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Biological Approaches to the Controlled Delivery of Drugs: A Critical Review*

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I. Introduction . . . 278
A. Overview . . . 278
B. Purpose and scope of the review . . . 278
C. Evaluation parameters . . . 279
II. Barriers to selective drug delivery . . . 279
A. Overview . . . 279
B. The endothelial barrier . . . 280
C. The basal lamina barrier . . . 283
D. The reticuloendothelial barrier . . . 283
E. Cellular barriers . . . 284
F. The targeting problem . . . 286
III. Technologies for controlled drug delivery . . . 287
A. Overview . . . 287
B. Methods of drug-carrier conjugation . . . 287
C. Sustained drug release systems . . . 295
D. Liposomal drug delivery systems . . . 296
1. Basic aspects . . . 296
2. Incorporation of drugs in liposomes . . . 297
3. Liposome-cell interactions . . . 298
4. Behavior of liposomes *in vivo* . . . 298
5. Targeting of liposomes . . . 299
6. Liposomal antineoplastic drugs . . . 300
7. Liposomal anti-infectious drugs . . . 302
8. Immunomodulation with liposomal drugs . . . 304
9. Summary . . . 304
E. Antibodies as drug/enzyme carriers . . . 305
1. Overview . . . 305
2. Early development . . . 305
3. Antibody-drug conjugates . . . 306
4. Immunotoxins . . . 308
5. Problems and prospects . . . 310
F. Cellular drug/enzyme carriers . . . 311
1. Erythrocytes . . . 311
2. Fibroblasts and leukocytes . . . 312
3. Encapsulated cells . . . 313
4. Semipermeable aqueous microcapsules or artificial cells . . . 313
G. Microspheres as drug carriers . . . 315
H. Macromolecules as drug/enzyme carriers . . . 317
I. Prodrug delivery systems . . . 320
J. New horizons in controlled drug delivery . . . 320
1. Targeting to cellular carbohydrate binding proteins (lectins) . . . 320
2. Pharmacologically active antireceptor antibodies . . . 322
3. Oligonucleotides as drugs . . . 323
IV. Conclusions . . . 324
1. Microparticulate carriers (liposomes, microspheres, red cells) . . . 324
2. Antibody carriers . . . 324
3. Prodrugs . . . 325

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4. Macromolecular carriers 325
5. Newer technologies 325

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"We must learn to aim, and to aim in a chemical sense."—P. Ehrlich, Address to German Chemical Society, 1909.

I. Introduction

A. Overview

RECENT advances in molecular biology and in pharmacology have allowed for the development of new classes of highly effective therapeutic agents. While drugs are now available to combat a wide range of genetic, malignant, and infectious diseases, the therapeutic efficacy of these agents is often diminished by their inability to gain access to the diseased site at an appropriate dosage. Two common cases which will be discussed at length in the following pages are the limitations associated with cancer chemotherapy and the difficulties in treating several genetic disorders manifested as enzyme deficiency diseases.

In the first case, there are many thousands of potential anti-tumor agents that exhibit excellent cytotoxic activity in the tissue culture dish. However, once work is initiated in appropriate animal models, several important limitations are invariably observed. To achieve a therapeutically relevant dose in the tumor cells, the amount of drug required usually proves to be toxic to normal tissue. In addition, difficulties arise due to the metastatic nature of many tumors thereby making it important that virtually total tumor cell kill be achieved. What is needed are vehicles capable of carrying the cytotoxic agent in a highly concentrated form to the tumor target, thus allowing for efficient tumor cell kill while largely sparing surrounding normal tissue.

In the second example where certain genetic disorders are manifested as enzyme deficiencies or defects, effective enzyme replacement is often problematic. In addition to the problems of hypersensitivity reactions mounted aginst a foreign protein, the enzyme deficiency is often intracellular and possibly intralysosomal such that any thought of enzyme replacement must consider the multitude of physical barriers that must be crossed before therapy can be achieved.

The concept of drug delivery systems is not a new one. Even before the discovery of antibodies in 1906 the great biologist Paul Ehrlich foreshadowed the possible use of "... bodies which possess a particular affinity for a certain organ ... as a carrier by which to bring therapeutically active groups to the organ in question." Ehrlich thus predicted the existence of antibodies, which were defined years later, and foresaw their use as site-specific carriers of therapeutic agents (see Ref. 119). Other more simplistic strategies for drug delivery have been described over the years ranging from the coated pill described by Wootton in 1910 (originally credited to Rhazes about AD 900), to the development of the drug capsule by Mothes in France in 1833 (see Refs. 280 and 281 for reviews), to the recent development of insulin pumps as a sustained release system to better control plasma glucose levels (38, 39).

The need for effective drug delivery systems is straightforward. For a drug to produce a specific pharmacological response it must gain access to its specific "site of action." The importance of drug delivery systems stems from an attempt to alter the pharmacokinetics of drugs to take into consideration specific cellular and subcellular sites of action. In the design of specific drugs and drug delivery systems particular attention must be given to: 1) the desired site of action of the drug, 2) the route of administration of the drug, and 3) the pathway or barriers that the drug must pass en route to its destination. Depending on the nature of the drug one must also ask whether there are specific regions of the body that the drug must avoid either because it is too toxic or because it may be rapidly degraded in that region. It is also important to be able to estimate what fraction of the desired dose of drug is required at the site of action. In the following pages these considerations will be discussed in terms of a variety of strategies and technologies for controlled drug delivery.

B. Purpose and Scope of the Review

The last decade has seen the emergence of a great many approaches to the problem of controlled drug delivery. Correspondingly, there have been several reviews dedicated to the consideration of individual technologies such as polymeric sustained release systems, liposomal drug carriers, and antibody drug carriers. However, rarely have the features of one approach been compared and contrasted to those of other possibly competing approaches. In this review we attempt to examine, evaluate, and compare a variety of strategies for controlled drug delivery and to point out their merits and liabilities with respect to each other and with respect to the ultimate goal of highly selective drug action.

Our emphasis will lie with biological approaches to the problem of selective drug delivery. There are several reasons for this; the first is simply that the background and research interests of the authors lie in this area. A second reason is that the evaluation of biological approaches, such as liposome technology or immunotoxin

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techniques, clearly still lies within the realm of basic research. By contrast, some of the chemical and mechanical approaches to controlled drug delivery (for example sustained release devices with osmotic pumps or erodible polymers) are currently moving into the areas of clinical and commercial evaluation and really lie beyond the scope of a research-oriented review. Thus, we will touch only lightly upon topics such as sustained release implants, transdermal delivery devices, minipumps, etc., where the technology used derives primarily from chemistry or engineering (187). Venter (539) in a review in this JOURNAL has recently covered the area of immobilized and insolubilized drugs and biomolecules. Instead we will attempt to review in considerable detail such topics as liposomal drug carriers, protein microsphere carriers, cellular carriers, and immunotoxins, where the approaches derive primarily from biological considerations. While the reference lists in these areas are quite extensive, it was not possible to deal with the many thousands of publications on these topics and thus we have surely slighted some excellent work. For this, we offer our sincere regrets.

One of the areas that we highlight is the use of enzymes as drugs. The potential of enzymes as therapeutic agents has been recognized for over a century since Purdon in 1871 used certain proteolytic enzymes to inhibit bacterial infection during surgery [see Westall and Cooney (557) and Poznansky (396) for reviews]. Sir Archibald Garrod in 1909 (157) first described the concept of inborn errors of metabolism, predicted the source as enzyme deficiencies, and foresaw the potential of enzyme replacement therapy. The use of L-asparaginase in the treatment of acute lymphocytic leukemia (ALL) was first conceived by Kidd in 1953 (see Refs. 82, 194, 399). Despite the widespread potential of enzymes as therapeutic agents (484) several important limitations have militated against their successful use in medicine (see Ref. 176 for a review): 1) Until recently many enzymes, especially in pure form, were not readily available. 2) Upon administration, enzymes as proteins are readily susceptible to rapid bioinactivation at 37°C or proteolytic degradation. 3) Enzymes as foreign proteins are highly immunogenic. 4) In the case of enzyme therapy for the treatment of lysosomal storage diseases (manifested as enzyme deficiencies) access to sites of substrate accumulation is limited. The development of modern techniques in solid state protein synthesis and recombinant DNA for the production of animal or human gene products may alleviate the first limitation but problems of bioinactivation, immunogenicity, and site-specific delivery remain. The widespread applicability of enzyme therapy depends on the development of delivery techniques to protect enzymes from bioinactivation and immunological reactivity while producing site-specific targeting. While specific cases of enzyme delivery techniques will be discussed in the following sections, many of the techniques developed as drug delivery systems hold equally well for applications in enzyme therapy and for the purposes of this review enzymes will be considered as drugs.

C. Evaluation Parameters

Throughout the following pages, we will attempt to evaluate each of the drug delivery systems considered on the basis of several different parameters:

1. *Selectivity*: To what degree is the delivery system capable of selectively conveying a drug to the target site, be that a specific organ, tissue, cell, or intracellular location?

2. *Load Factor*: Does the delivery system possess a sufficient capacity to permit a therapeutic level of drug to be achieved at the target site?

3. *Immunology*: Does the production of drug-carrier complexes produce any new antigenic determinants that can result in possible hypersensitivity reactions upon repeated administration?

4. *Toxicity*: Are the carrier and/or carrier-drug complex nontoxic in the contemplated dosages?

5. *Scope of Diseases*: What conditions are amenable to treatment with the described drug delivery system?

6. *Pharmaceutic Feasibility*: Does the specific drug delivery system make practical sense both in terms of production of the drug-carrier complex and in terms of its administration in the field?

These parameters will provide a basis for comparing and evaluating the various technologies for controlled drug delivery.

II. Barriers to Selective Drug Delivery

A. Overview

The goal of any controlled drug delivery system is to assure the transit of drug molecules from the circulation or other site of administration to a specific locus ("receptor") on or within a particular population of cells within a particular organ or tissue. Most of the delivery systems that have been proposed thus far are macromolecular or particulate in nature; some examples are antibodies, other biological macromolecules, liposomes, and polymeric microspheres or microbeads. As we shall explore in some detail in this section, there are a multitude of physiological barriers standing between a drug-carrier complex in the circulation (or other body compartments) and the ultimate target of the drug within a cell.

Several different routes of administration are available in the design of drug delivery systems: oral, intravenous, intraperitoneal, subcutaneous, intrathecal, and even direct injection into diseased or affected tissue. In the majority of cases, one might expect that it is best to utilize the body's own distribution network, namely the vascular system, to deliver a drug to the target tissue or organ in question.

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B. The Endothelial Barrier

The lumen of the vasculature is circumscribed by a layer of endothelial cells which serve to demarcate the vascular and extravascular compartments and to regulate the flow of solute molecules (especially macromolecules) between these compartments (20, 150). Most of the exchange of solutes takes place at the level of the capillary endothelium which, in aggregate, forms an enormous surface for exchange. In humans, for example, the total surface area at the lung capillary bed has been estimated at 40 m^2 while the systemic capillary bed has an estimated area of 60 m^2 (469). Three major types of capillary endothelia have been described: a discontinuous or sinusoidal endothelium found in the liver and spleen; a fenestrated endothelium found in endocrine glands, the renal glomerulus, and the intestinal mucosa; and a continuous endothelium found in most tissue such as the capillaries of all muscle, the central nervous system, lung, and bone. In sinusoidal capillaries, the endothelium and the underlying basement membrane both have gaps up to thousands of Ångström units in diameter (159, 469). Fenestrated endothelia such as those of the renal glomerulus are marked by a very thin cellular layer (0.05–0.1 μm) which is penetrated by transcellular circular openings (the fenestrae) of about 600 to 800 Å containing a thin diaphragm with an underlying continuous basement membrane. By far the most common capillary barrier is the continuous endothelium where the cells abut closely on each other and are joined mainly by continuous tightly occluding junctions also subtended by a continuous basement membrane of between 200 to 500 Å.

In the design of drug delivery systems an understanding of how the drug-carrier complex might leave the vascular space seems essential. The question of exactly how molecules penetrate from the vascular space across the vascular endothelium into tissue spaces has not been adequately established. The mysteries of the microcirculation and the mechanism by which molecules, especially larger peptides or molecules (or particles) in excess of 5,000 to 10,000 M_r, pass across the endothelial barrier into the tissue space and even their return to the circulation via the lymphatic system are intriguing. We have only a poor understanding of the mechanism by which solutes and water pass from the plasma into the tissue fluid spaces and whether in fact these processes are subject to any control mechanisms.

From a morphological point of view, the vascular endothelium resembles any simple epithelium except that it appears to have a higher permeability for water and for small water soluble molecules. In addition, unlike most normal epithelia, the vascular endothelium appears to have a relatively high permeability to large water soluble macromolecules ranging in size from the smaller plasma proteins like albumin (M_r 68,000) to large plasma-borne lipoprotein particles which have molecular weights in excess of 2×10^6 (474). In 1951 Pappenheimer (see Refs. 377, 378) proposed what has become known as the "pore theory of capillary permeability." Biophysical measurements carried out during the 1950s and 1960s led to the postulate that there existed two systems of pores which were defined as water-filled channels that connected the capillary lumen with the interstitial spaces of the surrounding tissue. Based on permeability measurements with molecular probes of various weights and dimensions (see Ref. 57 for a recent review), it was concluded that, on average, two different pore sizes with different pore densities might exist. The first with a density of 10 to 15 units/μ^2 was thought to be an equivalent cylindrical channel of 120 Å while the second larger pore (sometimes called "leaks") was thought to have a much lower density, as little as 0.05/μ^2, but to be much larger with a diameter of between 500 and 700 Å. Repeated attempts over many years to visualize these pores by light or electron microscopic techniques yielded few results. Landis and Pappenheimer (277), in what was the accepted standard in the field for many years, viewed the capillary system as a relatively undifferentiated continuous endothelium. They failed, however, to take into account the striking heterogeneity of the microvasculature. It has become obvious that the various vascular endothelia are really highly specialized, ranging from the tightness of the blood-brain barrier to the high porosity (and ability to withstand high pressures) of the vascular endothelium of the glomerular capillary bed. In spite of the failure of repeated attempts to identify pores morphologically, there continues to be support for the pore theory (333, 334) with several variations including one of an endocapillary layer providing a sort of selectivity filter in terms of both pore size and pore charge.

At odds with the strength of the pore theory, in the early 1960s several morphological studies began to describe the existence of a great many plasmalemmal vesicles in certain regions of the vascular endothelium (33, 371, 372). Fawcett (127) described these particles as micropinocytotic vesicles, producing a new tentative hypothesis suggesting that these vesicles might offer a transport mechanism for the movement of soluble plasma proteins between the capillary lumen and the interstitial spaces. The process of vesicular transport of large proteinacous macromolecules has now been described by many authors (245, 371, 373–375, 445, 465–469, 568). The plasmalemmal vesicles, one of the hallmarks of endothelial tissue, can be shown to be derived from the plasma membrane on the capillary luminal side by a process indistinguishable from pinocytosis. The process appears to be almost continuous and there sometimes appear to be chains of these vesicles reaching from the plasma membrane (hence the name) into the cytoplasm towards the tissue side of the endothelial layer. The development of tracer techniques in electron microscopy allowed Palade and coworkers (374) to demon-

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strate the transit of colloidal gold particles (diam. = 300 Å) and ferritin (diam. = 300 Å) exclusively within plasmalemmal vesicles. Within a few years these findings had been extended to include several other tracers including peroxidases and hemoproteins of various sizes (cytochrome *c*, diam. = 30 Å, myoglobin, diam. = 33 Å, horseradish peroxidase, diam. = 50 Å, and hemepeptides, diam. = 20 Å) showing the markers in the cell exclusively within cytoplasmic vesicles. In early reports some of these markers were also detected in intercellular junctions and spaces and it was proposed that open junctions might represent a solution to the "pore" theory. More careful attention to experimental technique, however, seemed to indicate that the junctions were in fact impermeable to hemepeptides and myoglobin (466) and that the plasmalemmal vesicle route appeared to be the only one accounting for the passage of macromolecules between the capillary lumen and the interstitial spaces. While the controversy has not abated entirely (see Refs. 543, 57, 77), it has become increasingly clear that protein molecules or macromolecules in excess of 10 Å cross the endothelial barrier, not by a series of pores, but by a process that has come to be called *transcytosis*. Figure 1 depicts this process demonstrating the internalization and externalization of transported moiety on the blood and tissue sides, respectively. The plasmalemmal vesicle is the intermediate particle responsible for the transport of the solute within the cytoplasm. An important feature of the system is that it appears to bypass the lysosomal system thereby avoiding any intracellular processing or degradation.

While the evidence for endothelial transport by transcytosis is becoming very strong we know much less about the specificity and/or selectivity of the system. Only very recently has the question of discrimination of solute particles by the capillary endothelium been approached. Another question that still has to be answered is the property of the endothelial barrier which allows for differences in permeability or transport characteristics. These are especially important if one is attempting to devise drug carrier systems to deliver drugs in macromolecular form to a specific site. The mechanism of transcytosis may give the endothelium the capability of transporting particles as large as 500 Å in diameter or even larger, but can it reflect any level of tissue specificity? Brigham *et al.* (52) studied the exchange of macromolecules in the pulmonary microcirculation and measured the equilibration of molecules between the plasma and lung lymph. They demonstrated that molecules penetrated as a function of size irrespective of chemical dissimilarities thus suggesting a passive nonspecific process. Figure 1 on the other hand presents the possibility that receptors, possibly tissue specific, may reside on the luminal face of the endothelial cell to lend specificity to the transport mechanism in much the same manner as has been shown for receptor-mediated endocytosis (173). This remains at this time simply an attractive hypothesis, there being no hard evidence to indicate that specific receptors exist for the express purpose of transporting proteins across the endothelial barrier by the process of transcytosis. Were this true, however, one might coin the term "receptor-mediated transcytosis" to define a process analagous to receptor-mediated endocytosis, but allowing for the plasmalemmal vesicle to be directed to the tissue side of the endothelial cell for externalization rather than intracellular processing.

The majority of evidence for specific interaction of protein molecules with the endothelium for the purpose of transport is morphological. The lack of biochemical data, until very recently, can be attributed to two facts. First, there has been no convenient model in which to gather biochemical data. Although a rat contains several grams of endothelial tissue, there is no established way to obtain biochemical data regarding the transport properties of the tissue. The establishment of cultured endothelial tissue is also in its infancy (143) and amounts available are insufficient for subcellular fractionation. The second point is that a distinction has to be made between receptor-mediated endocytosis and the process of transcytosis. Because both presumably involve an initial binding of the protein to the plasma membrane and internalization, it may be difficult to dissect the two processes at a biochemical level until an isolated sheet preparation of endothelium is available to carry out actual transport experiments.

In recent years, at the morphological level at least, biochemically differentiated microdomains have been described at various capillary endothelia and specific receptors have been identified on the luminal side of the endothelial cells. Simionescu and colleagues (468) have identified microdomains of anionic sites present to different degrees in various capillary beds (e.g. viscera, pancreas, jejunum) and have partially characterized these sites by the use of specific enzymes (e.g. neuraminidase, hyaluronidase, heparinase, trypsin). They have also identified the oligosaccharide components on the luminal side by using lectin-peroxidase conjugates. They speculate that these microdomains (which often appear clustered) represent a capillary endothelium with highly differentiated regions for the transport of macromolecules with specificity according to charge, size, or chemical nature. Other investigators have identified specific receptors on cultured endothelial cells including those for insulin and insulin-like growth factors (27) and low density lipoproteins (199). Simionescu *et al.* (468), using ferritin conjugates of histamine and histamine analogues, have identified histamine receptors on the capillary lumen side of endothelial cells *in situ*, and examined a range of arterial and venular endothelia. A major drawback in all of these examples is a failure to determine the function of the receptor on the endothelial cell. These cells require glucose, so they would be expected to have

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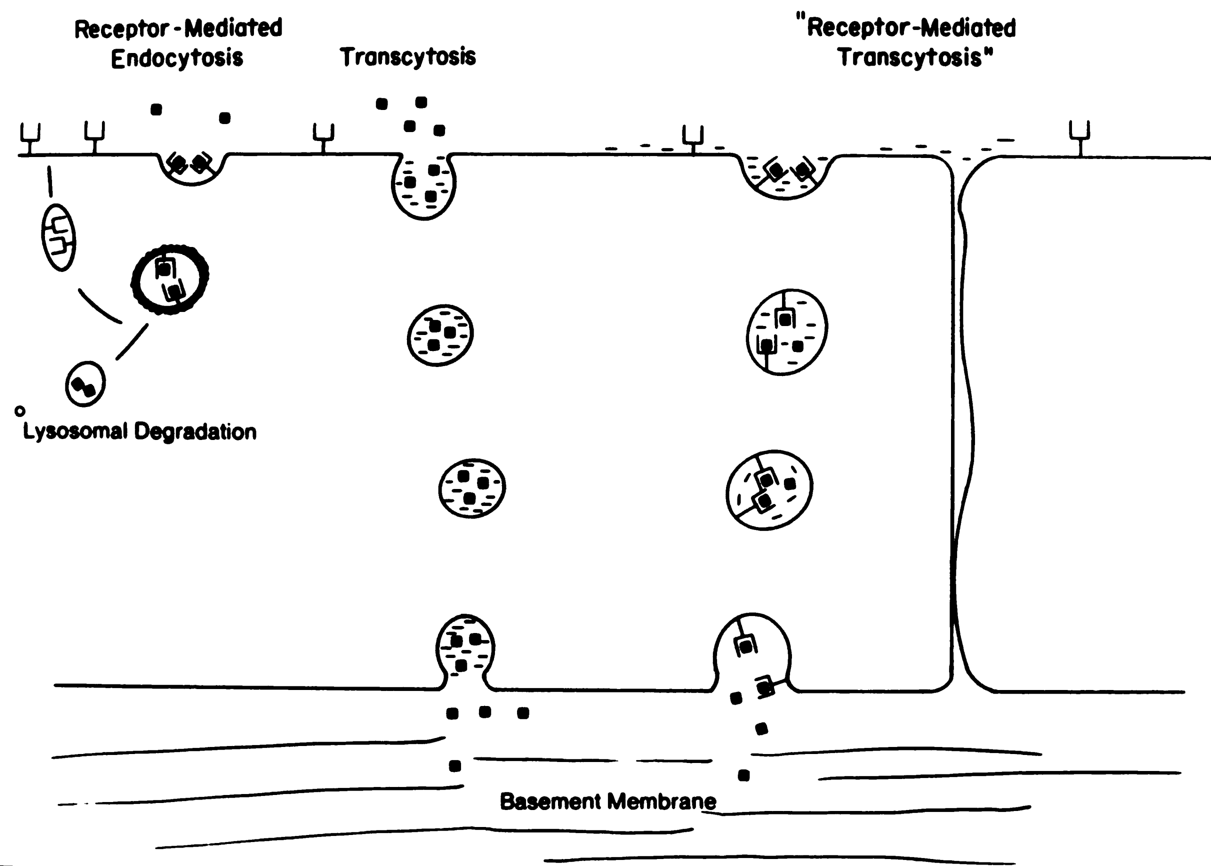

FIG. 1. Transcytosis in endothelial cells. This figure poses the question of whether a process combining the properties of receptor-mediated endocytosis and transendothelial peptide transport (transcytosis) might exist to produce a receptor-mediated and, hence, highly specific mode of macromolecular transport from the plasma to underlying tissue. Such transport might be important in the transport of proteins and even small peptide hormones across the most common continuous endothelia (as opposed to fenestrated or sinusoidal endothelia found in such specialized tissue such as kidney and liver). The possibility of using such a transport system for the targeting of drugs or drug-carrier systems from the circulation across the endothelial barrier to underlying target tissue is discussed in the text.

insulin receptors, and they possess contractile protein in their cytoskeleton, so it might not be surprising for them to have histamine receptors. An important question is whether these receptors function in the transport of these proteins or peptides across the endothelial barrier to underlying tissue, e.g. muscle, pancreas, gut, etc. Such receptors might be important in the process of receptor-mediated transcytosis but none have yet to be shown to function as such.

Vasile *et al.* (538) have recently demonstrated the binding, endocytosis, and transcytosis of low density lipoproteins (LDL) in the arterial endothelium *in situ.* They demonstrate two routes of LDL transport into the endothelial cells. The first is receptor-mediated endocytosis which is mediated by coated pits and delivers the LDL molecules to the lysosomes for processing and control of cellular cholesterol metabolism. The second pathway, accounting for most of the transported LDL, represents the LDL that moves across the endothelial cell by the process of transcytosis. It is carried within the cytoplasm by plasmalemmal vesicles presumably for the purpose of supplying the underlying tissue with cholesterol from the LDL particles. This appears to be a low affinity nonsaturable process that is markedly increased by high LDL concentrations. While it is clear that the plasmalemmal vesicles are responsible for the transport of LDL particles across the endothelial barrier, it has not been established whether this process exhibits any specificity (i.e. are specific receptors involved on the luminal side). It would be important to determine whether the system is under any regulatory control to determine the extent of LDL delivery to tissue sites below the endothelium. This process may be crucial in the development of atherosclerosis and the mechanism of control of cholesterol metabolism and cholesterol accumulation in peripheral tissue.

The tightness of the endothelial barrier surrounding the central nervous system, sometimes called the blood-brain barrier, is an extreme example of the heterogeneity of vascular beds. This particular barrier complicates the delivery of even small water soluble drug molecules to brain tissue, let alone more complex drug-carrier complexes (see Refs. 43, 49, and 53 for a review of the problems of drug delivery to the brain).

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On balance it would appear that the older established notions of how water soluble protein molecules larger than approximately 10 to 15 Å in diameter cross endothelial barriers are currently being replaced. Morphological evidence for the existence of pore structures appears slim while the process of vesicular transport, newly termed "transcytosis," is gaining acceptance. This bodes well for those investigators who are concerned with the transport of drugs from the circulation, across the endothelial barrier, to an underlying target tissue. A detailed understanding of the mechanism of this process and its control will allow for the development of carrier systems that are modified to utilize this transendothelial transport system. Thus, in an analogous manner to the introduction of drugs or enzymes into cells utilizing specific transport or endocytotic properties, one might be able to utilize the transcytosis pathway to cross what now appears to be a formidable barrier.

C. *The Basal Lamina Barrier*

In all capillaries other than those of the liver, spleen, and bone marrow, the capillary endothelium is subtended by a layer of dense fibrillar material which is termed the basal lamina or the basement membrane (317). The highly insoluble nature of this material has made analysis difficult, but in recent years (255) the biochemistry of this material has been investigated. A major component of the basal lamina is type IV collagen organized in microfibrillar arrays. In addition, the basal lamina contains laminin, a high molecular weight protein that promotes adhesion of cells, as well as several different proteoglycans especially those of the heparin sulfate type. Some basement membranes also contain fibronectin, another high molecular weight adhesive protein (310). The study of this tissue is still in its infancy although the use of cultured endothelial cells has greatly expedited the study of the biochemistry and bioassembly of the basal lamina (265).

