ANTICANCER RESEARCH *25*: 2345-2356 (2005)

Review

Contemporary Definitions of Tumor Specific Antigens, Immunogens and Markers as Related to the Adaptive Responses of the Cancer-Bearing Host

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Abstract. *This review describes clear parameters for designating the correct use of the term Tumor Rejection Antigen [TRA] to define the role of tumor cell constituents which activate adaptive anti-tumor immune reactions in the cancer-bearing host. This is important, especially in defining immunogens which activate the patient's cytotoxic T-cells that are important to immunotherapeutic applications in human cancer treatment. The focus of the review is to correctly delineate the immunogenic properties of 37kDa oncofetal antigen [OFA], one of only a few true TRAs expressed on human and experimental rodent cancers. The purpose of this review is to provide a background for publication reviewers, journal and text editors, and scientists reporting on TRAs to avoid creating further confusion that has proliferated in the cancer literature to imply traits of so-called tumor-associated antigens that do not qualify as TRAs.*

Whether cancer immunobiologists admit it or not, a persistent problem has emerged in the terminology commonly used in the field of tumor immunology to define tumor-associated antigens. This has produced considerable confusion and has increasingly corrupted the definition of tumor rejection antigens (TRA) in the modern literature. The confusion has arisen from an unfortunate disassociation of the concept of immunogenicity away from the property of antigenicity of various substances naturally present, reappearing, or appearing *de novo* in association with tumor cell development during active carcinogenesis in the autochthonous, tumor-bearing patient or host. Unfortunately, the confusion has finally seeped from the experimental literature into the fundamental consideration of primary tumor immunogenicity for the host as presented in medical lexicons and immunological textbooks published in the past decade.

The goal of this review of tumor immunology-related terminology is to hopefully provide a useful set of clarifying guidelines which will enable investigators, journal and text reviewers working in the field to agree to accurately designate the characteristic immunologic role of tumor-associated antigens / immunogens. Basically this involves being able to distinguish actual T-cell-inducing immunogens from other protein "tumor markers" or so-called "differentiation antigens", which are not auto-immunogenic for the tumor-bearing host and can only arouse an immune response in a foreign species or under other poorly understood tolerance-breaking methods (1-4).

The first illustration of the problem is found in the example set forth in "Dorland's Medical Dictionary" (5) and in "Basic Immunology" (6). Dorland's defined an immunogen stating that it is "a substance capable of inducing an immune response; in most contexts immunogen is synonymous with the term antigen; in some contexts, however, immunogen is used to draw a distinction between substances capable of inducing and reacting only with antibody (antigen or haptens) or to denote a form of

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Key Words: Tumor specific antigens, immunogens, tumor markers, cancer-bearing host, review.

antigen that induces an immune response as opposed to a tolerogen, a form that induces tolerance" (5). In other words, immunogen and antigen are synonymous only when the autochthonous host expressing the protein in its cells elicits an immune response directed against the protein in that host as opposed to a foreign host (5).

The text "Basic Immunology" (6), used widely to train medical and graduate students, defines "immunogen as an antigen that induces an immune response." It goes on to state, "Not all antigens are immunogens." Had the term immunogens used here been replaced with the term auto-immunogen, the confusion could have been greatly reduced. In neglecting this last point, some tumor immunologists in the last two decades have created confusion in the classification of tumor rejection antigens by failing to distinguish tumor-associated proteins as T-cell activating auto-immunogens in the primary host from those cell products that cannot serve as auto-immunogens in the autologous patient.

Many investigators today are on a quest to identify tumor rejection-inducing immunogens which activate T-cells in the host developing the tumor and are capable of killing tumor cells by recognition of true tumor rejection antigen(s) (TRAs) presented in association with human MHC's. This leads us to the first proposed Guideline in clearly defining a *tumor specific antigen / auto-immunogen*. If the protein under consideration as a TRA is not *immunogenic* for the primary tumor-bearing host as a T-cell-stimulating antigen, it should never be called a TRA. We suggest that all non-auto-immunogenic substances be called tumor-associated "markers" in keeping with the original designation for these protein substances. If a marker protein is involved in normal cell differentiation, it can be designated a Differentiation Marker Antigen (DMA) to acknowledge its ability to incite an antibody in a foreign animal host. Such proteins can often be detected in greater amounts in some tumor cells than in normal cells!

