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Application of Monoclonal Antibodies for the Diagnostic and Therapeutic Targeting of Human Tumors with a Necrotic Component

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Abstract—Due to a unique structure of tumors, TNT antibodies can bind with a great number of target cells in the hypoxic core of the injury zone. Long-standing laboratory and clinical research gave evidence for the utility of histone/DNA complexes as stable insoluble antigens available for binding with TNT antibodies in necrotic (but not in healthy!) cells. The ability of TNT antibodies to selectively accumulate in the necrotic cores of most solid tumors allows detection and killing of primary and metastatic tumors of any origin.

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General Principles of the Application of Antibodies in Diagnostic and Therapeutic Human Cell and Tissue Targeting

Over the past quarter of the century the idea to use antibodies and their derivatives for selective delivery of therapeutics and their binding with functionally significant targets in a living body (targeting) has made the way from purely scientific research to introduction to therapeutic practice.

The annual turnover of pharmaceutical products whose action is directly or indirectly based on the use of antibodies exceeds $10 billion and is constantly growing. The benefits immunoglobulins offer over low-molecular physiologically active substances (potential drugs) is the possibility of virtually unlimited modification of the binding site of the immunoglobulin molecule evolutionarily selected to fulfill the functions of recognition, binding, and activation of effector mechanisms for maintaining integrity of a unique multicellular organism.

The following criteria are useful to be taken into account when assessing the effect of affine protein complexes administered in the body [1].

Selectivity: Whether these complexes prefer to accumulate in a specific part of the body (when a lot of competitive binding sites are available)?

Efficacy: Whether the potential accumulation capacity of the protein complexes in the target site is sufficient for the therapeutic effect (or for diagnostic purposes)?

Immunogenicity: How strong the complex induces development of a delayed immune response upon repeated administration or some immediate immune hypersensitivity reactions in previously sensitized patients?.

Toxicity: Whether the affine protein complex injected at a calculated effective dose will exhibit nonspecific side effects?

Application: What are pathological states at which the protein complex in focus is reasonable to use instead of traditional therapeutic means [2]?

Expediency: Whether concrete dosage forms of affine protein complexes are reasonable to commercialize in terms of their prospective use in a targeted therapy?

Biodistribution of Antibodies

The specific feature of the biodistribution of macromolecular complexes (compared to traditional drugs) consists in a limited region of their free motion (in a stream of surrounding fluid due to diffusion or filtration through the tissue matrix) [1, 3]. The most active transport can be via the circulatory system upon intravenous injection of the protein complex. As a rule, when other traditional administration routes are used (oral, peritoneal, intrathecal, subcutaneous), a therapeutic agent is preferentially retained just in the injection site. In an extreme case, a solution or suspension is mechanically injected directly in the target site. Therewith, the ability of the protein complex to selectively bind with targets in the body is not a key issue, even though affine binding with the material of the carrier ensures slow, controlled release of the complex.

When an affine protein complex enters the blood stream (whatever is the infusion site), a powerful flow transport mechanism is switched on, which ensures uniform distribution of the injected substance in the circulatory system within a few minutes [1]. Therewith, an active interaction of the binding sites (epitopes) of the protein component with blood components (proteins, platelets, and white and red blood cells) and the coating of blood vessels (endo-thelium and basal membrane and reticuloendothelial system components) develops [4, 5].

Nonspecific contacts do not form strong bonds, provided affine protein complexes have been chosen correctly [6]. In should be borne in mind that the endothelium surface area in human lung capillaries is slightly less than 50 m^2, and the surface area of the endothelium in the capillary bed of the systemic circulatory system is 60 m^2. The total surface area of red blood cells is estimated at 2000 m^2.

It is easily seen that for at least a few percent of the injected protein complex dose to be accumulated in a target site, high-selectivity binding should be provided [7]. Therefore, in the present therapeutic practice, the choice of targets for antibodies is limited to circulating cells (platelets [8], some populations of white blood cells, cancer-modified stromal cells) [9]. An interesting and realistic alternative, viz. immune targeting of circulating stem cells, necessitates labeling of the corresponding site on the surface of the circulatory bed, edothelium, exposed subendothelium, fibrin deposits, or damaged vessel areas [7, 10]. It should be noted that the accessibility for the protein complex of the binding site from the blood stream side is more important for targeted delivery than the specificity of binding with components of this site (a parameter which is preferably evaluated *in vitro*) [11].

It might seem natural to accomplish organ-specific targeting by means vector molecules affine to a potentially inert target or components of the extracellular matrix. However, when labeled antibodies to type IV collagen (one of the structural elements of the endothelial monolayer which separates the principal tissues–muscle, nervous, bone, lung–from the blood stream) were infused into the blood stream, they rapidly accumulated in lungs, and this was associated with multiple lung damage. Labeled antibodies to the angiotensinase enzyme (present on the surface of lung epithelium), too, preferentially accumulated in lungs but caused no side effects. When infused into the blood stream of rats, labeled antibodies to type III collagen (present on the surface of fibrous atherosclerotic plaques) selectively accumulated in spleen [12]. Consequences of such an accumulation have never been studied.

