the immune response to vaccines. Besides the traditional skin test, the cell-mediated arm of the immune response has been measured by quantitating the *in vitro* proliferation of mononuclear cells, primarily lymphocytes, when these cells are placed in culture with the particular antigen being assessed. The proliferation of lymphocytes has traditionally used the incorporation of ^{3}H thymidine in dividing cells as a quantitative measure of memory cells to the antigen being assessed. A second *in vitro* measurement of cell mediated immunity, especially in cancer and viral immunity, is the quantitation of killer cell function mediated by lymphocytes previously sensitized to the antigens present on the target cells. Target cells are labelled with soluble intra-cellular compounds such as ^{57}chromium that are released into the culture medium upon cell lysis. The number of labelled target cells lysed by cytotoxic T-cells is a measure of immunologic memory to the target antigens in question whether they are cancer antigens or viral antigens expressed on the surface of the target cells.

The measurement of antibodies has been the most widely used surrogate marker of immunity. The most common antibody measure is quantitating the level of antibody present in serum to a particular antigen or pathogen. The quantity of antibodies in serum to such pathogens as diphtheria and tetanus has long been used to determine if an individual has sufficient levels of pathogen-specific antibodies to be protected from subsequent exposure to that pathogen. The functional activity of antibodies is also measured to determine efficacy of vaccines (Käyhty 1998). Examples of functional measures of humoral immunity include bacterialcidal assays (*H. influenza* type b, meningococcus), opsonophagocytic activity (*S. pneumoniae, H. influenza* type b), toxin or virus neutralization (tetanus, diphtheria, poliovirus) and IgG avidity (a measure of immunologic memory). In the latter example, the binding avidity of IgG to its specific antigen has been known to increase over time after the initial exposure of the host to the pathogen. As shown in Figure 1, Ashley-Morrow et al. (2004) demonstrated the increased avidity of IgG to the specific antigen of HSV-2 (gG2) over

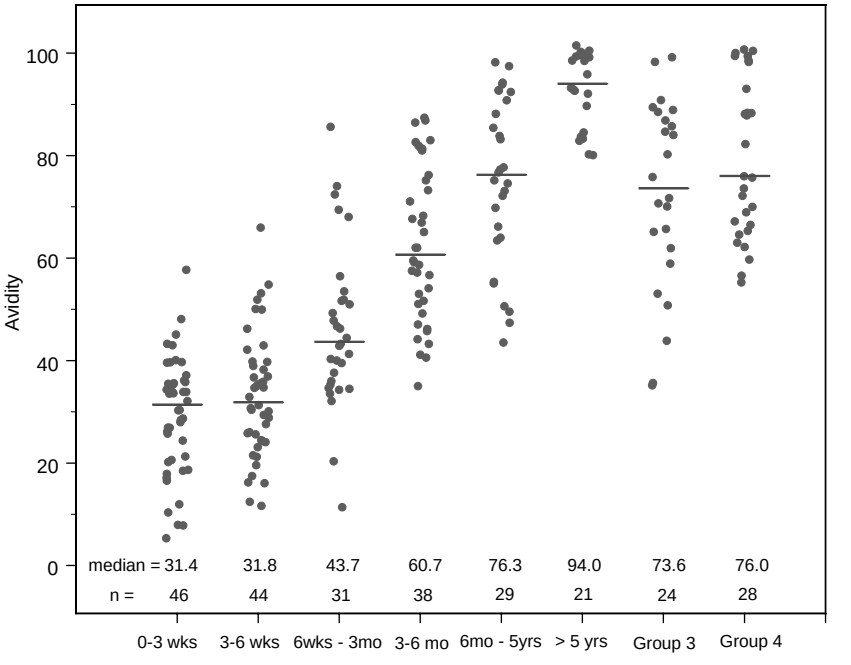

Figure 1. HSV-2 IgG avidity as a function of time after the primary genital herpes episode (Ashley Morrow et al. 2004).

time after the initial exposure to HSV-2 genital infection. It is now known that increasing avidity of IgG to a particular antigen is a function of immunologic memory in the host that is required for long-term protection (Usinger & Lucas 1999, Joseph et al. 2001).

Although all of the above markers of immune function have existed for decades, they are still today the primary markers used for infectious disease vaccine efficacy. As recently as 2003 (Jódar et al. 2003), published criteria that should be used to assess the next generation of conjugated pneumococcal vaccines. The primary criteria is the attainment of threshold levels of protective antibodies, the secondary criteria is the measurement of functional antibodies using the opsonophagocytic assay and the tertiary criteria was the measurement of immunologic memory by either measuring antibody avidity or detecting increasing antibody levels with booster injections.