An example that highlights the confusion that has now unfortunately emerged in the literature of tumor immunobiology centers on a variety of new textbook classifications which seek to distinguish Oncofetal Antigen (OFA) from other subclasses of so-called tumor "antigens" (6, 7). Dorland's Dictionary defines "Oncofetal antigens" as "antigenic gene products that are expressed during fetal (and possibly embryonic development), are partially or completely repressed in adult tissues and de-repressed in some tissues that have undergone neoplastic transformation". Then follows the unfortunate confusing statement about Oncofetal Antigens in Dorland's which espouses that "Carcinoembryonic Antigens (CEAs) and Alpha Fetoprotein (AFP) are thus useful tumor markers.". This definition involves a bit of "slight of hand", jumping from the term "antigen" to "marker" without acknowledging immunogenicity or lack thereof in the host where OFA is expressed in all primary tumors of rodents and humans we have tested to date (8-10). Thus, this supposed clarification is only partially true, but is clearly not relevant to defining OFAs nor any tumor-specific immunogen, as is implied in inappropriately conjoining the two distinct definitions of tumor-specific "marker" as an "OFA". The confusion grows when Dorland's separately defines "Tumor Markers" as "a biochemical substance indicative of neoplasia (tumor), ideally specific, sensitive and proportional to tumor load, used variously to screen, diagnose, assess prognosis, follow response to treatment, or monitor (the patient) for recurrence." Herein lies a significant and misleading "rub". Since OFA is represented as CEA and AFP as examples of tumor-specific markers with the traits outlined above for OFA, they would be assumed to be immunogenic antigens in the tumor-bearing host.

This is never true as reflected in the world literature. It can never be true since CEAs and AFP are classically "self-proteins" in normal adults in several non-cancer disease states (AFP or CEA associated cirrhosis) and in normal, proliferating hepatic cells and many other such cell types. After reviewing the vast literature historically relating to CEA and AFP, it is clear that both the many forms of CEA reported and AFP are expressed in significant quantities in "normal", multiplying, diploid human tissues and cells. It is obvious that Dorland's and other medical dictionaries lead to the inclusion of CEA and AFP as Oncofetal Antigens (OFA's). Our position is that CEA and AFP are never tumor-specific, nor auto-immunogenic in the tumor-bearing host, and their designation as "tumor markers' rather than true OFA's was intended to distinguish them from OFAs rather than intended to include CEA and AFP "markers" and other oncogene-encoded proteins as true OFA's.

We have consistently reported that OFA is a true tumor immunogen/antigen in the cancer-bearing hosts (11-17) as will be discussed in a subsequent section of this review. We have cited this fact since discovering a true OFA that is tumor-specific and auto-immunogenic in the cancer-bearing host (11, 12, 17). We recently described the normal maturation of OFAs to a non-immunogenic surface membrane-associated form in late fetal development (11, 12, 17). This results from a post-translational modification of the immunogenic OFA present in the membranes of late term fetal tissues and diploid adult cells and the subsequent loss of immunogenic expression of dimerized OFA in late fetal cells and in neonate and adult, replicating cells and tissues.

Abbas and Lichtman (6) have thus correctly defined OFAs as "Proteins that are expressed, as autoimmunogens, at high levels on cancer cells and in normal developing fetal but not adult tissues. As has become a common mistake, these authors then state that "Antibodies specific for these proteins (implying OFAs) are often used in histopathologic

identification of tumors or to follow the progression of tumor growth in patients. Once again these authors have switched to defining tumor markers like CEA, CD66 and AFP as OFA glycoproteins that are over-expressed by certain carcinomas, but are rarely expressed in excess in all carcinomas, even those of the same type (*e.g.*, colo-rectal, hepatocellular, pancreatic or breast carcinomas). Again, as stated in Dorland's, the definition of OFA is again rendered confusing and misleading. Thus, we again remind the reader that immunogenic / antigenic OFA is found in embryo and some early fetal human and rodent cells, but due to its modification in fetal development, is not antigenically / immunogenically expressed in term fetal cells in pregnant inbred rodents or in outbred human females, nor in neonate tissues or in adult tissues (17). As listed above, CEA and AFP are never antigenically expressed so as to induce antibody or T-cell subclasses in the human tumor-bearing host, unless apparent low affinity immunologic tolerance or ignorance are broken artificially, as is the case for certain members of the CEA family of molecules (4, 18-21). Antibodies referred to in their textbook's definition of CEA and AFP, needed to detect these marker proteins, must be made normally in rabbits or other xeno-species. Hence, CEAs and AFP are not auto-antigenic nor immunogenic components of tumor cells for the cancer-bearing host. Further, not all carcinoma cells or individual human tumors express AFP or CEA, whereas OFA is detected in all rodent and human tumor cells and tissues as an early, tumor specific, immunogenic protein not expressed on normal adult cells (8, 9, 22). OFA clearly arouses T and B cell responses in the tumor-bearing host (10).

