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Edited by

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HUMAN CANCER-ASSOCIATED ANTIGENS: PRESENT STATUS AND IMPLICATIONS FOR IMMUNODIAGNOSIS

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I. Introduction

The search for distinctive markers for neoplasia or, as tumor immunologists prefer to call them, tumor-specific antigens (TSA) is at least as old as immunology. Inspired guesses as to the role that immune phenomena might play in the natural history of cancer and, by implication, as to the likely existence of protective antigens in tumors go back to the beginning of modern biology. But it took over half a century of frustration and disappointments before irrefutable proof was finally demonstrated that carcinogen-induced tumors can indeed evoke specific antitumor immunity in mice (for historical notes see Hellström and Brown, 1979; Springer *et al.*, 1979; Gold and Goldenberg, 1980).

The successful demonstration of specific antigens in animal tumors provided a major impetus to the search for comparable antigens in human neoplasms. Initially, attention was confined largely to the study of cell-mediated immune responses of patients against their tumors (reviews:

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Hellström and Brown, 1979; Shuster *et al.*, 1980), but gradually the interest shifted toward serological analysis, culminating in a veritable avalanche of publications following the advent of the hybridoma technology (Köhler and Milstein, 1975). The uninitiated trying to find his way in the intricate jungle that the literature on human tumor-associated antigens (TAA) is today is bound to become hopelessly lost. There is first the sheer number of publications, which is enormous. But more importantly, a great deal of the published work is hardly relevant anymore. Armed with rudimentary technology and often with shaky factual data, but imbued with irresistible optimism, many of the earlier investigators were quite prepared to state, or at least imply, that the vague materials they were studying were indeed "tumor specific." This was said notwithstanding the crude techniques used in their assays, the scarce proofs of specificity, and the poor or complete lack of antigen characterization. It took a great deal of useless work before the realization seeped through that the use of sensitive techniques is a sine qua non requirement for tests of specificity and cross-reactivity, although, surprisingly, immunodiffusion is still being used for such purposes.

An extensive review of the antigens associated with human solid tumors, covering the period up to 1979, has been published by Gold and Goldenberg (1980). Their list of "Cancer Markers of Current Interest" has grown considerably in the intervening years (Waldmann and Herberman, 1982), but cancer markers of "current interest" appear to come and go at a relatively fast rate. The fact remains that only a few markers are actually used at present (Bagshawe, 1983), and none of them has reached the eminence still enjoyed by carcinoembryonic antigen (CEA), the first marker discovered. Furthermore, none of the markers used in clinical work is a true TSA.

The purpose of this review is to sift the considerable amount of data that have accumulated in recent years on TAAs of human solid tumors, with a view to assessing their *specificity* and their possible usefulness for the *early diagnosis* of cancer. In the absence of a clear-cut and universally accepted definition for TAA, the selection of material included in (or left out of) the discussion is bound to seem at times arbitrary. Unfortunately, the confusion regarding the TAAs starts with their very definition (Sulitzeanu and Weiss, 1981). Ng *et al.* (1983), for instance, define TAA as an antigen which is not expressed by the normal counterpart of a tumor cell. Order and his associates (1982) term TAA a normal tissue protein increased in concentration as a result of cancer, while according to Levine (1982), tumor antigens are "moieties associated with a tumor in an animal, that elicit an immune response in the host." The major problem is, of course, that TAAs are an extremely heterogeneous group of molecules, and the overlap with normal antigens is so great that it is practically impossible to define TAAs in such a way as to distinguish them clearly from ordinary cellular components. For

better or for worse, we have adopted a rather loose definition, according to which a molecule is a TAA if its presence renders the cancer cell quantitatively or qualitatively different from its normal counterpart.

It should be obvious to anyone acquainted even superficially with the subject that it is absolutely impossible to cite, much less discuss here, everything that has been published on human TAA. The most one can possibly do is to try to present a broad survey of the field, hoping that the reader will be a little less confused at the end than he was at the beginning. In attempting to do this, the approach has been to select a few representative papers for more detailed discussion and to refer the reader to additional articles for further information. Well-reviewed markers such as CEA and α-fetoprotein (AFP) are not included. For recent reviews consult Shuster *et al.* (1980), Waldmann and Herberman (1982), Bagshawe (1983), and Klavins (1983), markers in general; Beatty and Terz, (1982), NIH (1981), Fuks *et al.* (1980), CEA; Sell (1980), AFP; Horne and Brenner (1980), pregnancy proteins as tumor markers; Klavins (1981), markers of pancreatic carcinoma; O'Brien and Morrow (1983), placental proteins; Griffiths (1983), prostatic adenocarcinoma; Björklund and Björklund (1983), tissue polypeptide antigen; Goldenberg *et al.* (1980), and Wolf and Reid (1981); radioimmunolocalization of tumors. Studies dealing with cell-mediated immune reactions will not be discussed here, since the specificity of the antigens involved (our major concern here) is difficult if not altogether impossible to establish by such reactions.