The size of the immunoglobulin macromolecule or its fragments confines the distribution region of antibodies and their derivatives by that compartment, where their solution is infused into the body. In particular, this relates to direct infusion into the blood stream, when the distribution inside the circulatory system occurs within a few minutes due to vigorous mixing, and the natural half-excretion time by renal filtration comprises, even for fab fragments (fragments of the immunoglobulin molecule, which bind with antigens), one day or longer [3], whereas the degree to which antibodies and their complexes can penetrate to the interstitial (intercellular) space within a day is fairly low [13]. A much more essential mechanism of clearance involves entrapment of modified immunoglobulins by the liver reticuloendothelial system. In this case, the concentration of the injected substance in the blood stream can be decreased many times within as little as 10 min [14], which is of exceptional importance, for example, for labeling an object expected to entrap radioactive antibody derivatives in the blood stream. Naturally, liver therewith entraps (and then processes) the most part of the radioactive agent injected for diagnostic purposes, and this confines the region of localization of the site of affine binding of labeled antibodies by the requirement for it to be remote from liver [15].

Over two decades all known blood-transportable objects has been tested as targets for affine binding with antibody derivatives. Directing neurotropic viral particles got into the blood stream to a macrophage pathway of their elimination via incorporation in the immune complex formed [16] and blocking active enzymes (toxins), hormones, or other biologically active molecules have become practically significant routine approaches in immunotherapy. Formed blood elements (platelets, red and white blood cells) proved to be a more complicated object [8]. Thus, blocking the glycoprotein IIB/IIIA complex (fibrin/fibrinogen binding receptor) by antibody fragments allows one to suppress platelet aggregation for a period from a few hours (antibody fab fragments) to 2 days (fab-2 fragments) [17]. Drugs on the basis of such antibodies showed their clinical efficiency and are presently applied for thrombosis prevention. The destruction of defect red cells, accelerated by binding with immune complex, favors erythropoiesis and much improves hemodynamics. Biospecific antibody complexes allow lymphocyte targeting to circulating cells, such as transformed cells capable of forming metastatic tumors.

Antibody Targeting of Conjugated Plasminogen Activators

Research on the features of biodistribution of labeled antibodies to fibrin (a protein formed on blood clotting) in the framework of the problem of targeting conjugated activators of plasminogen (a precursor of plasmin) to a thromb-forming blood clot in the great vessel [18] revealed some regularities. First, antibodies rapidly distributed in the blood stream, not forming complexes with blood components or vessel coating [19]. Second, up to 20% of the label of the injected substance rapidly, within minutes, was found in liver. Presumably, this is a way how immunoglobulin molecules damaged during chemical modification and labeling are eliminated from the body.

The circulation time of the rest antibodies is a few days; radioactivity was preferentially accumulated in liver. Fab fragments of antibodies clear from blood 3 times as rapid and primarily through kidney. The binding of antibodies with a blood-swept fibrin clot is fairly efficient, but labeled antibodies (and their complexes with urokinase) are sorbed in a thin surface layer of the clot, not penetrating into its depth [20]. The diffusion permeability of the fibrin clot for macromolecules even comparing in size with immunoglobulin M (IgM) molecule is fairly high, but penetration into the depth of the clot is limited just by specific binding of antibodies in surface layers. These data show that the perspective to create thrombolytic drugs with selective affinity to fibrin is limited in terms of their applicability to microaggregates [21, 22]. Analogously, the applicability of labeled antibodies to fibrin for localization by means of γ-scintigraphy of arterial thrombosis after myocardial infarction, too, proved to be limited by physical accessibility of the target [21]. The accumulation of the radioactive label at the site of an artificial thromb is incommensurate with what is observed in the surgery zone, where are a lot of blood clots exposing antigen (fibrin) over a large surface.

Thus, for efficient selective targeting of macromolecules, not only a highly specific antibody (vector) is needed, but also it is necessary that the target is accessible within a reasonable time frame [13]. Accessibility is limited not only by the limiting physical pore size of membranes, separating organs, tissues, or their compartments, but also by diffusion distances in a macromolecule-permeable hydrogel [23]. In the case of sorbable macromolecules, an essential issue is the spatial distribution of binding sites in the target volume, since inner layers of the target prove in fact inaccessible in view of the efficient entrapment of the affine protein complex in the surface zone [22].

Forced Antibody Clearance

Attempted scintigraphic localization of thrombosis zone gave evidence showing that labeled antibodies fairly fast, within 1–2 h, ac-cumulated in a 3D target modeling a surgery site [24]. To “visualize” the label accumulation area (the label contained fractions of percent from the injected quantity) on the background of the distributed radiation from labeled antibodies present in the blood stream, forced clearance was applied [14].

Labeled antibodies bind with a protein ligand additionally injected to the blood stream, and the resulting complexes are absorbed by liver within a few minutes. As a result, the distributed radioactivity background falls by an order of magnitude within 10 min, which makes it possible to visualize the antibody accumulation area, provided it remote enough from liver.

At the Convergence of Chromatin and Cancer Biology

While the goal of developing monoclonal antibodies that specifically target tumors has been around

for about 35 years, the field has been hampered by technical problems, chief amongst them being the transient nature of many tumor "specific" antigen markers. But what if monoclonal antibodies were targeted NOT against the ever-changing expression pattern of tumor markers but rather against stably expressed antigens common to collateral structures?