For the purpose of devising and understanding drug delivery systems, we are concerned with the ability of the basal lamina to act as an ultrafilter for macromolecules and macromolecular structures (see Ref. 317). Charge and size characteristics are important in determining the rates of diffusion through the basal lamina. This has been carefully demonstrated in the basement membrane of the renal glomerulus and presumably holds true for other capillary basal lamina as well. Part of the charge discrimination of the basement membrane may be due to the proteoglycan component (317). It is also important to keep in mind that macromolecular components of the basement membrane have specific binding capacities for other macromolecules. For example fibronectin has binding sites for collagen, DNA, fibrin, actin, and other macromolecules (430). Thus the ability of macromolecular drug-carrier complexes to transit the basal lamina of capillary endothelia will depend not only on the size and charge of the carrier complex, but also on specific macromolecular binding characteristics.

D. *The Reticuloendothelial Barrier*

In order for a drug-carrier complex to successfully reach target cells within a certain tissue, it must not only be able to exit from the circulation, passing the endothelial and basal lamina barriers, but it must also be able to escape the grasp of the reticuloendothelial system, the body's disposal mechanism for foreign particles and macromolecules.

The reticuloendothelial system (RES or monocyte-macrophage system, as it is sometimes called) is comprised of a set of mononuclear phagocytic cells. These cells originate from precursors in the bone marrow, enter the circulation as monocytes, then pass into various tissues where they differentiate into macrophages (331) and begin to perform a variety of functions (13, 346). The macrophages are a crucial component of the host defense system; they are involved in antibody responses via the processing and presentation of antigens, they are responsible for secreting factors which regulate the functions of lymphoid cells (529), and finally the macrophages are themselves effector cells which can acquire the capacity to attack and destroy both pathogens and tumor cells (5). The complex host defense role of the reticuloendothelial (RE) cells must be kept in mind as we consider the actions of drug-carrier complexes, especially in terms of possible toxicity to the RES.

One of the simplest functions of the macrophages is the uptake of foreign particles and macromolecules. These cells, in fact, are sometimes called "professional" phagocytes. The cells most closely involved with this function are the Kupffer cells of the liver, and the splenic macrophages. By scanning electron microscopic techniques, it has become clear that the Kupffer cells actually sit astride the channels of the liver sinusoids and thus are in an ideal position to "catch" passing particles (266, 571). In the spleen, especially the red pulp, there are sheaths of macrophages bordering the sinusoids and these cells are also in an advantageous position to take up circulating particles (555). Macrophages are highly specialized cells which have a well developed phagocytic ability (464). A variety of foreign particles used as drug delivery systems including liposomes, (175, 181, 183, 234, 238), microspheres (561–563), and various other colloids (436) are rapidly taken up by macrophages *in vitro*, or by the liver and spleen macrophages *in vivo*. The efficiency of these phagocytic processes is extraordinary; for example, a mouse peritoneal macrophage can "eat" its own weight in liposomes in 1 hr (Juliano and Hsu, unpublished data). Macrophages take up not only particles, but also certain proteins when these are capable of interacting with receptors on the macrophage surface. The protein receptor complex is internalized into a vesicular structure which then fuses with lysosomes; the internalized protein is then usually degraded while the

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receptors may recycle to the cell surface (54), resembling the process of receptor-mediated endocytosis seen in cells with less active phagocytic function. The macrophage membrane contains a variety of receptors including two types of receptor for the Fc domain of IgG (359, 464), receptors for at least two complement components (276), receptors for mannosyl/fucosyl-terminated glycoproteins (481) as well as a binding site for fibronectin (207).

The receptor-mediated internalization system of the macrophage is extremely active. In addition to the uptake of soluble proteins, these surface receptors can also mediate the uptake of particles. Thus, coating a particle with proteins capable of interacting with macrophage surface receptors (a process termed "opsonization" by immunologists) can enormously enhance the uptake of the particle. For example, coating liposomes with IgG and thus promoting interaction with the macrophage Fc receptors enhances the rate of phagocytosis of liposomes 1000-fold (207). Similar enhancement would be expected with other types of particulate carriers. Sometimes the acquisition of an opsonic protein coat is unintentional but nonetheless occurs. Thus, gelatin microspheres inevitably acquire a coating of fibronectin upon injection into the circulation (437) and are cleared by macrophages.

Several types of drug-carrier complexes are likely to interact with and be taken up by macrophages of the RES. Particulate carriers such as liposomes and microspheres are known to be rapidly cleared from the circulation and be deposited largely within RES cells (237). While this process can be modified to a certain degree by manipulating the physical and chemical characteristics of the particles, it cannot be entirely avoided (389). One should keep in mind that not all particles in the circulation are rapidly cleared by macrophages. Healthy erythrocytes circulate for 110 days in man, suffering, in the interim, an astronomical number of collisions with liver and splenic macrophages and yet are not engulfed or destroyed; however, subtle alterations of red cell membrane structure caused by damage or senescence will lead to rapid uptake by the macrophages (192). Almost nothing is known about the signals that regulate red cell-macrophage interactions. It is conceivable that a detailed understanding of the subtle recognition properties of macrophages will eventually allow one to construct particles or drug delivery systems which can then evade uptake by cells of the RES.

Macromolecular carriers, as well as particulate ones, can be removed from the circulation via interaction with the RE cells. Antibody-drug conjugates where the antibody is of the IgG1 or IgG3 subclasses can bind directly to human macrophages via protease sensitive Fc receptors. Antibody-drug conjugates of other classes (IgG2, IgG4) can bind to the trypsin insensitive Fc receptor if they first bind to the antigen (556); a plausible scenario might involve shedding of some of the target antigen, binding of the antigen to the drug carrier in the circulation, and then clearance via the macrophage Fc receptors. Another aspect is that many of the proposed protein-type drug carriers are likely to be rather immunogenic themselves, leading to the formation (upon repeated use) of anticarrier antibodies and subsequent clearance of the carrier-antibody complexes by the RES.

Certain types of polymeric drug carriers, particularly complex carbohydrates, and highly negatively charged species may, in addition to their immunogenicity, activate the alternate pathway of the complement system (10, 83, 84). If activated complement components, particularly C3b, remain attached to the carrier, then uptake via the C3 receptors of macrophages may be promoted.

In summary, the action of the RES can result in the clearance and sequestration of a variety of particulate and macromolecular drug carriers. This has two unfortunate consequences: first it reduces the amount of drug available for interaction with target sites; second and perhaps most important, it poses the danger of selective destruction of macrophages, thus presenting grave consequences for the host defense system.

E. Cellular Barriers

After passing the endothelial and basement membrane barriers and escaping the RES, a drug-carrier complex must then reach its ultimate site of action within a specific cell population. The problem of drug "targeting," or of obtaining a selective interaction of the drug-carrier complex with a particular set of cells, has received a good deal of thought and attention. Some effective approaches have been devised and are discussed below. If the ultimate target of the drug is a surface receptor then a carrier that brings the drug into the proximity of the receptor has completed its task. However, if the ultimate site of action of the drug lies within the cell, then binding of the drug-carrier complex to the cell surface only marks the beginning of a complex process of intracellular migration.

The movement of proteins and other macromolecules between various compartments within the cell, the so called "sorting problem," has emerged as one of the central themes of cell biology. Here we can only lightly touch on selected aspects of this problem and try to point out how they impact upon the task of controlled drug delivery. A large number of reviews and research articles have been devoted to the processes of intracellular and intercellular movements of protein. Among the most timely reviews are those of Steinman *et al.* (485), Rothman (425), DeRobertis (104), Schatz and Butow (441), Pastan and Willingham (379–380), Farquhar and Palade (126), and Meyer (332). A detailed understanding of the fate of ligand and receptor following internalization will allow for the design of appropriate drug-carrier complexes to utilize the intracellular "sorting" process.

Cells possess at least three distinct processes for the uptake of particles and macromolecules. Perhaps the best studied process is that of phagocytosis where the cell

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internalizes a large particle (1000 Å or greater) by engulfing it into a membrane-bound vesicle which is then internalized and ultimately fused with lysosomes. Phagocytosis is largely an adaptation of specialized host defense cells such as macrophages and granulocytes (485); however, even fibroblasts may take up particles to a limited degree. The phagocytic uptake of a particle is a highly energy dependent process involving the action of cytoskeletal elements such as actin/myosin-containing microfilaments (464). After fusion of the phagocytic vacuole with lysosomes, the contents of the vacuole can be degraded (providing they are digestible by the lysosomal hydrolases) and the components of the internalized membrane can recycle back to the plasma membrane (485).

Cells can also internalize material by "nonspecific" or "fluid phase" pinocytosis. Here cells pinch off and internalize small fluid-filled plasma membrane vesicles of about 250 Å in diameter. These vesicles, like phagocytic vacuoles, ultimately fuse with and deliver their contents to the lysosomes. Fluid-phase pinocytosis is extensive in both professional phagocytes and in nonphagocytic cells. Thus, macrophages internalize 25% and fibroblasts 4% to 8% of their total cell volume per hour (485). The plasma membrane components involved in forming the limiting membranes of pinosomes seem to be a random sample of the overall composition of the membrane (329). The amount of plasma membrane material internalized in this process seems to be about 3% per minute in macrophages and 1% per minute in fibroblasts (485). Since the biosynthetic turnover time of membrane proteins is 10 to 20 hr, this implies that the membrane components involved in pinosome formation rapidly recycle to the cell surface. At this time the energy requirement and cytoskeletal involvements in fluid phase pinocytosis are not well resolved, while the role of "coated" structures (see below) is also still uncertain.

Recently is has become clear that the cellular receptors for a variety of polypeptide hormones, growth factors, and serum proteins undergo a process of continuous recycling from the plasma membrane to the cell interior and back again. This process has been termed "receptor-mediated endocytosis" (RME) and has been the subject of intense investigation in recent years (54, 328, 330, 379, 380). The LDL receptor system is perhaps the best studied and can serve as a paradigm. LDL receptors, like most membrane glycoproteins, are synthesized in the rough endoplasmic reticulum (RER), further processed in the Golgi apparatus, and then migrate to the plasma membrane where they are inserted randomly. At the cell surface, the receptor binds LDL and clusters into specialized regions of the membrane called "coated pits." The coated membrane regions are marked by a highly structured array of proteins on their cytoplasmic aspect. The major component of this array is the protein clathrin, which is capable of forming organized, basket-like structures (382). The vesicles that bud from the coated membrane regions quickly lose their clathrin coats. These vesicles are now termed "smooth endosomes" or receptosomes [379; see Helenius *et al.* (198) and Pastan and Willingham (380) for recent discussion]. At this point several important but poorly defined events occur. First the ligand (LDL for example) dissociates from the receptor; this may be a consequence of the acidification of the endosomes which occurs at about the same time the clathrin coat is lost (527). In addition a "sorting out" process seems to occur (159, 447, 54, 51). Thus the ligand remains free in the lumen of the endosome, while the receptors appear to cluster in tubular extensions of the vesicle which then bud off and return to the plasma membrane. The remainder of the endosome, with the ligand, continues on its path to the cell interior and eventual fusion with lysosomes and degradation of ligand (54, 485). The sorting or uncoupling of the ligand and receptor is an essential part of this process. Thus, studies of receptor turnover rates indicate that the LDL receptor traverses the entire cycle within 3 min. By contrast, the biosynthetic lifetime of the LDL receptor is several hours; thus the receptor may make over 100 round trips into the cell before being degraded itself. In this scheme, the ligand is carried along passively, entrained in an ongoing process of receptor recycling. However, in some cases the presence of ligand can regulate the process of receptor-mediated endocytosis. Thus ligand binding may incite the entry of diffusely distributed receptors into coated pits, thereby hastening the turnover. As an example, the binding of asialoglycoprotein to its receptor specifically depletes the surface pool of receptors for this ligand but not for other ligands (78). The receptors of other ligands such as epidermal growth factor and alpha-2-macroglobulin are initially diffusely distributed and only cluster (presumably in coated pits) a few moments after ligand binding (379). Apparent "down regulation" and internalization via coated pits of surface receptors has also been observed for insulin (246, 385). The best example of metabolic regulation of cell surface receptors is the demonstration that internalization and subsequent degradation of LDL, and the resultant increase in intracellular cholesterol causes LDL receptor synthesis to be inhibited. It also seems clear that the efficient recycling of receptors does not occur in all cases; the Fc receptor seems to be degraded along with its ligand subsequent to internalization (485).

One must keep in mind that not all cell membrane proteins are capable of shuttling between cell surface and cell interior via the coated pit mechanism. Bretscher *et al.* (50) have proposed that coated pits act as "molecular filters" separating "migrant" proteins, such as the LDL receptor, from other membrane components (e.g. histocompatibility antigens) which are not designed to recycle. Presumably each type of protein has implicit in its structure a signal that facilitates its recognition by

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coated pits; as yet, however, no distinctive differences between migrant and nonmigrant proteins have been identified (54). Nonmigrant membrane proteins may also enter cells by a mechanism that involves a contractile actinomyosin system and smooth vesicles rather than the coated pit-coated vesicle system (211). In fact, some membrane proteins are as likely to be shed into the extracellular space as they are to be internalized. Needless to say these would make a poor target for intracellular drug delivery.

A large variety of protein ligands are known to enter cells by receptor-mediated endocytosis. In addition to hormones and serum glycoproteins, whose recycling is presumably part of normal cellular physiology, several pathological entities can also gain entry to the cell in this fashion. Thus, certain enveloped viruses such as influenza and Semliki Forest virus (SFV) are internalized via receptor-mediated endocytosis (197, 558, 470) as is diphtheria toxin (111, 46) and perhaps other toxins as well. Studies of the internalization of viruses and toxins have led to the realization that certain types of proteins (e.g. influenza hemagglutinin) can undergo a pH-sensitive conformational change leading to the exposure of hydrophobic groups, a strong interaction with membrane lipids, and ultimately to the destabilization of the membrane. Thus not only can certain viruses and toxins "ride" the receptor-mediated endocytosis system, they may also escape from the endosomal compartment and enter the cytoplasm via a pH-sensitive interaction with the endosome membrane.

Recently it has become clear that there is also a precisely regulated traffic of proteins moving from their sites of synthesis in the cytoplasm or RER to their ultimate destinations in various subcellular organelles. The signals regulating this flow of proteins are built into the protein molecules themselves in the form of particular amino acid sequences called signal peptides (332) or sometimes as attached carbohydrate residues (348, 425). Signal peptides have been implicated in the export of proteins by mammalian cells (332) and by bacteria (559). Interestingly, bacteria can utilize mammalian signal sequences to direct export of proteins destined for secretion, indicating a high degree of conservation of the mechanism (502). Peptide recognition sequences apparently also direct the insertion of cytoplasmicly synthesized proteins into mitochondria (441) and chloroplasts (188). By contrast, the nature of the signals directing selective entry of proteins into the nucleus are unclear (104). In many but not all cases, proteins destined for incorporation into organelle compartments are synthesized as larger precursors and the insertion of the mature protein is accompanied by specific proteolytic cleavage (332, 441).

The ability of water soluble polypeptides to cross lipid membrane barriers on the way to their final cellular destinations is a rather startling finding. In the case of the chloroplast protein plastocyanin, three membranes are crossed during transit of the protein from its site of synthesis on cytoplasmic ribosomes to its ultimate functional site within the thyalkoid (188). The transit of polypeptides across membranes seems, in most cases, to occur via specific receptor-pore mechanisms (169, 104). However, the insertion of proteins into and even through membranes may be driven by simple thermodynamic forces as well (40, 559). In addition it is not clear at this time whether the gates that regulate the passage of proteins across endoplasmic reticulum, mitochondria, or nuclear membranes allow only unidirectional traffic or whether bidirectional flow is permitted.

Not only proteins, but lipids as well, undergo a sorting procedure during organelle formation, and a great deal of information exists documenting the unique lipid composition of various organelle membranes. The mechanisms underlying this intracellular separation of lipids are unknown. Recently, however, Pagano and his colleagues (366) have garnered evidence that exogenous lipids can also undergo sorting processes subsequent to cellular uptake.

Thus, our expanding knowledge of the subcellular traffic of proteins and their transit across membrane barriers at least suggests the possibility of developing drug carriers which could ride the machinery for protein sorting and thus be directed to particular subcellular compartments.

F. The Targeting Problem

One of the most fundamental barriers to selective drug delivery involves the so called "targeting" problem. Much of the intellectual excitement in the drug delivery area revolves around the concept of directing therapeutic agents to a particular cell population where desirable effects can be achieved without exposing other cell populations where toxic effects may occur. The most obvious example of this would be a toxic agent that can discriminate between neoplastic and non-neoplastic cells. However, many other examples related to infectious diseases and genetic disorders readily come to mind. Obviously the targeting paradigm is a direct descendant of Paul Ehrlich's concept of the "magic bullet."

Later in the article we will examine in detail several of the technologies which attempt to deal with the targeting problem. Here we will explore in a general way some of the difficulties involved. For the sake of uniformity we will consider the problem of targeting a cytotoxic agent to neoplastic but not to normal cells, although the general considerations would be similar for other targeting problems.

First this approach presumes the existence of something to aim at, that is, some molecular characteristic that differs between target and nontarget cells. This might be a surface receptor, a structural membrane protein, an intracellular enzyme, or an altered sequence in the genome. Clearly the first task is establishing that a

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discrete and accessible molecular difference does exist between target and nontarget cells. Until recently, this was rather problematic in the case of neoplastic cells; however, new developments in the characterization of oncogenes and their products and of monoclonal and polyclonal antibodies to tumor-associated antigens (504, 200, 256, 569), strongly suggest that it will be possible to establish distinct molecular differences between normal and tumor cells.

The second task is to develop a reagent that will show a high degree of selectivity in bringing a toxic drug into (or at least to) the neoplastic cell type. As we will see, this has been achieved in several cases in the *in vitro* situation; however, the extension of these selective targeting approaches to the *in vivo* situation is far from straightforward. Drug-carrier conjugates showing excellent discimination between target and nontarget cells *in vitro* often become involved in complex sets of nonspecific interactions and redistribution when administered *in vivo*. These are problems usually associated with the endothelial barrier and the efficiency of the RES.

Finally, one must realize that a neoplastic cell population is not a static entity, but rather a protean, everchanging one. All tumors tend to undergo progression and often this takes the form of moving from more differentiated to less differentiated states. Selective pressures (e.g. cytotoxic agents) can also hasten this rate of progression (384). Tumors display an amazing ability to escape or neutralize the actions of drugs or other therapeutic agents to which they were initially sensitive. Some common examples of this are: 1) the loss of specific receptors, 2) down regulation of tumor-associated antigens, and 3) shedding of antigens into the body fluids (81). Likewise, tumors may regulate the levels of various enzymes and even the number of genes coding for particular enzymes, via gene duplication, thereby frustrating the action of drugs directed against the enzyme (12). Thus targeted drugs which are initially effective may lose their effectiveness as the tumor cell population responds to selective pressures.

A possible circumstance where this would not occur is when the "target" of the toxic agent is an essential component of the cellular machinery involved in the maintenance or expression of the transformed state. Thus oncogenes themselves, regulatory genetic elements associated with oncogenes, or the initial transcription or translation products of oncogenes may be targets that are not subject to escape mechanisms. Some possible areas of research in this vein might be: 1) synthesis of inhibitors of oncogene coded proteins that do not also inhibit normal cell function, 2) development of antibodies that might recognize and inhibit oncogene coded proteins (or even RNAs), or, 3) development of synthetic oligonucleotides that might bind to and inactivate the oncogene itself.

III. Technologies for Controlled Drug Delivery

A. Overview

In the following pages we will attempt to critically evaluate a range of different drug carrier systems from lipid structures such as liposomes, to denatured albumin microspheres, to plasma proteins such as low density lipoproteins, to synthetic macromolecules. Less attention will be given to the specific drugs to be carried in terms of mechanism although desired sites of action will be stressed. For the purpose of this review a wide range of enzymes with therapeutic potential are being considered as drugs. While no attempt is being made to include all drug delivery systems in our review, we have chosen those systems whose description should prove instructive to those interested in the past and future development of drug delivery systems. Some of the systems described have already reached the clinical domain while others are barely past the stage of germination. Some systems show great promise whereas others have serious flaws and we will attempt to point these out. Throughout we attempt to utilize the evaluation parameters listed in an earlier section.

B. Methods of Drug-Carrier Conjugation

Except for the cases of encapsulation techniques, the production of drug and/or enzyme carrier systems invariably requires some means of conjugation. Some earlier studies with drug molecules such as daunomycin or methotrexate bound to antibodies or DNA molecules [see Trouet *et al.* (518, 519), for review] in a noncovalent manner have been reported, but the specific benefits of the conjugation have been questioned. Evidence now exists to demonstrate synergistic effects of the drug-carrier system as opposed to the use of the carrier as an actual delivery method.

The earliest attempts at producing conjugates utilizing proteins as carriers and drug molecules as haptens occurred, not as a result of the need for new techniques in drug delivery technology, but rather in the field of immunology. In 1917, Landsteiner (278) reported on the production of what he called "artificial conjugated antigens." The object of this synthesis was Landsteiner's belief that the antigenicity of molecules was dependent on the specific chemical constitution of proteins and that it was this specificity that caused the production of antibodies. He, therefore, started with a nonantigenic molecule such as serum albumin and conjugated to it small haptenic groups which themselves were not antigenic but when conjugated to the carrier protein became antigenic in their own right. In certain instances, they induced antibody formation to the carrier protein as well. Landsteiner also determined that the method of conjugation had to be covalent and established many new procedures for the attachment of small hapten molecules to proteins. His earliest work attached chlorides or anhydrides of several acids such as butyric, isobutyric, or

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TABLE 1
Overview of drug delivery systems

Carrier	Drug (Enzyme)	Comments	References
Liposomes	Anticancer	Poor tumor accessibility without targeting	391
Liposomes	Antiinfectious	Excellent penetration to parasites residing in reticuloendothelial system (RES)	14–17, 386
Liposomes	Immunomodulators	Exciting possibility of enhancing immune response by encapsulating macrophage activating factor (MAF)	130, 134, 142, 232, 257, 258
Liposomes		Targeting using antibodies and other ligands to specific cell surface receptors	158, 208, 455, 487
Liposomes	Chelating agents	Limited accessibility to parenchymal tissue, sites of iron storage	285, 406
Lipsomes	Gold products	Potential treatment of rheumatoid arthritis	79
Liposomes	Amphotericin B	Liposomes greatly diminish the generalized cytotoxicity of the antifungal agent	302, 307
Liposomes	Proteins	Use as adjuvants for enhanced immunogenicity	11, 15, 262, 263, 407, 453, 454, 513, 514, 536, 537
Liposomes	Enzymes	Immunoglobulin-mediated targeting	135, 136, 554, 210, 105
Liposomes	Interferon	Altered pharmacological properties	122, 311
Liposomes	Genetic material		146–148, 286, 305, 355, 500
Liposomes		General reviews (see also table 2)	128, 175, 181, 183, 234, 238, 389, 546
Antibodies	Methotrexate	First use of antitumor antibodies as a drug carrier system	320
Antibodies	Chlorambucil	Noncovalent conjugates	160, 163, 164
Antibodies	Chlorambucil	Demonstrate synergism between antitumor antibodies and drugs administered independently	428
Antibodies	Chlorambucil	Limited clinical study of immunochemotherapy demonstrates cross-reactivity of antibodies	164, 426
Antibodies	Adriamycin, daunomycin	*In vitro* and *in vivo* efficacy of drug-antibody conjugates	22, 215–219
Antibodies	Daunomycin	Use of dextran cross-bridges between drug and antibody	34
Antibodies	Diphtheria toxin	Early demonstration of the potential of immunotoxins	338, 340
Antibodies	Glucose oxidase	Utilizes the peroxidase-mediated iodination of cellular constituents to produce toxicity	267
Antibodies	α-Glucosidase, L-asparaginase	Targeting nonimmunogenic enzyme-albumin conjugates *in vitro* and *in vivo*	397, 399
Antibodies	Ricin or diphtheria toxin A-chain	Cytotoxicity of immunotoxin demonstrated in tissue culture only to cells bearing specific antigens	41, 62, 63, 166–168, 340, 541
Antibodies	Ricin A	*In vitro* tumor cell depletion for autologous bone marrow transplantation	274, 275, 541
Antibodies	Immunotoxins	Review	542
Antibodies	Ricin or diphtheria toxin	*In vitro* targeting using antitransferrin receptor monoclonal antibodies	522, 523
Antibodies	Ricin A	Targeting to a common acute lymphoblastic leukemia antigen (CALLA)	408–410
Antibodies	Pseudomonas exotoxin	Conjugate internalized by receptor-mediated endocytosis using antitransferrin antibodies	137
Antibodies		Review	116, 117, 289, 290, 112
DNA	Adriamycin, daunorubicin	Noncovalent complexes, lysosomotropic systems	518–521, 103
Albumin	5-Fluorodeoxyuridine	Lysosomotropic approach to antiviral chemotherapy	28, 138, 139
Albumin	Antifungal agents	Altered pharmacokinetics with a suggestion that the albumin acts as a slow release system	139
Albumin	Uricase	Increased stability of enzyme-albumin conjugate	370
Albumin	Uricase, L-asparaginase, α-glucosidase, cholesterol esterase, superoxide dismutase, glucose-6-phosphatase	Enzyme-albumin conjugates in a nonimmunogenic form for enzyme replacement therapy	395–401, 413, 578
Albumin microspheres	6-Mercaptopurine	Particulate, denatured albumin	271
Albumin microspheres	Doxorubicin	Use of an extracorporeal magnet to target magnetite containing microspheres	560–562

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TABLE 1—*continued*

Carrier	Drug (Enzyme)	Comments	References
Magnetic albumin microspheres	Various drugs	Review	563
Low density lipoproteins	α-Glucosidase	Attempt at enzyme replacement for Pompe's disease	567
Fibrinogen	Methotrexate	Suggest a preferential targeting to newly formed vascular beds associated with tumor growth	398
Collagen	L-Asparaginase	Produce an insoluble membrane matrix for implantation	360
Dextrans	Proteins, antibiotics, vitamins, insulin	Excellent cross-linking potential but possible complications due to immunogenicity	337
Dextran	Daunomycin		34
Dextran	Enzymes	Decreased allergic reactions	313, 314
Insulin	α-Glucosidase, L-asparaginase	Targeting agent for the delivery of enzyme-albumin conjugates	400, 401
Human chorionic gonadotropin	Ricin A	Targeting to a cell surface receptor	358
Epidermal growth factor (EGF)	Ricin A	EGF-ricin A is more effective than EGF-diphtheria fragment A	60
Human placental lactogen	Diphtheria toxin, fragment A	Enhanced cell surface binding	62, 63
Glycoconjugates	Albumin	Modify *in vivo* clearance of albumin by coupling to fetuin glycopeptides	424
Glycoconjugates (monophosphopentamannose)	Ricin	Targeting to the mannose-6-phosphate receptor	583, 584
Neoglycoprotein	Antiviral drugs	Targeting to galactose receptor on hepatocytes	138
Asialofetuin	Diphtheria toxin	Targeting to hepatocytes in culture	471
Lectin, concanavalin A	Trypsin, L-asparaginase	Effective binding to specific sugar residues	459
Lectin, concanavalin A	Diphtheria toxin, fragment A	Affinity targeting of toxins	166, 167
Lectin, concanavalin A	Dextranase		29
Lectin, *Wisteria floribunda*	Diphtheria toxin		530
Dipeptides	Daunorubicin	Concept of the "prodrug" dependent on amino-peptidase activity for drug release	318
Polyethylene glycol (PEG)	Uricase, L-asparaginase, catalase, and others	Polymer renders enzymes nonimmunogenic and enhances circulation times	2, 3, 439, 564
Poly(*l*-lysine)	Methotrexate	Increased toxicity in methotrexate-resistant cells; interesting use of pH-sensitive linkage	434, 435, 457, 458
Ficoll	Superoxide dismutase	Altered pharmacokinetics of the enzyme	398
Carboxymethylcellulose	Penicillin	Tolerance induction	109
IgG (self)	Haptens	Tolerance induction	47
Erythrocytes	Desferrioxamine	Promising preliminary clinical results in the treatment of iron overload in β-thalasemics	178–180
Erythrocytes	Enzymes, drugs, DNA	Review	220, 129, 212, 105
Erythrocytes	Glucocerebrosidase	Attempt to treat a patient with Gaucher's disease	35, 213, 507
Leukocytes	Enzymes	Bone marrow transplantation as a means of treating certain inborn errors of metabolism	201–204
Fibroblasts	Enzymes	Questionable use of fibroblast transplantation	94–97, 165, 361
Reconstituted Sendai virus	Membrane proteins, soluble macromolecules	Sendai virus induces fusion with target cell	31
Sustained drug release	Various drugs	Implantable drug depot systems showing excellent clinical promise	279–282, 38, 39, 402, 156, 1, 191
Microencapsulation	Intact cells	Develops the concept of "semipermeable aqueous microcapsules"	64, 68
Microencapsulation	Islet cells	Development of a technique for "pancreatic islet cell transplantation"	299
Microencapsulation	Lymphocytes, hybridomas	Enhanced production of interferon and monoclonal antibodies in high yield	356
Poly-DL-alanyl peptides	L-Asparaginase	Improved therapeutic and immunological properties	532, 5
Virosomes	Rubella vaccine		524
Lactic/glycolic acid polymers	Narcotic antagonists		448
Liposomes	Polymerized vesicles	Stabilized time-release carriers	411, 412
Liposomes	Radionuclide	Diagnostic imaging	30, 59, 364, 365
Liposomes		Assay for systemic lupus	227
Liposomes	Lithium		586

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cinnamic acid to free amino groups of serum albumin by way of a simple acylation reaction. Later he demonstrated that he could attach a wide range of diazonium compounds to histidine, tyrosine, or tryptophan residues on the protein. While his work did much to establish the rules for antibody response to haptenated protein molecules (nature of the hapten, number of hapten molecules/protein carrier, etc.) he also made significant contributions to the possibilities of attaching new determinant groups to protein molecules while retaining many of the original characteristics of the intact protein. We can learn from Landsteiner's work not only methods of conjugations but also the immunological dangers of attaching small haptenic drug molecules to large macromolecular carriers.