II. Approaches to the Identification of Human TAA

The work on human TAA was started with the tacit assumption that tumors of the same histological origin should possess cross-reacting antigens, an assumption which was in fact entirely gratuitious, as it had neither a theoretical nor an experimental basis. Of the three broad categories of experimental tumors, carcinogen induced, viral induced, and spontaneous, only the viral-induced tumors are cross-reactive. The carcinogen-induced tumors possess individual specific antigens (Old, 1981), although even this firmly established dogma is not without its heretics (Lennox, 1983). With regard to the spontaneously arising tumors of experimental animals, it is amazing that virtually nothing is known about their serologically defined antigens. They have been shown to lack protective antigens, however (Hewitt *et al.*, 1976).

Investigations on human TAA have been conducted with three types of reagents: heteroantisera (poly- or monoclonal), antibodies from sera of patients, and immune complexes. For the sake of convenience, our discussion will follow the same lines.

A. Studies with Heteroantisera

The classic approach to the study of TAA has been to prepare antisera, generally in rabbits, to absorb them extensively with normal tissues, and to test the absorbed sera for specific reactivity with the tumor cells. Investigations using this approach have declined sharply since the advent of hybridoma technology, but quite a few are still being published, although the authors tend to be more cautious nowadays when describing their antigens as "tumor specific."

A convincing demonstration of a true TSA would require ideally a strategy more or less along the following lines:

1. preparation of an antiserum reacting with cells of a given tumor;
2. purification and characterization of the antigen;
3. development of highly sensitive assay (preferably a competition radioimmunoassay) for the antigen; and
4. extensive search (by means of the radioimmunoassay) for the presence of the antigen in fetal tissues and in normal and malignant cells of as many types as possible.

A survey of the published work reveals that most investigations stopped after the completion of the first stage, and less than a handful reached the final stage. This is why truly convincing data on TSAs are almost nonexistent, despite the seemingly promising results presented in many of the initial reports.

Among the antigens detected by means of heteroantisera, the most thoroughly investigated and, possibly, the only one deserving the designation as tumor specific is the mammary tumor glycoprotein (MTGP) discovered by Edgington and his co-workers. MTGP was identified by Leung *et al.* (1978) in the cytosol and membranes of breast cancer cells by means of an antiserum prepared against a perchloric acid extract of metastatic breast cancer tissue. The cytosol MTGP, which exists in two major forms with a weight of 20 kDa was demonstrated in 76% of the 101 breast tumors tested (Leung *et al.*, 1979), but was not detectable in benign breast cells (Leung *et al.*, 1978). An exhaustive examination of normal tissues and of tumors of other than breast origin (76 samples altogether) showed them to be devoid of MTGP (Leung *et al.*, 1979). This conclusion was strengthened by further analyses with a sensitive radioimmunoassay. The antigen was not present in serum and urine of patients (Leung and Edgington, 1980). Subsequent studies (Leung *et al.*, 1981) revealed that whereas the membrane MTGP was contained by all mammary carcinomas, the cytosol form was not demonstrable in half of the breast cancer cell lines. The concentration of MTGP was estimated to be about 500 μg/kg tumor (Sundblad and Edgington, 1983).

The unusual thoroughness of these studies and the high degree of sensitivity achieved, down to 2 molecules per cell (Leung *et al.*, 1981), would seem to justify designating MTGP as one of the very few (if not unique) TSAs discovered so far. What is still needed to confirm these extremely interesting findings is independent corroboration.