It might appear odd to think of DNA as a "structure," however, it is a necessary one for the survival of any cell, including tumor cells. The sheer amount of DNA required to program a mammalian cell requires a system of organization and that is achieved by the presence of histone proteins [26]. There are five major families of histone proteins, with members from four of those families (H2A, H2B, H3 and H4) acting as a spool around which the DNA is wound. We refer to these members as "core" histones and the structure created by them and the DNA is referred to as a nucleosome. The members of the fifth family are known as "linker" histones (H1 is the major family and H5 is a minor variant) and they sit on the outside of the nucleosome to lock the structure in place [26]. Histone proteins, DNA and other auxillary nuclear proteins are referred colloquially as "chromatin."

Chromatin researchers have spent years utilizing antibodies in their study of nuclear organization in the cell. Generation of these antibodies has taken two approaches. The first involved immunizing animals with pure antigen against a very specific target. Examples of this approach includes antibodies created in the 1980s by Mendelson and Bustin against the avian linker histone, H5 [27, 28], and antibodies created in the 1990s by Parseghian and Hamkalo against human linker histones $H1^S$-1 through $H1^S$-4 [29, 30]. The other approach has focussed on using nature's own creations isolated either from auto-immune mouse models [31] or from aging mice [32]. This second approach highlighted a trend, also noted by auto-immune researchers, that most autoantibodies created in nature target the H1 linker histones found on the outside of the nucleosome, a target that is easily accessible [33]. Confirmation of this came when researchers would inject uncharacterized samples of nuclear extract into mice and generate antibodies against immuno-accessible chromatin components [34], chief amongst these accessible structures being histone H1 [35].

Isolation of these antibodies led some groups to investigate their use as tumor targeting agents. The laboratory of A.L. Epstein isolated a series of antibodies they termed TNT-1, TNT-2 and TNT-3, with the "TNT" standing for Tumor Necrosis Therapy [35, 36]. Tumor Necrosis Therapy is a strategy that takes advantage of stably expressed markers that are the result of a growing cancer. Solid tumors have several distinguishing characteristics from normal tissues, including an under-developed vasculature that delivers much of the oxygen and nutrients to those cells located in the periphery of the tumor. The growth of any solid tumor over a 4 mm diameter is soon accompanied by a hypoxic environment in the center [37]. Furthermore, the difficulty of surviving in such an environment is compounded by the fact that solid tumors generally have a poor lymphatic system as well, hence, leading to a slow clearance of waste and debris from the tumor microenvironment [38]. All of this generally leads to a large number of dead and dying cells (necrosis) at the center of any solid tumor [39, 40]. The poor lymphatic system results in an extended presence of these necrotic cells; whereas, normal tissue can quickly dispense with any necrosis occurring in its midst.

The laboratory of V.P. Torchilin, having isolated nucleosome targeting antibodies from aging mice [41], has used a monoclonal they have termed 2C5 to detect nucleosomes on the surface of tumor cells grown *in vitro* [42]. Exposing tumor cell cultures to a number of chemotherapeutics, the researchers demonstrated chromatin released from necrotic cells could bind the surface of surviving tumor cells accompanied by a 50-fold increase in the binding of 2C5 antibody [43]. They have suggested a strategy similar to the Epstein laboratory, that these chromatin targeting antibodies could make good tumor targeting molecules *in vivo* and this targeting can be enhanced in combination with tumoricidal agents. To that end, a team of US researchers are investigating the 2C5 antibody as a targeting molecule and delivery vehicle for brain tumors, however, that work is still in the research phase [44]. As we shall see, the TNT technology is already in the clinic.

The migration of chromatin fragments from necrotic cells to the surface of living tumor cells is not a prerequisite for the success of this therapeutic strategy. Unlike apoptosis, necrotic cells develop a permeability to large molecules early in the process of disintegration. Hence, necrosis exposes the highly conserved nuclear structures of a cell, such as histone proteins and DNA, and allows for the targeting of

these structures by monoclonal antibodies even when the structures are still within the dead or dying cells[1]. The advantages of this strategy include (1) the targeting of universal antigens that are found in all human tumor types, (2) the targeting of antigens that are highly conserved in structure and can be applied to a wide array of animals as well, (3) the targeting of an abundant, stable and insoluble structure at the center of solid tumors, and (4) the targeting of antigens that CANNOT cease expression in any viable tumor cell, indeed, in any cell, precluding the ability of a tumor cell from developing resistance to the antibody.

By marrying the fields of cancer and chromatin research, Tumor Necrosis Therapy seeks to use the most stable antigenic structures found at the very center of a tumor as the target for delivery of a wide variety of diagnostic and therapeutic molecules. As delivery vehicles, these antibodies can be used in combination with any necrosis generating procedure, including chemotherapy and radiation therapy. The more necrosis, the more anchorage for the TNT antibodies at the center of a solid tumor.