While the immunologists were the first group of investigators interested in the production of protein-hapten conjugates, new advances in the use of drug delivery systems have made the area of covalent bonding of drug and/or enzyme molecules to proteins or other macromolecular structures extremely important. The topic of chemical cross-linking or conjugation of proteins or other macromolecular structures with sugars, drug molecules, lipid or other proteins has been reviewed extensively in the past few years [see Means and Feeney (324); 21, 32, 123, 124, 533, 573]. In the following pages we will briefly review several strategies and chemical cross-linking techniques that have been used in the production of conjugated drug/enzyme-carrier complexes.

Prior to consideration of the cross-linking step, a detailed understanding of the available reactive groups on the carrier and drug or therapeutic agent is essential. For instance, on bovine serum albumin there are at least five available groups: 1) epsilon amino groups of lysine residues; 2) alpha amino groups found on many different proteins; 3) phenolic hydroxyl groups of tyrosine residues; 4) sulfhydryl groups of cysteine residues; and 5) imidazole groups of histidine residues. The other reactive groups found on either carrier systems or on drug molecules include vicinal hydroxyl groups, phenols, free hydroxyl groups found on many alcohols, and terminal carboxyl groups. Even in places where free carboxyl groups are not available, hapten or drug molecules frequently possess reactive groups onto which carboxyl groups can be attached. The following diagrams depict several possible reactive groups that may be available on drug/hapten molecules and the possible means by which they might be conjugated to macromolecular carrier systems such as simple polypeptides, polysaccharides, synthetic polyanions or polycations, and lipidic structures such as liposomes.

The object of any conjugation procedure is to effect the attachment without altering the desired properties of the ligand and carrier particle. For example, in the use of antibodies as carriers of drugs it is necessary that, following conjugation, the antibody retain its specificity towards its binding site while the drug retain its required activity. It might be that the drug does not possess its therapeutic action while still conjugated to the carrier and that cleavage of the bond might be a necessary step for drug action. Under such circumstances the bond must be sensitive to endogenous breakdown if the drug is to be released following delivery. Consideration must be given to the cross-linking conditions to assure that carrier and drug maintain the desired properties. Conditions of the cross-linking such as temperature, pH, time, and protection of sensitive sites (as in the active site in considering enzyme cross-linking) must be considered. The conjugation procedure must be gentle enough so as not to destroy either drug or carrier activity. Lesser concerns such as the possibilities of biodegradation of the product, its final disposal or elimination, and possible contamination with toxic side products must also be considered. In choosing a cross-linking procedure, while there is a certain amount of empiricism involved, a knowledge of what side groups are available remote from the "business" regions of both drug and carrier is beneficial. For example, in one case derived from one of our laboratories (M. J. P.) it was our intention to covalently bond the iron chelating drug desferrioxamine to albumin (36). The drug molecule has a free amino group attached by a short alkane chain to a cyclic peptide, the carbonyl groups of which are said to form a cage to carry the iron molecule. We therefore guessed that it might be possible to cross-link the drug molecule to albumin by using the apparently free amino group on the drug without affecting its iron-chelating ability. We picked a reagent that utilized such a side group. Glutaraldehyde was effective as a bifunctional reagent in cross-linking the amino group on the drug molecule to primary amino groups on the carrier albumin molecule. The water soluble carbodiimide (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, EDCI) could also be used as a cross-linking agent by first reacting it with free carboxyl groups on the albumin molecule followed by conjugation to the free amino group on the drug. This second reaction was not as efficient as the glutaraldehyde reaction since in the second step there existed a direct competition between the free amino groups on the drug molecules with amino groups on the protein molecules resulting in protein-protein cross-linking, and intraprotein cross-linking, in addition to the desired drug-protein cross-linking.

The covalent attachment of ligands, most often immunoglubulins, to lipid strutures such as multilamellar or unilamellar vesicles, appears to pose special problems. It is not clear why this is so, but it may be that the reactive groups associated with the lipid structures (glycolipids, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol) are not stable in the bilayer structure following conjugation to large protein ligands such as antibody molecules. Papahadjopoulos and coworkers (195, 196, 315, 316) have devised several new

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strategies for the stable conjugation of immunoglobulins and immunoglobulin fragments to preformed lipid vesicles. Some of these utilize traditional cross-linking reaction schemes such as periodate oxidation utilizing vicinal hydroxyl groups and primary amino groups (195) which show good stability. Other authors have utilized cross-linking agents such as glutaraldehyde (515) and carbodiimides (113) where the stability of the ligand-vesicle complex is poor and the targeting efficiency is low. Early reports utilized gamma globulins attached in a noncovalent, ill-defined manner to the surface of lipid vesicles (553). No follow-up work has been reported and efforts to utilize this procedure for targeting purposes have not met with success. A recent procedure described by Martin et al. (315) utilizes the N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) reaction (described below, Equation 8) to attach Fab′ (immunoglobulin) fragments to the surfaces of lipid vesicles containing phosphatidylethanolamine, by way of disulfide bonds. Leserman and colleagues (296) have used a similar disulfide bridge reaction to cross-link thiolated IgG and protein A to small unilamellar vesicles. While the cross-linking efficiency of this reaction is high, there is some question whether the bond can, in fact, withstand the reducing conditions found in serum (see Ref. 315). Martin and Papahadjopoulos (316) have recently described a new irreversible coupling procedure for the attachment of immunoglobulins to preformed lipid vesicles utilizing a new sulfhydryl reactive phospholipid derivative. The reaction scheme is one of those listed and described below.

The following represents some examples and possibilities in the dictionary of cross-linking procedures that might serve to bind drug molecules to macromolecular carriers.

1. *Glutaraldehyde* (*Eq. 1*). This is one of the most gentle of the cross-linking reactions. It conjugates free amino groups at temperatures between 4°C and 40°C at neutral pH (6.0 to 8.0) and in a wide range of buffer solutions as long as none of the buffer components contain free amino groups. The reaction works by Schiff base formation between the aldehyde group on the glutaraldehyde and the primary amine on the protein. The fact that glutaraldehyde is a dialdehyde allows for the reaction to occur directly between two primary amines. The Schiff bases may then be reduced with either sodium borohydride or sodium cyanoborohydride both of which stabilize the bond. Depending on the desired stability of the bond the reduction step may sometimes be omitted.

GLUTARALDEHYDE

$$OHC-CH_2-CH_2-CH_2-CHO$$

$$\text{Carrier}-NH_2 + OHC-(CH_2)_3-CHO + NH_2-\text{Drug/Enz.}$$
$$\Downarrow$$
$$\text{Carrier}-N=CH-(CH_2)_3-CH=N-\text{Drug/Enz.} \qquad (1)$$

PERIODATE OXIDATION

Carrier–C(H)–C(OH)(H)–C(OH)(H)–C(H)–CH_2OH (ring O linking first and fourth C)

$\Downarrow$ $+IO_4^-$

Carrier–C(=O)–C(H) C(H)(=O)–C(H)–CH_2OH (ring O)

$\Downarrow$ pH 9.0 - 9.5, $+NH_2$ - Drug/Enz. (2)

Carrier–C–C–OH HO–C–C–CH_2OH (ring O), N–Drug/Enz.

$\Downarrow$ $+NaBH_4$

Carrier–C–C–H H–C–C–CH_2OH (ring O), N–Drug/Enz.

2. *Periodate Oxidation* (*Eq. 2*). This reaction is suitable for the attachment of free amino groups to a wide range of sugar residues. Like the glutaraldehyde reaction it is also gentle, working at normal pH and reasonable temperatures. It is a two-step reaction first utilizing sodium periodate ($NaIO_4$) to cleave the diol of the sugar residue which opens and allows for the formation of a three-membered transition ring necessary for the reaction to take place with the primary amine. Once this transition state is produced, then the primary amine from the other

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CARBODIIMIDES

R-N=C=N-R'

Carrier-COOH + R-N=C=N-R'

⇓

Carrier-C(=O)-O-C(=N-R')-NH-R (3)

⇓ + NH_2 - Drug/Enz.

Carrier-C(=O)-NH-Drug/Enz. + R'-NH-C(=O)-NH-R

MIXED ANHYDRIDE PROCEDURE

R-O-C(=O)-Cl

Carrier-COOH + R-O-C(=O)-Cl

⇓

Carrier-C(=O)-O-C(=O)-O-R + HCl (5)

⇓ NH_2-Drug/Enz. pH 9.5

Carrier-C(=O)-NH-Drug/Enz. + CO_2 + ROH

CIS-ACONITIC ANHYDRIDE

Drug-NH_2 + C(COOH)=C-C(COOH)-COOH

⇓ pH 9.0 (4)

Drug-NH-C(=O)-C=C-C(COOH)-COOH

$\xrightarrow{\text{Carrier-NH}_2 \text{ + EDCI (Carbodiimide)}}$

Drug-NH-C(=O)-C=C-C(COOH)-C(=O)-NH-Carrier

DIAZO LINKAGES

Drug/Enz. - NH_2 + N_2Ar

⇓

Drug/Enz. - NH - N=N-Ar

⇓ + Carrier (tyrosine) (6)

Drug/Enz. - NH - N=N - (C$_6$H$_3$-OH) - CH_2 - CO-CH-NH-Carrier

protein or drug molecule is attached by Schiff base formation, generally at pH 9.5. The oxidation requires the presence of a vicinal hydroxyl group which forms a dialdehyde with which the primary amino group reacts. The Schiff base may be stabilized as with glutaraldehyde reaction.

3. Carbodiimides (*Eq. 3*). After glutaraldehyde, the carbodiimides appear to be the most common and successful of the cross-linking agents being used. The use of carbodiimides for cross-linking has been known in the pharmaceutical and organic chemistry fields for some time except that few of the cross-linking compounds were water soluble and the reactions had to be carried out in organic solvents, environments harsh to most protein carrier systems. The development of a water soluble carbodiimide, EDCI (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide HCL), made this a very attractive reaction for the conjugation of free primary amino groups to free carboxyl groups. The reaction is a gentle one, being carried out at neutral pH even in the cold. Protein and an excess of hapten (e.g. drug) can be mixed along with the EDCI and allowed to react for up to several days. Unused EDCI and drug molecules can readily be separated from the carrier-drug conjugates by dialysis. Care must be taken to ensure that changes in the desired properties of the carrier molecules do not take place. It has been shown that carbodiimide treat-

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SPDP (N-succinimidyl 3-(2-pyridyldithio) propionate)

MeOH
Triethylamine

PE in Vesicles
+Fab′-SH
pH 8.0

Lipid Vesicles

(7)

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ment of antibodies produces new antigenic determinants and may alter the binding specificity of the original antibody.

4. *Cis-Aconitic Anhydride* (*Eq. 4*). This is an interesting cross-linking mechanism (see Ref. 458) since it produces a conjugate that is stable at neutral pH but which is cleaved at acid pH (4.0 to 5.0). It has the potential to produce an ideal lysosomotropic complex which, following internalization by a cell and deposition within a secondary lysosome, may cause release of the drug from the complex due to the acid conditions. This may have important advantages as to whether the drug is to work within the lysosome or whether it is destined for some other cytoplasmic locale. The reaction is carried out in two steps with a water-soluble carbodiimide in the second step. The reaction sequence is thought to be as follows. The amino-containing drug is substituted into the alpha-carboxyl group at pH 9.0. The gamma carboxyl group can then be conjugated to the carrier protein by using carbodiimides (EDCI). Reducing the pH to 4.0 causes a spontaneous cleavage of the drug-amide bond linked via the N-cis-aconityl group to the protein carried.

5. *Mixed Anhydride Procedure* (*Eq. 5*). This procedure was originally used in peptide synthesis and has been utilized for the conjugation of steroid hormones to proteins. The reaction scheme is simple and does not require the preparation and isolation of active derivatives. The drug molecule or hapten containing a terminal and free carboxyl group is converted into an acid anhydride which can then react directly with a primary amino group on the carrier protein. This procedure has been used to conjugate cortisone-21-hemisuccinate to protein as illustrated below under conditions where an aqueous-acetone solution is used to accommodate the steroid. The procedure, however, can be adapted to be used in purely aqueous solutions.

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CYANURIC CHLORIDE (2,4,6-trichloro-5-triazine)

$$\text{Carrier-CBH}\}\text{-OH} \xrightarrow{\text{cyanuric chloride}} \text{Carrier-CBH}\}\text{-O-}C_3N_3Cl_2 \xrightarrow{NH_2\text{-Drug/Enz.}} \text{Carrier-CBH}\}\text{-O-}C_3N_3Cl\text{-NH-Drug/Enz.} \quad (8)$$

IMIDOESTER
(Diethylmalonimidate)

$$\text{Carrier-NH}_2 + C_2H_5O-\overset{Cl^-H_2N^+}{\overset{\|}{C}}-CH_2-\overset{N^+H_2Cl^-}{\overset{\|}{C}}-OH_5C_2 + \text{Drug/Enz.-NH} \Downarrow \text{Carrier-NH-}\overset{NH_2^+}{\overset{\|}{C}}-CH_2-\overset{NH_2^+}{\overset{\|}{C}}\text{-NH-Drug/Enz.} \quad (9)$$

6. Diazo Linkages (*Eq. 6*). This reaction is carried out readily when the drug/hapten molecule possesses an aromatic amine or diamine which can then be converted to diazonium salts by the addition of nitrous acid. The diazonium salt can then conjugate directly to the protein at alkaline pH (9.0). The reaction occurs primarily with tyrosine, histidine, and tryptophan residues although lysine residues have also been shown to react with lysine residues as well. Care must be taken in this reaction to minimize intraprotein and protein-protein conjugation, but this can be done by producing the diazonium salts in the absence of the carrier protein molecule.

7. SPDP [*3-(2-pyridyldithio)proprionate, N-succinimidyl*] *Derivative* (*Eq. 7*). The object of the reaction is to add free SH groups to both reactants and then to form the conjugation by the formation of disulphide bridges between the two. The beauty of the SPDP molecule is that the N-succinimidyl is an excellent leaving group producing a very electrophylic centre which can react with primary amino groups. Once the primary amino group on the protein or drug molecule attacks the electrophylic centre of the dithiopyridal group on the SPDP, a strong electron donor like dithiothreitol (DTT) or 2-mercaptoethanol (2ME) can be used to break the S-S bond and the reactants can then be linked spontaneously by the formation of a disulphide bridge, care being taken to remove the electron donor before this step. An alternative reaction is the attachment of dithiodiglycolic acid to the primary amine of the drug or protein using a water-soluble carbodiimide (EDCI) followed by breakage of the S-S group with DTT and followed by disulphide bond formation between the two reactants. (SPDP is a product supplied by Pharmacia Fine Chemicals.)

8. Cyanuric Chloride (*Eq. 8*). This reaction scheme is used to attach carbohydrates to either enzymes or other proteins. The reaction can also be used to transform alcohols into chlorides. This reaction has been used extensively by Abuchowski and colleagues (3) in the production of nonimmunogenic conjugates of enzymes and polyethylene glycols. The chloride moiety on the cyanuric chloride molecule can readily be substituted by alcohols (thereby attaching it to the sugar carrier) while primary amino groups on proteins or drug molecules can substitute into another of the chloride positions on the bound cyanuric chloride derivative.

9. Imidoesters (*Eq. 9*). This is a multistep reaction. Proteins can be conjugated to the imidoester in aqueous solution at slightly alkaline pH whereby the primary amine from the protein is inserted and linked to the same carbon group on the imidoester where the amino group and the ester oxygen are linked. Furthermore if the imidoester possesses an SH group then it can be used as a bifunctional agent to join two proteins; the first attached to the imidoester as described above and the second possessing an SH group being attached to the SH group on the imidoester by forming a disulphide bond under oxidative conditions.

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THIOETHER LINKAGE (N-succinimidyl 4-(P-maleiminidophenyl) butyrate

MeOH
Triethylamine

PE in Vesicles
+Fab′-SH
pH 6.5

Lipid Vesicles

S-Fab′

(10)

10. *Thioether Linkage* (*Eg. 10*). This reaction scheme was devised by Martin and Papahadjopoulos (316) for the express purpose of binding immunoglobulin fragments to lipid vesicles in a stable nonreducible form. The reaction occurs between a sulfhydryl-reactive phospholipid derivative N-[4-(*p*-maleimidophenyl)-butyryl] phosphatidylethanolamine (MPB-PE) and the sulfhydryl group on each Fab′ fragment. The addition of the Fab′-SH to the double bond of the maleimide moiety of the MPB-PE molecules present on the lipid vesicles results in a stable thioether cross-linkage. The linkage is stable to the reducing conditions of human serum for several hours. The vesicles remain intact (they do not leak their contents under these conditions) and the binding specificity of the Fab′ fragment is retained as determined by the binding of the antibody-conjugated lipid vesicles to a specific cell-surface antigen.

C. Sustained Drug Release Systems

We have chosen not to review in any detail the many dramatic advances made in drug administration utilizing sustained drug-release systems. These advances have been extensively reviewed in recent years (38, 39, 76, 279–283) and are rapidly entering the phase of clinical and commercial utilization. Sustained release systems do not represent efforts to effect target-specific drug delivery as much as they are devices for the continuous release of drug into a particular compartment, such as the blood, gastrointestinal tract, skin, eye, etc.

There are several distinct technologies for promoting sustained drug release. This would include mechanical or osmotically driven pumps, membrane limited diffusion controlled devices, erodible and nonerodible polymeric matrix devices and various combinations of these (see reviews, Refs. 506, 76, 280). Perhaps the most exciting

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use of a sustained drug release device has been the attempted construction of a closed loop artificial pancreas with a built-in glucose sensory linked to a continuous and variable insulin infusion system. This would be a major advance in the treatment of diabetics, who, in spite of insulin, still suffer from a diminished "quality of life." They have continued difficulties in maintaining proper glucose levels and most importantly they suffer from the development of related diseases of the microvasculature such as glaucoma and atherosclerosis. These are believed to be a result of the poor level of control of plasma glucose. While there have been a number of serious drawbacks in the development of an effective closed loop system (problems associated with the production of stable implantable glucose electrodes), the development of an open loop system utilizing an implantable insulin pump has proceeded (7) and clinical trials have been ongoing for some time now. Although there have been many approaches to the long-term sustained release of insulin using polymeric (nonrechargeable) implants (see Refs. 93, 279, 280, 282), the use of an implantable rechargeable (38) device for the long-term intravascular infusion of drugs seems the most exciting, except for cost limitations, at the present time. Rupp *et al.* (429) and Buchwald *et al.* (56) have reported on preliminary clinical trials on the use of this pump for insulin infusion in patients with type II diabetes and for continuous heparin infusion in ambulatory patients with recurrent venous thrombosis. A second approach initiated by Folkman (see Refs. 144, 282), utilizes a nonrechargeable polymer implant with slow release properties to achieve sustained drug release. This system has a much higher load factor than the rechargeable pumps since the drug within the implant is not in solution and is only solubilized and released as the polymer is hydrated. Though this approach is at a much earlier stage of development than the rechargeable unit described by Blackshear and colleagues (39), it has the advantage of having no mechanical parts; it is much less expensive and although nonrechargeable, a single implanted pellet might suffice for up to a year or more of sustained release. Langer (280) has recently described a system where the rate of release of drug from the implanted polymer pellet may be modulated by using magnetic steel balls embedded within the polymer. Application of an external oscillating magnetic field can modulate the release of drug as much as 30-fold. This device is indeed at a very early stage of development but Langer (280) points out that new electronic technology might make such a system practicable. The sustained drug delivery system is of course only a means of slow or sustained release into a particular compartment and does nothing for the delivery of material to a particular site of action.

One of the first sustained release devices used clinically was designed to solve a problem in ocular disease. The Ocusert system is a membrane-limited reservoir which is placed under the lower eyelid and releases pilocarpine at a fixed rate for about 1 week. In many respects this device is a substantial improvement over conventional multiple-dose (4 times daily) pilocarpine therapy for glaucoma; nonetheless the device has not attained widespread use because of expense and poor patient compliance (280, 281).

One of the most widely studied applications of controlled-release systems has been in the area of contraception (76, 281). Several reservoir-type and bioerodible-polymer-type devices for sustained intravaginal or intrauterine release of contraceptive steroids are now available. A number of oral sustained release preparations are now also available. These include erodible matrix systems for iron and potassium salts, an oral osmotic pump system for indomethacin, and polymeric sustained release systems for theophylline. Although these systems all give rise to desirable (sustained, uniform) pharmacokinetic profiles, their ultimate roles in therapy are poorly defined at present.

The sustained release approach that currently stands out in terms of technological, clinical, and commercial success involves the transdermal administration of nitroglycerin for treatment of angina. Since nitroglycerin is subject to extensive first pass metabolism but has a high degree of skin permeability, this drug seems ideal for a transdermal delivery approach. Several devices have been developed by using either matrix-type or membrane-type techniques; these devices are applied as adhesive patches to the skin and release nitroglycerin at a fixed rate (2.5 to 10 mg/day in various devices). The three commercially available entities are Transderm Nitro, NitroDur, and Nitrodisc; each of these products seems to be enjoying considerable success in terms of sales and patient acceptance (76, 280, 281).

D. Liposomal Drug Delivery Systems

1. Basic Aspects. One of the more popular experimental approaches to controlled drug delivery has been the use of liposomes, or phospholipid vesicles. In this section we briefly review some of the basic chemical and physical characteristics of liposomes and also touch upon their interaction with cells *in vitro*. Since there is an abundant literature on these topics, rather than citing individual references, we direct the reader to several recent comprehensive reviews (234, 238, 497, 236, 181, 183, 389, 391).

Phospholipids dispersed in water can form a variety of structures depending on the molar ratio of lipid to water. At relatively low ratios the liposomal type structure consisting of aqueous compartments bounded by lipid bilayer membranes is the predominant one (88). There are several distinct types of liposomes available to the investigator (Figure 2). The most common preparation is the MLV (multi-lamellar vesicle) an "onion-like" structure of concentric aqueous and lipid layers; these structures are heterogenous in size but range up to sev-

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	SUV	MLV	REV (LUV)
Size (nm)	20-50	400-3500	200-1000
Capture Volume (μl/mg)	0.5	4.1	13.7
Encapsulation Efficiency (%)	0.5-1.0	5-15	35-65

FIG. 2. Types of liposomes. Liposomes have now become an almost generic formulation but in fact come in many different varieties encompassing differences in chemical composition and physical characteristics including size and homogeneity. This figure describes three major "types" of liposomes although even within each type, production methods, chemical composition, and physical characteristics such as fluidity, charge, and stability may vary greatly. (SUV, small unilamellar vesicle; MLV, multilamellar vesicle; LUV, large unilamellar vesicle; REV, vesicles made by a process of reverse phase evaporation) (See Ref. 96).

eral microns in diameter. Sonication can reduce MLVs to SUVs (small unilamellar vesicles) which are single walled vesicles of 200 to 500 Å diameter. It is also possible, by various means, to construct LUVs (large unilamellar vesicles), where a single large aqueous compartment is bounded by a single bimolecular lipid membrane; these structures are usually about 1 μ in diameter. Typically the thickness of a lipid bilayer membrane is about 50 Å.

The physical properties of liposomes depend, in part, on their chemical composition; thus it is possible to use a variety of different types of saturated or unsaturated phospholipids, to include sterols, fatty acids, or a variety of other charged or uncharged amphiphilic compounds. The physical characteristics of the bilayer membranes of liposomes are also highly dependent on temperature, pH, ionic strength, and the presence of divalent cations. In response to temperature changes (or in response to other types of environmental perturbations), bilayer membranes undergo phase transitions, shifting (as the temperature is raised) from a "gel" state in which the fatty acyl side chains of the membrane lipid are closely packed and relatively ordered, to a "fluid" state in which the side chains are capable of more rotational motion. This thermotropic phase transition can have major effects on the stability, permeation properties, and overall behavior of the lipid vesicles (234). Discrete phase transitions are observed only in liposomes containing one or a few species of homogeneous phospholipids; natural lipids, which are mixtures of species containing heterogeneous fatty acyl chains, display broad, ill-defined phase transitions. Cholesterol has a very interesting modulatory effect on the phase transition behavior of bilayers composed of homogeneous phospholipids. This molecule serves as a "fluidity buffer," since below the phase transition cholesterol tends to make the bilayer less ordered, while above the transition it tends to increase the order in the membrane; thus the presence of cholesterol damps out dramatic phase change behavior of bilayers containing homogeneous phospholipid species.