The literature is replete with publications describing additional TAAs identified with heteroantisera (Table I), but none of these TAAs has been studied with a thoroughness remotely approaching that of MTGP. Yet, in isolated instances, the data presented look quite impressive. Ghose and his co-workers (1979), as an example, prepared an antiserum against renal cell carcinoma which stained (by immunofluorescence) 21 of 22 human renal car-

TABLE I
TAAs OF HUMAN TUMORS IDENTIFIED BY MEANS OF HETEROANTISERA

Tumor	Reference
Bladder	Highashi and El-Asfahani (1983)
Breast	Yu *et al.* (1980)
	Kamiyama *et al.* (1980, 1982)
	Edgington and Nakamura (review, 1982)
Cervix	Ibrahim *et al.* (1979)
Colon	Higgins *et al.* (1983)
	Chakrabarty *et al.* (1983)
Lung	Sega *et al.* (1979)
	Veltri *et al.* (1980)
	Yamada *et al.* (1980)
Melanoma	Birkmayer (1981)
	Galloway *et al.* (1981)
	Gaynor *et al.* (1981)
	Carrel *et al.* (review, 1982a)
	Heaney-Kieras and Bystryn (1982)
	Steplewski and Koprowski (review, 1982)
Neuroblastoma	Seeger *et al.* (1979, 1980)
Ovarian cancer	Gerber *et al.* (1977)
	Bhattacharya and Barlow (1978)
	Imamura *et al.* (1978)
	Dawson *et al.* (1980)
	Cantarow *et al.* (review, 1981)
	Knauf and Urbach (1980, 1981)
	Bissary *et al.* (1983)
Pancreas	Schultz and Yunis (1979)
Prostate	Nadji *et al.* (1981)
	Wang *et al.* (1982)
Stomach	Wang *et al.* (1983)

cinomas but failed to stain an imposing collection of normal tissues (including normal kidney, perirenal fibroblasts, peripheral blood cells, and fetal kidneys) as well as unrelated tumors (transitional cell carcinoma of the bladder, adenocarcinomas of the breast and colon, squamous cell carcinoma of the lungs, malignant lymphoma, and melanoma). This performance is certainly on a par with that of some of the best monoclonal antibodies, but in the absence of follow-up studies no definitive conclusions can be reached from this and similar investigations.

B. Studies Using Human Sera: Autoantibodies in Human Sera

A major difficulty in the search for antibodies against TAA in sera of patients with cancer has been the presence in all human sera of immunoglobulins with affinity for normal tissue antigens (Mackay, 1983). Some of these are specific for fetal antigens, as they bind to normal cells in culture and can be removed by absorption with first-trimester human fetal tissues (Thorpe *et al.*, 1977; see also brief review by Gupta and Morton, 1983). Others react with ordinary cellular and serum proteins as shown in a most convincing work by Avrameas *et al.* (1981; see also Guilbert *et al.*, 1982). These workers used immunoadsorbents to purify from normal human sera antibodies specific for seven common antigens, including even antibodies to human serum albumin! Other investigators have shown that autoantibodies binding specifically to heart, liver, and brain homogenate can be found in practically every normal serum (Daar and Fabre, 1981). However, when cancer and normal sera are compared, a higher incidence of autoantibodies is generally found in the former. As an example, 65% of sera of patients with lung tumors, as compared with 17% of normal sera, contained antibodies to nuclear antigens, smooth muscle, mitochondria, thyroid microsomes, gastric parietal cells, and reticulin (Ruffatti *et al.*, 1983). Autoantibodies in cancer sera which react with these and other antigens have been described in numerous other publications [Humphrey *et al.* (1977, antibodies to altered IgG in breast cancer), Kurki *et al.* (1977, antibodies to smooth muscle, reticulin, bile canaliculi, and nuclear antigens in urogenital diseases), Turnbull *et al.* (1978, autoantibodies in breast cancer), Storch *et al.* (1980, antibodies against myocardium, smooth muscle, and nuclear antigens in various cancers), Zauli *et al.* (1980, antibodies to smooth muscle in neuroblastoma), Dube *et al.* (1982, red blood cell cold agglutinins and B lymphocyte cytotoxins in breast cancer), Thomas *et al.* (1983, antinuclear, antinucleolar, and anticytoplasmatic antibodies in melanoma)]. These findings should make it abundantly clear why it is imperative to absorb patient sera as extensively as the heteroantisera before drawing conclusions as to their specific reactivity with tumor antigens.

C. Antibodies to TAA in Patient Sera

The search for antibodies in sera of patients with malignant diseases was initiated with the hope and mistaken belief that antibodies found to react with the tumor cells would be specific for the tumor. Antibodies binding to tumor cells were found indeed in numerous investigations, although the frequency of the positive samples varied widely according to the technique and target cell used and perhaps according to the optimism of the investigator. It became increasingly clear, however, that these antibodies were more often than not normal autoantibodies, as they could be removed by extensive absorptions with normal tissues. This is not surprising, considering the widespread occurrence of similar antibodies even in normal sera (see preceding section).