Some authors (6, 7) list distinct tumor rejection antigens subclasses without regard for whether the peptides present in the designated proteins are non-self, auto-immunogenic substances or not. Abbas and Lichtman (6) suggested that the substances comprising the types of "tumor antigens" recognized by host T-cells are: a) mutated self-proteins like those appearing on carcinogen or radiation-induced animal tumors or on human melanomas; b) products of oncogene or mutated tumor suppressor genes like mutated Ras, Bcr/Abl fusion proteins, or p53, respectively; c) over-expressed self-proteins like tyrosinase, gp100, MAGE or MART proteins; or, d) viral-encoded transforming proteins like SV40 TSTA or human papilloma virus gene products like E6 or E7 proteins. Janeway *et al.* (7) lists the following tumor rejection antigen subclasses as: a) embryonic proteins restricted to immune-privileged specific organs like the testis in normal, cancer-free humans where they incite T-cell responses under special circumstances when appearing outside the testes in malignant tumors. These proteins include MAGE -1 and MAGE-3 (note that CEA and AFP are not listed); b) differentiation antigens like Tyrosinase (note that CEA and AFP are not listed); c) mutated oncogene (Ras) or suppressor gene products (p53); d) abnormal post-translational modification of non-self peptide epitopes like MUC-1; and e) oncoviral encoded proteins like HPV 16 E6 and E7 proteins expressed in cancerous warts. Note also that OFA protein is missing from both classifications, we believe, primarily, due to the unwillingness of the authors to consider true OFA (8-17).

The improper inclusion of CEAs and AFP as OFAs is due, in part, historically to miss-naming them as examples of "embryonic antigens" when it was noted many years ago that CEA, AFP and other "marker proteins" which are never immunogenic in the cancer-bearing hosts due to their presence in normal, replicating tissues and identity as "self" proteins, and the requirement to detect them with antibody prepared in xeno-species hosts (*e.g.*, rabbits). Clearly, the examples of OFAs used by Dorland's are referring to "tumor markers" and DMAs, not tumor "autoantigens". Tumor "markers" and DMAs are never immunogenic for the host (arouse neither antibody nor T-cell responses in normal humans nor when expressed in higher levels in some but not all cancer patient's tumors). Tumor markers are not tumor *versus* normal adult cell-specific. The unfortunate decision by the Dorland lexicon to give an accurate general description of OFAs in humans with and without cancer and then to use the term "tumor markers" and give the examples of CEA and AFP as OFAs negates their classification as true OFAs.

Further, in the search for novel tumor rejection auto-immunogens that can be used to arouse anti-tumor T-cell responses which promote tumor rejection, this issue is further complicated by the needlessly confused definitions of "self" *versus* "non-self" proteins detected in primary tumors which do not or do elicit autologous host T-cell responses, respectively (23). This has led to a disturbing literature which leaves the reader of these reports in a quandary because of the confusion of some investigators as to the auto-immunogenicity of differentiated cell components in activating T-cells *in vivo*.

Immune Responses against Tumors

All manner of studies seeking to identify and characterize TRAs have been reported since the 1950's when serious studies in inbred rodents were first attempted (24-26). In the years prior to 1950, much that was assumed to be true about the characteristics of cancer immunobiology was defined in outbred rodents. Potent T-cell activating antigens detected to be present in such non-syngeneic rodent model tumor systems were, in fact, indistinguishable from MHC determinants present on the tumor cells; hence, the rejection data generated was valuable only in defining histoincompatibility determinants, not tumor-specific auto-immunogens (18). Later studies were conducted with inbred mice, hamsters and rats and clarified this problem and

mandated that TRAs had to be identified in syngeneic rodents (25-27). Since the term "antigen" generally implied any protein that was observed to elicit an immune response in a test host, the term became loosely used in the cancer immunology literature. Subsequently, genuine tumor-specific rejection immunogens (TRAs) were detected to be present on a variety of cancer types in syngeneic or autochthonous hosts. Viral-encoded immunogens (v Oncs) expressed on virus-induced neoplasms (28), authentic oncofetal antigen (OFA) (11-17, 29), mutated p53 proteins in human cancers (30) or mutated proteins chaperoned to the cell surface membrane are examples of true TRAs.