Proof of Principle

To demonstrate the utility of such a strategy several lines of investigation were followed. Preliminary work involved the injection of mice with several tumor models, including ME-180 (a cervical carcinoma), HT-29 (a colon carcinoma), A375 (a melanoma) and HS Sultan (a myeloma). The tumors were injected subcutaneously and allowed to grow prior to having the mice treated with Iodine-131 (^{131}I)-labeled F(ab')$_2$ fragments of TNT-1. Analysis of the antibody distribution 3 days after its administration indicated a tumor to blood ratio ranging from 3 : 1 all the way up to 20 : 1 for the various solid tumors. Of the four tumor models, only HS Sultan showed a tumor to blood ratio of 1 : 1. Such a result was validation of the principle behind the technology. HS Sultan is not a solid tumor, it was lacking in a necrotic core and so no antibody accumulation occurred with the TNT-1 F(ab')$_2$ fragments [36].[1]

The possibility of antibody diffusion into regions of hypoxia and necrosis was questioned at first because of the high pressure gradient at the center of any significant tumor mass caused by lack of a properly organized lymphatic system [38] and the structure of stromal fibroblasts and extracellular matrix within and around the tumor [45]. Despite these claims, antibody diffusion was proven back in 1990 by tracking TNT antibodies, at various timepoints, in large human tumors xenotransplanted on the hindquarter of mice [46]. In brief, mice bearing the ME-180 cervical carcinoma were intravenously injected with 60 μCi of Iodine-125 (^{125}I)-labeled TNT-1 for every 50 μg of TNT per mouse. The mice were sacrificed at varying time points and the tumors sliced for analysis by hematoxylin/eosin staining and by autoradiographic exposure to film (Figs. 1a–1d). Most solid tumors develop a hypoxic core, rich in necrotic cells, once the tumor is greater than about 4 mm in diameter [37]. The tumors generated in [46] were ~1 cm in diameter before being used, so they not only presented numerous sites for TNT binding, they also should have had a large interstitial pressure gradient for the antibodies to overcome. As Figs. 1a–1d illustrate, the labeled TNT-1 antibody injected into the tail vein of mice localizes to the tumor periphery within 1 h after injection (Fig. 1a). Antibody penetration into the necrotic core of the solid tumor occurs over the course of the next two days (Figs. 1b, 1c). To counter arguments that the presence of antibodies in the core of the tumor is not stable, mice were injected with ^{125}I-TNT-1 and sacrificed 7 days after administration, demonstrating localization in the necrotic core of the tumor and absence from the blood pool and normal tissues, including the liver and kidney shown here (Fig. 1d). Experiments with control antibodies targeting a lymphoid cell surface antigen were reported to clear from the system within 24 h (data not shown).

While macroautoradiography of tissue samples revealed a targeting of the necrotic core, closer examination of the samples indicated a high level of discrimination for cells undergoing necrosis, even in its preliminary stages. Indeed, the anti-histone antibodies appear to target cells undergoing necrosis even before it is possible to detect the phenomenon histologically [36].

To further investigate the potential of these reagents in the early detection of necrosis, artificial infarctions were generated in mice [47]. The study involved clamping one kidney for 3 h, in order to restrict blood flow to the renal tissue, while leaving the contralateral kidney surgically untouched. After only 3 h, the clamp was removed and radiolabelled TNT-1 F(ab')$_2$ frag-

[1] Living cells continue to maintain an intact plasma membrane and are not permeable to the antibodies, hence, the ability to differentiate intact normal cells from tumors using this strategy.

