Edited by
Eugene P. Goldberg

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TARGETED DRUGS

Edited by

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Series Preface

This new series, *Polymers in Biology and Medicine*, presents the important developments in individual, separately identifiable fields where polymer science (especially in the area of synthetic polymers) interacts with biology and medicine. We intend, therefore, to provide a bridge between polymer science, with its subdisciplines of chemistry, physics, and engineering, and biology, pharmacology, toxicology, medicine, agriculture, and other disciplines of the life sciences.

Rapid progress in research and the use of polymers in biology and medicine has taken place over the last few years. These developments ha resulted in a sharp increase in published papers and patents that are associated with polymers in biology and medicine or, more generally, with the interaction of polymer science and the life sciences. In addition, and even more important, recent national and international symposia, such as those dealing with modified polymers and polymeric drugs, represent the direction this field may well take.

When we undertook to edit this series, our objective was to provide a mechanism for transmitting knowledge and understanding in this interdisciplinary field. But we found, when trying to make literature searches, that the chore of discovering the proper correlations, or even all the necessary references, in journals of different fields was difficult and exasperating. We will, consequently, have each volume co-edited by a volume editor who is one of the leading experts in a well-defined and recognized field. Each book, which will contain chapters written by experts in the field, should provide easier access to the interdisciplinary literature. We hope that the accessibility to the literature, the focus on the disciplines involved, and the presentation of a better-defined field will stimulate ideas and further define and enhance the study of polymers in biology and medicine.

Ultimately, we hope that this series will be so effective that the development of new products such as drugs, pesticides, and prostheses, will be stimulated and will be of great benefit for the health and well-being of all people.

The first volume of this series deals with Anionic Polymeric Drugs, with Raphael M. Ottenbrite as the volume editor.

Two additional books, *Targeted Drugs* and *Polymers in Agriculture*, are in preparation. Subsequent volumes may deal with such subjects as toxi-

cology of monomers and polymers, polymeric artificial organs, plasma expanders and other topics relevant to *Polymers in Biology and Medicine*.

We are obliged to our wives, Jane and Jeanne, for their patient understanding of the time we spent in editorial work rather than with them.

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Preface

Targeted Drugs, as the second volume in the series Polymers in Biology and Medicine, was designed to provide an overview of that rapidly emerging field of biomedical polymer science which is concerned with the synthesis, properties, and clinical use of macromolecular drug carriers for *targeting* or localizing pharmacological activity. Although a single book could not possibly be comprehensive in addressing this topic, brought together here for the first time are original contributions by many of the pioneers in this still very young interdisciplinary field.

The basic concepts and guiding principles for targeted drug delivery and specific chemotherapy or immunotherapy are to be found in the brilliant works of Paul Ehrlich at the turn of this century. Even in his inspired 1878 dissertion on histology and specific ("targeted") stains, important ideas relating molecular structure and tissue localization were set fourth.

Medical and polymer science began to merge only in the mid-1960s and has rapidly produced such singularly important biomedical polymer developments as affinity chromatography and solid phase polypeptide synthesis. These advances, which now permit exquisitely precise separation and synthesis of complex biologically active molecules, place us at the threshold of the field of affinity or local therapy envisioned so long ago by Ehrlich.

This volume surveys many different aspects of this new field. Especially significant are chapters by Ghose et al., Arnon and Hurwitz, and Rowland, who have led the way in research on antibody targeted polymeric drugs.

The advent of hybridoma technology for production of monoclonal antibodies now makes clinical possibilities for targeted affinity therapy even more promising. The importance of fostering further collaborations between polymer and biological scientists is also especially clear for antibody directed drug delivery. Polyclonal and monoclonal antibodies will initially be used clinically for drug conjugates having targeting properties. However, in the future the relatively short polypeptide sequences which are responsible for biospecific complexing will be identified, synthesized, and utilized for the preparation of wholly synthetic polymeric affinity drugs.

Also important and included here are authoritative discussions on (a) hormone receptor targeting, (b) cell membrane–polymer interactions, (c) various neutral and ionic soluble polymeric drug carriers, (d) drug-containing microspheres and blood cells, and (e) tissue binding polymeric drugs. Phys-

ical targeting by magnetic guidance and by direct injection are also covered. Liposome carrier systems, which have received adequate attention elsewhere in the literature, are not emphasized. However, relevant discussion of drug-containing liposomes does appear in a few chapters. Although many types of localized therapy are considered, it is perhaps natural that most authors have focused on cancer therapy because of the great need to minimize toxic drug effects.