The major development in the first 20 years of studying tumor-associated immunogens /antigens in inbred animals was the concept of "Immune Surveillance" (31, 32). Three lines of clinical / pathological evidence that the human or other mammalian host immune systems can be aroused against TRAs appearing on tumors and not normal tissues are:

1. Lymphocytic infiltrates developing around primary tumors contained activated, immunogen-specific T-cells in immunocompetent syngeneic hosts (33-35).
2. T-cell lymphocytes are routinely activated against tumors in inbred experimental animals and humans by direct vaccination with syngeneic tumor cells from another syngeneic animal or in the autologous patient, respectively, or were detected subsequently following successful tumor removal and elimination by surgery. The immunity mediated by these antigen-specific T-cells can passively transfer immunity (adoptive immunity) to naive, inbred, syngeneic rodents when the animals receiving the immune T-cells are subsequently challenged with syngeneic tumor cells expressing the tumor-specific immunogen. Control, non-immune mice receiving adoptive transfer of non-immune T -cells are not similarly resistant to the same challenge with a variety of tumor cells including carcinomas, leukemias or sarcomas (36-38).
3. T-cell-deficient humans and animals experience an increased incidence of certain types of cancer such as lymphoma/leukemia (39, 40).

Host Cytotoxic T-cell Responses

Shared OFA or unshared, tumor clone unique tumor-specific transplantation-like antigens (TSTAs) are capable of arousing the required CD8 + CTL or Tc responses that can recognize and kill emerging tumor cells *in vivo*. Both types of immunogens are present on rodent and human cancer cells (10, 12-17, 41-44). TRAs are endogenously synthesized cytosolic proteins which are displayed at the tumor or early fetal cell surfaces in conjunction with class I MHC-associated proteins on early transforming as well as fully-transformed malignant cells or some embryo cells or early fetal cells. These antigens are recognized by class I MHC-restricted CD8+CTLs whose function is to attach to and subsequently kill tumor cells expressing the immunogen. CD8+CTLs are detected in rodent tumor challenge models and in human (41) where tumors are induced by chemicals, radiation or oncogenic viruses (*e.g.*, vONCs-mutated, normal cell proteins, *etc.*) (10, 12-17, 41-44).

CTLs are activated against tumors by recognition of MHC-bound peptides of tumor-specific immunogens like viral encoded v Oncs or cellular-encoded TSTAs and by universally expressed OFA on the surface of host antigen-presenting DCs or other professional antigen-presenting cells (APC's). APC's ingest tumor cells or their antigens and present these antigens to two T-cell subclasses (*i.e.*, CD4+helper T lymphocytes (Thl) and naive CD8+ precursor T lymphocytes) (10, 12-17, 41-44). The CD8 precursor cells differentiate into tumor antigen-specific CD8+CTLs or Tcss. The CD8+ T-cell activation is promoted by APC interaction of co-stimulator B7 determinants present on the APCs with Tc-expressed CD28 subsequent to Tc T-cell receptor binding of class I MHC-bound tumor antigen peptide. Class II MHC :tumor antigen peptide-activated CD4+ Thl cells promote differentiation of the CD8+ precursor Tc-cell into fully activated tumor-specific CD8+ CTLs by cytokine secretion. Presentation of processed tumor antigen to both class I MHC-restricted, tumor antigen-specific T-cells by dendritic cells which took up tumor antigen is called "Cross-priming or Cross-presentation" (45-47). The activated tumor-specific, effector CTLs that develop and kill tumor cells express the class I MHC-bound homologous tumor immunogen peptide without either the costimulator or CD4 Thl helper interaction. However, the central role of CD4+ T-cells in the anti-tumor immune response should not be underestimated (48).

Thus, CTLs are induced by cross-presentation of a tumor immunogen by host APCs and are then effective in killing the tumor cells. Some immunologists have reported that CD4+ Thl helper cells can kill tumor cells to a limited extent directly (49, 50) but, if true, this is very limited killing. Antibodies to some tumor antigens which require Th2 helper cell interaction have also been implicated in tumor cell killing, but this too results in marginal killing of tumor cells, if it occurs. Likewise, activated macrophages and natural killer cells (NK) have been linked to the killing of some tumor cell types *in vitro*, but the role of such anti-tumor antigen reactivity has not been effectively established to operate significantly *in vivo*.

It is thus understandable that many investigators have incorrectly deduced that primary tumors, which may display one or several potential tumor immunogens, can only induce "weak" tumor resistance (26, 27, 51-53). Such immunity is presumably easily overwhelmed by challenge with high levels of tumor cells (12, 13, 15, 16, 44). Since

Table I. *Reported types of mammalian tumor antigens distinguished by their auto-immunogenicity or lack thereof in the tumor-bearing host.*

Name	Immunogenic in cancer-bearing host	Example
Mutated Cellular Self-Proteins[1]	+	TSTA
Cellular Oncogene Products (c Onc)[2]	--	Abl
Viral-Induced Oncogene Products (v Onc)[3]	+	SV40 polyoma; ADV T-antigenn
Differentiation Antigens[4]	--	CEA, AFP
Heat Shock Protein-Peptides Complexes[5]	+	Gp96-chaperoned peptides
Oncofetal Immunogen[6]	+	37kD OFA/iLRP
Unmasked Mucin Immunogen[7]	+	MUC-1

