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Short Review

Antibodies as Specific Carriers for Chemotherapeutic Agents

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Introduction

One of the therapeutic needs in cancer treatment is the development of a pharmaceutical agent that possesses not only potent toxicity but also specificity for the neoplastic cells. Many attempts have been made to achieve this goal. The demonstration of tumor antigens on malignant cells [13, 39] led to high hopes of production of specific antitumor antibodies that, according to the known function of antibodies to bacteria, would be able to recognize and destroy tumor cells. Thus, workers anticipated that antitumor antibodies might be useful for serotherapy in cancer. Indeed there are examples showing that passive transfer of immune serum to tumor-bearing hosts was capable of causing tumor regression in some animal tumor systems [11, 20]. Unfortunately, these favorable results are not universally observed. In other systems, especially in human cancers [35, 21, 10, 54], convincing evidence of the efficacy of serotherapy in the treatment of human cancers remains to be demonstrated.

In spite of this uncertainty about the function of antitumor antibodies, there is some solid evidence demonstrating that antitumor antibodies can be eluted from certain tumor cells, and that these eluted antibodies can be specifically bound to the corresponding tumor cells in vitro [8, 19, 48]. These findings indicate that, at least in certain types of tumors, specific antitumor antibodies against them have been induced and were subsequently bound to the tumor cells in vivo. Although the presence of antibodies on tumor cells was clearly demonstrated, the reason for the lack of cytotoxicity of these antibodies to tumor cells remains obscure. Nevertheless, the fact that antitumor antibodies can be specifically bound to tumor cells provides an unique tool for targeting the tumor cells with tagged chemotherapeutic agent on the antibodies. It is hoped that with this strategy the maximum therapeutic index will be achieved, since the tagged cytotoxic agent would be 'homed' exclusively to the target tumor cells and its nonspecific detrimental effect on the host would be minimized.

The concept of using specific carriers such as antibody for delivering toxic agents to target cells has gained popularity in recent years because many results, especially from studies of tumor suppression, have demonstrated the possible use of this strategy for clinical trials of immunochemotherapy. It should be noted that several nonspecific carriers such as hormones [52], serum proteins [50], and DNA [51] have also been tested for the same purpose. However, in this short review, only the potential application of the conjugates of chemotherapeutic agents and antibodies will be discussed.

A. Antibodies Specific to Tumor Antigens as Carriers

A pioneering study with this approach was performed by Mathé et al. [34]; the conjugate was formed by coupling between methotrexate (MTX) and antibodies specific to murine L1210 leukemia, achived by means of a diazotization process. It was found that in leukemia-bearing mice treated with MTX-antibody conjugates, survival was significantly better than in those treated with either antibody or drug alone. It was also demonstrated later [40] that conjugates derived from carboxamide formation between the carboxyl residue of MTX and the amino group of antibody were of slightly more therapeutic value in the suppression of tumor growth than the conjugate made by azo linkage of the same materials.

To produce more potent conjugates, it is desirable to synthesize a conjugate with a high molar ratio of drug to antibody, provided that the antibody activity is not al-

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tered. Burstein and Knapp [4] reported that when a modified mixed anhydride procedure was used for the formation of conjugate between MTX and rabbit antibodies raised against mouse ovarian carcinoma, the molecular ratio of MTX to antibody in this conjugate could be as much as 120 : 1. Mice bearing the ovarian carcinoma were treated with these MTX-antibody conjugates, and their survival times were significantly increased over those in several control groups.

Daunomycin, a potent antineoplastic agent, has been covalently linked to antitumor antibodies by two different procedures [22, 32]. The first procedure involved the oxidation of drug, followed by coupling of the oxidized drug with antibody and then reduction of the product. In the second procedure a bridge agent, glutaraldehyde, was used to link the drug and antibody, forming a stable conjugate. These daunomycin-antibody conjugates have been shown in vitro to retain both the drug and the antibody activities. In studies along the same lines, in which Fab dimers of antitumor antibodies were used as carriers to couple with daunomycin, a cytotoxic activity of the resulting daunomycin-$(Fab')_2$ conjugates against tumor cells was demonstrated [23]. The advantage of using Fab dimers instead of intact antibody molecules for drug carriers is that the Fab dimers are less immunogenic for a xenogeneic host, since they do not possess the Fc fragment, which is highly antigenic.