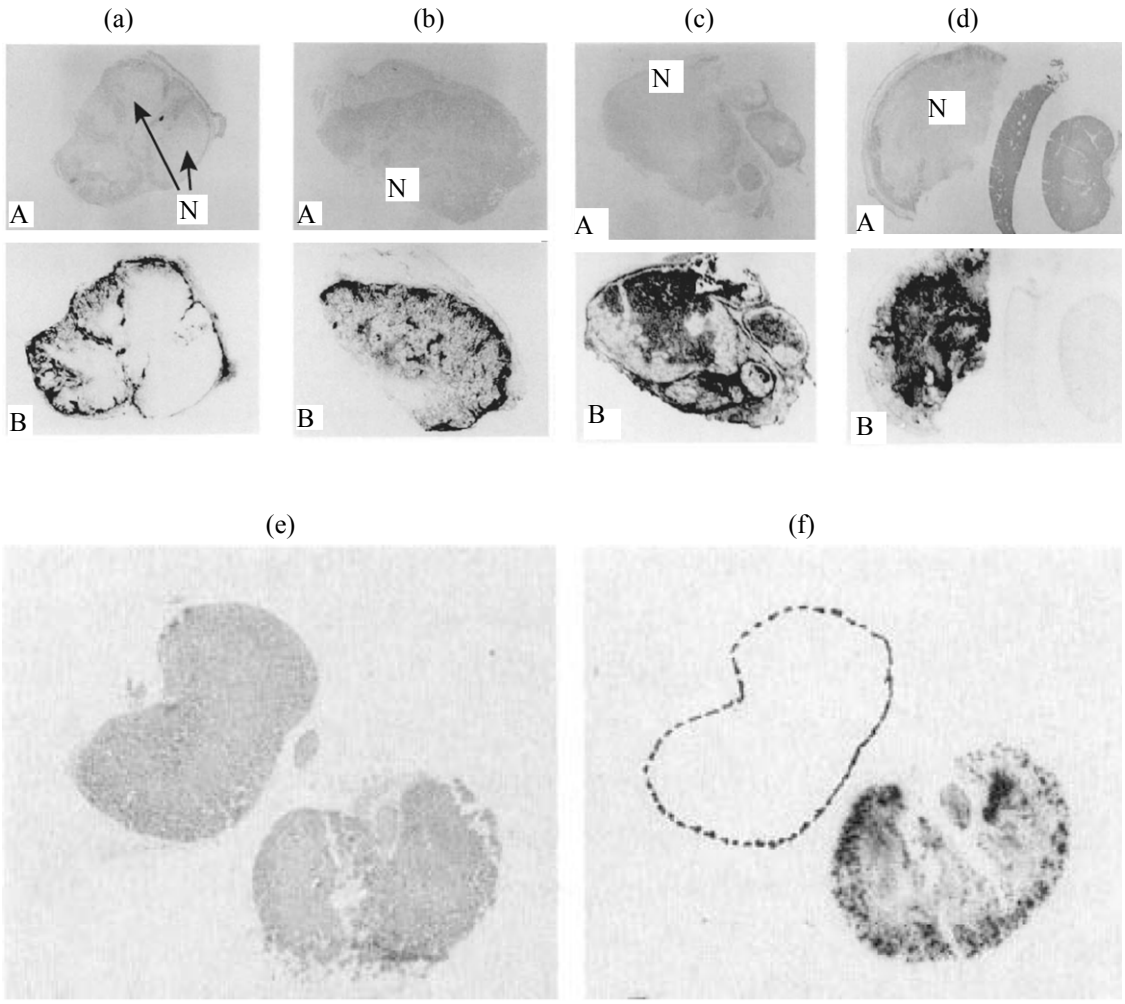


Fig. 1. ME-180 cervical carcinoma showing (A) hematoxylin and eosin (H&E) staining of tissue and (B) their corresponding macroautoradiographic slices. Mice bearing large ME-180 tumors were sacrificed at (a) 1 h, (b) 6 h, (c) 2 days, and (d) 7 days after injection with ^{125}I-labeled TNT-1 antibody in order to track its distribution. H&E staining differentiates viable cells (dark staining) from necrotic cells (light staining). Regions of necrosis are marked by "N." A high concentration of labeled antibody remains at the tumor core 7 days after injection despite complete clearance from the liver and kidney shown here (d). (Adapted from images published in [46] and reprinted by permission of the Society of Nuclear Medicine). Early detection of necrosis in an experimental kidney infarction. (e) H&E staining does not reveal significant regions of necrosis either in the unclamped kidney (upper tissue) or the clamped one (lower tissue). (f) Macroautoradiography reveals accumulation of the radiolabeled TNT antibody in the clamped kidney only, as evidenced by the silver grains in the lower tissue sample. The unclamped kidney lacked any signal and was penciled in (dotted line). Both (e) and (f) photographed at a magnification of 4X. (Adapted from images published in [47] and reprinted by permission of the FASEB Journal).

ments were intravenously injected. The distribution of the antibodies were analyzed 48 h later. Inspection of the kidneys involved extraction and gamma counting of both pairs of organs from five mice prior to macroautoradiography of tissue slices exposed to film. Figures 1e and 1f illustrate the ability of TNT antibodies to target even the minimal amount of necrosis that occurred during the 3 h that renal ischemia was induced in one of the kidneys. Tissue slices from both kidneys show no significant differences when exposed to hematoxylin staining (Fig. 1e); however, autoradiography reveals the presence of the TNT antibody fragment in only the clamped kidney, indeed, the contralateral kidney had to be penciled in for the reader's benefit (Fig. 1f). A comparison of the counts per minute per gram of tissue emanating from each organ revealed a 59 fold increase in label bound to the clamped kidney, on average.

Clinical Results

The savvy reader is aware that animal models provide proof of principle, however, clinical validation in the human animal is often quite another story. That is not the case here. This strategy is already being successfully employed in clinical trials of a chimeric TNT antibody (chTNT-1/B) labeled with Iodine-131 (^{131}I) and being developed in the US for the treatment of recurrent glioblastoma multiforme (GBM) under the tradename Cotara® (for a recent review see [48]). Iodine-131 is a therapeutic isotope whose high-energy beta and gamma emissions kill any living tumor cells surrounding the hypoxic core of a solid tumor. Furthermore, a second ^{131}I-labeled chimeric TNT antibody (chTNT-3) has already been approved for the treatment of advanced lung cancer in China [49].

Both antibodies target the histone/DNA complex and are being used to concentrate therapeutic doses of radiation at the center of a tumor in order to kill the tumor from the inside out. A wide variety of therapeutics can be conjugated to these antibodies, however, radioisotopes were originally chosen for the first generation of clinical products because of the potential to dramatically improve drug uptake in subsequent rounds of treatment. In brief, using TNT antibodies as radiotherapeutic delivery vehicles has been shown to cause increased necrosis of any viable cells surrounding the dead core of a tumor, thus, leading to more necrosis and more binding targets for subsequent administration of the antibody [50].