Since coming into the biomedical polymer field in 1975, I have been intrigued by the rapidly growing possibilities for safer and more effective localized drug delivery, which must come from the marriage of polymer and medical science. This book is therefore "targeted" at an audience of both physical and medical scientists. It has been edited with the pleasure and enthusiasm that comes from feeling so strongly that this field will revolutionize medicine in the future.

I am indebted to Otto Vogl and Guy Donaruma, the editors of this series, for their encouragement and for the preparation of the indexes. A special note of thanks is also due the State of Florida Biomedical Engineering Center of Excellence program and the Department of Materials Science at the University of Florida, which have provided the fertile and supportive environment for undertaking this task.

Gainesville, Florida
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Eugene P. Goldberg

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CHAPTER ONE

Antibody-Directed Drug Targeting in Cancer Therapy

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1 Introduction

Attempts to exploit antibodies for targeting or delivering drugs specifically to tumor cells stem from observations that chemotherapeutic agents themselves are limited in their ability to act selectively; all actively proliferating cells are damaged by their administration as free antitumor agents. This

nondiscriminating action severely limits the dose that can be given, in most cases to a level that will not effect a cure.

The practicability of targeting with antibodies depends on the presence of new antigens on tumor cells and the ability to obtain specific antibodies against them. It is now accepted that when neoplastic transformation occurs, new and specific antigenic components not detectable in normal untransformed tissue generally appear.[1] Increasing evidence shows that human tumor tissues synthesize substances that are either absent from, or produced in extremely limited amounts by the original normal parent tissue. Because these substances are not easily detectable in nontumor tissue, they have been called tumor markers or tumor-associated antigens (TAAs). TAAs constitute a heterogeneous group of antigenic materials, many of which have not yet been isolated and characterized. Antibodies of high specificity and affinity, however, can be produced against virtually any cell constituent ranging from polypeptides, proteins, and complex proteoglycans to small haptenic moieties.

Antibodies fulfill many of the criteria of an ideal carrier for targeting drugs.[1] If the purpose of targeting is destruction of a cell, carrier antibodies themselves could possibly contribute by their independent or synergistic cytotoxic action. They could also promote endocytosis of bound or dissociated drug, either through events following capping or by rendering the membrane of the target cell more permeable. In a previous article, we reviewed the literature on production and purification of antibodies to TAAs and their coupling to various cytotoxic agents.[1]

This chapter presents our experience with targeting of drugs by antibodies. We begin with the selection of appropriate methods to link prototypes of different classes of anticancer agents, first to a model antibody against a well-defined antigen and then to antibodies against TAAs. We also consider appropriate ways to assess drug–antibody conjugates for retention of drug and antibody activities as a function of molar incorporation of drug. An outline is then given of the experimental design for assay of tumor inhibition by drugs linked to antitumor antibodies in both *in vitro* and clinically relevant *in vivo* tumor models. Finally, we present the results of this approach using members of four different classes of cancer chemotherapeutic agents. These agents are: two non-cycle-specific alkylating agents, chlorambucil and Trenimon; a phase-specific antimetabolite, methotrexate; two cycle-specific antibiotics, adriamycin and bleomycin; and two surface-active agents, phospholipase A_2 and cardiotoxin.

2 Preparation of Antitumor Globulins for Use as Carriers

Tumor-specific antibodies usually have been produced by immunization with whole tumor cell preparations or fractions purported to contain TAA. Antisera produced in this manner must be absorbed with normal tissue prep-

arations, and their specificity then established by demonstrating reactivity with immunizing tumor cells and lack of reactivity with normal and unrelated tumor tissues from the tumor host. In the absorption method, considerable amounts of specific antibodies are lost and unwanted antibodies persist, elicited by normal tissue antigens contaminating the immunizing preparation. The persistence of antibodies directed against vital host tissues, such as bone marrow, could contribute to toxicity of conjugates prepared from these immunoglobulins.[2,3] When such immunoglobulins are used for therapy, they contain a mixture of target-directed and nonspecific conjugate molecules. To achieve the required therapeutic dose at the target site, an excess of drug bound to irrelevant immunoglobulin molecules has to be administered; such an excess would also contribute to toxicity.