The potency of inhibition of tumor growth by daunomycin-antibody conjugates has been demonstrated in vivo in two murine tumors [24]. For suppression of plasmacytoma development in mice, these conjugates showed better results than either antibody alone or daunomycin coupled to irrelevant antibodies, but neither was better than free drug. It was noted that the highest efficacy for tumor suppression was observed when daunomycin was given in a mixture with specific antibodies without covalent linkage between them. In a lymphoma system, the daunomycin was linked to specific antibody via a dextran bridge, to give a higher molar ratio of drug to antibody in the conjugates. Again, the best results were obtained in a group of animals that had received the treatment with a mixture of daunomycin and antibody. However, in a low dose range, the advantage of daunomycin-antibody conjugates over either free daunomycin or daunomycin coupled to normal immunoglobulin was observed. The overall impression from the results of these studies is the superiority of the mixture of free antibody and drug over daunomycin-antibody conjugates; the latter, however, are still better than the free antibody or free drug alone. A possible deleterious effect of the conjugation procedure could conceivably account for the loss of some potency of the conjugates, since their activities with respect to either drug or antibody were reduced by about 20%.

The alkylating agent trenimon (2,3,5-trisethylenimino-1,4-benzoquinone) was also used to conjugate covalently with partially reduced antitumor antibodies [33]. This conjugate exhibited both alkylating and antibody activity and was shown in vitro to have specific cytotoxicity for the tumor cells that were used to raise the antitumor antibodies. These results would be more convincing if some control groups, including animals treated with free trenimon alone, antibody alone, and mixtures of the two, were also evaluated.

Although the conventional methods for coupling of drug with antibody by cross-linking agents are simple, the number of drug molecule that can be covalently bound to the antibody by these methods without affecting its activity is limited, and those conjugates with low substitution of drug on antibody are frequently therapeutically ineffective. Attempts to increase the drug substitution by varying the method, e.g., by increasing the concentration of cross-linking agent or prolonging the reaction time, usually produce a conjugate with a complete loss of antibody activity. To overcome this limitation in the methodology of coupling with a high molar ratio of drug to antibody, a multivalent 'intermediate' with a high capacity for drug substitution was introduced to provide a link between the drug and antibody. This conjugate with high drug content has been shown to retain the antibody activity. A polyanion, polyglutamic acid (PGA), has been used for this purpose. It was shown that a nitrogen mustard derivative, *N,N*-bis-(2-chloroethyl)-*p*-phenylenediamine (PDM), could be linked to rabbit antibody against mouse EL4 lymphoma via PGA. The PDM-PGA-antibody conjugates with high PDM content effectively suppressed the growth of EL4 lymphoma in mice in vivo [41].

However, it was found later that the reaction using PGA as a intermediate bridge between PDM and antibody for the formation of PDM-PGA-antibody conjugates were difficult to control, as these products varied in the degree of coupling of drug-PGA to antibody in different preparations. Water-soluble dextran was introduced as an alternative intermediate bridge to overcome this drawback. In one study [42], primary amine groups of PDM were attached to dextran that had previously been activated by CNBr, and the PDM-dextran conjugates were then linked to antibodies specific to EL4 lymphoma by glutaraldehyde. In another study, dextrans of various sizes were used as intermediate bridges for linking daunomycin to antitumor antibodies [24]. Daunomycin was attached to dextran-antibodies by a series of chemical reactions, which involved initial periodate oxidation of the dextrans to polyaldehydes, which were then reacted with daunomycin and finally with antibody. The resulting Schiff bases were reduced with sodium borohydride to form stable conjugates. The daunomycin-dextran-antibody conjugates synthesized by this method have high molar ratios of drug to anti-

body, ranging from 5 to 40 moles per mole of antibody. They were more potent than free daunomycin in the suppression of the growth of YAC lymphoma in A/J mice. It is interesting to note that, in a collateral study [3], the daunomycin-dextran conjugates that lack antibody can be as efficient as the daunomycin-dextran-antibody conjugate in preventing the growth of YAC lymphoma in mice. However, severe hemorrhagic and allergic reactions have been observed after the administration of high doses of dextran in experimental animals; any attempt to use dextran as a intermediate bridging agent for drug and antitumor antibody in clinical study should therefore take this undesirable effect into consideration.