Next generation of biomarkers for vaccines

The traditional biomarkers outlined above measure the final immunologic outcome or protective capability of vaccines. As knowledge of immunologic mechanisms expands, the next generation of vaccine biomarkers allows one to measure vaccine efficacy at the individual immune cell level rather than measuring the total immune response. The new biomarkers almost exclusively measure T-cell function at the memory, helper and effector levels, as well as T-cell interaction with other cells of the immune system such as dendritic or antigen presenting cells. These biomarkers are designed to attain information at the individual immune cell level, incorporate individual cell phenotypic information together with cell function, assess memory and function at the T-cell

receptor level and measure function via cytokine production at the intra-and extra-cellular levels (Elahi et al. 2003, Gans et al. 2003, Ovsyannikova et al. 2003).

Figure 2 is a very simplified schematic representation of the immune system where the T-cell has a central role in both regulatory and effector functions of the immune response. The immune response is controlled by numerous soluble factors and immune response mediators such as tumour necrosis factor (TNF-α), interferon-gamma and numerous interleukins (IL). Described below are three methods that either measure cell function via the measurement of the soluble immune factors (cytokines) at the individual cell level or monitor cell receptor-antigen receptor interaction. The methods include the binding of tetramers to cell surface receptors, measurement of epitope immunoreactivity at the individual cell level using the ELISPOT and the simultaneous measurement of intra-cellular cytokine production and cell phenotype using flow cytometry.

Figure 3 illustrates the interaction between dendritic cells and T-cells where the dendritic cell is responsible for presenting to the T-cell the specific antigen (epitope) in a proper orientation so as to be recognized the T-cell receptor (TCR). The proper antigen orientation includes not only the epitope itself but also the appropriate major histocompatibility complex (MHC) molecules and other cell surface molecules such as CD80/86. T-cells possess a large repertoire of TCR and the presentation of the correct epitope/MHC/CD molecules that 'fit' into the TCR will initiate the subsequent immune cascade leading to the development of T-cell memory, Th1 or Th2 regulatory cells or some other effector cell function. For vaccines, therefore,

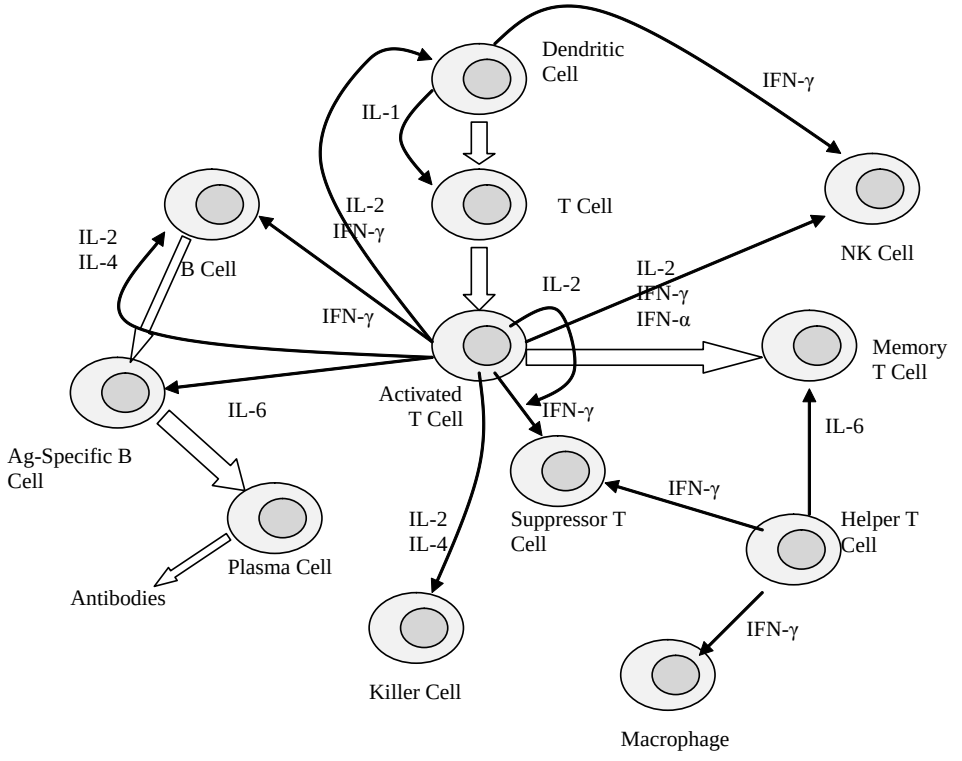

Figure 2. Schematic of T-cell interaction.