[1]Mutated self-proteins which are rendered immunogenic to the tumor-bearing host. Chemical carcinogen, radiation-induced or spontaneous mutations in cellular genes encoding these altered proteins all express these individually specific, immunogenic proteins which are unique to each tumor clone. These unique immunogens are termed tumor-specific transplantation-like antigens (TSTAs) which are functional TRAs. TSTAs are unshared with other tumors even when primary tumors induced by the same chemical or radiation carcinogen appeared on the same host animal (70-72). Tumors of rodents and humans may express both a unique TSTA and a shared oncofetal antigen, both of which are immunogenic in the host, as well as several other "marker proteins" that are not actually immunogenic in the host (12, 13, 15, 16, 44).

[2]Cellular encoded Oncogene proteins (c Onc). These non-immunogenic, normal cell proteins are linked to normal regulation of growth properties of the cell and are not tumor specific. These self-proteins are generally over-expressed by tumor cells (*e.g.*, the Abl gene product) (73). This over-expression is frequently due to altered mutations induced by carcinogens in one of several cellular encoded Tumor Suppressor genes (*e.g.*, p-53 protein) which produce proteins which regulate the cONC expression levels by normal cells. Cellular or cONC genes may also be translocated on the chromosome during cell transformation to alter their expression levels, but additional control mechanisms have been observed to impact their expression levels (74, 75).

[3]Oncogenic viruses encoded transforming auto-immunogenic gene products (v ONCs) introduced by abortive infection of host cells with tumor-producing viruses. Examples are: Human papilloma virus (HPV) type 16, expresses E6 protein in cancerous warts, E7 protein in cervical carcinoma (21, 76, 77); EBNA proteins in EBV-infected cells (78), SV40-like or Adenovirus encoded T-antigen proteins in various human cancers induced by these oncodnaviruses (79) including human papovaviruses like BK or JC polyoma viruses. Such v ONCs are classically shared immunogenic proteins to the host when expressed in viral-transformed human and animal cancer cells by a given virus such as HPV or SV40. These v ONC products may also be expressed on non-cancer cells infected permissively with these oncogenic viruses, but such cells are routinely lysed or damaged when producing infectious oncogenic virus.

[4]Over-expressed differentiation associated antigens (DAs) or non-mutated self-proteins (*e.g.*, CEA (36, 80, 81), AFP (4, 18-20), HER-2/neu (82), human telomerase reverse transcriptase (hTERT) (49, 59, 83), tyrosinase (84) , gp100 (85, 86), tryrosinase-related protein (TRP)-1 (87), TRP-2 (88), *etc.*) are non-immunogenic, differentiation markers present in many primary tumors and are routinely expressed on normal tissues in adults or which appear in immune priviledged sites (*e.g.*, testes).

[5]Heat Shock Protein-Peptides Complexes (HSP-PCs): HSPs (gp96, hsp70, hsp90, calreticulin, hsp 110 and grp 170) are a family of inducible but also ubiquitously and constitutively expressed self-derived protein chaperones involved in assisting protein folding and unfolding in the cells. Because of their ability to chaperone cellular peptides, HSPs, derived from animals with cancers, elicit protective immunity specific for the cancer from which the HSPs are purified. This property allows CTL activation without the need to identify the corresponding antigen and provides the basis for a new type of cancer vaccine. HSPs-chaperoned peptides can be presented to CTL by dendritic cells (DCs) in the context of MHC class I molecules through receptor-mediated endocytosis. CD91 (also known as α-2 macroglobulin receptor) has been recently identified as one of these receptors (reviewed in 89, 90). In a pilot study, 16 patients with different types of cancer were vaccinated with autologous tumor-derived gp96, which is known to be immunogenic in mice (91). Six of 12 patients that could be tested developed MHC class I-restricted tumor-specific T-cell response (92). A similar study performed on metastatic melanoma patients receiving autologous tumor-derived gp96-peptide complexes led to an increase in specific T-cell responses against melanoma antigens in 48% of patients and to clinical responses in 18% of patients (93).

[6]True Oncofetal Antigen (OFA)/immature Laminin Receptor Protein (37kDa OFA/iLRP). This protein is immunogenic when re-expressed on all mammalian cancers examined to date, yet remains undetected and rendered non-immunogenic when expressed in term fetus, neonate tissues, and in the inbred and outbred pregnant host's adult tissues due to dimerization, acylation and other post-translational maturational changes (11-13). OFA/iLRP is a shared, universal tumor rejection antigen/immunogen (TRA) (12). OFA/iLRP can also induce immunoregulatory CD8+ T suppressor cells (iLRP-specific T-cells secreting IL-10) which disables CTLs directed against any tumor rejection antigen (TRAs including OFA/iLRP) (10, 14, 41-43).