The most intensive and revealing work on the reactivity of patient sera with cancer cells has undoubtedly come from the laboratory of Old (for reviews see Old, 1981; Houghton *et al.*, 1981; Carey, 1982). To eliminate errors inherent in the use of allogeneic serum–target cell combinations, a new technique was devised—autologous typing. This consists of establishing tumor and fibroblast cell lines from each patient so that the serum can be tested in parallel against the autologous neoplastic and normal cells. Binding tests were performed by means of highly sensitive techniques, and the specificity of the antibodies was checked by appropriate absorptions. Using this approach in studies of patients with melanoma, the investigators were able to identify three classes of melanoma TAA: class I antigens (found in 4 tumors) specific for each individual tumor; class II antigens (5 tumors), expressed by a proportion of the melanomas tested; and class III antigens, present in a wide range of normal human and animal cells. Further studies with melanoma (Livingston *et al.*, 1981), astrocytoma (Pfreundschuh *et al.*, 1978), and renal cell carcinoma (Ueda *et al.*, 1979) disclosed a similar distribution pattern, with very few of the sera defining the more specific class I and class II antigens.

Class I antigens are reminiscent of the individual specific antigens of carcinogen-induced mouse tumors, and their inability to induce formation of antibodies (only six class I antibodies encountered in 150 melanoma patients, Mattes *et al.*, 1983) could be taken to constitute another point of similarity. Initial difficulties with the immunoprecipitation of class I antigens have delayed investigations on their biochemical nature, but the problem seems now to have been solved (Real *et al.*, 1983) and rapid progress in this field can be anticipated.

Antibodies to AH, the prototype class II TAA of melanoma (Watanabe *et al.*, 1982), have also been identified only infrequently, and sera containing similar antibodies have been found as well in patients with astrocytoma

(Pfreudschuch *et al.*, 1978) and even in healthy individuals (Houghton *et al.*, 1980). The demonstration that AH is a normal, autoantigenic GD2 ganglioside (Watanabe *et al.*, 1982) goes a long way toward explaining why antibodies against it are so rare and why it is so difficult to induce them even after immunization—only 1 of 20 patients injected with an AH-bearing allogeneic melanoma vaccine made anti-AH antibodies (Livingston *et al.*, 1983). A much better response of melanoma patients receiving immunotherapy with melanoma vaccine was reported, however, by Dent *et al.* (1982), with 7 of 10 patients producing antibodies that reacted with melanomas or melanomas and brain tumors (class II-like). However, whereas the Livingston group had tested their postimmunotherapy sera on a single cell line, Dent and colleagues used a panel of 10 lines, and their analyses indicated that different patients had responded to different "class II-like" antigens.

Brown and co-workers (1982) were unable to confirm the existence of the three antibody classes in melanoma. They conducted their experiments with allogeneic and autologous melanoma cells and fibroblasts, using a complement-dependent microcytotoxicity assay. The only antibodies they detected were directed against fetal fibroblasts (as shown by the ability of these cells to remove completely the reactivity against the tumor cells). Whether these discrepant results can be accounted for by the inability of the microcytotoxicity assay to detect other than class III antibodies remains an open question.

The autologous typing technique enabled for the first time the beginning of some sort of order in the previously chaotic state of human TAA. Unfortunately, as pointed out by Oettgen and Hellström (1982), this technique is fraught with considerable difficulties, a major one being the establishment of cell lines from a large group of patients. Modified autologous typing methods which avoid this problem were devised by other investigators. Roth and Wesley (1982) carried out binding experiments with autologous sera, using butanol extracts of freshly removed sarcomas as antigen. The inhibition of binding by tumor but not by normal tissue extracts was taken as indication of tumor-specific reactivity. Interestingly, 14 of 16 sera showed preferential binding to extracts of the primary tumors as compared with extracts prepared from the metastases, suggesting antigenic differences between the two preparations. Gupta *et al.* (1979a) purified antibodies from melanoma sera on affinity columns charged with a membrane-rich fraction isolated from autologous tumors. The purified antibodies reacted in complement fixation tests with preparations derived from melanomas, sarcomas, and carcinomas, but not with similar preparations from normal liver, lung, or lymphoblastoid cell lines. These findings, together with additional results obtained by cross-absorption experiments, led to the conclusion that the patient sera contained

antibodies against melanoma TAA as well as against oncofetal antigens (OFA). An oncofetal antigen, which elicits antibodies in both cancer patients and normal individuals, was subsequently obtained in a partially purified form from spent culture medium (Gupta and Morton, 1983) and identified as a glycoprotein of 60–70 kDa. (For further work with autologous sera see Carey *et al.*, 1983; Kornblith *et al.*, 1983.)