Targeting the histone/DNA complex has been validated for safety in Phase I and II clinical trials completed in the US and India for the treatment of solid tumors, largely in glioblastoma multiforme, colorectal cancer and its metastasis to the liver. Overall, 140 patients have been treated, including about 100 patients with GBM. GBM is a stage 4 brain cancer and patients who have a recurrence of the disease have a median survival time of 24 weeks [51, 52]. In clinical trials for the treatment of recurrent glioblastoma, patients had Cotara® administered intra-tumorally via catheter, allowing for the localization of 300X more radiation at the tumor site compared to the surrounding normal brain tissue (Fig. 2a) [53–55]. A series of superimposed SPECT/MRI images from one patient illustrates the confinement of ^{131}I-labeled TNT antibody to the tumor microenvironment despite its close proximity to the ventricles (Fig. 2b) [56]. The drug's safety has been proven in GBM trials at the University of Utah, Case Western University, the Barrow Neurological Institute, the Medical University of South Carolina and the University of Pennsylvania, as well as a Phase I colon cancer trial at Stanford University. Median survival time (MST) from the Phase II studies recently completed in the US and India show an increase in the MST for patients from 24 to 41 weeks [48], with a significant number of patients showing survival greater than 1 year [54]. At a site in New Delhi, patients with recurring GBM at first relapse have achieved an MST of 86 weeks and at a site in Salt Lake City, two patients were still alive 10 years after treatment [57] and are still expected to be alive as of this writing (12 years).

The Value of Having a Sensitive Necrosis Detection Agent

It goes without saying that detection of significantly large necrotic lesions in the body, whose source is tumorigenic or not, should be of interest to the medical community. Researchers have long investigated the potential of TNT antibodies as diagnostic agents using Technetium-99m (^{99m}Tc) labeled murine TNT [58] and Indium-111 (^{111}In) labeled chimeric TNT-3 [59]. They have even engineered antibody fragments for this purpose [59, 60], however, no recent advancements have been reported about these particular reagents.

Clinical trials in humans have provided tantalizing evidence of how useful it would be to have a necrosis detection agent that binds histones and DNA giving physicians a clearer picture of what is happening inside the tumor. Since the goal of current chemotherapeutic, biologic and radiation regimens is to eradicate tumors by establishing sufficient necrosis, anti-histone and DNA antibodies have the potential of being developed as monitoring agents for new, as well as conventional, therapies. Despite the maturing of PET and MRI technologies, oncologists often still have to wait a significant amount of time to determine if a new treatment modality is working in their patients before making the decision to switch modalities or continue the course of treatment. Evaluating tumor shrinkage is a common metric used in the decision making process and, if the tumor is largely necrotic on the inside, it can be an erroneous metric. Using anti-histone antibodies to measure the extent of necrosis caused by a treatment modality would help physicians better evaluate the effectiveness of that treatment modality.

As part of an FDA approved protocol, patients in a dose-escalation Phase I study in the US for recurrent GBM were first treated with a 3 mCi imaging dose of

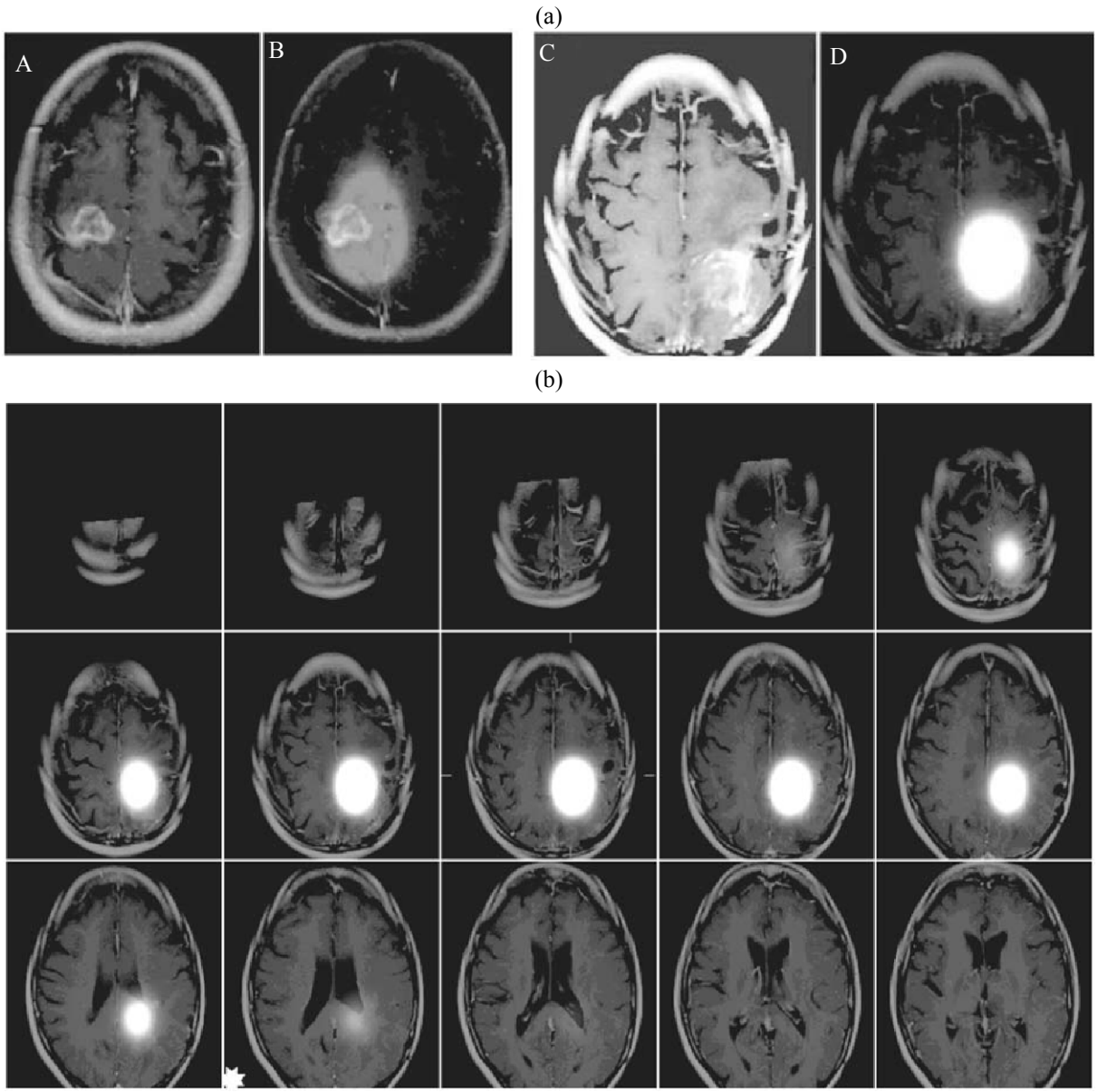
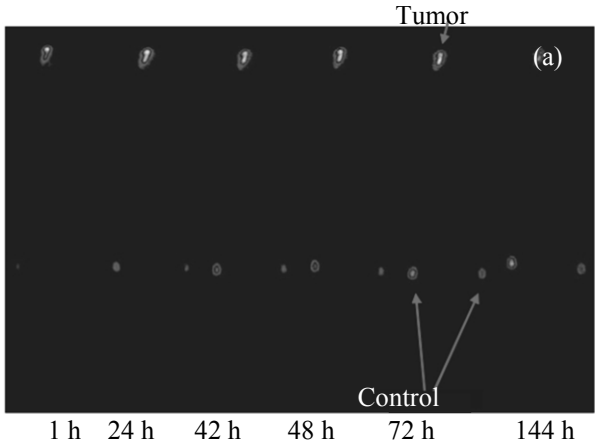