Phospholipids which undergo phase transitions at distinctly different temperatures or in response to different environmental perturbations have the potential of undergoing phase separation or sorting out in the plane of the bilayer. Thus, an initially random mixture of two lipids can, under appropriate conditions of temperature, pH, or divalent cation, sort out to form domains enriched in one or the other individual lipid (see Refs. 55, 86, 88, 100, 154, 226, 231, 288, 291, 306, 312, 404, 460, 488, 540, 565, 582, 587 for various discussions of membrane lipid and liposome properties).

2. *Incorporation of Drugs in Liposomes*. The problems and characteristics of formulating drugs in liposomes have been reviewed in detail elsewhere (98, 236–238). Here we briefly deal with some very basic considerations.

It is clear that polar or hydrophilic drugs will be largely found in the internal aqueous compartments of liposomes, although some degree of binding to the bilayer is also a possibility. By contrast, lipophilic or amphipathic drugs can intercalate into the liposome membrane. This behavior can be demonstrated very graphically by briefly sonicating liposomes containing entrapped polar or lipophilic drugs; the polar molecule is quickly and completely released as the liposome membrane is breached, while the lipophilic drug remains associated with the liposome membrane (483).

For lipophilic drugs, the maximal incorporation into liposomes simply depends on the amount of lipid and the solubility of the drug in the lipid. Drug/lipid molar ratios of 1:10 are usually readily achieved without disrupting bilayer structure (235). For polar drugs, the entrapment depends on the solubility of the drug in water and on the volume of water encapsulated per mass of lipid. This latter parameter can differ substantially among different liposome types (495–497). Thus, encapsulation ratios can vary from about 0.5 l/mol for SUVs to in excess of 15 l/mol for LUVs. One must keep in mind, however, that the encapsulation efficiency is only one of the parameters to be considered in the choice of a liposome type and composition for a particular application; stability, lack of toxicity, or special biochemical characteristics may also be important.

Ordinarily, loss or efflux rates of highly polar or of highly lipophilic drugs from lipid vesicles tend to be low (less than 1%/hr). Amphipathic drugs with an oil/water partition coefficient near 1 will readily leak out of the vesicles unless special steps are taken (e.g. charge complexation) to stabilize the drug-liposome association. Efflux rates of both polar and lipophilic drugs tend to be highest near or at the bilayer phase transition (236).

Generally speaking, a variety of drugs are easily incorporated into liposomes. However, the problems of commercial scale formulation of liposomal drugs are just

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beginning to be addressed. There may be several obstacles to achieving sufficiently high drug/lipid ratios, narrow size heterogeneity, and good stability of chemical stability during prolonged storage. The problem of stable incorporation and storage of polar drugs which reside in the entrapped aqueous compartment may be difficult, while stable incorporation of lipophilic drugs is less of a problem.

3. Liposome-Cell Interactions. Liposomes can interact with cells via four basic mechanisms, namely: 1) absorption to the cell surface, 2) endocytosis, 3) fusion, and, 4) lipid exchange. These mechanisms need not be mutually exclusive. There is a large, complex, and often controversial literature on this subject and the reader is referred to the reviews by Pagano and Weinstein, and by Juliano (369, 234) and to the papers cited therein for detailed analysis. References 224, 272, 292, 354, 367, 368, 388, 493, 498, 499 510, 511, 535 deal with some cases of liposome-cell interactions including the interaction of liposomes containing antitumor agents and tumor cells.

Although early work suggested that vesicle-cell fusion might be important, more recent evidence suggests that, for most vesicle compositions and most cell types, either adsorption or endocytosis are the dominant mechanisms of liposome-cell interaction. Solid (i.e. gel state) vesicles tend to strongly adsorb to cell surfaces, while this is less true of fluid vesicles. Cells which are "professional" phagocytes such as neutrophils and macrophages tend to engulf and internalize vesicles by an active, energy dependent, actinomyosin-related phagocytic process. Less specialized cells such as fibroblasts tend to internalize vesicles (particularly SUVs) by using the coated pit vesicle endocytotic mechanism (487). The presence on the liposome surface of protein or carbohydrate ligands capable of interacting specifically with certain cellular receptors (e.g. Fc receptor on macrophages) can markedly enhance the rate and extent of cellular uptake of liposomes (207). The multiple nonspecific mechanisms of liposome cell interaction may pose considerable problems in those cases where more specific interactions are desired.

4. Behavior of Liposomes in Vivo. There have been many studies on the *in vivo* fate of liposomes and liposome encapsulated drugs; for discussion of some of the older literature the reviews by Juliano and Layton (234) and by Patel and Ryman (381) are useful. From the outset it has been clear that the primary sites of liposome uptake are the liver and spleen, both in terms of total uptake and uptake per gram of tissue. As discussed above, the liver and spleen are particularly well suited for the clearance of circulating particles since: 1) they contain an abundance of phagocytic reticuloendothelial cells (i.e. macrophages), and 2) their capillary endothelium is fenestrated thereby permitting the egress of comparatively large particles.

The behavior of liposomes *in vivo* can be modulated to a considerable degree by altering physical characteristics such as size, surface charge, and stability [see Gregoriadis *et al.* (185) for review]. Early studies of the clearance kinetics of lipid vesicles demonstrated that: 1) large vesicles were cleared more rapidly than small ones, and 2) with vesicle populations similar in size, negatively charged liposomes were cleared far more rapidly than neutral or positive ones (234). Since vesicles can interact with and sometimes become destabilized by plasma lipoproteins, manipulations that reduce these interactions and increase vesicle stability will also prolong the lifetime of vesicles in the circulation (8, 381, 152, 184, 233, 321, 343, 452). References 26, 46, 87, 189, 259, 336, 341, 345, 393, 427, 478, 494, 588 deal with the nature of liposome-protein interaction with specific reference to plasma proteins, including serum lipoproteins, clotting factors, and complement.

A number of recent studies have explored the role of the reticuloendothelial (RE) cells in the clearance of vesicles from the circulation. Based on experience with other types of colloids (436) it was known that the RES could be blockaded. Two forms of blockade are possible; one results from a supersaturation of the endocytotic capabilities of the tissue macrophages, the other is the result of depletion of circulating protein factors (opsonins) which aid the macrophage in particle uptake. Several laboratories have now demonstrated that strategies known to cause RE blockade can reduce the clearance rate of circulating liposomes (477, 242, 422, 48, 149, 121). This seems to result from a diminution of the uptake capacities of macrophages (especially the Kupffer cells of the liver) rather than as a result of the depletion of opsonic factors (242, 422). Although marked alterations of liposome clearance rates can be obtained by RE blockade techniques such as the injection of carbon particles or latex beads, this is not accompanied by very marked alteration of the ultimate tissue distribution of the vesicles; thus the liver and spleen remain the primary sites of uptake (477, 242, 389). The RE system must have an extraordinary capacity to recover from insult and go on to perform its role in particle clearance. While it seems likely that endocytosis by RE cells is a major factor in the clearance of both large and small liposomes (242) a certain fraction of the vesicle population may bind to capillary endothelium and be diffusely distributed in a variety of tissues (477).

Although much of the early work (133, 342, 381) suggested that liposomes could interact with the parenchymal cells in various tissues, there was really scant documentation of this contention. As discussed above, it is now generally believed that liposomes cannot readily pass out of the circulation in most tissues because of the tight structure of the capillary endothelium. Recently Scherphof and his colleagues have made a convincing series of studies (443, 444) where they show that liposomes with a diameter of less than 1000 Å can pass

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through the fenestrations of the liver sinusoids and interact with the hepatic parenchymal cells. Larger vesicles are retained within the sinusoids and interact primarily with Kupffer cells. In addition to this direct process, lipids from vesicles taken up by Kupffer cells can redistribute to parenchymal cells by diffusion; presumably lipophilic drugs can behave in the same way.

Thus liposomes seem ideally suited as a means to deliver drugs to the macrophages of the RE system. Smaller liposomes may also direct drugs to the parenchymal cells in tissues (liver, spleen) where the capillary endothelium is fenestrated. However, the egress of liposomes and liposomal drugs into the parenchyma of other tissues is an unlikely proposition.

Although many investigators have used liposomes with little evidence of acute toxicity, consideration must be given to the question of the chronic toxicity of these preparations (58, 193, 301, 389, 391). While it is quite possible to "poison" animals with liposomes by inappropriate choice of lipids (4), it is also quite clear that high doses of neutral or negatively charged liposomes can be tolerated on an acute basis. Chronic studies of liposome toxicity, liposomal drug toxicity, or the immunogenicity of liposome-drug complexes have not been published to our knowledge. It has been known for some time that liposomes can serve as excellent adjuvants for protein antigens (503, 536, 537) and that immune responses may also be mounted against many of the lipid components that have been used in liposomes (14, 528, 534). We are not aware of any reports of antibodies elicited against drugs administered in liposomes, but this possibility must be considered.

5. Targeting of Liposomes. An attractive but elusive approach in the liposomal drug delivery area has been the notion of coupling antibodies to vesicles so as to "target" drugs to specific cell populations. This topic has been reviewed recently by Gregoriadis *et al.* (184), by Weinstein *et al.* (548), and by Finkelstein and Weissmann (136). Some of the early work on this subject has been reviewed by Juliano and Layton (234). In considering the problem of targeting two important concepts must be kept in mind. First, the specific association of antibody-linked vesicles with cells is not a guarantee of effective intracellular delivery of drug molecules from within the vesicle. Second, the attainment of specific targeting is much more easily accomplished *in vitro* than *in vivo*, where a variety of processes may frustrate the specific liposome-cell association. Early work [review by Juliano and Layton (234)] indicated some prospects for promoting specific liposome-cell association by simply coating the liposome with antibodies or with lectins. An important development was the use of carboxyfluorescein (CF) by Weinstein and his colleagues (see Ref. 548 for review) to study both specific binding of vesicles and transfer of vesicle contents. This was followed by use of encapsulated methotrexate, a water soluble drug which must reach the cytoplasm in order to exert its effect. In an interesting series of studies, this group demonstrated that a variety of approaches could cause a specific cross coupling of vesicles to cells. However, only in the case of Fc receptor positive, phagocytically competent cells, was there effective transfer of encapsulated marker to the cell cytoplasm (294–296, 548).

More recently, several groups have chemically coupled monoclonal antibodies or affinity purified Fab or F(ab')2 fragments to vesicles. With these reagents, a high degree of specific cell-vesicle association has been attained, at least *in vitro* (170, 190, 208, 305, 472). Perhaps the most elegant studies to date are those of Leserman and his colleagues (308, 309, 293). These workers have investigated both specific association of monoclonal coupled liposomes with T and B lymphocytes and the effectiveness of transfer of encapsulated methotrexate to the cells. An important finding is that binding of a multivalent antibody-liposome complex to the cell surface does not assure effective transfer of liposomal drug. Thus while anti H-2Kk liposomes bound well to both T and B cells, methotrexate transfer only occurred in T cells. By contrast, B cells were much more sensitive to methotrexate transfer with anti-Ia-liposomes. Thus, both the nature of the target antigen and the characteristics of the target cell population are likely to be important factors in the development of strategies with antibody targeted liposomes. This may be contingent on the relationship of particular surface receptors to cellular mechanisms for protein internalization, as discussed above (section II E).

Another form of targeting involves the incorporation of glycolipid determinants into the liposome membrane so as to promote specific association with lectin-like receptors on cell surfaces (348). Early work in this area was reviewed by Juliano and Layton (234). An approach that has attracted a certain amount of attention involves the incorporation of aminomannosyl derivatives of cholesterol into liposomes (331, 575–577). This tends to enhance uptake by macrophages, although the mechanism involved is unclear (macrophages have a receptor for mannosylated glycoproteins but it is uncertain whether this interacts with aminomannosyl compounds). This approach would seem to enhance the spontaneous uptake of liposomes by macrophages but not to offer additional possibilities for targeting.

In a totally different approach to "targeting," two groups have used vesicles coated with an antigen to specifically manipulate certain aspects of immune function (566, 489–491). Both of these groups have shown that liposomes coated with myelin basic protein suppress allergic encephalomyelitis in animal models; this effect was not obtained with free protein. Presumably the antigen-coated vesicles somehow divert cytotoxic T cells from attacking myelin. This model may eventually provide an exciting approach for the specific suppression of

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a variety of autoimmune or allergic processes by "targeting" vesicles to specific lymphocyte populations.

Despite a number of *in vitro* successes, the problem of antibody-mediated targeting of liposomes *in vivo* is just beginning to be addressed. There would seem to be many formidable barriers to overcome in this attempt; thus if one wished to target liposomes to solid tumors, the problem of passing the endothelial barrier comes into play. Targeting to tumor cells in the circulation may be more likely; however, even here one would have to overcome nonspecific clearance by and possible toxicity to the RE system. Clearly the selective targeting of liposomes *in vivo* will likely remain an elusive goal for some time to come.

6. Liposomal Antineoplastic Drugs. A few years ago liposomes were hailed as a major new approach to the chemotherapy of cancer. The idea underlying this enthusiasm (which in retrospect seems rather simplistic) was that one might be able to "target" the liposomes and their payloads of cytotoxic drug to tumor cells by manipulating the physical or immunological characteristics of the liposomes. While it is quite clear that one can indeed manipulate the *in vivo* behavior of lipid vesicles to a certain extent and that one can promote specific liposome-cell associations *in vitro*, it is, at present, far from clear as to whether these approaches will indeed permit specific delivery of liposomal drugs to tumors.

A major conceptual problem is that the putative targeting of liposomes, at least to solid tumors, would seem to violate current understanding of the barriers to controlled drug delivery which we discussed at length above. Thus in order for effective antitumor targeting to take place, liposomes injected into the circulation must cross the endothelial barrier, cross the basement membrane barrier, and be effectively taken up by neoplastic cells within the tumor; meanwhile these liposomes must also evade capture by the phagocytic cells of the RES since delivery of drug to RE cells might cause a substantial degree of toxicity and would be a diversion of drug from its intended target site. It seems rather unlikely that one could design a lipid vesicle, with or without coating with monoclonal or conventional antibody, which would simultaneously attain all of these objectives. The reader will recall that particles in the size range of lipid vesicles are constrained from exiting the circulation except at certain specialized sites such as the sinusoids of the liver. thereby effectively prohibiting direct contact between liposomes and cells in solid tumors. Even if the vesicles did come into contact with the tumor cells, they would deliver their payload of drug only if the tumor cells were actively phagocytic. Although this may be true in some cases (151, 433, 434, 521), it is by no means universally true. Finally, at this juncture, there is no known way of avoiding uptake of injected liposomes by the RE cells (389, 242). Hence, we must conclude that the "targeting" of liposomes, as we currently know them, to solid tumor cells is an unlikely eventuality. This analysis does not exclude the possibility of targeting liposomes within the blood compartment by antibodies or by other means. However, this approach is just beginning to be addressed and it is difficult to evaluate its prospects for success.

The above analysis does not imply that the deployment of cytotoxic drugs in liposomes for therapy of cancer is completely senseless; rather it indicates that the initially attractive concept of "targeting" is likely to be a difficult if not intractable problem. However, there are several other approaches with liposomal drug delivery systems which may, in the long run, turn out to be of considerable merit. For example, it has been suggested that liposome encapsulation may be useful in overcoming drug resistance; we will examine current data on this possibility below. Another consideration is that the deployment of cell cycle specific cytotoxic drugs in vesicles may serve as a sustained or controlled release system. The incorporation of cytotoxic drugs in vesicles may, in some cases, alleviate toxicity to the host without loss of antitumor effect. This may be due to alterations of drug kinetics, distribution, or metabolism. Finally, drug-containing liposomes may be deposited in direct proximity to tumor cells by using various strategies for localized drug delivery (271, 376). The extent to which these possibilities are validated by experiment will be discussed below.

Early results on the pharmacokinetic and therapeutic behavior of liposomal antitumor drugs have been reviewed at length in the volumes edited by Papahadjopoulos (376) and by Gregoriadis and Allison (183). A selected representation of more recent *in vitro* and *in vivo* studies of liposomal antitumor drugs is presented in Table 2. A very salient critique of many of the early studies is presented in the review by Kaye (251), who points out that, in many cases, investigators considered the therapeutic and toxic effects in isolation and did not adequately evaluate the impact of liposomal encapsulation on the ratio of therapeutic/toxic effects (i.e. therapeutic index). As seen in Table 2 no consistent pattern emerges from the more recent studies; in some cases major successes have been claimed, while in other cases disappointing results are reported. Clearly drug delivery in lipid vesicles will not be a panacea for cancer therapy; however, some reasonably promising applications are beginning to emerge.

A. UPTAKE OF LIPOSOMES BY TUMORS. Early investigations (186, 90, 287, 414) suggested that substantial uptake of liposomes by solid tumors might be possible. However, other studies in animals [review by Kaye (251); Kimelberg and Mayhew (261)] and in man (414) failed to indicate any preferential uptake of liposomes by tumor tissue. Possibilities for enhancing tumor-specific uptake by using liposomes coated with monoclonal antibodies have been discussed by Gregoriadis *et al.* (182, 184); however, there is presently little experimental validation of these possibilities. A degree of selective deposition of

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TABLE 2
Effects of liposomal antitumor drugs

Drug	Liposome type*	Route	Tumor type	Outcome	Reference
Adriamycin	Neutral, negative, MLVs, SUVs	i.v.	Mouse	Reduced cardiotoxicity with maintenance of antitumor effect	Gabizon et al. (153)
Adriamycin	Negatively charged MLVs	i.v.	Mouse L1210 leukemia	Reduced cardiotoxicity with maintenance of antitumor effect	Olson et al. (362)
Adriamycin	Negatively charged MLVs	i.v.	Mouse L1210, P388 leukemias	Reduced cardiotoxicity with enhanced antitumor activity	Forssen and Tokes (145)
Adriamycin	Positive SUVs	i.p. or i.v.	Mouse P388 leukemia	Reduced cardiotoxicity with maintenance of antitumor effect	Rahman et al. (405)
CIS-platinum	Neutral SUVs plus hyperthermia	i.v.	Mouse sarcoma 180	Enhanced tumor drug uptake and delayed tumor growth compared to free drug	Yatvin et al. (580)
Lipid soluble alkylating agent	Negative SUVs	i.v.	Mouse PCG Myeloma	Reduced toxicity and equivalent anti tumor effect compared to "free"	Babbage and Barenbaum (25)
Nitrosourea alkylating agent	Various	i.v. i.p.	Mouse P388 leukemia, lewis lung carcinoma	Effective against metastatic foci but not local tumor	Inaba et al. (223)
Nitrogen mustard, alkylating agent	Neutral	i.p.	Mouse Ehrlich ascites carcinoma	Liposomes potentiate action of the drug	Ritter et al. (418)
Neocarzinostatin	Various	i.p. i.v.	Mouse Ehrlich ascites carcinoma	Enhanced survival under some circumstances	Shinozawa et al. (461)
Actinomycin D	Negatively charged SUVs	in vitro	Drug-resistant cell line	Liposomal encapsulation overcomes drug resistance	Poste and Papahadjopoulos (394)
Actinomycin D	Negatively charged SUVs	i.p.	Mouse AKR ascites tumor	Prolonged survival compared to free drug	Gregoriadis and Neerunjun (186)
Actinomycin D	Positively charged SUVs	i.v.	Mouse ROS tumor	Reduced toxicity and reduced antitumor effect	Kaye et al. (253)
Cytosine arabinoside	Various types of vesicles	i.p. i.v.	Mouse L1210	Survival prolonged compared to free drug long-term survivors	Mayhew et al. (322)
Cytosine arabinoside	Various	i.p.	Mouse renal tumor	Antitumor effects less than free drug	Kedar et al. (254)
Cytosine arabinoside	Neutral MLVs	i.v.	Mouse L1210 leukemia	As effective as free drug given by sustained infusion	Mayhew et al. (322, 323)
Cytosine arabinoside triphosphate	Negatively charged MLVs	in vitro	Cytosine arabinoside sensitive and resistant mouse lymphoma cell lines	Liposomal AraCTP overcomes resistance to AraC	Richardson et al. (415, 416)
Cytosine arabinoside	SUVs LUVs	in vitro	Cytosine arabinoside resistant L1210 cells	Liposomal AraCTP fails to overcome resistance to AraC	Rustum et al. (431, 432)
Cytosine arabinoside and its nucleotide analogues	Negative and positive SUVs	in vitro	S4A mouse lymphoma, transport deficient variants	*In vitro* cytotoxicity of liposomal drug mediated by leak of free drug	Allen et al. (9)
Methotrexate	Positive SUVs	i.v. i.p.	Mouse L1210 leukemia	Liposomal drug no more effective than free drug	Kimelberg and Atchison (260)
Methotrexate	Positive SUVs	i.v.	Mouse ROS tumor	Enhanced antitumor activity and enhanced toxicity	Kaye et al. (252, 253)
Methotrexate	Neutral SUVs	i.v. plus local hyperthermia	Mouse L1210 tumor (solid form)	Greater control of tumor growth compared to free drug and hyperthermia	Weinstein et al. (547, 549, 550, 581)
Methotrexate-8-aspartate (nonpenetrating derivative)	Neutral SUVs conjugated with anti-H2 K^k antibodies	in vitro	L929 mouse fibroblasts	Targeted liposomes enhanced drug uptake 10-fold	Heath et al. (195)

* Abbreviations used are: MLV, multi lamellar vesicle; SUV, small unilamellar vesicle; LUV, large unilamellar vesicle.

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cytotoxic drug in tumors has been attained by a combination of heat-sensitive liposomes and local hyperthermia [review by Yatvin and Lelkes (579)]. This approach is limited to instances where the tumor is readily accessible to the manipulations involved in generating local hyperthermia, and is not really applicable to the problem of disseminated disease. There is little clear cut evidence at this point to suggest that liposomal antitumor drugs are capable of being selectively deposited in solid tumors.

B. OVERCOMING DRUG RESISTANCE. In an exciting early report, Poste and Papahadjopoulos (394) noted that the transport-mediated resistance of an actinomycin-D-resistant tumor cell variant could be overcome by presenting the drug in lipid vesicles. Although this finding seems quite reasonable for a case in which a high level of membrane impermeability to free drug is coupled with at least a modest degree of phagocytic capacity by the tumor cell, its general applicability to the problem of tumor drug resistance is questionable. Thus, a series of more recent investigations present a rather mixed perspective on this approach. Allen *et al.* (9) were unable to overcome drug resistance in AraC transport deficient variants, of mouse S49 lymphoma cells by using liposomal AraC. Likewise, Rustum *et al.* (432) failed to overcome AraC resistance in an L1210 variant with liposome encapsulated AraCTP (the active anabolite of AraC). By contrast, Richardson *et al.* (415), working with a mouse lymphoma resistant to AraC, were able to overcome this resistance with liposomal AraCTP *in vitro*. However, in further studies of the same tumor *in vivo*, liposomal AraCTP had little effect (416). While the concept of using liposomes to overcome tumor drug resistance is a reasonable one, its applicability seems to depend on the idiosyncracies of particular drugs and particular tumor lines. Recently this general approach has been extended by Heath et al. (195) who used liposomes conjugated with cell-type specific monoclonal antibody to promote uptake *in vitro* of a methotrexate derivative which is normally not taken up by cells. This may be the harbinger of a trend to use drugs that are specifically designed for use with a liposomal carrier, rather than trying to use liposome technology to ameliorate problems of conventional drug therapy.

C. SUSTAINED RELEASE. An interesting early development in the use of liposomes a a carrier for cytotoxic drugs was the finding that a single dose of liposomal AraC is quite effective in prolonging the lives of mice having L1210 leukemia (247, 322), whereas single-dose treatment with free drug is totally ineffective [review by Kimelberg and Mayhew (261)]. Since AraC is a cell cycle specific agent, it requires prolonged exposure of growing tumor cells to the drug in order to obtain optimal therapeutic effects. Thus at least part of the therapeutic enhancement caused by liposomal encapsulation is probably due to the "depot" or sustained release action of the liposomes. Indeed, Mayhew *et al.* (322) have demonstrated that the drug in liposomes is at least as effective as a sustained infusion of the free drug. Under some circumstances, the liposomal drug produced more long-term survivors than AraC infusion. However, the basic conclusion of these studies is that liposomal encapsulation of AraC does not uniformly lead to an increase in the therapeutic index of AraC as compared to optimal treatment schedules with the free drug.

Other studies indicating superior therapeutic efficacy of liposomal methotrexate may also result from the sustained release effects of the liposomes (251). Although administration of a cell cycle specific drug in lipid vesicles might be simpler and more convenient than a sustained infusion, there is really no convincing evidence that the liposomal approach provides a superior therapeutic index. Other more precise technologies for sustained drug release are available (section III C, above), and it is unlikely that liposomes will compare favorably with these systems in this regard.

D. REDUCTION OF TOXICITY IN THE HOST. Perhaps the most interesting application of liposomes in tumor therapy may not stem from sophisticated "targeting" but rather from the less glamorous task of diminishing the dose-limiting toxicity of anticancer agents. This seems to be particularly true for a number of lipophilic drugs which are sequestered in liposomes and distributed to the tissues in such a manner as to reduce toxicity to the host. Several groups (405, 145, 153, 362) have now accumulated quite compelling evidence showing that liposomal encapsulation of Adriamycin can reduce its toxicity to the myocardium with full maintenance of antitumor potency. The mechanism underlying these findings is obscure; low uptake of encapsulated drug by the myocardium may be a part of the story (362, 153), while alteration of the relative ability of the drug to interact with membranes as opposed to DNA might also be contributory. Another example of this type concerns the reduced toxicity and enhanced therapeutic index obtained when certain lipophilic alkylating agents are used in conjunction with liposomes (25, 241, 284, 417, 418).

In summary, the use of liposomal delivery systems in experimental chemotherapy has not lived up to overly optimistic early assessments of the potential of this technology. There is, at present, scant evidence that liposomes are preferentially taken up by tumors or indeed, that they can gain access to the parenchyma of solid tumors. Use of liposomes as a sustained-release system is reasonable, but many alternative technologies are available. The most promising aspect of the liposomal approach seems to be the reduction of the toxicity of certain antitumor agents when these drugs are used in a liposomal formulation.

7. *Liposomal Anti-Infectious Drugs*. An area that has received little attention until lately concerns the possible use of liposomal drugs to combat infectious disease. At first glance, the side effects of most anti-infectious agents

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are mild compared to antineoplastic drugs, so the need for utilizing a drug delivery system seems less pressing. However, a number of the agents used for therapy of fungal or parasitic infections are, in fact, quite toxic and, in some cases, these infections are rather refractory to conventional drug therapy. Exploration of the use of drug delivery systems in these situations seems a sound prospect. Another potentially important avenue which we will mention is topical application of liposomal anti-infective drugs (475).

The first highly successful use of a liposomal carrier system in infectious disease occurred in connection with leishmaniasis. This protozoan parasite, which is endemic in many tropical and subtropical areas, has a rather unusual life cycle which makes it particularly susceptible to liposomal drug therapy. The organism enters and colonizes macrophages, managing to live within endocytotic vacuoles without being destroyed. Since liposomes are avidly internalized by macrophages, they clearly afford a convenient means of "targeting" drug directly to the site of parasite infestation. The antimonial drugs used in treating leishmaniasis are quite toxic; thus attempts to "target" drug action would seem very desirable. Three groups have pursued this approach and have examined the effects of liposomal drugs on the visceral form of leishmaniasis (37, 352, 17, 18). These early studies indicated that the incorporation of antimonial drugs into liposomes could enhance their potency several hundredfold. Although the toxicity of the liposomal drug was also greater than the free drug, the net effect was a 30 to 40-fold improvement in therapeutic index. More recently this approach has been extended to the cutaneous form of leishmaniasis as well (350). In addition to antimonials, a number of other antileishmanial agents show an enhanced therapeutic index when incorporated into liposomes; this includes amphotericin B (351) and aminoquinolines (14, 18, 19). Recently the expectation that liposomes actually accumulate in parisitophorous vacuoles has been confirmed by electron microscopic observations (14).