Most of the work with patient sera has been and still is carried out with allogeneic targets. A large-scale study of Davis *et al.* (1981) can be taken as representative of the kind of conclusions reached in this type of investigation. The authors tested 344 sera of patients with breast cancer for binding to four breast cancer, four nonbreast cancer, and three animal cell lines, using both an indirect radioimmunoassay and a competition radioimmunoassay with specifically purified, labeled antibodies. Thirty of the sera gave significant binding, but the binding patterns differed from one serum to another, indicating clearly that a number of specificities were involved. After appropriate absorptions, only 2 of the sera bound exclusively to the breast cancer cells, suggesting perhaps that class I or II antigens may also be present in breast cancer. Plain and co-workers (1981, 1982) examined the specificity of the antibodies in breast cancer sera by means of antibody-dependent cellular cytotoxicity (ADCC) with a panel of 10 breast cancer cell lines as targets. Preferential reactivity of the breast cancer, as compared with the normal control sera, could only be demonstrated with 2 of the 10 cell lines employed, showing how critical the choice of a cell line can be. Absorption experiments again indicated that a diversity of antigen–antibody systems was involved, arguing strongly against a unique breast cancer-associated specificity.

A strange, well-documented, although still unexplained relationship exists between human breast cancer and its mouse counterpart, the virus-induced mouse mammary tumor. This relationship is evidenced both by the demonstration of antibodies with affinity for mouse mammary tumor virus (MMTV) components in sera of patients with breast cancer and in breast cyst fluids, and by the finding of an antigen in some of the breast cancers which cross-reacts with the gp52 protein of MMTV. The topic has been reviewed extensively (Moore *et al.*, 1983).

Numerous other reports have been published purporting to show the presence in patient sera of antibodies reacting selectively with the donor type tumor, but the evidence for specificity—the critical question—has generally been less than convincing. Often, the techniques used were patently nonsensitive (e.g., immunodiffusion) or not sensitive enough (immunofluorescence), the number of sera examined too small, the "specific" antigen detected was not characterized, at least to the extent of SDS–PAGE

analysis, and, finally, no follow-up studies were published in many instances, making it impossible to assess the validity of the preliminary findings.

In contrast to the many publications describing positive results, there is understandably only a trickle of reports on negative findings. Higuchi and colleagues (1980) tested 352 postoperative sera of patients with breast cancer, some of whom had also been treated with a breast cancer vaccine. The vaccination increased considerably the incidence of positive samples (15.6% in the nonvaccinated versus 72.3% in the vaccinated group), but no breast cancer specific reactivity remained after absorbing the sera extensively with normal adult, fetal, and melanoma cells. The authors concluded that all the antibodies in the sera were directed against oncofetal antigens. Similar negative results were described by Martin-Achard *et al.* (1980) for the glioma system.

Considering the abundance of publications on supposedly tumor-specific antibodies in patient sera, it is hard to understand why the next logical step was so rarely taken, namely, the use of such antibodies to prepare immunoadsorbents for the isolation and analysis of the antigens. A determined effort to do just that was reported by Thomson *et al.* (1980). Patients with breast or colon cancer who gave a positive reaction in the leukocyte adherence inhibition (LAI) test were selected as serum donors. Affinity columns prepared with the IgG fraction of the sera were used to isolate the antigens from the urine of patients, and the specificity of the eluates was checked in the LAI tests. Both the breast and colon cancer eluates showed bands of 38–40 kDa. In previous work, the same group had isolated from four different tumors (breast and colon carcinoma, melanoma and hepatoma) antigens seemingly associated with β_2-microglobulin, as they could be coprecipitated with anti-β_2-microglobulin antibodies (Lopez and Thomson, 1977; Lopez *et al.*, 1978; Thomson *et al.*, 1978, 1979). The antigens from all four tumors gave similar band patterns on SDS-PAGE, yet each was specific for the tumor of origin, as shown by ability to induce LAI with leukocytes from the relevant donors only. The interesting implications of this series of papers are that human tumors may contain TAAs of very similar structure, although of different antigenic specificity, and that these TAAs might actually be modified HLA antigens. The significance of these intriguing findings remains uncertain, as no independent corroboration has been forthcoming. However, further evidence in support of the "β_2-associated antigen" concept has been provided by the same laboratory (Brenner *et al.*, 1982). TAA purified from lung tumors by anti-β_2-microglobulin affinity chromatography was employed to raise monoclonal antibodies. These were tested by immunofluorescence with lung tumors and normal tissues and found to react strongly with squamous cell lung carcinomas, but not with oat cell car-