Fig. 2. (a) Localization of Cotara® in human patients being treated for glioblastoma multiforme. (A) and (C) are baseline MRI scans from two different patients showing the location of the stage IV glioma (white area). (B) and (D) are the same MRI scans superimposed with a 14-day SPECT image showing localization of the ^{131}I radiolabeled antibody to the tumor region. Adapted from an image published in [53] and reprinted by permission of LWW publishers. (b) Superimposed MRI and SPECT images from the patient in (C) and (D) illustrate the localization of Cotara® to the tumor in a series of transaxial scans from the top of the head toward the brain stem. Note the proximity of the lesion to the ventricles, and yet, no signal is detected within that cavity. Adapted from an image published in [56] and reprinted by permission of the author and the Society of Neuro-Oncology. All rights reserved.

Cotara® and monitored with whole body imaging, every 24 h, for an entire week (Fig. 3a) [59]. This study was undertaken to evaluate the *in vivo* stability of the ^{131}I isotope conjugated to the chimeric TNT-1 antibody. FDA concerns that the ^{131}I isotope may dislodge from the antibody and accumulate in the thyroid, an organ known to accumulate iodine, were allayed when the thyroid was either not visualized or faintly so in the evaluated patients [59]. However, it is important to note that all patients in this study were provided a solution of non-radioactive potassium iodide to minimize thyroid uptake.

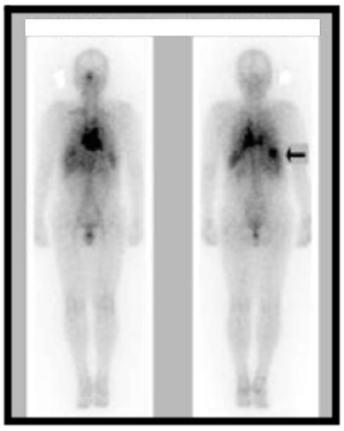


(b)

Side effects of treatment

Side effect	Observed in patients	Percentage of patients	Phase I	Phase II	Phase III	Phase IV
Fatigue	8	38.1	4	4	0	0
Nausea	4	28.3	3	1	0	0
Thrombo-cytopenia	4	19.0	2	0	2	0
Vomit	3	14.3	2	1	0	0
Vertigo	1	4.8	1	0	0	0
Headache	2	9.5	1	1	0	0
Muscle pain	2	9.5	1	1	0	0
Neuropenia	3	14.3	1	0	2	0

(c)



(d)

Fig. 3. (a) Whole body imaging of a patient for 6 days showing specific localization of Cotara® imaging dose in the brain tumor. Imaging standards were placed about 5 cm outside of the patient's knee to calibrate the amount of Cotara® in the tumor. Adapted from an image published in [61] and reprinted by permission of the author and the American Society of Clinical Oncology. All rights reserved. (b) Table of treatment related adverse events for a Phase I safety study in 21 colorectal cancer patients at Stanford University. (c) Whole body image of a colorectal cancer patient obtained 6 days after treatment with Cotara® showing the site of a liver metastasis (arrow). (b) and (c) were adapted from [62] and reprinted by permission of Mary Ann Liebert, Inc. Publishers. (d) A patient with hepatic metastases of a colon adenocarcinoma was treated using radiofrequency ablation followed by a dose of Cotara®. CT (middle panel) and SPECT (upper and lower panels) imagery further highlight the utility of having a necrosis detection agent allowing physicians to monitor the effectiveness of a treatment modality. The RFA treated sites are difficult to locate on the CT, however, they are clearly visualized on the SPECT images, showing colocalization of the radiolabeled TNT antibodies to the RFA treated sites. Adapted from an image published in [63] reprinted by permission of Gene Therapy Press.