Another very promising example of therapy of an intracellular parasite with liposomal drugs involves the use of liposomal aminoglycosides to treat brucellosis. This disease is widespread among cattle in areas having a hot climate, and remains a problem in human disease as well. As in the case of leishmaniasis, the Gram-negative bacteria of the Brucella family tend to colonize the endocytotic vacuoles of macrophages. Because of its intracellular location, the organism responds rather poorly to common antibiotics. When aminoglycoside antibiotics, such as streptomycin, are incorporated into liposomes, they are readily delivered into the endosomes of macrophages, thus "targeting" the drug to the site of infection. This approach provides a remarkable enhancement in the therapeutic index of aminoglycosides and seemingly, results in the cure of experimental Brucella infections in rodents and in larger animals (M. Fountain, personal communication, 1983).

A third potentially important application of the liposomal delivery approach in infectious disease involves the therapy of systemic fungal infections with polyene antibiotics incorporated into vesicles. Systemic mycoses represent a challenging therapeutic problem; they occur most often in immunocompromised individuals such as cancer patients or renal transplant patients, where they are sometimes life-threatening and always difficult to treat (585). The drug of choice for most systemic mycoses is amphotericin B (AMB), a potent but extremely toxic agent. AMB, a polyene antibiotic, interacts with ergosterol in fungal cell membranes thus creating transmembrane channels which permit the escape of vital ions and metabolites. The drug also interacts with cholesterol found in mammalian cell membranes causing similar permeability changes. This is probably the basis of amphotericin's profound toxicity to the kidney, CNS, and hematopoietic system.

In the course of exploring the possible uses of liposomal drugs against leishmaniasis, New *et al.* (351) found that the toxicity of AMB could be considerably reduced by incorporating it into ergosterol-containing liposomes. Recently two other groups have explored the use of liposomal AMB against systemic fungal infections. Working with ergosterol-containing liposomes, Graybill *et al.* (177) demonstrated that encapsulated AMB was less toxic to mice but retained good efficacy against murine cryptococcus infections. Lopez-Berestein *et al.* (302) showed that the incorporation of AMB in simple phospholipid vesicles markedly reduced its toxicity with retention of excellent therapeutic and prophylactic effects against *Candida albicans* infections in mice. A limited series of studies in patients with *Candida* and *Aspergillus* infection indicate that the promising results obtained in animals will also be true in man.

Although macrophages do take up fungal cells during the course of systemic infections, the organisms are by no means exclusively located in these cells (118). Therefore, the enhanced therapeutic index of liposomal AMB cannot be ascribed solely to spontaneous "targeting" of the drug to macrophages as seems to be the case for the liposomal treatments of leishmaniasis and brucellosis. This was made more apparent by recent studies of Mehta *et al.* (327), showing that the incorporation of AMB in liposomes reduces its *in vitro* toxicity to mammalian cells but not to fungal cells. Therefore putting AMB into liposomes seems to result in a fundamental alteration of the ability of the drug to interact with mammalian cell membranes. This may in fact be the primary basis for the enhanced therapeutic index of liposomal AMB. At this point the degree to which this approach can be extended to infections caused by other classes of fungal organisms is unclear as is the question of whether other

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polyene antibiotics will display an enhanced therapeutic index when incorporated into liposomes.

The utilization of liposomal carrier systems for the treatment of intracellular parasitic disease and for treatment of systemic fungal infections seems to represent a major advance over previous therapeutic modalities. Deployment of appropriate drugs in liposomes results in a 30 to 40-fold enhancement of the therapeutic index in the case of leishmaniasis and brucellosis, and 10 to 20-fold for fungal infections. Moreover, the use of a liposomal delivery system in these cases is founded on a rationale which is consistent with the known behavior of liposomes *in vivo* and consistent with the pathogenesis of the disease process in each case.

8. Immunomodulation with Liposomal Drugs. Since the intravenous injection of lipid vesicles is followed by a spontaneous "targeting" of these particles to the macrophages of the RES, the use of lipid vesicles to selectively convey drugs to macrophages seems a very sensible approach. Macrophages are multicompetent cells which play important roles in several aspects of the host defense system (5). In particular, macrophages seem to be one of the primary barriers against the growth and metastatic spread of neoplastic cells. Macrophages can be "activated" so as to become cytotoxic to tumor cells. This occurs as a result of exposure to a variety of immunomodulating substances including MAF (a T-cell-derived lymphokine), lipopolysaccharide (from Gram-negative bacteria) and certain synthetic compounds, muramyl dipeptide being the best known example.

In a provocative series of studies, Fidler, Poste, and their colleagues have explored the use of liposomes to selectively deliver immunomodulators to macrophages for the purpose of enhancing host defenses against metastases (130, 132). These studies began with the observation that MAF incorported into liposomes was far more active than free MAF in activating macrophages in vitro (392). This was quickly followed by a demonstration of the antimetastatic effects of encapsulated MAF by using the B16 murine melanoma as a model (131, 134). This group also explored the characteristics of the liposomes required for optimal effects and claimed that the use of relatively large multilamellar vesicles (MLVs) bearing a negative charge resulted in maximal liposome deposition in the lung (133, 446); this would be a desirable situation since the lung is a major site of metastatic disease. Some evidence suggests that blood monocytes may engulf circulating liposomes arrested in lung capillaries and then escape the vasculature into the surrounding tissue spaces. These monocytes may then be the cells involved in the suppression of lung metastases (390).

Although some very interesting effects were observed with liposomal MAF, the crude and heterogeneous nature of this material militates against the practical development of this approach. Many of the effects of MAF can be mimicked by the muramyl dipeptide (MDP) class of compounds (75). These agents, which are synthetic analogs of structures found in bacterial cell walls, have potent adjuvant and immunoregulating activities. Sone *et al.* (476) showed that muramyl dipeptide incorporated into liposomes was at least as effective for activating rodent macrophages *in vitro* as was MAF. This was followed by a demonstration of the efficacy of liposomal MDP in therapy of metastases in the B16 system (134). Although the antimetastatic effects of liposomal MDP in these studies was clearcut, the role of macrophages in the process remained in question. However, in a later study, the same group has marshalled a good deal of evidence supporting a central role for macrophages as the effector cell involved in the antimetastatic effects of liposomal MDP (132). In more recent studies, other groups have demonstrated that liposomal MDP can activate human monocytes (303), that the site of action of liposomal MDP is within the interior of the macrophage (325), and that liposomal C-reactive protein can have antimetastatic effects (102).

These studies have opened the way to a rather novel approach to the therapy of metastatic disease. The approach, however, is not without its limitations. First, the inherent antitumor capabilities of the macrophage system are limited. There are simply not enough macrophages in the available pool to cope with large tumor burdens; this necessitates the use of chemotherapy or radiation to reduce tumor load prior to immunotherapy. Unfortunately, these approaches may reduce the capabilities of the macrophage system as well. Second, there is an acute lack of fundamental information on both the overall process of macrophage activation and on the mechanism of action of substances such as MDP; this works against a rational planning of therapeutic approaches. Third, the actions of currently known immunomodulators such as MDP are rather nonspecific. Thus, activated macrophages play a role in inflammatory processes as well as in host defense. Protracted treatment with liposomal immunomodulators may lead to generalized inflammatory responses such as polyarthritis and polyvasculitis. Finally, protracted use of liposomes containing polypeptide substances such as MAF or even MDP may lead to the generation of immune responses (24). Some of the problems and prospects for use of liposomal immunomodulators in tumor therapy have been reviewed by Fidler and Poste (132).

9. Summary. Clearly liposome technology is not a panacea for all problems in controlled drug delivery. However, a judicious matching of the characteristics of the liposome technology with particular drugs and particular therapeutic problems can lead to interesting and useful results. This is exemplified by the use of liposomal drugs for treatment of certain infectious diseases, and the potential use of liposome-entrapped immunomodulators. Questions remain about the ability to formulate stable, reproducible preparations of liposomal drugs on

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a pharmaceutical scale. Although liposomes have been used by many investigators with little evidence of acute toxicity, the questions of chronic toxicity and chronic immunogenicity of liposomal preparations have yet to be properly addressed.

E. Antibodies as Drug/Enzyme Carriers

1. Overview. Although Ehrlich anticipated the use of antibodies as effective targeting agents to introduce drugs to specific tissues, it was more than 50 years before such an attempt was actually made. In 1958, Mathe and co-workers (320) were successful in treating a mouse leukemia (L1210) with diazo-linked conjugates of the antitumor drug methotrexate and hamster antibodies prepared against the L1210 cells. The methotrexate-antitumor antibody conjugates were more effective in reducing tumor size and prolonging survival than any combinations of free drug, free drug injected following injection of antitumor antibodies, or drug conjugated to a nonspecific antibody (hamster). Unfortunately it is not possible to determine from this early report whether the antibody was actually delivering the drug to the tumor site or whether it was simply altering the pharmacokinetics of the drug delivery. No mention is made as to the routes of administration of the drug and tumor cell population. A problem which occurs repeatedly in the literature on drug delivery systems is the case in which the drug is given at the same time or following (by a day or so) delivery of the tumor cells into the same compartment, usually the peritoneal cavity. In such cases it is difficult to see whether specific targeting has, in fact, been achieved or whether that compartment, say the peritoneal cavity, is being used as an elaborate test tube. Therefore, the sites of delivery of drug carriers and tumor cells in working with models of cancer or certain infectious agents, must be considered in the evaluation of drug delivery systems. This relates to the question of the ability of the drug carriers to cross the various barriers to seek out the tumor cells. A second question that must be addressed is that of drug-antibody (carrier) synergism. Is the increased efficacy of the treatment a result of the specific delivery of the drug to the site of action by the antibody or is the tumor-specific antibody somehow increasing the toxicity of the drug molecule by altering the properties of the tumor cell (making it more susceptible to the drug), by altering the pharmacokinetics of the attached drug, or a combination of the above. In general, a number of questions have to be addressed in considering the development of drug-antibody conjugates: 1) Does the linkage of the two retain the drug and antibody activities? 2) What is the nature of the linkage and is it stable? 3) What is the *in vitro* activity of the conjugate? 4) Is the conjugate actually working by a delivery route or is there a degree of drug-antibody synergism that is independent of the delivery of the drug to the tumor cell by the tumor-specific antibody? 5) What is the *in vivo* activity of the conjugate compared to equivalent doses of: a) free drug, b) drug conjugated to nonspecific antibody, c) free drug injected before or after equivalent amounts of antitumor antibody, or d) equivalent amounts of tumor-specific antibody without conjugated drug. 6) Are the routes of administration of the drug conjugates and the tumor cells appropriate to the disease model being studied (551, 552)? Or is the therapeutic model being set up as an elaborate *in vitro* model, e.g. use of the peritoneal cavity? Most of these questions pertain equally well to other drug delivery systems where specific targeting mechanisms are invoked.

2. Early Development. The possibility of antibody drug synergism was investigated in the 1970s and has been used to explain some of the data obtained. Some earlier studies demonstrated that conjugating the alkylating agent chlorambucil in a noncovalent fashion with antitumor antibody produced a complex that prevented the growth of transplanted tumors (160, 163, 225) in conditions under which equivalent amounts of free drug or free antibody were ineffective. These experiments were verified by Flechner (141), and by Davies and O'Neil (91, 92), but these latter authors also showed that protection against tumor growth could be achieved if the drug and antibody were injected separately. These experiments were further supported by the data of Rubens and Dulbecco (428) who showed that the addition of free chlorambucil and free antitumor antibody were as effective in preventing growth of BHK cells in tissue culture as noncovalent complexes of the drug and antibody. It was, therefore, possible to postulate that in the in vivo experiments, the noncovalent complexes (and even the covalent complexes following proteolytic cleavage) of drug and antibody dissociated in vivo and acted synergistically rather than as a specific drug antibody conjugate. This synergism is not limited to chlorambucil. Davies (91) demonstrated that the antitumor agent, cytosine arabinoside, when given up to 96 hours prior to the administration of tumor specific antibodies, resulted in a 100% protection of mice against lymphoma cells at concentrations in which either free drug or free antibody resulted in only minimal increases in survival. This synergism has not been demonstrated with other anticancer agents such as methotrexate and daunorubicin (428), although Ghose and Cerini (162) demonstrated that X-irradiation was potentiated by the administration of antitumor antibodies in several experimental systems. The mechanism of this synergism is still not well understood. One possible explanation put forward by the experiments of Segerling *et al.* (450) suggests that cells exposed to inhibitors of RNA or protein synthesis were subsequently more susceptible to lysis by either cell-specific antibody or by complement. Another hypothesis suggests that the enhanced susceptibility of cells to cytotoxic agents is a result of an increased proportion of the cells passing through the cell cycle as a result of the antibody treatment. This hypothesis has been supported by the dem-

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onstration (see Refs. 398, 300) that antitumor antibodies enhance the incorporation of tritiated thymidine into DNA in certain cell lines in culture.

The drug antibody synergism approach was used a number of years ago in preliminary clinical trials. Newman *et al.* (353) reported on a trial for bronchial carcinoma where he compared straight combination chemotherapy with a regimen of combined chemotherapy and immunotherapy. The immunochemotherapy group received chemotherapy just prior to the administration of antibodies against a resected portion of the primary tumor. During the time course of the trial, the chemotherapy group showed a 60% recurrence rate along with a 41% death rate whereas the immunochemotherapy group had a 25% recurrence rate along with a 16% death rate. The data were not deemed to be very significant although it was interesting to note that no reactions against the goat antisera to the primary tumor were observed. It is not clear whether the time course of the administration of the serum was too short to elicit an immune response or whether the immunosuppressive effects of the cytotoxic drugs increased the patients' tolerance to the foreign immunoglobulin. Ghose's group also reported on a limited study on the efficacy of immunochemotherapy (164). In a paper, controversial at the time, Ghose and coworkers demonstrated that a heterologous antimelanoma antiserum produced in goats or rabbits against one patient's tumor could cross-react with the cytoplasmic and surface antigens of the melanoma cells from other patients. This was an extremely important finding since the degree of cross-reactivity of specific tumor cells between patients would dictate the generality and ease of the use of antibodies not only in treatment (as in immunochemotherapy) but also in the use of antibodies for diagnostic imaging (161). Ghose *et al.* (164) reported on the use of chlorambucil noncovalently linked to an antitumor antibody used in the treatment of a melanoma patient. Following injection of the complex, there appeared a significant decrease in the number of observable skin metastases. Unfortunately, immunochemotherapy had to be stopped since the patient developed an anaphylactic reaction to the foreign immunoglobulins. Although chemotherapy was continued, many of the lesions which had almost completely disappeared, reappeared and continued to progress. It was interesting to note that one of the nodules, which had not regressed and another which appeared during the combined immunochemotherapy, contained many cells that did not cross-react with the cell surface antigen on the tumor cells against which the initial antibody had been directed. Although this represents data from only a single patient, the question of cross-reactivity and the possible diversity of antigen characteristics of tumor cells is an important one. This must be closely considered especially with the advent of monoclonal antibodies directed against a single antigenic determinant.

3. Antibody-Drug Conjugates. By the late 1970's, although it was clear that some benefits might be derived by the use of immunochemotherapy (whether the drug-antibody conjugates were covalently linked or not), there was a great deal more concern about the nature of the tumor inhibition and the possibility of devising more effective systems. This was stimulated by the demonstration of the existence of tumor specific antibodies (171, 256, 363, 383). More sophisticated methods of conjugation and more concern for the testing of the conjugates were pursued. Perhaps the most active group was from the Department of Chemical Immunology at the Weizmann Institute of Science in Israel [see Arnon and Sela (22) and Hurwitz (215) for reviews]. They used glutaraldehyde, carbodi-imides, and periodate oxidation to attach daunomycin to antitumor antibodies by using either the free amino group or the sugar residue on the drug molecule to bind to either free amino groups or free carboxyl groups on the immunoglobulin molecules. They also utilized dextran molecules as bridges between the drug molecules and the antibodies. The rationale behind this approach was that: a) it allowed for the conjugation of more drug molecules per immunoglobulin and b) it offered the possibility that the spacer would produce less steric hindrance between the antibody molecule and the drug molecules thereby enhancing the activity of both. In the use of dextrans as combined carriers and spacers, it was more efficient to oxidize the dextran (by periodate oxidation) and then link on the daunomycin molecules by using the free amino group. Attempts to conjugate adriamycin to antibodies via dextran bridges did not work, since the drug molecules were inactivated. The alternative was to leave the NH_2-sugar on the drug molecule free and to bind the drug to a hydrazine derivative of dextran via its keto group. This allows the antibody to be attached to the hydrazone dextran derivative utilizing glutaraldehyde as a bifunctional cross-linking agent. The authors tested the pharmacological properties of the conjugates by measuring inhibition of cellular RNA synthesis in vitro. The conjugated drugs retained their inhibitory properties although they appeared to act more slowly. It is not clear whether this was a result of the drug molecules having to become detached from the antibody molecule or whether it was a question of transporting the complex into the leukemia cells. The retention of the antibody-binding properties to the tumor cells depended on the number of drug molecules substituted per antibody molecule. At 2 moles of drug per mole of antibody, 64% of the antibody activity was retained whereas at 6 moles per mole the activity was reduced to 24%. The use of dextran bridges allowed the authors to attach up to 30 moles of drug per mole of antibody while retaining up to 70% of the antibody activity, greatly enhancing the load factor. The same authors demonstrate that it is possible to cleave the Fc fraction of the antibody molecule and conjugate the drug

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molecules to the (Fab′)² fragment while retaining the antigen-binding properties of the intact immunoglobulin. This procedure has two important advantages: 1) The Fc portion is nonspecific and binding to nontumor cells that possess Fc receptors should be avoided; and 2) the Fc portion is the more immunogenic portion of the antibody molecule so that in principle if the use of nonhuman antibodies is contemplated for therapy, then the use of a less immunogenic preparation (i.e. the (Fab′)² will have definite advantages. The authors (see Ref. 215) in fact were able to demonstrate that the (Fab′)² fragment alone was less toxic in mice than the intact IgG when the mice had been inoculated with tumor cells against which the antibodies were directed. This has an important experimental advantage since the lower background toxicity of the antibody fragment makes it easier to determine the advantages of conjugating the drug molecules.

In 1978 Lewis and co-workers (298) observed that antibodies directed against major histocompatibility antigens on lymphocytes were able to enter the cell and upon entry to bind to the nucleus. Hurwitz and colleagues (218) were able to demonstrate that both free daunomycin and its antibody conjugates (antibodies against a cell surface antigen on YAC cells, a maloney-virus-induced lymphoma) were taken up by the cells and could be found associated with a nuclear fraction. In describing the effects of drug-antibody conjugates, this step is crucial in understanding the mode of action. The experiments of Hurwitz *et al.* (219) did not determine whether the drug-antibody complex was approaching the nucleus directly or whether it was being processed through lysosomes, following uptake, thereby freeing the drug molecules to inhibit RNA synthesis. Their experiments with labelled dextrans in the cross-bridge between the antibody molecules suggested that the conjugate (i.e. dextran included) actually reached the nucleus. The mechanism by which this might come about has not been explained.

The suggestion that it is easy to cure cancer in a test tube is never more obvious than in examples using drug delivery systems. The acid test of course is to determine the ability of the drug-antibody conjugate to "home" to the cancerous tissue in vivo. Hurwitz *et al.* (219) demonstrated that upon intravenous injection, ^{125}I-labelled immunoglobulin fractions of syngenic anti-3LL (Lewis lung carcinoma) serum, "homed" preferentially to metastatic lung tissue in metastases-bearing mice when compared to an immunoglobulin fraction of normal mouse serum. The degree of localization was enhanced when a monoclonal antibody preparation against the 3LL cells was used. In this case, however, the monoclonal antibody also localized in the lungs of normal mice, suggesting that a common antigenic determinant was to be found in the normal lung tissue and in the 3LL carcinoma cells. Arnon and Sela (22) tested the efficacy of daunomycin-antitumor antibody conjugates in the YAC lymphoma system. The cells were injected intraperitoneally (IP) and the drug conjugated to the antibodies via the dextran link was injected intravenously 2 to 5 days following implantation of the tumor cells. At lower drug doses only the drug conjugated to anti-YAC antibodies via the dextran link was effective. At higher doses free drug as well as drug conjugated just to dextrans or conjugated to normal antibodies were also effective. The effect of the drug-antibody conjugate was striking, producing a number of apparent complete cures. It was interesting to note that the use of monoclonal antibodies against the YAC cells did not increase the efficacy of the treatment. The reason for this is not clear. It may be that the monoclonal antibodies are less efficient as carriers because of their ability to recognize ony a single antigenic determinant. A polyclonal preparation of antibody on the other hand which may recognize more than one antigen on the tumor tissue might therefore be more efficient at binding and internalization. The second possibility is the fact that the monoclonal antibody is an IgM preparation which may prove to be a less effective drug carrier than the IgG class of immunoglobulins. The same authors have also investigated the use of daunomycin-antibody conjugates against a rat hepatoma cell line which produces alpha-fetoprotein (AFP). They used an antibody preparation against the AFP. In earlier studies Tsukada *et al.* (525, 526) showed that when both the rat hepatoma cells and the conjugates were injected IP, the daunomycin-antitumor antibody conjugate was the most efficient in increasing survival times. This experiment suffered from the criticism of the simultaneous IP injections suggesting an elaborate *in vivo* culture dish. However, these same authors have also now demonstrated that the intravenous injection of anti-AFP antibodies conjugated to daunomycin were considerably more effective in inhibiting tumor development than equivalent amounts of free antibody or free drug and that the intravenous (IV) route of administration was much more effective than the IP route. In the case of the antibodies against the AFP, there did not appear to be any difference between the monoclonal antibodies produced by the hybridoma technique compared to polyclonal anti-AFP produced in horses. This was especially so in the case of the IV route of administration whereas in the IP situation the horse anti-AFP antisera seemed to be the more effective. The increased efficacy of the IV treatment is hard to evaluate since it appears that the IV dose was 5 times higher with respect to both daunomycin and antibody. Nevertheless, the fact that the IV injections produced so many survivors among rats receiving the AH66 hepatoma cells is very encouraging. This suggests that the anti-AFP antibody is indeed targeting the drug to the tumor site in a manner not found for the drug alone, the antibody alone, or the drug and antibody given without benefit of conjugation. These results also suggest than an IV injected antibody preparation can have good access to tumor cells within the parenchyma of solid tumors. IgG molecules

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(~160,000 M_r) are known to exit from the circulation and enter interstitial fluid at a fairly rapid rate (see section II B).

4. Immunotoxins. In addition to the use of anticancer drugs such as daunomycin, methotrexate, adriamycin, chlorambucil, etc., other cytotoxic agents have been coupled to antibodies in order to direct them specifically to tumor cells. Some earlier attempts were reviewed by Poznansky and Cleland (398). These include diphteria toxin first conjugated to immunoglobulins in 1970 by Moolten and Cooperband (338, 339). They also incude certain enzymes such as glucose oxidase, phospholipase C, and, recently, antibody conjugated L-asparaginase (399). Other authors also demonstrated the possibility of using antibodies to direct isotopes to tumor cells for either diagnostic or therapeutic purposes. Studies with the use of antibodies to direct isotopes to tissue for the purpose of irradiation have not been successful, except in the case of treatment of the thyroid where the use of antibodies is not even necessary. These studies were performed before the advent of monoclonal antibodies and specificity was poor. These experiments probably deserve additional attention especially if the generalized cytotoxicity proves to be less than that of many of the antitumor agents currently in use.

The advent of hybridoma technology (268) now allows for the production of unlimited amounts of pure, homogeneous antibody preparations with well-defined specificities. Mouse monoclonal antibodies are widely available and readily prepared while techniques of producing human hybridomas and therefore monoclonal antibodies of human origin are currently being developed.

Levy and Miller (297) and Trowbridge and Domingo (523) have recently reviewed the area of tumor therapy utilizing monoclonal antibodies reporting on both animal model studies and recent clinical trials. Early trials were meant primarily to establish the toxicity and problems associated with the use of monoclonal antibodies (449). Toxicity to mouse monoclonal antibodies has not been shown to be a problem with doses as high as 1500 mg. Immunogenic responses have been noted in more than half of the patients receiving mouse immunoglobulin. Although no instance of a severe anaphylactic type reaction has been recorded, it should be noted that most or all of these patients have impaired immunological systems due to previous treatment with immunosuppressive agents. The use of mouse IgG is further limited by the demonstration that the antibody response to the mouse IgG in at least one patient (297) neutralized the therapeutic effect of the monoclonal antibodies. In this case the reduction of Leu-1 positive tumor cells achieved by successive doses of mouse monoclonal antibodies (anti-Leu-1) was diminished as levels of circulating antimouse Ig antibodies rose. The therapeutic effects of the treatments have been promising but far from spectacular, although in no case has adequate consideration been given to what doses of monoclonal antibodies might be required. Clearance of leukemia cells from the circulation following antibody infusion has been observed in most studies but the responses have been temporary.

An interesting aspect of monoclonal antibodies reviewed by Levy and Miller is their cases of autologous bone marrow transplantation. The rationale is as follows. Bone marrow toxicity represents the major limiting factor in the treatment of many leukemias and lymphomas. The success of bone marrow transplantation is greatly hindered by the high incidence of graft versus host disease even when donor and host are deemed to be "well matched." The alternative is therefore to take the patient's own bone marrow prior to therapy, cryopreserve it and then use it to repopulate the patient's own hematopoietic function following supralethal whole body treatment with either chemotherapy, radiation therapy, or both. Monoclonal antibodies against the tumor cells may be used to eliminate, *in vitro*, the contaminating tumor cells from the bone marrow. This treatment has many advantages over in vivo therapy since problems such as toxicity, antigenic modulation, and absolute antibody specificity are eliminated as long as the hematopoietic stem cells are spared. In addition, multiple treatments can be achieved to effect total tumor cell kill and heterologous complement can be added to lyse antibody-coated cells. Preliminary clinical trials are very promising. Ritz *et al.* (419, 420) and Kaizer *et al.* (240) both demonstrated that bone marrow treated with antibody and complement *in vitro* and cryopreserved is able to effectively reconstitute hematopoietic function. This has been achieved in a number of patients with either common acute lymphocytic leukemia or lymphoblastic lymphomas. These reports and others utilizing monoclonal antibodies (107, 269) are too preliminary to determine whether the treatment was effective in eliminating all tumor cells which were presumed to be present in the bone marrow.