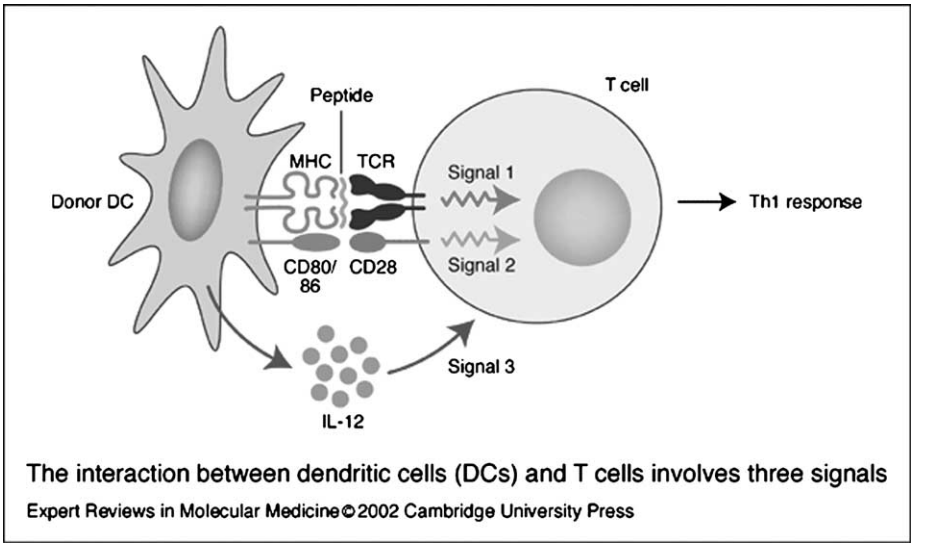

Figure 3. The interaction of dendritic cells and T-cells involving the T-cell receptor (TCR), major histocompatibility complex (MHC) antigens and inter-cellular signalling molecules.

finding the best epitope to stimulate the desired immune response is critical. Tetramers are synthetic versions of the dendritic cells' antigen presentation apparatus including the MHC molecules. Tetramers (Figure 4) are four single synthetic peptides/MHC complexes bound by a ringed structure (beta-2 microglobulin) to form a 'four-plex' or tetramer. The four-plex is more efficient and has a higher avidity than a single-plex molecule to bind to TCR *in vitro*. The tetramer also incorporates a fluorescent label so that when the tetramer binds to a T cell receptor *in vitro*, the binding event can be measured by flow cytometry.

Tetramers have several uses, but typically they are used identify the peptide sequence or epitopes that bind to the highest number of TCR in a naïve individual as well as identifying the phenotype of the T-cell to which the tetramer binds. Tetramers

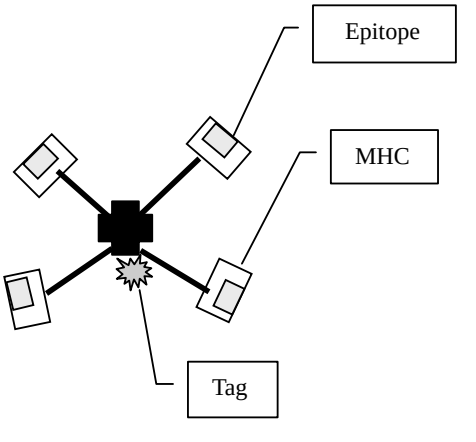

Figure 4. Structure of tetramer. Monomer consists of a synthetic molecule containing a peptide representing an epitope of interest, MHC molecule and beta-2 microglobulin.

can also measure the changes in the number of T-cells displaying a particular TCR before and after vaccination. Tetramers are now synthesized with both Class I and Class II MHC molecules so that binding to either Th1 or Th2 T-cells, respectively, can now be accomplished. Since tetramer binding is measured in a flow cytometer, the phenotype of the T-cell can be identified by the addition of monoclonal antibodies containing a fluorescent tag different than that present on the tetramer. The limit of detection of tetramer binding T-cells is one tetramer-binding cell per 8000 cells. After vaccination, a 2–5-fold increase in tetramer-binding cells typically occurs. Tetramers have been used for both infectious disease applications as well as identifying cancer vaccine candidates for melanoma, leukaemia, lymphoma and other cancers. Although tetramers are useful, the results can be difficult to interpret, since these measurements require high precision instrumentation due to the rare event analysis that is required to detect cell binding tetramers (Hoffman et al. 2000, Terajima et al. 2003, Whiteside et al. 2003, Walker et al. 2004, Ye et al. 2004).