Phase I studies to evaluate the intravenous administration of Cotara® in colorectal cancer patients at Stanford University [62] and in liver metastasis patients at the Mayo Clinic [63] further confirmed the safety of targeting histone-DNA complex systemically in the body and the potential of these types of reagents as tumor necrosis detection agents. The intravenous dose escalation study in colorectal cancer patients yielded a maximum tolerated dose of 1.57 mCuries of ^{131}I per kilogram of patient body weight, and revealed no major side effects (Fig. 3b, see grades III and IV) [62]. Besides Cotara®'s therapeutic potential, the identification of a liver metastasis in a colorectal cancer patient during whole body imaging (Fig. 3c, arrow) supports a potential role for TNT antibodies as detectors of metastatic lesions containing a necrotic component.

The limitations of CT scanning are illustrated in published data from a Chinese pivotal trial that led to the approval of ^{131}I-chimeric TNT for the treatment of advanced lung cancer [49]. The same patient was

imaged by X-ray and CT scan and while the CT is more informative than the X-ray regarding the size and location of the lung tumor, neither method conveys any information about necrosis within the cancer. X-ray and CT only provide anatomic information. SPECT imaging of the radioactive TNT antibody allows the physician to evaluate the amount of necrosis present and whether repeated treatments are creating more necrosis or not (see Figs. 2h, 2i, 2j, and 2k in [49]).

Physicians at the Mayo Clinic have already shown the synergy between TNT antibody technologies and other treatments that cause necrosis, in their case, using radiofrequency ablation technology (RFA). RFA is a surgical procedure which uses thermal energy at the tip of a radiofrequency catheter to induce a 1–5 cm zone of necrosis at the tumor site [69]. Cotara® was administered as an adjuvant therapy to help destroy any residual tumor cells, however, patients were also subjected to SPECT imagery helping researchers evaluate the localization of the TNT antibodies *and* the effectiveness of their RFA procedure at the surgical sites, something not easily seen with a simple CT scan (Fig. 3d).

Future Potential

TNT antibodies have shown their potential as imaging agents, however, the studies conducted so far in the clinic have been tangential because of the use of intact antibodies and their conjugation to a therapeutic isotope of iodine (^{131}I). Intact antibodies can have clearance times on the order of weeks [64], whereas, a proper imaging agent needs to clear from the blood stream rapidly so that the patient can be imaged in a matter of minutes or h. To develop an imaging agent, first, one must generate antibody fragments that will localize at the lesion site and clear quickly from the bloodstream [61] and second, attach isotopes (^{99m}Tc, ^{111}In, ^{123}I, ^{124}I) or paramagnetic particles (Gd, Fe, Mn) designed for detection with an appropriate imaging system.

One must consider the production scale necessary for such a technology given the vast number of individuals who would require early diagnosis of benign or metastatic cancer. Imaging isotopes have shorter half-lives than therapeutic ones, therefore, large production processes must be implemented to supply enough diagnostic reagents for what would be two overlapping patient populations: patients being diagnosed for cancer and patients undergoing treatment whose response must be monitored. To that end, one of us (MHP) has worked for years developing improved methods of TNT antibody production and radiolabeling.

Large scale production of histone-targeting antibodies, indeed chromatin-targeting antibodies in general, can be complicated by the release of antigen into the cell culture supernatant as necrotic cells accumulate in a bioreactor. Processes have been developed to remove any antigens that would "hitchhike" with the antibody at its binding sites during the purification process [66, 67]. These hitchhiker antigens must be removed during the purification process, otherwise, they give the final product the appearance of a diminished potency with a significant number of antibodies unable to bind their targets in vivo because their binding sites are already occupied [66].

To address the need for large-scale radiolabeling of antibodies, a whole new method of conjugation, known as In-Line Radiolabeling, has been devised and patented [68]. Rather than label the antibodies in a reaction vessel which concentrates the radioisotope and oxidizing reagent at levels high enough to be detrimental to the antibody, we have perfected a labeling system that replaces the reaction vessel with a silicone reaction tube. The labeling occurs as the reaction components flow through the tube. Only a fraction of each reagent is mixed together at any given time, hence, the deleterious effects of the concentrated radioactivity and oxidizing agent (e.g. chloramine T) on the antibodies can be minimized. The cost effectiveness of this procedure has been demonstrated by labeling nearly a gram of antibody with 13 Curies (481 Giga-Becquerels) of ^{131}I during a single 30 min reaction run [68]. Theoretically, a limitless amount of protein can be radiolabeled by simply adjusting the dimensions of the reaction tube and the flow rate of the reactants. The method has been validated and was used in the production of most Cotara® batches in the US and India during the Phase I and II GBM trials.

Concluding Remarks

The unique nature of the tumor microenvironment provides antibodies access to an abundant target at the hypoxic core of every solid tumor. Years of research at the lab bench and in the clinic have validated the histone and DNA complex as a stable, insoluble antigen accessible in dead and dying cells but not healthy ones. This ability to differentiate regions of

necrosis found in most solid tumors gives these antibodies the versatility to identify both benign and metastatic lesions regardless of origin.

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