The proportion of specific antibodies against TAAs in antitumor globulin preparations can be increased by affinity techniques.[4] These techniques can be applied in a sequential or successive step approach for incremental purification of both TAAs and anti-TAAs: an absorbed anti-TAA fraction is immobilized to purify a TAA and then the TAA so obtained is immobilized to purify anti-TAA antibody, without need for absorptions. Immunization with an antigen purified by this or other techniques should minimize the presence of antinormal tissue antibodies in the initial antiserum. For example, monospecific antisera against prostatic acid phosphatase have been raised in rabbits and mice, using the affinity-purified enzyme as the antigen.[5] TAAs such as oncofetal antigens and ectopic hormones are now available in a chemically well-defined form suitable for immunization.

Another way to increase the content of specific anti-TAA antibody molecules in immunoglobulin preparations for drug coupling is by using monoclonal antibodies.[6–8] Monoclonal antibodies against several human tumors (e.g., colonic carcinoma, melanomas, mammary carcinoma) have already been produced.[9,10] If well-defined TAAs for immunization and screening are not available, the specificity of monoclonal antibodies also must be established by their reactivity with immunizing tumor cells and lack of reactivity with any normal tissue component of the host. If well-characterized purified TAAs, such as oncofetal antigens, were used to produce monoclonal antibodies, it might be easier to identify appropriate antibody secreting clones and to establish specificity via sensitive radioimmunoassay procedures employing these TAAs.

The extent of localization of administered immunoglobulin preparations in target tumor tissues increases with the content of specific anti-TAA antibody molecules in the preparations.[11] Tumor localization might be further augmented if appropriate immunologically active fragments, such as F(ab) and $F(ab)_2$, were used; these fragments would be more amenable to transcapillary passage. (Permeability in the tumor bed might also be enhanced by ionizing radiations, or vasoactive agents such as histamine.) When the Fc moiety is absent, these fragments are less immunogeneic to xenogeneic tumor hosts. The general strategy of linking cytotoxic agents to these frag-

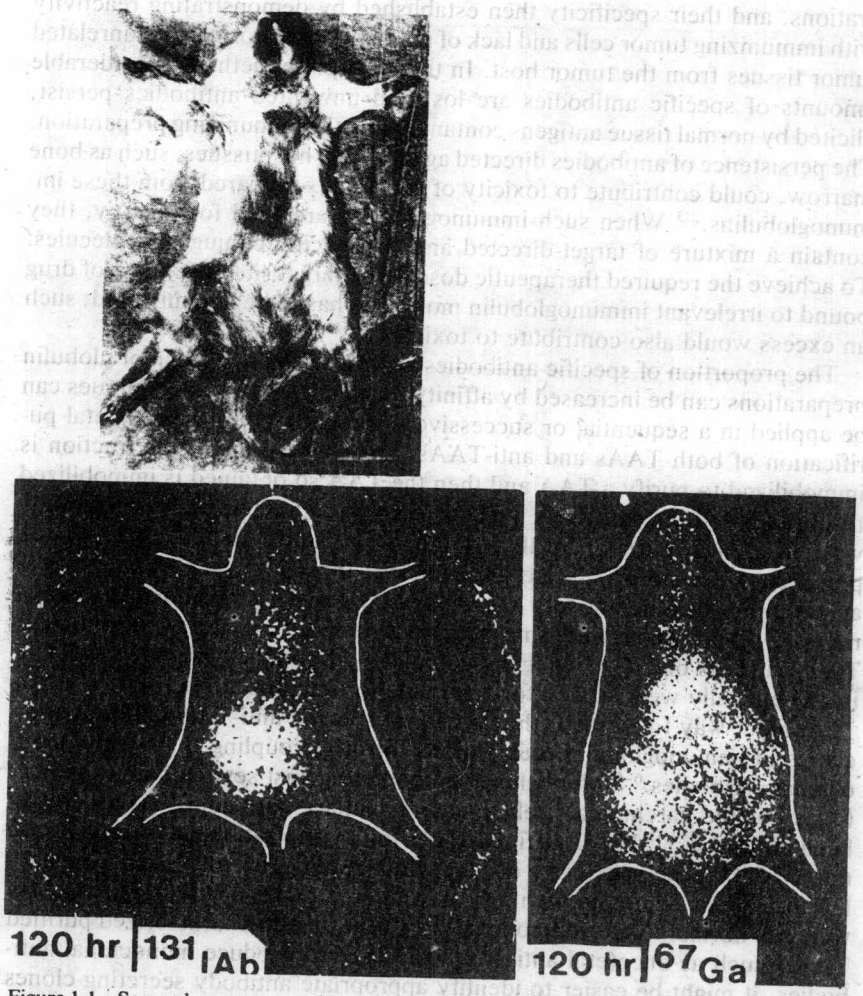

Figure 1.1 Scans show patterns of localization of [^{131}I]antihepatoma globulin and [^{67}GA]citrate in two mice, each bearing a H6 hepatoma in the right flank and a Freund's adjuvant-induced granuloma in the left flank. Scans were performed 120 hr after injection of the imaging agent.