In the 1990s, the ELISPOT technique was standardized and shown to correlate with the standard assay of the day to measure T-cell effector function, namely the chromium-release cell-mediated cytotoxicity assay. The ELISPOT gave the added benefit that it could measure individual soluble factors such as interferon-gamma, IL-4, IL-10 and TNF-α that are secreted by T-cells in response to an immunologic stimuli, i.e. measuring post-vaccine immune responses (Arlen et al. 2003). The ELISPOT technique utilizes 96 well micro-titer plates coated with a monoclonal antibody to the specific soluble factor being measured (INF-gamma, IL-4, etc.) where it functions as a capture antibody. The lymphoid cell population being investigated is then added to the microtiter wells containing the captured antibodies in a limiting dilution pattern. The specific antigen is also added to the wells and the cells are cultured for 6–48 hours. The cells and antigen are then washed from the well and replaced with a second monoclonal antibody containing an enzyme label to the specific soluble factor being measured. By developing the microtiter plate with the appropriate chromogen, spots will appear in the bottom of the microtiter well wherever a cell was present in the primary culture that secreted the soluble factor being measured. Since the number of cells originally added to the micro-titer well is known, it is possible to quantitate the number of cells responding to the antigen present in the cell population being studied.

As shown in Figure 2, measuring different soluble factors allows the investigator to assess different pathways of the immune system when activated by various vaccine regimes. For example, IFN-gamma is often measured to quantitate the number of activated T-cells present in the cell population being investigated. ELISPOT, like tetramer binding studies, is capable of identifying the presence of antigen reactive cells in very low concentration, as low as one cell in 50 000 (Terajima et al. 2003, Whiteside et al. 2003, Hudgens et al. 2004). Since ELISPOT reagents are now commercially available, this technique to dissect the immune response at the individual cell level is now a common practice.

The final method to assess immune reactivity to vaccines at the individual cell level is cytokine flow cytometry (CFC). CFC is similar to ELISPOT in that it allows one to measure the generation of specific cytokines in response to a particular antigen stimulus; however, CFC will also identify the phenotype of the cell stimulated to produce intra-cellular cytokines. Briefly, lymphoid cells are stimulated by a specific antigen in short-term cultures, usually less than 24 hours. After the short-term

cultures, the presence of intra-cellular cytokines is detected by fluorescein-labelled monoclonal antibodies. A second set of monoclonal antibodies with different fluorescein tags are used to identify the phenotype of the lymphoid cell stimulated to produce the intra-cellular cytokines. Although CFC does not measure cell function, it is very sensitive in detecting antigen reactive cells with a limit of detection of the one cell in 50 000. Thus, CFC can detect the presence of various T-cell sub-populations that will respond to a vaccine stimulus. This information can be used to modify both antigens and adjuvants to stimulate the T-cell populations desired, such as having an adjuvant that favours the stimulation of Th1 rather than Th2 cells (Whiteside et al. 2003, Mangada et al. 2004)

Practical applications of vaccine biomarkers

Recently, Wang et al. (2004) used nearly all the biomarkers available to develop a malaria vaccine strategy combining recombinant antigens and DNA vaccines. The investigators used the newer techniques such as IFN-gamma ELISPOT and tetramers, together with the traditional biomarkers of *P. falciparum* antibody levels and cell mediated cytotoxicity assays to investigate each arm of the vaccine strategy. They found the recombinant protein vaccines produced antibodies, but only short-term protection and no IFN-gamma producing cells. The DNA vaccine given separately did not give protection or tetramer-binding T-cells, although cytotoxic T-cells were detected. Using this biomarker information they developed a combined recombinant protein/DNA vaccine strategy that produced antibody, IFN-gamma producing cells, cytotoxic T-cells and increased protection.

Finally, Whiteside and Gooding (2003) recently reviewed the use of biomarkers to monitor the efficacy of gene therapy to enhance the immune response to residual disease in leukaemia and lymphoma patients. They found that cell population-based assays such as cytotoxicity and proliferation assays were not sensitive enough to be used to measure cancer vaccine or gene therapy efficacy. They also found that the single cell based assays such as tetramer binding, ELISPOT and CFC lack standardization and typically did not correlate with each other. Even though these single cell assays were able to detect reactive cells at levels of less than one cell per 10 000, this level of sensitivity was still not useful for cancer vaccine studies. For their studies in cancer gene therapy, they recommend the use of skin testing with appropriate cancer cell derived antigens to measure therapy efficacy. One hundred and fifty years later, Dr Koch would be proud.

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