While their use in the therapy of malignant disease seems obvious, it is equally clear that we do not yet know how best to make use of their properties. 1) Even if monoclonal antibodies are able to identify and bind to all tumor cells, it is not clear that there will be a sufficient number of effector cells to eliminate the antibody-coated cells (462). This may be especially true in immunosuppressed patients and it may be necessary to do quite the opposite from immunosuppression and actually augment the effector cell response. 2) The immunogenicity question of mouse IgG must be addressed and this may be solved either by inducing tolerance to the antigen or else by the use of human hybridomas or perhaps a combination of the two. 3) Finally, the use of monoclonal antibodies linked to cytotoxic agents might prove more effective as antitumor agents than antibodies alone. The question of immunogenicity of the complex might also be important from the point of view of the haptenated

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immunoglobulin (572) as well as the toxicity which now might be expressed against the antibody producing B cell as well as against the tumor target cell. Questions of the mechanism of action of the antibody also must be answered. Tumor cells even in vitro can be shown to escape antibody therapy. Antigenic modulation as a result of antibody exposure may give rise to a redistribution of cell surface antigens and their subsequent internalization or shedding, thus negating the effects of the antibody. Note that this may not be a drawback if the antibody is linked to a cytotoxic agent whose site of action happens to be extracellular or at the membrane level. The possibility of immunoselection is innate to the concept of immunotherapy and immunochemotherapy as it refers to the growth of tumor cells which fail to express a specific antigen following treatment with the respective antibody. This along with the knowledge of tumor cell antigenic diversity begs the question whether tumor cell specific antigens which "define" the cancerous nature of the tumor, and which therefore, by definition, cannot escape, should continue to be sought.

In the following paragraphs we discuss some of the recent experimental efforts to utilize conjugates of toxins and monoclonal antibodies as therapy. Gilliland *et al.* (166–168) have coupled the A chain from diphtheria toxin or from ricin toxin to a monoclonal antibody directed against a colorectal carcinoma tumor-associated antigen by a disulfide linkage. They were able to demonstrate that the conjugates were cytotoxic to the colorectal cells in culture but were not toxic, in the same concentration range, to tumor cell lines which lacked the specific antigen. They were, in fact, able to demonstrate virtually 100% tumor cell kill under conditions where other cell lines were unaffected. Although the authors do not present any *in vivo* experiments to demonstrate that the conjugate can in fact target to tumor cells *in situ*, the great specificity of their system and the fact that cells which lack the antigen are unaffected, is promising. The limitation with the use of a monoclonal antibody preparation against a single antigenic determinant is the danger that some metastases may not possess or may have lost that specific antigen, thereby escaping the antibody-drug conjugate. Some of these questions of antigen diversity may be alleviated by a more detailed understanding of the antigenic properties specific to tumor cells. The possibility of defining determinants that are more common or essential to the tumor cell would offset the problem of tumor cell diversity and allow for the use of one or a few monoclonal antibody preparations to be used to target agents for either therapeutic or diagnostic use.

Krolick and co-workers (274, 275) have reported on a novel *in vitro* use of antibody-toxin conjugates which might have important implications for autologous bone marrow transplantation. One method of treatment of patients with disseminated neoplasia, especially human chronic lymphocytic leukemia (CLL), involves vigorous chemotherapy and radiation therapy along with autologous bone marrow rescue. The authors demonstrate that an anti-immunoglobulin (Ig)-ricin A chain conjugate is effective in deleting tumor cells from infiltrated murine bone marrow. By using mice that were inoculated with a BCL 1 tumor, which is somewhat analogous to a prolymphocytic variant of human CLL, they showed that conjugate-treated cells were able to repopulate the hematopoietic system of lethally irradiated mice and that in most of the mice the tumor did not recur. This is a unique case since the tumor cells could be treated *in vitro* with ricin A chain conjugated to an antibody specific for the variable region of the surface immunoglobulin molecules expressed on the tumor cells but not on the normal hematopoietic stem cells which populate the bone marrow. Such a positive response for *in vitro* targeting in the use of autologous bone marrow transplantation, of course, requires the definition of such a surface antigen or immunoglobulin.

Krolick *et al.* (274) have also examined the use of antibody-ricin A chain conjugates [see Vitetta *et al.* (542) for review] *in vivo* for the treatment of a murine B cell tumor (BCL_1) which is known to infiltrate the bone marrow. Purified ricin A chain was conjugated to the tumor-specific antibody utilizing SPDP to form a disulphide bridge as described previously. Following purification procedures to separate conjugated immunotoxin from unreacted toxin or antibody, the product was quantified by radioimmunoassay and evaluated to determine antibody activity by in vitro toxicity of the complex to relevant target cells. Since the A chain has been shown to be nontoxic until it enters the cytoplasm and inhibits protein synthesis, the complex would be expected to be nontoxic to nonphagocytic cells lacking the specific antigen against which the antibody is directed. The fact that the immunotoxin is more effective when the antibody is in its $F(ab)'^2$ form than in the $F(ab)'$ form indicates that cross-linking and endocytosis is an important aspect of the cell killing. The fact that some authors have observed that immunotoxins prepared with some monoclonal antibodies have poor toxicities (228, 239, 508, 509) may be due to the inability of certain antibodies to induce endocytosis due to low binding affinities or because the target antigen may be one which is either shed or not readily internalized (see discussion in "Cellular barriers" above). By using anti-idiotype antibodies, Vitetta and colleagues (274) were able to demonstrate the total elimination of BCL_1 cells from bone marrow, a procedure described previously that may have important benefits for autologous bone marrow transplantation. This is probably a more promising approach than transplanting allogeneic marrow from which T cells have been eliminated in an attempt to avoid graft versus host disease (349, 542). In the studies of Krolick *et al.* (275) tremendously large tumor burdens were used to mimic

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the human situation. The burden was reduced by up to 95% with nonspecific cytoreductive methods such as fractionated total lymphoid irradiation and splenectomy. Control immunotoxin (ricin A chain linked to a nonspecific antibody) was without effect in prolonging survival while animals receiving immunotoxin directed against the tumor cells appeared tumor free 12 to 18 weeks following treatment. No tumor cells could be detected in the blood of the treated mice (as indicated by the lack of tumor production in passive transfer experiments to normal recipients). Tissues, however, transferred from immunotoxin-treated mice to normal mice some 25 to 30 weeks following treatment did result in tumor growth thus suggesting that host resistance had developed. The authors attribute the partial success of the treatment to the fact that the nonspecific cytoreduction had decreased the tumor burden to such an extent that the remaining tumor cells could be effectively controlled by administration of nonlethal doses of the immunotoxin. The lesssons learned from this exercise appear to be: 1) reduce the tumor burden to as large extent as possible without sacrificing the immune system entirely; 2) utilize the immunotoxin to kill additional cells; and 3) allow an intact immune system to keep the few remaining tumor cells permanently in check.

Several other authors have recently used essentially the same approach as the Vitetta group (228, 349, 408–410) with similar results, using different tumor systems and, of course, different target antigens. Raso *et al.* (408) have conjugated the ricin A chain to a monoclonal antibody against the common acute lymphoblastic leukemia antigen (CALLA) by using a disulfide bond linkage. The conjugate was shown to be cytotoxic *in vitro* only to CALLA-positive cell types. It is important to note that conjugates using $F(ab')^2$ as the carrier were 70-fold more potent than like-conjugates formed using the univalent Fab′ fragment as the carrier [see below work by Masuho *et al.* (319)]. A limiting point to this work is the demonstration in *in vitro* experiments that the conjugate appeared much less toxic than the free ricin and that not all CALLA-bearing cells appeared to be equally sensitive to the conjugate. The question of how the particular cell deals with the antibody-toxin complex still has to be addressed. Fitzgerald and colleagues (137) have constructed conjugates of *Pseudomonas* exotoxin and antitransferrin receptor antibodies which gain access to cells by the well-defined mechanism of receptor-mediated endocytosis involving coated pits. While the conjugate itself is toxic to monolayer cultures of KB cells, the investigators make the somewhat strange observation that the toxicity of the conjugate is enhanced some 100- to 300-fold by the presence of adenovirus. The authors speculate that the adenovirus and the toxin-antibody complex are cointernalized via the same coated pit and that the escape of the adenovirus into the cytoplasm is accompanied by a disruption of the receptosome thereby releasing the contents of the vesicle into the cytosol allowing the toxin to shut down protein synthesis. Although it is somewhat difficult to see how this particular scenario might be used directly as a means of drug or toxin delivery, the cell biology involved presents an elegant demonstration of how drugs might gain access to intracellular locales by using the pathway of receptor-mediated endocytosis and the mechanisms of subsequent intracellular handling of receptosomes or endosomes. Some of these concepts were covered n section II above and have also been reviewed by Brown and colleagues (54).

5. Problems and Prospects. A number of important unanswered questions and problems remain. Considering the extremely high potential toxicity of the toxins (especially the ricin A chain) the therapeutic index of the immunotoxin must be improved if any significant tumor burden is to be challenged. The question of the pharmacokinetics of the immunotoxin remains an important one as we consider how the complex is to penetrate the various barriers to diffusion and escape the clutches of the RES. The lack of a well-defined stable tumor-specific antigen also remains a serious limitation. Vitetta and colleagues (542) also question the accessibility of solid tumors to immunotoxin therapy in respect to the dense connective tissue component of the solid tumor. One novel proposal to increase accessibility is the possible use of bound vasodilators, such as histamine, to facilitate the complex's penetration of the blood-tissue barrier. This is certainly an intriguing but little explored possibility. Another possible drawback which holds equally for immunotherapy, for immunochemotherapy, as well as for immunotoxin therapy, is the presence of circulating tumor-specific antigen which may bind antibody or antibody-drug complexes in the serum before they even have a chance to attack the target tumor cells. It is not known whether levels of circulating antigen might be sufficient to pose such a problem although the presence of circulating CEA (carcinoembryonic antigen) has made monoclonal antibodies against CEA difficult to use in diagnostic situations (307).

Hara and co-workers (319) have carried out an extensive series of experiments to better define the importance and nature of the cross-linking bond between the ricin A chain and the antitumor antibody and the importance of the antigen-binding valency on the effect of the conjugate as an antitumor agent. They compared the relative potencies of conjugates made by disulphide bond formation and determined whether it was important that the bond be cleavable by sulphydryl reagents. While these experiments were all carried out in vitro with murine L1210 cells and IgG molecules from a rabbit antiserum to the cells, the examination is important in terms of defining the essential parameters in the design of drug delivery or carrier systems. These authors demonstrated that divalency in the carrier [$(Fab')^2$ as opposed to Fab′] increased the potency and that it was highly desirable

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for the bond between the drug and antibody to be cleavable by sulfhydryl reagents. In a cell-free system the cleavable and noncleavable conjugates had about equal potency with free ricin A chain in their ability to inhibit protein synthesis suggesting that the cleavage is not a requirement in terms of ribosomal interactions. The fact that cleavage of the bond appears to be important in the intact cell system suggests that the free chain can reach the ribosome only after binding of the conjugate to the cell-surface and internalization. This would then be followed by a processing step that must include bond-cleavage and which may be lysosomal in nature although the authors do not suggest this as a possible mechanism.

The topic of antibody carriers of drugs and toxins in tumor therapy has recently been covered in an entire volume of *Immunological Reviews* (vol. 62, 1982). Although other drugs are covered, the topic of "immunotoxins" is dealt with by several different authors.

F. Cellular Drug/Enzyme Carriers.

1. Erythrocytes. Chang's early demonstration (64, 65) that red blood cells could be encapsulated within semipermeable aqueous microcapsules while retaining some of their inherent properties suggested that in an analgous manner red blood cells might be used as carriers in their own right [Ihler *et al.* (221)]. It has been known for many years that osmotically insulted cells could be resealed to their original permeability characteristics and that their ability to circulate in the blood stream for prolonged times following reasealing could be restored. Ihler and coworkers (221) first utilized these characteristics to demonstrate the possibility of enzyme loading of red blood cells for use in enzyme replacement therapy. Ihler's initial publication demonstrated the feasibility of loading red cells with alpha-glucosidase and beta-galactosidase by rapid osmotic hemolysis of the red cells in the presence of these enzymes. They demonstrated that small protein molecules and proteins as large as 180,000 could similarly be entrapped while retaining some of the original physical chacacteristics of the red cell ghosts, at least in terms of mean cell volume and permeability.

The rationale for the encapsulation of enzymes, and now drugs, is well established. Some of these will be described below. An important question is whether the drug/enzyme is to act within the red cell or whether the red cell is to be used as a vehicle to target the encapsulated agent to phagocytic cells of the liver and spleen. In the first case one has to consider whether the red-cell-entrapped enzyme will be accessible to substrate which may be accumulating, as in the case of enzyme deficiency diseases. In the second, it has to determined whether the red cell will in fact be taken up by the appropriate cell and, as a consequence of this capture, will the enzyme be released in an active form to act upon accumulating substrate? There have been several reviews on methods of enzyme and/or drug encapsulation (see Refs. 214, 220) including hemolysis, dialysis, and electric field breakdown. Each of the procedures produces varying amounts of enzyme entrapment and varying degrees of cell damage, an important factor in terms of circulating half-lives and sites of uptake of enzyme-loaded cells. DeLoach and coworkers (99) examined the possibility of using enzyme-loaded erythrocytes as a means of enzyme replacement for inherited lysosomal storage disease. In *in vitro* experiments they examined the uptake of glutaraldehyde-treated red cells by bone marrow macrophages in tissue culture. The entrapped enzyme, along with partially degraded red cells, could be localized in cell vacuoles presumed to be secondary lysosomes. An important question that has yet to be answered, is whether the "targeted" enzyme can resist proteolytic degradation within the lysosome in order to demonstrate enzyme activity and substrate breakdown. Immobilization of the enzymes within the erythrocytes (e.g. by glutaraldehyde) may function to protect the entrapped enzyme from rapid degradation following phagocytosis. Dale and coworkers (89) have utilized gamma globulin-coated enzyme-loaded resealed erythrocytes to deliver exogenous glucocerebrosidase to monocytes from Gaucher's disease patients. The uptake proved to be far superior to uptake of free enzyme, enzyme in uncoated resealed erythrocytes, or liposome-entrapped enzyme. The manuscript brings home the importance of the delivery system-target cell interaction but provides no information regarding the *in vivo* handling of the complex.

There have been a number of reports in the literature on the use of enzyme or drugs entrapped within red cells in preliminary clinical applications. The first, reviewed by Beutler *et al.* (35), reported on an attempt to treat a patient with advanced Gaucher's disease with glucocerebrosidase-loaded erythrocytes. There was no evidence of any clinical improvement or even a positive effect from the enzyme. These experiments however, were probably premature. The authors did not ascertain that an adequate amount of enzyme had been administered. At the time there was little understanding of how the entrapped enzyme might reach the site of substrate accumulation or whether the delivered enzyme would be active. The second clinical trial proved much more positive. Green *et al.* (178) entrapped the iron-chelating agent, desferrioxamine, within human red cell ghosts and demonstrated that, in patients suffering from iron overload, mostly beta-thalassemics, the drug was more effective in increasing urinary iron output when entrapped within human erythrocyte ghosts compared to equivalent amounts of free drug. There was some criticism of this approach in that it was suggested that the ghosts themselves, being not completely devoid of hemoglobin, might themselves add to the problem of the iron overload (473). Green *et al.* (179) rebutted this criticism with a demonstration that the increased urinary iron output was more than an order of magnitude higher than could be accounted for by the quantity of iron in the ghosts alone.

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This demonstration by Green of the efficacy of using drug-loaded red cells as carriers in both animal experiments (180) and preliminary clinical trials (178) is an exciting demonstration of the potential of such carrier systems. It should be noted, however, that the "engineering" involved is minimal since the natural site of uptake and degradation of the "loaded ghosts" would appear to be the natural first site of iron accumulation in thalassemic patients, namely by the RES cells of the liver and spleen.

In respect to the use of red cells as enzyme carriers, a more rigorous demonstration in an animal model is required to demonstrate that the entrapped enzyme is either available to accumulating substrate for breakdown or else that the red cell ghost is capable of delivering the enzyme/drug to the appropriate site in an active form. Ihler and colleagues (222) have shown that uricase-loaded erythrocytes are effective in lowering uric acid levels as rapidly as the substrate can get into the cell. Updike *et al.* (531) examined the potential of L-asparaginase-loaded red cell ghosts as antitumor agents. While the enzyme was active under the described conditions, it was not clear that L-asparagine levels could be reduced sufficiently in sensitive tumor cells without inducing asparagine synthetase activity in normal cells, thus offsetting the benefits of the therapy (194). These experiments did provide some interesting data measuring the ability of the red cells to remain in the circulation following ghost formation. They suggest that at least as much damage is done to the red cells in the handling as is done through the hemolysis step. Sprandel and co-workers (479) have suggested that a dialysis method for enzyme loading of erythrocytes by reversible hypo-osmotic hemolysis produces resealed hemoglobin-containing ghosts loaded with enzyme and having excellent retention of the transport properties for sodium, L-phenylalanine, and uric acid. It is suggested that the reduction in the number of washing steps in this procedure and the retention of the hemolysate within the cells may make them more viable with respect to their ability to remain in the circulation. Chalmers and co-workers (209, 480) have investigated the *in vivo* and circulatory properties of several different resealed ghost preparations. Although Ihler and colleagues (220) have shown that the red cells might be partially targetable by judicious membrane damage (e.g. mild glutaraldehyde treatment), the targets would probably be limited to phagocytic cells of the RES. An additional concern remaining is the question of how much enzyme can be loaded and whether the permeability barrier of the cells is not too limiting a factor in terms of substrate conversion. Red cells may be an interesting tool for experimental manipulation in enzyme replacement therapy. However, the complex biological nature of this carrier makes the problem of development in a clinical and commercial fashion a formidable one.

2. Fibroblasts and Leukocytes. For some years now, Dean and coworkers (95–97) have been examining the possibility of using fibroblast transplantation as a means of enzyme replacement therapy. This topic is included in this review since the fibroblasts are acting as an enzyme delivery system. By transplanting fibroblasts from HLA identical normal donors into patients with Hunter's syndrome, Dean *et al.* (96) observed increased catabolism of accumulating glycosaminoglycans as well as increased levels of previously deficient lysosomal enzyme, iduronate sulfatase. More recently, Dean *et al.* (94, 95) have reported on the use of fibroblast transplantation for the treatment of three patients with Sanfilippo A syndrome, an inherited disorder of connective tissue resulting in mucopolysaccharide accumulation and incomplete catabolism of dermatan and heparan sulphates. The authors report a very significant improvement in the biochemical picture of these patients in terms of substrate breakdown, but no clinical improvement in terms of mental development, joint mobility, or liver and spleen size. The rationale for this therapy is not simply substrate breakdown by the transplanted fibroblasts. The secretion of normal lysosomal enzymes by the transplanted fibroblasts and their subsequent uptake by adsorptive endocytosis into the deficient fibroblasts, restoring enzyme activity, is postulated as the mode of action. A similar approach has been demonstrated for the transfer of normal enzymes from leukocytes of normal patients into enzyme-deficient fibroblasts. The most recent report on clinical trials with patients with mucopolysaccharidoses (165) has been much more negative, concluding "that fibroblast transplantation is not therapeutically useful in the diseases studied."

Hobbs and colleagues (see Refs. 201–204) have demonstrated the possibility of using total bone marrow transplantation (BMT) as a means of treating certain inborn errors of metabolism. Their approach is based on their success in the use of BMT for the treatment of certain types of leukemias and their relatively high rates of graft acceptance following new techniques of "pregraft conditioning" and success in decreasing "graft-versus-host disease." The rationale for this treatment is that if the metabolic error is expressed in leukocytes, then the transplanted leukocytes will repopulate the recepient, displace the abnormal bone marrow and thereby provide a lasting source of leukocytes synthesizing the missing normal enzyme. It is Hobbs' view that the circulating normal leukocytes will provide the enzyme to other deficient tissue as a result of the normal breakdown of leukocytes in tissue spaces and the subsequent uptake of the normal enzyme (from the graft leukocytes) into enzyme-deficient tissue. The rationale is based on the work of Neufeld and others regarding the secretion recapture phenomenon of certain lysosomal enzymes (347, 438) and is not dissimilar to the rationale put forward earlier for fibroblast transplantation. Hobbs' contention is that the graft will produce sufficient leukocytes to, in effect,

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produce an enzyme therapy to the affected enzyme deficient cells. Hobbs *et al.* (204) produce some convincing preliminary data (both clinical and biochemical) that features of Hurler's syndrome (a mucopolysaccharidoses manifested by alpha-L-iduronidase deficiency) are decreased following BMT. The criticism that such a cure may be worse than the disease is probably unfair. Although bone marrow transplants are still considered high risk in terms of graft-versus-host disease, it should be pointed out that these patients, unlike leukemics, have not been immunosuppressed previously and the prognosis for a successful graft is probably better. The conditions being proposed for treatment by BMT are tragic, painful, usually untreatable, and have high mortality rates. Nevertheless, the evidence that leukocyte transfer achieves any enzyme replacement in any other tissue is not yet available in spite of the early positive clinical signs described by Hobbs' group. Hobbs (201) anticipates the possibility of treating a fairly wide range of lysosomal storage diseases by the method of displacement BMT. There is, however, little reason to assume that other inborn errors of metabolism which Hobbs listed as candidates for treatment by BMT will be amenable to this treatment. These lipid and carbohydrate storage conditions are not limited to leukocytes. They are usually fatal as a result of substrate accumulation in brain, muscle, and liver (often hepatocytes as opposed to phagocytic Kupffer cells) and, even if grafted leukocytes do "dump" the deficient enzyme, there is no indication that they will be taken up by appropriate deficient cells. The fact that Hobbs has been able to demonstrate some biochemical and clinical improvements in the Hurler's patients is encouraging, although it would be nice if the treatment had a better rational basis so that its applicability to other inborn errors of disease could be understood. Questions such as how much leukocyte enzyme might be expected to be delivered to different affected tissue would give the possible use of bone marrow transplantation as a viable treatment for many inborn errors of metabolism greater credibility.

3. Encapsulated Cells. Chang (64) first described the encapsulation of intact cells some 20 years ago when he was able to incorporate intact red cells within nylon semipermeable aqueous microcapsules. Whereas he recognized the advantages of enclosing intact cells to avoid proteolytic inactivation and immunological rejection upon *in vivo* administration, it is only in the past few years that investigators have begun to take advantage of this procedure. Sun and colleagues (299) have microencapsulated pancreatic islet of Langerhans cells in order to produce an artificial pancreas. Islet cells were encapsulated with poly-L-lysine and polyethyleneimine following the immobilization of the islet cells in a sodium alginate and calcium chloride gel form. Following membrane formation, the encapsulated material could be liquified by placing the microcapsules in a sodium citrate solution which dissolves the sodium alginate. In tissue culture, both encapsulated and nonencapsulated cells continue to be responsive to glucose levels (in terms of insulin production) over a period in excess of 60 days. Lowering glucose levels from 300 mg/100 ml to 100 mg/100 ml produced rapid increases in insulin output by either set of cells. Rats that had been rendered diabetic chemically were treated by IP injection of the encapsulated islet cells. Following implantation of the cells, the diabetic rats showed a decrease in plasma glucose levels from 325 mg/100 ml to less than 100 mg/100 ml in less than 2 days. The plasma glucose levels remain low in these animals for up to 50 days following a single injection of 3,000 encapsulated islet cells. No immunological reaction to the encapsulated islet cells was detected in spite of a major histocompatibility barrier between the source of the donor cells and the receipient's type. This represents an extremely encouraging finding which has important implications not only in the treatment of diabetes, but in the treatment of a wide range of metabolic disorders where cell transplantation might be considered were it not for the problems associated with major histocompatibility barriers.

Nilsson and colleagues (356) have reported on a different technique which allows for the encapsulation of a variety of protein-secreting cells within an agarose bead. The procedure is gentle in that it produces a membrane which is permeable to large polypeptides, such as monoclonal antibodies, and the cells appear to grow and produce secreted product at a rate much higher than found for the cells in culture. While the cells appear active and productive, a major objective of cell encapsulation, that is, protecting the cells from immunological attack and proteolytic inactivation is lost by using this procedure. The authors' more immediate objective here is *in vitro* use of these cells for the synthesis of secreted polypeptides.

4. Semipermeable Aqueous Microcapsules or Artificial Cells. Techniques of microencapsulation have been used in industry, especially in the printing business, for over half a century. It was only in the early 1960's that the possible use of microencapsulation techniques for biomedical purposes was envisioned. Chang first described the techniques whereby enzymes, detoxifying agents, and even intact and live cells could be encapsulated within a controlled aqueous environment [see Chang (68) for a detailed review]. Typically, the microcapsules Chang first described were composed of ultrathin membranes made from either nylon or collodion. They ranged in diameter from several microns to a few millimeters with a degree of control over the permeability such that large protein molecules were absolutely impermeant whereas smaller molecules such as glucose or urea could penetrate with ease. Chang realized the problems associated with the introduction of encapsulated drugs or enzymes *in vivo* and the multitude of barriers that had

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to be crossed and to a certain extent limited himself to the use of microcapsules placed in an extracorporeal shunt. This allows the pasage of biological fluids, usually plasma, over and through the microcapsules which remain trapped by small screens within the shunt chamber. The tremendous surface area to volume ratio of these microcapsules and the high density of the drug, enzyme, or detoxifying agents achievable in these microcapsules allow for the use of very small volumes. An important distinction between the microcapsules being used both experimentally and clinically by Chang and the drug carriers discussed elsewhere in this review is, that for the most part, the therapeutic agents in Chang's system remain within the semipermeable aqueous microcapsules to act upon permeant molecules. In the next section on other types of microcapsules and microspheres, the particles are used more as vehicles whereby the drug or therapeutic agent is meant to leave the capsule or particle to have its therapeutic action.

Chang's earlier studies demonstrated the possibility of using microencapsulated enzymes as a means of enzyme replacement therapy for the treatment of enzyme deficiency diseases [Chang and Poznansky (73)], as a means of treating certain types of leukemias using the enzyme L-asparaginase (66), as a means of producing an alternative to dialysis in the treatment of uremia (67, 69), and, most recently, as a means of treating certain types of liver disease (69). These microcapsules, or what Chang calls "artificial cells," have now been used in clinical trials as a hemoperfusion system for the treatment of drug overdose, liver failure, and uremia (70, 72). The central feature of this system, that has proven so successful in clinical trials, is the use of microencapsulated activated charcoal. Several review articles are available to describe these clinical trials showing the benefits of this system for the treatment of theophylline poisoning (drug overdose) in children (71) and for the treatment (at least temporarily) of patients with grade IV liver coma. Chang's development of a microcapsule artificial kidney is now complete in terms of the removal of creatinine, uric acid, and other toxic molecules, as well as the control of water and ion composition. The removal of urea has, however, been problematic. In 1968, Chang demonstrated the possibility of including the enzyme urease in the microcapsule system, but no efficient method for the removal of the resultant ammonia was available. Alternative methods for the removal of nitrogenous metabolites (e.g. ion exchange resins for the removal of ammonia) have been suggested. Gardner *et al.* (155) have described the use of encapsulated adsorbents for metabolite removal via oral ingestion. None of these approaches has proven to be sufficient. Chang and coworkers (74) have recently described a microencapsulated multienzyme system capable of converting urea and ammonia to simple amino acids which may enter the normal amino acid pools. Figure 3 describes such a

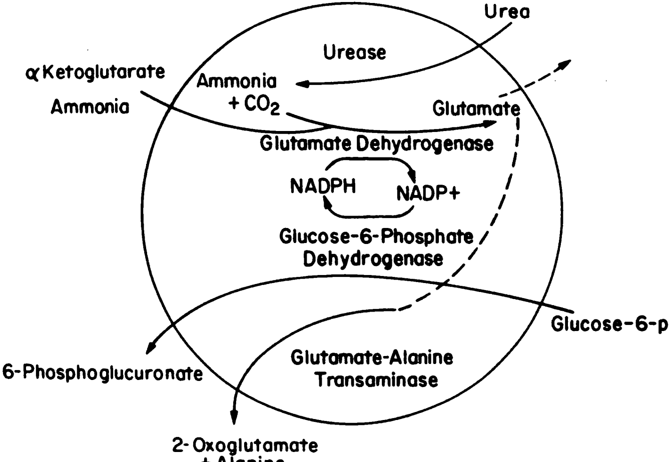

FIG. 3. Multienzyme system to convert urea and ammonia to simple amino acids. Schematic representation of a multienzyme system for the conversion of toxic waste metabolites into harmless metabolic by-products. Such a system has been used in preliminary clinical trials [see Chang et al., (74)] for the treatment of renal failure and analagous multienzyme systems have been described by Chang for the support therapy in liver failure and for detoxification in cases of drug overdose.

multienzyme system. The glucose dehydrogenase system is included as a means of co-factor regeneration since levels of NADPH in plasma would be insufficient to support the reaction. Another alternative that Chang and coworkers have devised is the use of immobilized cofactors such as soluble conjugates of dextran and NADPH which restricts the movement of the cofactor out of the microcapsule, thereby solving the problem of cofactor replenishment. Control of the permeability of the microcapsule membrane is another possibility that he discusses to limit the loss of cofactors and/or necessary intermediates.