[7]Unmasked Mucin Proteins. The mucin family of glycoproteins is characterized by a variable number of amino acid tandem repeats and extensive O-glycosylation at serine and threonine residues. To date, at least eight mucin genes, encoding the protein backbone of these glycoproteins, have been identified. One of these genes, MUC-1, codes for a membrane-bound mucin (94). In some carcinomas, MUC-1 has frequently been found to have increased, unpolarized expression as well as under-glycosylation, allowing for greater immunogenicity by revealing otherwise masked epitopes in its protein core (95). Such unmasked MUC-1 proteins are immunogenic when processed endogenously by host APCs (96, 97). Natural antibodies exist against cryptic carbohydrate epitopes such as Tn and TF which become exposed in tumor cells due to under-glycosylation (98).

emerging cancer cells actually express several *de novo* immunogenic macromolecules in the host, all these immunogenic proteins could be considered "weak" antigens or immunogens in the host since primary tumors emerged successfully in the immune-competent animal, yet the host developed cytotoxic T-cells against these immunogens . In other words, all these immunogenic proteins produced an ineffective host T-cell response to eliminate the immunogenic tumor. The concept that unfortunately emerged was that all tumor rejection immunogens are "weak" or "ineffective antigens" (51, 53, 54). We have suggested that this view reflects an incorrect assumption

(12, 13, 15, 16, 44), because the investigators claiming such weak immunogenicity did not recognize that immunoregulatory mechanisms involving a newly discovered subclass of CD8 T-cells called T-suppressor cells can be activated by a regulatory tumor immunogen such as OFA (10, 14, 41-43). OFA may be unique in this activity since Ts cells are specifically activated by OFA, but the IL-10 secreted by these Ts cells inhibits Tc cell killing directed to any TRA (10-14).

When OFA-specific Ts cells are activated as the predominant subclass in animals responding to these new immunogenic macromolecules, the OFA-activated Ts lymphocyte subclass may be down-regulating the tumor-bearing host's T-cell mediated immunity in the presence of high levels of certain tumor-specific immunogens (10, 14, 41-43). Since this is often true, the concept of "weak" TRAs becomes erroneous and misleading. In addition, some tumors may also utilize several other immunologic "tricks" that prevent maximal impact of the T -cell immune system on the developing cancer. Some examples are tumor cell secretion of immunosuppressive cytokines such as TGF-β and IL-10, loss of MHC determinants needed for effector T-cells to recognize tumor T-cell immunogens, and mutation or reduced expression of the immunogenic tumor protein. The latter may be of importance because tumor antigens expressed at levels sufficient for cross-presentation by bone marrow-derived stromal cells appears to overcome immunological "ignorance" to solid tumors (55). Some researchers reported that tumors of humans and animals are weakly immunogenic. Increasing the immune reaction may have little effect or may actually stimulate rather than inhibit the growths of these tumors in their primary host (52, 56). This too is incorrect (10, 14, 41-43)!

How Does OFA Differ from CEA, and AFP and Other So-called Carcinoembryonic Proteins?

As stated above, CEA and AFP are "Differentiation Antigens "(DAs), and are not *tumor rejection immunogens/antigens* (*TRAs*). Only 37kDa OFA actually warrants the designation "Oncofetal Antigen". Sir Peter Medawar, together with the first author of this article (JHC), coined the term OFA in the early 1970s (16, 29). These key criteria were that true OFA is only expressed in immunogenic form in the developing mammalian embryo and early fetus during pregnancy and is re-expressed in all malignant tumors of mammals tested by us and others to date (12, 13, 15, 16, 44). Most important, 37kDa OFA is not expressed in auto-immunogenic form in normal adult and /or normal regenerating cells (see details below). In contrast, CEA, and AFP are not immunogenic in the pregnant female or in the cancer-bearing host and are present on many, but not all, normal, regenerating cells of several organs. 37kDa OFA ceases to be synthesized as an immunogen for the pregnant host in late term fetus and in normal adult animals after birth due to the maturational process to the mature, 67kDa non-immunogenic OFA previously described.