ments, either directly or through intermediaries, is essentially the same as the strategy for linking to intact immunoglobulins.

Ideally, localization of the proposed carrier (antibody or its fragments) in the tumor should be established before drug–antibody conjugates are produced and administered. Tumor localization of anti-TAA antibodies has been demonstrated in mouse tumor models and clinical cancer.[1,3,11–14] We have established the specificity of such localization by demonstrating that the antibody was not localized in antigenically unrelated tumors or in inflam-

matory swellings in the same host, and that the antibody was more localized in tumor tissue than in normal tissue of origin of the tumor. Figure 1.1 shows the localization pattern of ^{131}I-labeled antihepatoma globulin and of a currently used radiopharmaceutical, [^{67}Ga]citrate, in mice bearing subcutaneous transplants of the H6 hepatoma in one limb and a Freund's adjuvant-induced granuloma in the other limb. In the mouse given [^{67}Ga]citrate, most of the radioactivity was diffusely spread throughout the abdominal cavity, tumor, and granuloma. In the mouse given antihepatoma globulin, most of the radioactivity localized in the tumor, demonstrating the requisite specificity.

Another useful approach for establishing the specificity of such localization is the "pair label" technique developed by Pressman and described by Day,[15] using ^{131}I-labeled specific antibody and the corresponding ^{125}I-labeled "normal" immunoglobulin from unimmunized animals, for example. This technique has been applied in our studies on human renal cell carcinoma.[11]

3 Development of Methodology for Binding Anticancer Drugs to Immunoglobulins

To produce effective drug–antibody conjugates, binding methods must be developed that retain chemical groupings essential for drug and antibody activities and, at the same time, allow maximal drug incorporation. Appropriate reactive groups may have to be introduced into drug, antibody, or linking intermediary. Among reagents commonly used for coupling drugs to proteins are those of the homobifunctional type, such as glutaraldehyde and toluene diisothiocyanate (TDIC).[1] Application of these reagents may cause self-polymerization of the protein or drug and intramolecular cross-linkage. It may be possible to avoid cross-linking of protein (or of drugs containing appropriate functional groups) by exploiting differential reactivities of the functional groups of a reagent such as TDIC. There is also a risk that protein will cross-link when an agent such as the carbodiimide ECDI is used in a one-step procedure, since immunoglobulins contain both carboxyl and amino groups.

A more controlled approach involves a two-step procedure in which a reactive derivative of the drug is first prepared and then allowed to react with immunoglobulin or a spacer molecule with the appropriate reactive group. For example, we used *N*-hydroxysuccinimide to prepare the corresponding active ester derivative of methotrexate (MTX), which reacts with immunoglobulins without causing protein to aggregate (see Section 6.2). Avoidance of unwanted cross-linkage in conjugating other proteins to immunoglobulins has been achieved by the use of *N*-succinimidyl iodoacetate and *S*-acetylmercaptosuccinic anhydride to introduce a thioether link,[16] and by the use of the heterobifunctional reagent *N*-succinimidyl 3-(2-pyridyldithio)propionate to introduce a disulfide link.[17]

Reactive groups that are usually exploited for drug linkage can be scattered throughout the protein molecule, including the antigen-binding site. Many factors contribute to loss of antibody activity, especially on conjugation at high drug incorporation ratios. Two of these are substitution of the drug within the antigen-binding site, and changes in the tertiary structure of the immunoglobulin molecule, which result from substitution at multiple sites. Thus only a fraction of the conjugated molecules may possess the steric and other necessary properties for effective tumor inhibition. Separating active from inactive conjugates in such a mixture is a formidable problem. However, it is by eliminating nontumor inhibitory molecules and those that may be toxic that the full potential of this approach to cancer therapy can be realized.