In a fashion analogous to his development of a new artificial kidney, Chang has also demonstrated the feasibility of using enzyme- and detoxicant-loaded microcapsules as an artificial liver, or at least as a support system for a regenerating liver. Early clinical trials using only the activated charcoal microcapsules for the treatment of type IV liver coma patients brought the patients out of the coma temporarily but did not provide a permanent recovery (69). Chang's recent studies have centered around the microencapsulation of multienzyme systems to remove high levels of ammonia found in patients with liver failure. The glutamic acid formed within the microcapsules by the glutamate dehydrogenase can be converted to other amino acids (for instance by including glutamate-pyruvate transaminase in the multienzyme system or by including activated charcoal in the microcapsules to adsorb the glutamic acid formed). The beauty of this system is that the microcapsules remain in a small extracorporeal shunt which may be removed or recharged at any time. In the case of the use of the microcapsule artificial kidney, the patient may be hooked up to the shunt for 1 to 2 hours twice a week compared to the three 12-hr shifts required by traditional

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dialysis. Kjellstrand and colleagues (264) reported on the clinical use of microencapsulated zirconium phosphate-urease in the treatment of patients with chronic uremia by using the oral route of administration of the microcapsules. This approach has obvious practical advantages over the use of an extracorporeal shunt utilizing an arteriovenous fistula. The urease converts urea to ammonia which is then bound by the zirconium phosphate compound in exchange for sodium. Clinical trials under careful nitrogen balance conditions indicate an important decrease in blood urea nitrogens (BUN). There were no adverse effects, although changes in certain plasma electrolytes such as potassium, calcium, and magnesium, supposedly as a result of binding to the zirconium microcapsules in the digestive tract, necessitated supplementation of these electrolytes in the diet. The dose of microcapsules required to maintain acceptable BUN levels in these patients with chronic uremia was 100 gm/day, an amount associated with the feeling of fullness, but no other adverse effects.

The use of intact cells, either native or reconstituted as in the case of red cell ghosts, for drug/enzyme delivery, represents a highly experimental approach. The complexities involved in bringing such systems to use in the "field" are enormous and are not likely to be solved unless the benefits over other modes of treatment become so overwhelming that further development to more practical states are warranted. The question of physical barriers depends on the desired site of action of the therapeutic agent. The use of desferrioxamine-loaded erythrocyte ghosts for treatment of transfusional iron overload (440), results in ghost uptake by the RE system, the very site of initial iron accumulation. The use of fibroblast or leukocyte transplantation does not solve the problem of how secreted, or released enzymes will reach the appropriate site of accumulating substrate—often lysosomal and often in cells of specific tissues. The use of microencapsulated islet cells circumvents the "barrier problem" because of the high permeability of both glucose and insulin moving to and from the encapsulated cells. Chang's use of microencapsulated enzymes and/or detoxicants requires that the substrate or toxic moiety in question be sufficiently mobile to find its way into the blood stream (or peritoneal fluid) ad hence be exposed to the contents of the microcapsules in the extracorporeal shunt. These examples represent a departure from the drug delivery systems we have been examining but probably represent realistic alternatives to some of the other approaches we have described.

G. Microspheres as Drug Carriers

The literature terminology is somewhat confusing in this area. Generally speaking, the semipermeable aqueous microcapsules described by Chang (68) and now used widely, refer to envelopes that contain an aqueous solution of some therapeutic agent. In most cases, the agent remains within the capsule and acts upon permeant material. In the cases to be discussed in the following section, the microparticles are being used either as sustained release systems or else as vehicles for delivery of specific therapeutic agents to particular sites for local release by either permeation or by degradation of microcapsular material.

Kramer (271) first described the production of heat-denatured albumin microspheres capable of entrapping certain drugs (he used mercaptopurine) and delivering them *in vivo*, primarily to RES cells. Widder and Senyei (see Refs. 561–563 for reviews) have devised a technique for using albumin microsphere-entrapped drugs while avoiding the almost obligatory clearance by fixed macrophages of the liver and spleen. In addition to serving as a vehicle for the chemotherapeutic agent, the microspheres, composed of a denatured human serum albumin matrix, contain ultrafine particles of magnetite (Fe_3O_4), making the microspheres susceptible to the effects of an externally applied magnetic field. This system allows for a degree of target site specificity, as the magnetically responsive microspheres can be retained in capillaries surrounding the tumor site by placing an external magnet over the tumor. The applied magnetic field allows for a very high retention percentage (approaching 100% in some cases) which is crucial: a) to allow the drug to diffuse out of the microspheres at or near the tumor site: and b) to allow microspheres to move from the vascular space into extravascular space, thus establishing an extravascular drug depot producing a sustained drug release in the area of the tumor. Widder and colleagues (560, 562) have demonstrated the efficacy of such a system in several different cases. They were able to selectively target albumin microspheres containing doxorubicin and magnetite to Yoshida sarcoma tumors in rats by using an extracorporeal magnet. The tumor cells were injected in the tail of the rats and the microspheres or control treatment (free drug, microspheres without drug or without magnetite) were infused proximal to the tumor. The tumor was allowed to grow until it was at least 9 by 45 mm, at which time treatment was initiated with a single injection of the test sample. Of the 12 rats treated with the magnetic microspheres containing drug, nine exhibited total remission of the tumor (which were initially as large as 60 mm in diameter). Of the remaining three rats, there was marked tumor regression, and no deaths or tumor metastases were noted. In contrast, in all of the control groups, whether using free drug or using microspheres containing drug but no magnetite, there was a progressive increase in tumor growth, evidence of tumor metastases, and a very high mortality rate. These experiments suggest that magnetic microspheres show excellent promise in the treatment of solid neoplasms that can be partially isolated using an externally applied magnetic field. The use of this system requires a well defined, fairly solid, and well vascularized tumor that has not undergone any metastases. The question of how

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a magnetic field might be focused if a deep tumor is involved may appear formidable but can probably be resolved with sophisticated electromagnetic focusing devices. The major advantage of this system is its apparent high efficiency. A single dose of microspheres totally eliminated the tumor. This does away with problems such as eliminating excess magnetite, and any immunological complications due to the denatured albumin if repeated injections of the microspheres was to be contemplated. The fact that such a high percentage of the dose of antitumor agent can be localized at or near the tumor site by the magnetic field (560), with little if any drug appearing in normal organs, alleviates the problems associated with the use of highly potent antitumor agents that also exhibit generalized cytotoxicity. No therapeutic advantage would be expected against tumor metastases that have migrated from the solid neoplasm.

Kato and colleagues (248–250) have utilized the technique of transcatheter embolization to deliver microencapsulated mitomycin C (MMC) to particular organs while sparing the rest of the organism from high concentrations of the cytotoxic drug. The MMC was encapsulated by using ethylcellulose, resulting in particles with a mean particle size of 224 μm containing (by weight) 80% drug and the rest polymer. The microcapsules were infused into the renal artery following arterial catheterization and became lodged in small arteries mainly of the corticomedullary region of the kidney. The kidneys receiving the microcapsules retained active drug for more than 6 hours and showed extensive necrosis in the region 5 days after the infusion. Infusion of free MMC resulted in rapid excretion of the drug, little histological change in the kidney, and fairly high levels of drug in the blood and other organs. The benefits of such a system is that, as with the magnetic microspheres, the selective infusion of the microencapsulated drug into tumor-supplying arteries could facilitate high dose chemotherapy at the region of the tumor while minimizing systemic side effects due to the exposure of normal tissue to high drug doses. Kato *et al.* (248) have also utilized the technique of transcatheter arterial chemoembolization to treat patients with renal cell carcinoma. They utilize an absorbable gelatin sponge as a means of embolizing the organ and diminishing the blood supply. In patients receiving microencapsulated MMC along with the gelatin sponge, there was a marked decrease in tumor size compared to a second group of patients receiving the gelatin sponge with free MMC. Histological analysis also showed a marked necrosis in the tumor tissue from patients receiving the MMC in the microencapsulated form. The authors have now extended these clinical trials to several hundred patients, including primary or metastatic hepatic carcinomas, urinary bladder carcinomas, prostatic carcinomas, cervical or ovarian carcinomas, rectal carcinomas and a number of other tumors in addition to the renal cell carcinomas (250). Measureable reduction in tumor size was detected in only 50% of the cases. Tolerance to the MMC in the encapsulated form was high compared to previous use of free drug. Approximately 25% of the tumors failed to respond in any measureable way to the therapy. As expected, patients treated in this manner who had evidence of recurrent or disseminated tumors did not fare well from the point of view of survival (less than 25% survived up to 40 months), although their localized tumors appeared to regress for a time. Patients who were treated and who had only locally advanced tumors with no evidence of distant metastasis fared much better, there being an 80% survival rate up to 40 months following treatment. The Japanese have undertaken a widespread clinical trial in many different centers to ascertain the efficacy of this therapy. Unfortunately in many of the cases, as with most novel treatments, very advanced cancers are being included in the trials, somewhat biasing the data in a negative manner. Kato and colleagues (248) have also made magnetic MMC microcapsules in an effort to localize the encapsulated drug at a particular site following intra-arterial infusion. This system might differ somewhat from the Widder approach (561) in that the transcatheter embolization step is the crucial one in this therapy. As with other therapies utilizing the strategy of sustained, localized release, success might only be expected in cases of highly localized tumors with no evidence of tumor metastases.

Sjoholm's group in Uppsala (114, 115, 120) have demonstrated the possibility of immobilizing macromolecules, especially enzymes, within a macroporous structure of cross-linked polyacrylamide. The microparticles are meant as a means of enzyme therapy in the treatment of certain lysosomal storage diseases and for other forms of therapy as in the use of L-asparaginase against certain types of tumor cells. As in the case of the soluble enzyme-albumin and enzyme-polyethylene glycol (PEG) conjugates described in the next section, the macroporous beads stabilize entrapped enzyme. However, an appreciable amount of protein is exposed on the surface of the bead and is potentially immunogenic, although the polyacrylamide material of the microparticles itself is inert. Depending on the route of administration of the microparticles, they may or may not act as immunological adjuvants to enhance the immune response to the bound or trapped enzyme. In the case of L-asparaginase-polyacrylamide microbeads, an intramuscular route of administration is the most efficient in terms of both reducing plasma L-asparagine levels and minimizing the immune response to the enzyme. In a mouse tumor model system, the authors show an enhanced antitumor effect of L-asparaginase immobilized in either polyacrylamide or polyacryldextran microbeads when compared to equivalent amounts of free L-asparaginase. The major advantage of the beads is the ability of the immobilized form of the L-asparaginase to prolong the depressive effect on plasma L-asparagine levels. This form of administration

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lowers the dose of enzyme required, thereby minimizing the chances of severe immune reactions to the enzyme. As expected, the microparticles, once injected intravenously, are rapidly removed by phagocytic cells of the RES including liver, spleen, and circulating macrophages. Intramuscular injection not only reduces the immune response to the entrapped enzyme, but also allows the particles to avoid phagocytosis by macrophages for a longer period of time. Following cellular uptake, the particles can be found associated with secondary lysosomes within the phagocytic cells, so in this respect they are lysosomotropic. It is difficult to see what benefit they might have in the treatment of lysosomal storage diseases where accumulating substrate was occurring in cells other than those of the RES.

Tokes and colleagues (423, 512) have recently described a novel formulation of adriamycin coupled to polyglutaraldehyde microspheres and investigated the cytostatic activity of the polymer-bound drug in several different murine and human leukemias and a murine sarcoma cell line. Not only do the microspheres retain the drug effect, but they appear to be able to overcome drug resistance demonstrated in a rat liver cell line. In the latter case the covalent attachment of adriamycin to the microspheres increased the cytostatic activity of the drug 1000-fold. The complex shows a great deal of stability with better than 99% of the drug remaining attached to the microsphere over a 24-hr period under tissue culture conditions. This approach has the important advantage in that it offers a lower toxicity to normal cells (423). While the exact mode of action of adriamycin as an antineoplastic agent is not known, it has been widely accepted that its anthracycline ring binds to and intercalates into DNA molecules resulting in the inhibition of DNA replication or RNA binding (108). However, Tokes' results suggest that the covalently bound adriamycin does not have to enter the cell to produce its cytostatic effect. This suggests that the mode of action of the drug might reside solely at the level of the plasma membrane. In addition to decreasing the generalized cytotoxicity of adriamycin and overcoming drug resistance in some cell lines, the microsphere formulation working on the cell membrane has the possibility of providing multiple and repetitious sites for drug-cell interactions. A possible increase in the therapeutic index also occurs since the drug in the conjugated form is protected from loss by simple clearance and/or metabolic degradation. These experiments, however, remain strictly at a tissue culture stage and mechanisms to direct these microspheres to appropriate sites of action have still to be worked out.

Tritton and colleagues (463, 516, 517) have also examined the mechanism of action of adriamycin. By using similar principles to Tokes *et al.* (512) they have also come to the conclusion that adriamycin can be actively cytotoxic without even entering the cell. It this is true, then the use of some form of nonpenetrating adriamycin derivative targeted to tumor tissue might be used to avoid metabolic repercussions and high cardiac toxicity associated with administration of the drug in its free form.

H. Macromolecules as Drug/Enzyme Carriers

Soluble macromolecules acting as drug carriers have an important advantage over the many particulate carrier systems that have been described. Their solubility and smaller size allow them to gain access to regions not available to larger insoluble particles while at the same time avoiding rapid clearance from the organism via the kidneys. Depending on the chemistry (reactive groups available) of the carrier, it has the potential of assuming a very large load factor in terms of drug units carried per unit volume. The number of such potential carriers is enormous, dependent mostly on the investigator's imagination. Virtually every plasma protein, ranging from albumin to fibrinogen to low density lipoproteins (LDL), has been proposed at one time or another as a potential carrier of drugs and/or enzymes (396, 398). Biological macromolecules such as antibodies, lectins, and certain glycoproteins, such as asialofetuin which have particular affinities for specific cell surface receptors, are dealt with in separate sections of this review. Other synthetic macromolecules such as polyethylene glycol (PEG), poly(N-vinylpyrrolidone) and preparations of polyamino acids such as poly-L-lysine have been used to form soluble drug carrier conjugates.

Trouet and colleagues (520, 521) have for many years been interested in the development of "lysosomotropic" carrier systems to enhance the chemotherapeutic selectivity of certain antitumor drugs. The rationale is that the desired target cell should recognize the carrier system, and activation of the drug system should occur following endocytotic uptake of the drug-carrier complex and intralysosomal release of the drug from the carrier. This might occur as a result of the lysosomal acid pH or as a function of the lysosomal hydrolases. Trouet and colleagues (519) have made extensive use of DNA as a carrier of several antitumor drugs, most often the anthracycline derivatives, daunorubicin and adriamycin. The drugs are prepared (521) as noncovalent complexes with high molecular weight DNA. The complexed drug exhibits a number of important advantages compared to free drug. Both complexed daunorubicin and adriamycin are much more effective antitumor agents in a murine leukemia (L1210) than an equivalent amount of the free drug (103). Adriamycin toxicity is greatly reduced when administered IV in the complexed form with DNA and much less drug is lost via excretion. Trouet's group has carried out clinical trials on more than 700 patients with different types of leukemias. The major advantage of the DNA-drug complexes is the greatly reduced cardiotoxicity of the complex when compared to equal amounts of the free drug. While this is an important advantage, there is no indication that DNA enhances the antitumor

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activity of the drugs themselves. A major difficulty with this system is that little is known about the nature of the DNA-drug interaction. It is known, however, that the stability of the complex in the bloodstream is poor and there is no evidence that the DNA renders the drug any more tumor cell-specific. The use of DNA as a noncovalent carrier must be limited to drugs like the anthracycline derivatives which have high spontaneous affinities for DNA. Trouet and his colleagues also have considerable experience with the use of lipsomes and proteins as lysosomotropic agents. Masquelier *et al.* (318) have prepared mono- and dipeptide derivatives of daunorubicin in an effort to produce prodrugs which may be activated in the region of tumor tissue known to have higher extracellular aminopeptidase activity. In a second publication the authors demonstrate the superiority of the amino acid and depeptide derivatives of daunorubicin against L1210 leukemia cells *in vivo*. Again, the exact mode of action of these complexes is unclear. The authors suggest that it may be due to the greater hydrophobicity of the drug derivatives as well as to the hydrolysis of the complex *in situ* by hydrolytic lysosomal enzymes secreted by the tumor cells or present on the tumor cell surface.

Ryser and coworkers (434, 435, 457) have made extensive use of poly(L-lysine) as a lysosomotropic agent to carry methotrexate into cultured cells which exhibit drug resistance as a result of transport deficiencies for the drug. Shen and Ryser (457) had demonstrated that a 6,700 M_r fragment of poly(L-lysine) conjugated to human serum albumin or to horseradish peroxidase enhanced cellular uptake of the proteins greater than 400-fold by a process resembling pinocytosis. The authors demonstrate that the toxicity of the methotrexate-poly(L-lysine) conjugate in methotrexate-resistant cells is a result of the intracellular release of the drug following uptake of the conjugate. In a more recent publication, Shen and Ryser (458) have demonstrated a model of a pH-sensitive linkage which may be useful in releasing drugs from lysosomotropic conjugates. They conjugated daunomycin to poly(D-lysine) by using cis-aconitic acid as a spacer conjugated to a carbodiimide-linked poly(D-lysine). The authors conclude that the conjugate enters the cells and reaches the lysosomal compartment, at which time the acid milieu causes the cis-aconityl spacer to release the daunomycin from the poly(D-lysine). Under acid conditions, the daunomycin molecule is uncharged (see Ref. 487) so that it can readily leave the lysosome to exert its toxic effect within the cell.

Molteni (337) has proposed the use of dextrans as drug carriers. He has reported on the covalent attachment of a wide range of therapeutic agents, ranging from insulin to vitamins to antitumor agents and a number of enzymes. The use of dextrans is attractive because of their repeating sequence and multitude of cross-linking possibilities. Although short-chain dextrans have seen much use in medicine as plasma expanders, it should be pointed out that longer chain dextrans are highyl immunogenic and the attachment of haptens, either small drug molecules or more complex proteins such as enzymes, may be counterproductive in terms of increased immunogenicity. Poznansky and Cleland (398) describe the use of a number of other synthetic polyamino acids as drug and enzyme carriers.

Plasma proteins, including albumin discussed in greater detail below, offer attractive possibilities as enzyme and drug carriers. A potentially valuable point is the fact that they are natural plasma constituents and as such their conjugation to other proteins or drugs may, under some conditions, allow the foreign hapten to remain in the plasma either as a "family member" of plasma proteins or else in the best tradition of the "Trojan horse." Serum proteins other than albumin and immunoglobulins have been used largely as carriers of cytotoxic antitumor agents in order to avoid the rapid removal from the organism via the kidney. Several authors (see Ref. 398 for a review) have examined the potential use of plasma fibrinogen as a carrier of antitumor drugs such as methotrexate and phosphoramide dichloride in an effort to produce a preferential deposition of the drug-conjugate in the region of the tumor cells. They demonstrated an increased therapeutic index for the conjugates and suggested that the fibrinogen may target to regions of increased tumor cell growth as a result of an increased fibrin content of newly formed vascular beds associated with rapidly growing tumors. We have not been able to find any follow-up to this published work.

Low density lipoproteins (LDL) have been proposed as potential drug carriers to direct therapeutic agents to specific cell types (extrahepatic and nonreticuloendothelial tissue) possessing specific high affinity receptor sites for the LDL molecule. Williams and Murray (567) have examined the feasibility of using LDL-bound alpha-glucosidase as a means of enzyme replacement in a patient with Pompe's disease (type II glycogenosis). Although their clinical trial in a terminal patient was not successful in terms of increasing the life span of the patient or indeed in observing any significant decrease in muscle (either cardiac or respiratory) glycogen levels, they did make several interesting observations. They detected no antibody formation to either the LDL molecules or to the enzyme (from human liver), although the number of infusions was limited. It might therefore be premature to conclude that no new antigenic determinants were formed as a result of the cross-linking procedure. Following the third infusion of enzyme-LDL conjugate, the authors reported a threefold increase in the muscle enzyme, but this represented only 13% of the normal muscle enzyme activity. It is virtually impossible to assess this approach to enzyme therapy on the basis of a single series of infusions in a single patient. It would be important to determine the targeting efficiency of

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such a system in animal experiments to better define dose requirements and administration regimens.

One of us (M. J. P.) has been especially interested in the use of enzymes in medicine and the limitations resulting from the highly immunogenic nature of most foreign proteins (see Refs. 396, 501, 539, 545 for reviews of enzyme therapy and immobilized proteins). Rapid bioinactivation following administration and problems associated with delivery to specific sites are also major concerns. Paillot and colleagues (370) were able to conjugate uricase and L-asparaginase with an excess of albumin to form a stable product which showed increased resistance to heat denaturation. Carrying on from these experiments, we were able to show that in addition to stabilizing the enzyme, the albumin was also able to mask the antigenic determinants on the conjugated enzyme. In our first report (413), we stressed that the loss of antigenicity and immunogenicity of hog liver uricase in the conjugated form with albumin did not mean that this was a general rule applying to all enzymes similarly conjugated. However, we have now conjugated eight different enzymes with albumin, and in each case we were able to mask the antigenic sites of the enzyme while retaining enzyme activity (396, 399–401, 574). The conjugated albumin is also able to alter the pharmacokinetics of the conjugated enzyme. To take the most dramatic case, the enzyme superoxide dismutase, a potential candidate for the treatment of certain types of rheumatoid arthritis, has a half-life in the circulation following intravenous administration of less than 1 min. Following conjugation with an excess of albumin, approximately 10 albumin molecules per enzyme molecule, the conjugate has a half-life in the circulation as high as 12 hr (574). Such a system, however, would primarily benefit enzyme deficiency diseases manifested by substrate accumulation in the plasma. Many enzyme deficiency diseases are in fact lysosomal in nature, resulting in intracellular accumulation of substrate in what are believed to be secondary lysosomes.

We have made attempts to direct enzymes to specific cell types by the use of ligands, such as antibodies and hormones, which bind to specific cell surface receptors [see Poznansky (396) for a review]. Antibodies produced against rat hepatocytes could be conjugated to alpha-glucosidase-albumin polymers and used to direct the conjugate to rat hepatocytes *in vivo* (397). Similarly, L-asparaginase-albumin conjugates have been targeted to RI tumor cells possessing the H-2k histocompatibility antigen by the use of monoclonal antibodies directed against that locus conjugated to the polymer (399). Targeting of the antibody-albumin-enzyme conjugate was demonstrated in both tissue culture and *in vivo* experiments (395, 396). More recently, we have demonstrated the use of insulin conjugated to either free enzyme or enzyme-albumin conjugates to target cells possessing insulin receptors (400, 401). The lysosomotropic nature of the antibody or insulin conjugates with enzyme have been verified by localizing the enzyme with a lysosomal fraction following uptake by cells both in culture and *in situ* (396, 401).

Albumin has also been used as a drug carrier (28, 138–140) in an attempt to use a lysosomotropic approach to antiviral chemotherapy. Like many other cytotoxic agents, these inhibitors of DNA synthesis are highly toxic to rapidly dividing cells such as stem cells of the bone marrow and cells of the digestive tract. The rationale in the use of albumin-drug conjugates was the assumption that the conjugates would be localized to cells with a high protein intake, that is cells like macrophages where the DNA viruses would also be concentrated. This might then spare rapidly dividing normal cells whose rate of exogenous protein uptake is generally slower. The authors demonstrate that drug-albumin conjugates are effective in inhibiting virus growth in liver macrophages at drug doses at which free drug is ineffective. In more recent experiments, this same group (139) has coupled antiviral agents to asialofetuin in an effort to target the drugs to hepatocytes via the Ashwell receptor. This would then inhibit virus growth in the parenchymal cells of the liver as the albumin-drug conjugates appeared to be able to arrest viral replication in the Kupffer cells of the liver. The authors demonstrate excellent inhibition of virus DNA synthesis in both *in vitro* and *in vivo* experiments with Ectomelia-virus-infected mice. Bhardwaj *et al.* (36) have cross-linked the iron chelating drug, desferrioxamine (DF), to albumin in an effort to avoid urinary clearance of the DF while delivering it to cells of the RES, the initial site of iron overload in patients suffering from transfusion iron overload.

Abuchowski and coworkers (2, 3) have reported on the use of polymeric conjugates of a large number of enzymes with PEG. These results are very similar to those demonstrated by Poznansky (396) for albumin-enzyme conjugates. The conjugates are nonimmunogenic and highly resistant to heat denaturation and proteolytic degradation. The enzyme kinetic parameters are only slightly changed (decrease in V_{max} and some increase in K_m) following conjugation with PEG and the ability to remain in the circulation may be greatly enhanced. The pH profiles of the immobilized (but soluble) enzymes may be altered as a result of their conjugation with either PEG (2) or albumin (395) in a manner demonstrated by Goldman *et al.* (172) for the conjugation of papain to solid supports. Both Abuchowski (2) and Poznansky (396) have been especially concerned with the question of the immunological reactivity of administered enzyme, often, although not nessarily, from foreign sources. Two possible solutions have been suggested. The first simply attempts to mask the antigenic determinants of the foreign protein so as to suggest to the recipient organism that the complex is really "self" by virtue of the fact that the "foreign" antigenic determinants are hidden. The

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second seeks to induce tolerance by suggesting to the recipient that the foreign antigen attached to a known "toleragen" is in fact "self" and should be recognized as such so that no immune response should be mounted against it. The question of tolerance is complex and has not yet been adequately explained. The toleragenic nature of several biological and synthetic macromolecules has been established [PEG, Abuchowski *et al.* (3); Sehon and Lee (451); carboxymethyl cellulose, Diner *et al.* (109); IgG, Borel (47); albumin, Poznansky (396)]. It may be that the use of such toleragens may be necessary if the repeated administration of foreign protein molecules or even small haptenic groups is to be considered.