Background Summary of OFA/iLRP

Immature laminin receptor protein (iLRP) is a 37kD, monomeric, non-acylated, true oncofetal immunogen in the strictest sense (11, 12). It is immunogenic for the mother being actively expressed on the surface of cells comprising part of the embryo and early fetus *in utero* (12, 13, 17, 44). In contrast, mature 67 kD laminin receptor protein (mLRP) is rendered non-immunogenic in pregnant hosts before birth of the mature fetus because it is dimerized and acylated to mature LRP (mLRP) in the newborn and is often linked non-covalently to β-galectin (11). Mature LRP is expressed at varying levels on some normal adult cells, which function in the adult host to enable these differentiated cells to egress through laminin basement membranes in normal tissues and vessel walls. That is, OFA/iLRP, the 37 kD immature fetal-restricted molecule, unlike mLRP, can serve as an "auto-immunogen". Following early neoplastic cell transformation, OFA/iLRP is re-expressed universally on cancers induced by viral, carcinogen and radiation-induced cancers as well as spontaneous cancer cells arising from other mutations in DNA in the host (11-12). OFA/iLRP is never expressed on any normal or differentiating cell in the neonate, juvenile or adult mammal. Both mature 67kDa and immature 37kDa iLRP attach to laminin (11).

To be a true autologous immunogen (capable of inducing an immune response in the adult host) a protein must be recognized as foreign to its own thousands of other so-called self-proteins which comprise that animal's normal tissues and cells. For example, AFP and CEA are expressed on normal dividing adult liver cells and a few other organ-specific cells in the animal at birth and on normal fetuses of that animal (10-12, 14, 41-43). When the normal adult liver makes new cells to replace damaged liver, and other cells in the adult animal from undifferentiated normal liver precursor cells that give rise to replacement liver cells, they classically express lots of AFP. When these precursor cells are fully differentiated into normal liver cells, they continue to express small amounts of the AFP. AFP is over-expressed on several types of normal adult cells during normal cell replication and is generally present in high levels in some tumor clones, but it is never normally immunogenic for that tumor-bearing host.

The host mammal expressing Differentiation Markers (DMs) is usually immunologically incapable of recognizing DMs as *non-self* proteins. Why? T-lymphocyte precursors undergo a process called "thymic maturation". During thymic

maturation, precursor T-cells (thymocytes) that develop T-cell antigen receptors which bind self-antigens presented in the thymus too well are deleted by apoptosis to render the host mostly incapable of responding to its own tissue, thereby preventing auto-immune disease. Most importantly, human CEA and AFP, unlike 37kDa OFA/iLRP, are most easily detected with antibodies made in a foreign host like a sheep or goat. Antibodies against 37kDa OFA/iLRP are induced in autologous tumor-bearing hosts or by immunizing inbred mice with syngeneic early fetal cells expressing OFA/iLRP (17).

Self-antigens and Tumor Immunotherapy Trials

To date, the large majority of the known human tumor "antigens" used in cancer immunotherapy are non-mutated self-proteins which are over-expressed by tumors (57). However, some researchers still find them to be promising for the purpose of diagnosis and immunotherapy. This may explain the confusion in the field between an "antigen" and an "immunogen", because any promising antigen is immediately pursued experimentally as a possible tumor-specific immunogen in clinical immunotherapy trials in an attempt to control tumor growth by activating T-cells. The apparent success in these trials depends on the following:

1. Despite the thymus being exposed to these proteins during embryonic development, some peptide-specific T-cells probably escape deletion during the ontogeny of the immune system. Activation of these T-cell clones is achieved by presenting these epitopes in an immunostimulatory context, such as by engineered dendritic cells (4). When DCs are transduced with a recombinant adenovirus (Adv) vector encoding AFP, they will process and present epitopes in the context of MHC class I antigens and can induce modest AFP-specific protection (4, 18). Similarly, DCs pulsed with agonist CEA peptide epitopes induce CEA-specific effector T-cells and block thyroid carcinoma progression (21, 58). Also, immunization of CEA-expressing cancer patients with a canary poxvirus vector encoding CEA and B7.1 has produced disease stabilization and increased CEA-specific effector T-cells in 37% of patients in a small clinical trial (21).
2. However, most of these trials are based on the use of high affinity dominant epitopes as targets for specific CTL responses (59-62). Despite some positive results, there is increasing evidence to indicate their clear lack of efficacy, is probably due to tolerance to dominant determinants on self-antigens (1, 61). Breaking tolerance to dominant epitopes on these self-antigens is, indeed, becoming one of the major goals of tumor immunologists in the preparation of vaccines (1-3). To circumvent immunologic tolerance to dominant epitopes, cryptic epitopes are used for tumor immunotherapy provided that they are efficiently presented by tumor cells (36, 63, 64).
3. The use of high-affinity heteroclitic variants of low affinity epitopes to mobilize CTL targeting low affinity epitopes, provided that they are presented by tumor cells efficiently enough to be recognized by CTL, poses an interesting avenue for immunotherapy. However, low levels of expression of low affinity epitopes should be a barrier for their use in immunotherapy, since CTL effectors require a small number of peptide /HLA complexes on the target surface to be activated (65). However, the use of the non-mutated self-antigens in tumor immunotherapy raises the risk of autoimmunity, due to the expression of these antigens on normal cells, including the thymus. This could be harmless if the antigen is present in an immuno-privileged organ, shielded from an autoimmune response, as in the case of MAGE-A-expressing testis, but in other cases could be a great problem, as when using the hTERT protein, which is expressed in activated T- and B-cells and in the CD34+ hematopoietic progenitors (66).