We have been investigating whether the antigen-binding site can be protected during conjugation by binding the immunoglobulin to antigens immobilized on appropriate affinity matrices, such as CM Biogel A or cyanogen bromide-activated Sepharose. After carrying out the coupling reaction, the drug–antibody conjugates can be dissociated from the immobilized antigen by a variety of agents, including NaSCN and low pH buffers.[18] The eluted conjugate should display maximal drug incorporation with minimal loss of antibody activity. Dissociation of low affinity antibody during conjugation and the irreversible binding of antibody to antigen by the conjugation reaction could cause difficulties. We did not encounter this latter problem in our model system when we used anti-bovine serum albumin (anti-BSA) antibody bound to immobilized BSA. In any case, the risk of irreversible binding can be minimized by avoiding bifunctional cross-linking reagents such as glutaraldehyde and ECDI. Drugs such as chlorambucil and MTX can be converted to the corresponding active ester intermediate, which will react only at one site.

Masuho et al. illustrated another approach, designed to avoid interference with the antigen-binding site, when they prepared diphtheria toxin–antibody conjugates.[19] They first obtained $F(ab)_2$ by splitting the original antibody with pepsin. This fragment was then reductively cleaved to yield monovalent F(ab) with its free —SH group located away from the antigen-binding site. Reaction of F(ab)—SH with the *S*-sulfonated fragment A of diphtheria toxin produced a disulfide-linked hybrid that possessed both antigen-binding capability and cytotoxic potential. This methodology should be applicable to other cytotoxic drugs and peptides or proteins that either contain, or can be modified to contain, appropriate sulfur substituents.

Drugs may be bound either directly to antibodies or through appropriate spacer molecules or multivalent coupling intermediaries. Binding of drugs directly to immunoglobulin has two advantages: less chemical manipulation with reduced risk of side reactions, and minimal increase in the size of the molecule. However, the limited number of available reactive groups for linkage in immunoglobulin restricts the extent of drug incorporation, particularly as the integrity of the binding site for antigen must be maintained.

Coupling intermediaries of particular interest in this context are polymeric molecules, such as polylysine, polyglutamic acid, dextran, and their derivatives.[20–22] Linkage of an intermediary that has multiple drug molecules attached can increase drug incorporation without extensively modifying residues in an immunoglobulin molecule; loss of antigen-binding capacity is thus minimized. The risk of losing the immunologic reactivity of the resulting conjugate can be reduced further by protecting the antigen-binding site during the coupling procedure (e.g., by immobilization on an antigen affinity matrix). A spacer incorporating drug-binding groups of appropriate chain length might also overcome steric hindrance with drug and antibody activities. Special properties of a spacer or intermediary could be exploited when conjugates are designed. Polylysine, for example, might facilitate intracellular transport.[23] However, gross alteration in the size and charge of the conjugated antibody might lead to diversion from the target tumor tissue.

Diversion could result from altered hemodynamic properties, impaired transmembrane passage, or increased susceptibility to phagocytosis. The potential toxicity of intermediaries themselves must also be considered. For example, dextran is widely used as a plasma expander but there have been reports that it causes sensitization[24] and facilitates metastasis formation.[25] We are currently investigating a series of polylysines as intermediaries in coupling MTX to immunoglobulins.

Occasionally, a cancer chemotherapeutic drug may bind sufficiently tightly to an immunoglobulin or linking intermediary through noncovalent interactions providing a conjugate suitable for therapeutic application. Chlorambucil is one such drug. Its noncovalent binding also inhibits alkylation and hydrolysis, that is, it preserves the drug's activity before the drug reaches the tumor tissue.[26] Polymers, such as polylysine, that contain multiple charged groups might bind ionic drugs of opposite charge sufficiently strongly so that antibody-conjugated polylysine would mediate their transport to a target site.[27]

4 Assay of Conjugates for Incorporation of Drug and Retention of Antibody and Drug Activities

It is useful to initially assess the effectiveness of linking procedures by using antibodies to well-defined antigens, such as BSA or ovalbumin, that are readily amenable to quantitative analysis. Retention of antibody activity should be measured as a function of the extent that the active drug is incorporated in the conjugate. The optimal coupling method emerging from these studies can then be applied to anti-TAA antibodies.

During model studies of drug binding employing well-defined antigens, we have generally used radial immunodiffusion because of its ease and simplicity. Conjugates are compared with equimolar amounts of unreacted immunoglobulin and of immunoglobulin preparations exposed to the coupling