Albumin and other plasma proteins, as well as certain synthetic polymers, appear to offer several important advantages as drug/enzyme carriers. They appear to have the capacity to avoid rapid clearance from the circulation via the RES, a fate that comes rapidly to many of the other carrier systems such as liposomes, microcapsules, and even albumin microspheres. This property not only allows for a prolonged circulation lifetime for the various conjugates, but in avoiding cells of the RE system, the possibility of reaching other targets such as tumor tissue, muscle or other parenchymal tissue is enchanced. The immunological problems of drug or enzyme-carrier conjugates have been dealt with successfully in studies with albumin and PEG and this might be very important if repeated treatment with highly immunogenic enzymes or haptens is contemplated. The question of targeting these complexes beyond the vascular space has been examined by Poznansky (396) by using both monoclonal antibodies and hormones with some limited success. It is clear, however, that a more detailed understanding of the transport mechanisms whereby biological macromolecules normally leave the vasculature for underlying tissues will make the design of such carrier systems more practical and efficient.

I. Prodrug Delivery Systems.

A prodrug is an agent which must undergo biotransformation prior to exerting its pharmacological effect (6). If the pharmacodynamic characteristics of the prodrug are different (and more desirable) than those of the drug itself, then the prodrug can constitute a "drug delivery system." There is extensive literature on prodrug development and several excellent reviews have been devoted to this topic (486, 357, 42). In most cases, prodrug development has occurred with the aim of solving specific pharmaceutic or pharmacological problems. This could include improvement of drug esthetics (taste, odor), improved water solubility, or enhanced absorption via the oral route. The use of the prodrug approach in connection with these problems has been discussed extensively by Stella *et al.* (486).

The problem of site specific drug delivery with the prodrug approach is only now beginning to be considered, but nonetheless, is clearly an exciting prospect. The relatively simple problem of enhanced local delivery of drugs has received the most attention thus far. Beta-adrenergic prodrugs have been used to enhance sympathetic mydriatic effects in the eye. Prodrug approaches have also been used to enchance dermal permeation of corticosteroids and other drugs and thus facilitate the use of the transdermal route for delivery. These topics have also been reviewed by Stella *et al.* (486).

Site specific delivery of a drug from the systemic, circulation is the most challenging and potentially the most important problem that may be susceptible to the prodrug approach. An extremely clever and apparently successful example of this has recently been described by Bodor and his colleagues (45, 44). These workers have used a chemical approach to achieve brain specific delivery of neuroactive drugs such as dopamine and phenylethylamine (see figure 4). This approach involves preparing a quaternary adduct of the drug and then reducing this so as to improve its lipophilicity and thus its ability to penetrate the blood-brain barrier. The reduced quaternary compound is then enzymatically reoxidized *in vivo* (both systemically and within the CNS); the resultant quaternary compound is rapidly excreted from the systemic circulation, but, because of its ionic character, is trapped in the CNS. Enzymatic liberation of the drug from its quaternary adduct is ensured and a sustained, CNS-specific delivery of active compound may be achieved. This is an extremely interesting approach and one wonders whether it is susceptible to some generalization. It may be possible to design prodrugs to take advantage of unique characteristics of particular organs (permeability, enzyme levels, pH) and thus create other, novel, organ-selective drug entities.

J. New Horizons in Controlled Drug Delivery

In this section we discuss several recent, tentative, but potentially exciting, approaches for the development of site specific controlled drug delivery.

1. Targeting to Cellular Carbohydrate Binding Proteins (Lectins). An exciting idea of relatively recent vintage is the notion of using oligosaccharides to target drugs to particular cell types via the carbohydrate-binding proteins (lectins) known to exist on mammalian cell surfaces. Carbohydrate-binding proteins of plant origin have been know for many years and have been widely used in cell biology and hematology (174, 459). In the last 10 years, however, it has become apparent that mammalian cells also possess lectin-like proteins which can avidly bind specific carbohydrate determinants [review by Neufeld ad Ashwell (348)]. These mammalian lectins are present in the plasma membrane and can engage in endocytosis as well as binding of appropriate ligands. At present six to seven vertebrate lectins have been described including: 1) the galactose (asialoglycoprotein)-binding lectin of mammalian hepatocytes (23); 2) the N-acetylglucosamine lectin of avian hepatocytes (304); 3) the mannosyl-6-phosphate (M-6-P) lectin of fibroblasts

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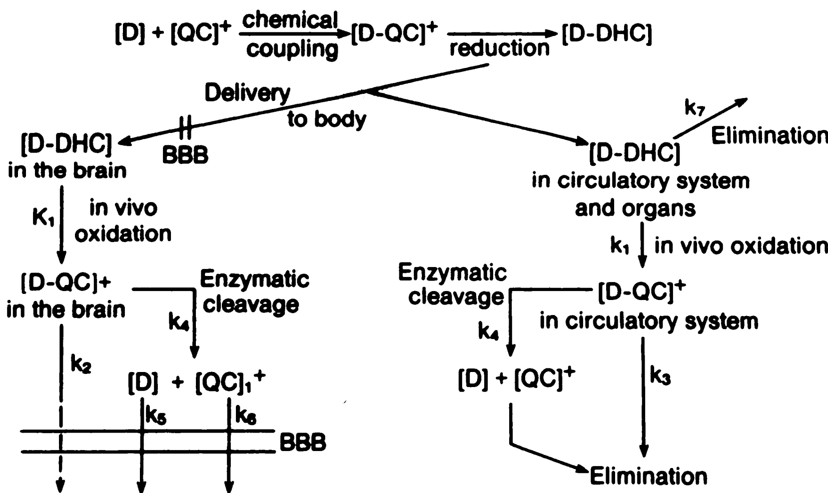

FIG. 4. Prodrug approach to brain specific drug delivery. This figure describes the production of a prodrug D-QC (D, drug; QC, quaternary carrier) which when reduced to a dihydroform (D-DHC) becomes lipid soluble allowing for penetration of the blood-brain barrier (BBB). In vivo oxidation (K_1) of the reduced carrier will return the complex to its original ionic, hydrophilic state thus in effect trapping it within the brain. The complex then can act as a sustained release system following enzymatic cleavage (K_4) and release of free drug and carrier. Once cleaved the drug is of course free to move past the blood-brain barrier (K_5) but at a rate dependent on its own permeability coefficient. [From N. Bodor *et al.* (44a)]

(243); 4) the mannosyl-N-acetylglucosamine-binding lectin of RE cells (482); 5) a fucose-binding lectin of hepatocytes (403); 6) several galactose (Gal)-binding lectins (which may be related) have been described in mammalian lung and heart (106), chick myoblasts (101), chick brain, and electroplax organ (505). The true biological functions of these lectins is unknown at present, but several possibilities have been suggested. The M-6-P lectin may be involved in the packaging of hydrolytic enzymes into lysosomes (438); the Gal-binding lectin of hepatocytes may be involved in the clearance of circulating glycoproteins and glycopeptide hormones (23); and finally there is a good deal of suggestive evidence pointing to an involvement of mammalian lectins in the cell-cell recognition processes which are important in development.

Irrespective of the biological role of these cell surface lectins, the idea of using them as "targets" for drug delivery has considerable appeal. Presumably, a drug conjugated to a specific oligosaccharide structure might bind and be internalized in cells having a complementary membrane lectin, but not in other cells. A positive aspect of this approach is that lectins do display considerable specificity towards carbohydrate structures. Although lectins are usually classified in terms of their binding to the terminal sugar residue of a saccharide structure (e.g. Con A is a mannose-binding lectin, while ricin is a galactose-binding lectin), they, in fact, discriminate the sequence and anomeric linkages of several subterminal sugar residues as well. Thus, while Con A will bind many mannosyl-terminated oligosaccharides, it binds much more avidly to saccharides with a biantenary structure than to those with tri- or tetra-antenary structures (344). Therefore, although several tissues might have lectins with the same general specificity (e.g. Gal-binding lectins in liver, brain, heart, lung) it may be possible to construct an oligosaccharide that would bind to one but not all of these lectins.

Another positive feature of this approach is that the drug carrier would be relatively compact. Oligosaccharide structures typically range in molecular weight from 500 to 5000 daltons; the presence of one or more conjugated drug molecules would add another 500 or so daltons. This contrasts with molecular weights of 160,000 for immunoglobulin or millions for liposomes and polymeric microspheres. Because of the relatively small size of the hypothetical oligosaccharide drug carriers, they would not be as greatly affected by the barriers to drug delivery presented by the capillary endothelium and basement membrane, nor would they be likely to be taken up nonspecifically by RE cells.

A major limitation of this approach derives from the formidable chemical complexities involved in the preparation of oligosaccharide drug carriers. At present the stereospecific synthesis of complex oligosaccharides is not at a practical state and at best only mono- or disaccharide-drug complexes, or complexes between drug and simple repeating polymers (e.g. dextrans) have been achieved (see Ref. 398 for earlier references on drug-dextran conjugates). Another approach to the generation of oligosaccharide carriers might be their preparation by exhaustive protease digestion of glucoproteins; here one would, however, encounter the problem of microheterogeneity of glycopeptide structures (270). Another difficulty with this approach is that the repertoire of cell type specific membrane lectins is currently rather limited. Biochemical investigation of lectin-like activities in a variety of normal and tumor cells would seem to be a

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prerequisite to intelligent use of oligosaccharide-mediated targeting for cancer therapy for example.

Despite the above-mentioned limitations, several workers have used conjugation with carbohydrate residues to alter the cellular uptake and/or *in vivo* distribution of proteins or drugs. An early report of this type was that of Rogers and Kornfeld (424) who modified the *in vivo* clearance behavior of proteins by coupling them to fetuin glycopeptides. Subsequently Lee and colleagues (273) used thiol analogs of sugars to produce "neoglycoproteins" multiply derivatized with simple sugar residues. These neoglycoproteins have been used by several investigators to modify the cell uptake and *in vivo* behavior of proteins (570, 481). Fiume *et al.* (138) have targeted antiviral drugs to hepatocytes *in vivo* by coupling the drug to a neoglycoprotein consisting of albumin deriviatized with galactose; this material apparently binds avidly to the hepatic galactosyl lectin. Youle, Neville, and their colleagues have conjugated ricin, a potent toxin, with monophosphopentamannose thus obtaining toxin uptake into fibroblasts *in vitro* via the M-6-P receptor (583, 584). Herschman and his colleagues have used toxins conjugated with asialofetuin to effect selective killing of cultures hepatocytes through uptake of the conjugate via the hepatic galactosyl lectin (60, 61, 471). Doebber *et al.* (110) have enhanced the uptake of glucocerebrosidase by macrophages *in vitro* and *in vivo* by conjugating the enzyme with trimannosyldilysine, a synthetic glycopeptide that can interact with the macrophage mannoysl lectin. For a more complete discussion Shen and Ponpipom (456) have reviewed the possible use of synthetic glycopeptides to target drugs.

Although this approach is at a very preliminary stage of development, it seems to warrant further exploration since it offers the possibility of high selectivity with avoidance of some of the barriers that constrain the use of high-molecular-weight particulate carriers.

2. *Pharmacologically Active Antireceptor Antibodies.* The basis of much of pharmacology revolves around the interactions of drugs with their specific receptors. While some drugs seem to bind selectively to only one type of receptor, the more common situation is that a drug whose primary action is on one class of receptor will also interact with other receptor classes as well (244). Common examples of this might include the cholinergic actions of antihistamines and the effect of epinepherine on both alpha- and beta-adrenergic receptors. In contrast to the relative lack of receptor specificity of most drugs, antibodies can be exquisitely sensitive to subtle alterations in protein structure and can readily discriminate changes as small as a single amino acid substitution. Thus the use of antibodies to explore drug binding sites on receptors and perhaps to aid in the preparation of more specific drugs would seem to be an intriguing proposition. Until recently, however, the path to obtaining antireceptor antibodies involved first isolating (or at least partially purifying) the receptor by laborious biochemical procedures. Now increased understanding of immune regulatory phenomena suggests that investigators should be able to "build" antibodies reactive with the active sites of receptors by working backward from the structure of the drug molecule itself. This approach is based on the regulation of immune response by anti-idiotypic antibodies.

Immunologists have known for some time that the unique heavy- and light-chain variable regions, which are part of the antibody combining site for a particular antigen, can themselves serve as immunogens and elicit additional antibody production. The unique determinants of the antibody combining sites are called idiotypes and the antibodies they elicit are termed anti-idiotypes (for review see Ref. 421). The idiotype-anti-idiotype response can take place between animals that are syngeneic or that are closely matched in terms of allotype, as well as across species lines. Since anti-idiotype antibodies are reactive with determinants in the antibody combining site, they are, at least in some cases, complementary in structure to that site; that is, the anti-idiotype combining site stereochemically resembles the original antigen. For example, if one were to raise, in rabbits, antibodies to the dinitrophenol antigen (DNP), affinity purify these antibodies on a DNP-agarose column, and then inject these antibodies (let's call them Ab1) into a closely related (i.e. allotypically matched) rabbit, the antibodies produced (let's call them Ab2) would largely be directed against the DNP-combining site of Ab1 and thus be anti-idiotype antibodies (see Figure 5). In some cases the Ab2 molecules would be able to compete with DNP itself for binding to Ab1, that is the combining site of Ab2 would "mimic" the structure of DNP. It whould be noted, however, that it is not really necessary to physically separate Ab1 and reinject it in order to get an anti-idiotypic response. The injection of an antigen and consequent antigen-specific antibody production will often elicit the production of additional antibodies that react with the idiotopes on the first antibody. In fact Jerne (230) has postulated that the overall immune response is regulated by a network of interactions among lymphoid cell clones producing primary antibody, anti-idiotype antibody, anti-anti-idiotype, etc., etc.

Several groups have now prepared antibodies to the active sites of drug receptors by using variations of the following strategy, First an analog of the drug of interest is synthesized which can be covalently linked to a protein carrier. The drug-protein conjugate is then used to immunize animals to produce antidrug antibodies which are subsequently purified on a drug affinity column. These antibodies are then used to immunize other animals to produce anti-idiotypic antibodies which minic the drug and bind to the drug receptor. Thus Wasserman *et al.* (544) have used a potent cholinergic agonist to prepare anti-acetylcholine receptor antibodies in rabbits.

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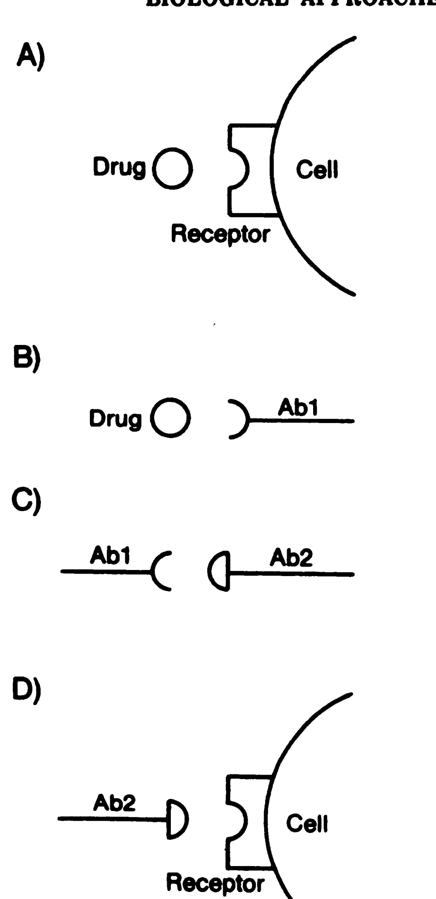

FIG. 5. Molecular mimicry by the immune system: Antiidiotype approach to receptor site specific antibodies. Anti-idiotype antibodies (Ab2) may be used to either mimic the action of a drug or ligand or else to bind to the drug (may in fact be a hormone or other peptide) receptor. The antibodies may thus act as effector substances themselves or as highly efficient targeting agents even without the necessity of purifying or identifying the specific receptor. The rationale for this approach is found in the text.

These antibodies bound to several purified acetylcholine receptor preparations, and also seemed to produce a myasthenic syndrome in some of the rabbits. The anti-idiotype route has also been used to prepare antibodies to adrenergic receptors by Homcy *et al.* (206) and by Strosberg *et al.* (492). An interesting finding from Strosberg's group (85) is that the antireceptor response is quickly followed by an anti-anti-idiotype response. Furthermore, these tertiary antibodies bind the drug, and the anti-idiotype and anti-anti-idiotype responses seem to cycle alternately.

Anti-idiotype approaches have also been used to obtain antibodies to peptide hormone receptors. In some ways this is a simpler proposition since no special conjugations are needed to produce an immunogenic molecule as is the case for low M_r drugs. On the other hand, a peptide is likely to have several immunologically active determinants, whereas only some of these will overlap with the portion of the peptide that is involved in binding to the receptor. Farid *et al.* (125) have raised an anti-thyrotropin receptor antibody which inhibited thyrotropin binding activity and stimulated adenylate cyclase in thyroid membranes. In a fascinating study, Schechter *et al.* (442) showed that mice immunized with insulin develop both anti-insulin antibodies and (presumably via an anti-idiotype response) antibodies to the insulin receptor. The latter mimicked the action of insulin, including stimulation of glucose oxidation and inhibition of lipolysis in fat cells.

Recently a report has appeared concerning the preparation of monoclonal anti-acetylcholine receptor antibodies by an anti-idiotype approach (80). This is likely the first of many such interesting developments.

The work done in this area is still at a very primitive stage and several fascinating questions remain: 1) Will antireceptor antibodies made by anti-idiotypic mimicry of an agonist have only agonist activity or will structures with antagonist activity arise as well? 2) Can this technique generate antibodies with higher affinity for the receptor than the original drug molecule? 3) Can this technique be used to generate antibodies which are more selective in stimulating (or blocking) a particular receptor than the original drug molecule? Most of the work to date has been with highly purified drug or hormone as the starting material. However, one could conceive of a "leapfrog" approach using partially purified hormone, growth factor, lymphokine, or neuropeptide as immunogens to generate neutralizing monoclonal antibodies. These would presumably react with sites on the peptide involved in receptor binding; the first monoclonal could then be used to raise polyclonal or monoclonal anti-idiotype antibodies which might be reactive with the receptor for the peptide.

Even based on the relatively small amount of data available thus far, it seems quite clear that the anti-idiotype approach will provide a fascinating array of reagents to probe pharmacologically interesting receptors. This may also add to our understanding of the molecular basis of drug specificity. The question of whether antireceptor antibodies (or antibody fragments) could serve as highly selective druglike molecules awaits future development.

3. Oligonucleotides as Drugs. Our rapidly expanding knowledge of mammalian molecular biology has allowed us to explore some of the most fundamental aspects of cell function and differentiation. Subtle distinctions between normal and neoplastic cells, or between subsets of functionally differentiated cells are now seen to involve the replication and expression of particular sequences of nucleotides, including both structural genes and controlling elements. If one could develop agents that react with specific nucleotide sequences within the genome, then these might be able to regulate cell function at the most basic level.

One approach to this problem involves the development of oligonucleotide analogs as drugs. These analogs

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would have to meet several very stringent criteria including: 1) absolute retention of Watson-Crick base pairing specificity; 2) lipophilicity for easy penetration of cell membranes; and 3) resistance to degradative enzymes. These goals have been pursued very vigorously by P. Ts'o and his colleagues, who have synthesized a number of nonionic nucleic acid methyl phosphonate analogs (229, 335). Although none of these agents could be considered to be "gene specific" thus far, several of them seem to have interesting biological effects which relate to their ability to bind to cellular nucleic acids. In one case (229), a set of oligonucleotides complementary to a sequence found on bacterial 16S ribosomal RNA was able to inhibit protein synthesis in an *Escherichia coli* system but not in a rabbit reticulocyte system.

This approach entails several difficult synthetic and experimental problems; nonetheless it represents an attempt to target agents at the most fundamental level of cell function and deserves further exploration.

IV. Conclusions

In this review we have considered a variety of strategies and technologies for the controlled, selective delivery of drugs. Clearly the problem of controlled drug delivery has fascinated scientists from a variety of backgrounds and disciplines and has elicited many ingenious responses. In examining this rich store of information we can come first to some very general conclusions and then to a consideration of the virtues and liabilities of some of the individual approaches. First, it is very clear that no one technology will prove to be a panacea with near universal applicability to the general problems in drug delivery. Rather, a successful outcome will no doubt often be a matter of matching up a particular therapeutic problem or a particular class of drugs with the most appropriate delivery system. Second, all of the approaches discussed above are limited to some degree by the various barriers to selective delivery which we considered in section II. These limitations may include physical exclusion of the drug from certain body compartments, inappropriate cellular uptake or processing of the carrier, biological modulation of the target site, or problems due to toxicity or immunogenicity of the carrier or drug-carrier complex. Third, there has been surprisingly little attention given to the pharmaceutical characteristics or suitability of most of the more sophisticated biologically oriented drug delivery systems. Questions of long-term stability, reproducibility, cost of manufacture, and so forth, have really not yet been addressed in the literature although they are of great importance in ultimately translating concepts of controlled drug delivery into therapeutic and commercial realities. Perhaps investigators should, at a fairly early stage of their work, give some consideration as to how their "pet" system would be deployed in a clinical setting rather than in the research laboratory.

Let us now consider some of the systems and technologies described above with respect to our evaluation parameters (section I), namely potential for selectivity, load factors, immunogenicity, toxicity, applicability to disease processes, and pharmaceutical feasibility. We will also consider the "barriers" that are most limiting for particular approaches.

1. Microparticulate Carriers (Liposomes, Microspheres, Red Cells). In many respects these technologies can be considered together, although each clearly has some unique characteristics. All of the microparticulate carriers are constrained by the endothelial barrier to remain within the circulation. They are cleared by the phagocytic cells of the RE system at a rapid rate. Thus they seem ideally suited for delivery of drugs to phagocytic cells for purposes of immune modulation or treatment of infectious disease. Microparticulate carriers are also quite suitable for localizated therapy such as intra-arterial infusion to particular organs, or injection into joints or other body spaces. It seems rather unlikely that a high degree of target selectivity can be built into these particles (aside from RE system uptake), although gross manipulation of regional distribution may be possible by using techniques such as "magnetic steering."

Liposomes, protein microspheres, and cells are all relatively innocuous in terms of short-term toxicity, although questions of chronic toxicity still have to be seriously addressed. Clearly all microparticulate materials have the potential to be immunogenic and to elicit responses both to the drug and to the carrier moiety. Use of "self" type materials (e.g. common neutral phospholipids in liposomes, syngeneic proteins for microspheres) should reduce, but not eliminate immunogenic potentials.

Microparticulate carriers are likely to enjoy wide use in a variety of infectious and neoplastic conditions as well as some forms of enzyme replacement therapy. It should be possible to formulate stable, uniform preparations of protein microspheres and liposomes on an industrial scale, although some concerns about size heterogeneity and stability of drug trapping remain for liposomes. Large quantities of drugs can be incorporated per unit weight of carrier, especially for the protein microspheres. Cellular carriers are unlikely to be amenable to industrial scale development and are likely to remain largely a research tool or, at best, limited to experimental clinical settings.

2. Antibody Carriers. Antibodies are capable of exquisite selectivity and thus may appropriately be used to target highly toxic drugs. Unlike the microparticulate carriers, antibodies can leave the circulation, by transcytosis or other means, and enter the interstitial fluid and lymph. Although antibodies (particularly monoclonal) can bind with great selectivity to molecular "targets" in certain cells, this may or may not constitute an effective means for drug delivery. Target antigens may be shed from the cell or undergo other types of modulation.

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Another concern is the degree to which the target antigen is linked to cellular mechanisms for endocytosis and protein sorting, since as we have seen, some surface antigens are much better targets for drug delivery than others. Another problem of the antibody as carrier concept concerns the load factor. It may be difficult to couple adequate amounts of drug to an antibody without denaturation. The use of toxins rather than conventional drug solves this problem, but may be too much of a good thing since the nonspecific uptake of even tiny amounts of the toxin can be lethal.

Conjugates of drug or toxins and antibodies are likely to be highly immunogenic, indeed this has been the primary limitation of this type of therapy thus far. This will continue to be true even when human monoclonals become available, since the conjugated drug or toxin will still be highly immunogenic even if the antibody carrier is not.

The prime arena for antibody carriers will likely be in cancer therapy, particularly the difficult problem of treating metastatic disease. Since antibodies have long been manufactured commerically for diagnostic purposes, the development of suitable formulations of therapeutic antibodies should not be a major problem.

3. Prodrugs. In some respects the prodrug approach is one of the more attractive concepts in the drug carrier field. Small prodrug molecules would be expected to have excellent penetration into most tissues avoiding the limitations presented by the endothelial, basal lamina, and RE barriers, which markedly restrain the distribution of microparticulate carriers. At present, there are few reported attempts of utilizing biological information to produce selective prodrugs in terms of specific binding to cellular or molecular targets. However, there is every reason to expect that a fusion of chemical and biological expertise can lead to the evolution of prodrugs with a high degree of target specificity.

Another attractive feature of the prodrug approach is the load factor; thus active drug usually comprises a very appreciable fraction of the mass of a prodrug entity. This contrasts sharply to the tiny mass fraction of active drug in an antibody-drug conjugate. Since prodrugs are usually small molecules there is no reason to expect them to be immunogenic, unless reactions with host proteins occur. Evaluation of the toxicity of a prodrug will necessitate consideration of both the active drug moiety and the "targeting" moiety as well. One of the most positive aspects of the prodrug approach is that it represents an extension of conventional medicinal chemistry and pharmaceutical development. The process of formulating and manufacturing prodrugs should pose no more problems than conventional drug entities.

4. Macromolecular Carriers. This approach seems most highly developed in the area of enzyme replacement therapy where conjugation of certain enzymes to serum proteins can greatly enhance their desirable physiological and pharmaceutical characterisitics. Larger synthetic polymers or macromolecular aggregates (greater than 500 Å diameter) are likely to be constrained by the endothelial barrier and be cleared by phagocytic RE cells, while small macromolecular carriers may have good penetration into tissue fluids and be relatively unaffected by RE clearance. Reduced immunogenicity and toxicity seem to be among the major benefits of conjugating exogenous enzymes to syngeneic serum proteins. Attempts are currently underway to enhance the selectivity of macromolecular carriers by the coupling of specific ligands, such as hormones or antibodies.

5. Newer Technologies. Some of the newer approaches, such as use of antireceptor antibodies or sequence-specific polynucleotides, are exciting because they seem to go to the heart of the question of drug selectivity. The key to selectivity is the design of agents which will intervene in pathogenesis at a very fundamental level and permit discrimination between subtly different cellular macromolecules. The closer one moves to the fundamental pathogenic lesion, the more likely one can divise a truly selective therapy. As we learn to recognize genomic sequences essential to the maintenance or expression of neoplastic transformation, we can begin to identify drug targets that are fundamental to malignancy and not just peripheral manifestations. As we more precisely discriminate similar but unique receptor molecules, we can begin to manipulate endocrine or paracrine processes in specific cell populations. Thus use of the tools of molecular biology and of molecular immunology to delineate the structure and function of fundamentally important proteins and nucleic acids may constitute the true frontier in the field of drug delivery systems.

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