Summary of Tumor-associated Antigens *vs* Tumor Rejection Immunogens

True tumor rejection antigenic immunogens (TRAs) (our term preference) must first and foremost be recognized as non-self proteins to the extent that these proteins arouse detectable T-cell-mediated immune responses directed against the auto-immunogenic protein(s) expressed on the emerging cancer in the host. To be effective in arousing a protective, host T-cell-mediated adaptive response which is capable of destroying the tumor cells, this immune response to any TRAs present on a cancer cell must activate CD4+ helper T-cells (TH1) and CD8+ effector cytotoxic T-cells (Tc) stimulated by CD83+dendritic cells (DC) or other antigen-processing cells (APCs) (4, 10, 14, 41-43). A cardinal feature of the adaptive immune response against tumors is its "exquisite specificity (of T-cells) for distinct macromolecules and ‘memory’, which is the ability to respond vigorously to repeated exposures" to a non-self immunogen present on cancer cells (6).

True tumor rejection inducing TRAs thus must be capable of activating Tc-mediated immunity in the autologous host. Autochthonous antibody induction against TRAs is widely reported to play at least a minor role in tumor rejection (10). The use of the term "immunogen" to characterize non-immunogenic markers like CEA, AFP and c Onc products, which appear on normal as well as cancer cells, must be avoided. If the protein is not immunogenic in the primary cancer-bearing host and must be detected with antibody made in a foreign species (most correctly called a "xenoantibody"), such a protein is not a true TRA for the cancer-bearing host, but is simply a "cancer marker" to denote its association with abnormal cell replication and differentiation. This is a fundamental requirement to

differentiate normal cell components from tumor-specific components which activate tumor rejection. Markers are usually expressed in greater concentration on tumor cells than normal cells, but are never tumor-specific, since they are also expressed on normal adult cells and tissues.

Thus, the second criterion that must be determined to warrant the status of being ranked as a TRA is that the cancer-associated protein or other component must not be expressed in immunogenic form by normal adult tissues of the host. The bottom line in this terminology distinction (Antigen *vs.* Immunogen) is that the historical term "tumor antigen", as reflected in the immunological literature, does not automatically imply its immunogenicity in the autochthonous host.

Unfortunately, most modern reviews of tumor immunology historically do not make this important distinction (6, 67, 68). The seeds of this confusion in terminology rest in a classic dogma that has allowed certain proteins expressed on normal diploid cells of an animal or human to be classed as "Tumor Antigens" even if they lack immunogenicity in the host with the cancer. Such misnomers give the reader the impression that these "antigens" are true tumor "immunogens" for the host when, in reality, they are only present in higher concentrations on tumor cells than normal cells of the host. Likewise, the cancer-bearing host is unable to make a T-cell response to these self-macromolecules.

Normal adult tissues of a human generally lack any immunogenic determinant(s) which can arouse a host T- or B-cell-mediated response due to *deletion of precursor thymic cells* which can "recognize" self-antigens during maturation in the normal thymus or inactivation of those which escape deletion. If this were not so, mammals would have tremendous auto-immunity problems. Hence, the most important so-called "tumor rejection-capable" immunogens likely to be useful in creating an effective and specific anti-cancer vaccine or be useful to detect malignant cancers are those few TRAs which are absent from normal adult tissues and are processed as T-cell immunogens in the cancer-bearing host. Some, but a very few, human cells located in immunologically "privileged sites" (*i.e.*, anterior chamber of the eye, testis and central nervous system) may express an auto-immunogenic "self-antigen(s)" in the host, but such cells are restricted to the sequestered anatomical site. Cancer/testis (CT) antigens are examples of tumor components normally expressed and restricted to male germ cells in the testis, but are not present in adult somatic tissues. Spontaneous humoral and cell-mediated immune responses have been demonstrated against several CT antigens, including NY-ESO-1, MAGE-A and SSX antigens (69). Table I gives a summary of the different types of tumor-antigens and their immunogenicity in the tumor-bearing host.

Acknowledgements

This work was sponsored by the NIH National Cancer Institute grant ROI CA 82603, USA.

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Received February 24, 2004
Accepted March 23, 2005