It is generally thought that for drugs to be delivered to the tumor site by antitumor antibodies, they must be linked to the antibody carriers with stable covalent bonds so that they will not be split off from the conjugates during their passage to the target site. However, several studies have shown that mixtures of drug and antitumor antibodies, or complexes of drug and antitumor antibodies without any covalent linkage between them were also capable of suppression tumor growth. Ghose and his associates [14, 15] reported that administration of a complex of the chemotherapeutic agent chlorambucil and heterologous antitumor antibodies led to a significant prolongation of the life of mice with Ehrlich ascites carcinoma (EAC) or EL4 lymphoma. These studies were based on an earlier report [25] that chlorambucil and serum proteins formed a non-covalent complex after incubation for 24 h, and this complex retained both the specific activity of antibody and the alkylating activity of chlorambucil. The beneficial effect of chlorambucil-antibody complex in the treatment of EAC was also observed by Flechner [12], who reported, in addition, that mice that had rejected the first IP inoculation of EAC were able to resist a second lethal dose challenge of EAC. These results thus indicated that tumor-specific immunity had been conferred on the mice by the treatment with chlorambucil-antibody complex. Similarly, Smith et al. [47] also showed that chlorambucil-antibody complex was able to inhibit the growth of Novikoff hepatoma in rats. However, the result was not impressive, since about 50% animals in several control groups also survived inoculation with the same number of tumor cells.

Other chemotherapeutic drugs admixed with antitumor antibodies have also been shown to have antitumor effects. It was shown that a mixture of daunomycin and antitumor antibodies was significantly better than either antibody or daunomycin given alone for conferring protection from the development of plasmacytoma on mice [24]. Similarly, Davies [5] has shown that other antitumor drugs, such as cytosine arabinoside and melphalan, were as effective as chlorambucil when they were used consecutively with antitumor antibodies for the protection of mice against EL4 lymphoma.

The mechanism of this cytostatic effect of non-covalent drug-antibody complexes against tumor cells is still unknown. However, it has been shown that the same degree of protection against EL4 tumor growth in the mice could be achieved by injection or chlorambucil and antitumor antibodies, either separately or as a non-covalent complex [6]; therefore, it appeared that the pre-incubation of drug and antitumor antibodies to form a non-covalent complex was not essential to augment the effect of drug by means of antibody. In addition, there was in vitro evidence [43] that the chlorambucil-antibody complex dissociated rapidly in equilibrium dialysis in the presence of other proteins. Therefore, it seemed that the antitumor antibodies would not function as specific carriers for drug in this model.

In a series of experiments aimed at elucidating the mechanism of the synergism between drug and antitumor antibodies for tumor suppression, it was shown [6] that the best protection was obtained when the drug was given 1 h before the antitumor antibodies. This result thus indicated that the drug may increase the susceptibility of tumor cells for the subsequent immune attack by antitumor antibodies. This explanation is supported by a study [44] which showed that rabbit antibodies specific to guinea-pig hepatoma were not cytotoxic to the hepatoma cell even in the presence of complement. However, after the hepatoma cells had been cultured with either metabolic inhibitors or chemotherapeutic agents, they were susceptible to the action of antitumor antibodies and complement. The question as to why either drug or antitumor antibodies alone cannot exert the same degree of cytotoxicity is still unanswered.

Ghose and his associates [15—17] have used chlorambucil-antibody complexes in clinical trials for patients with disseminated malignant melanoma. The antitumor antibodies were produced by goats or rabbits after several IM immunizations with viable human melanoma cells plus Freund's complete adjuvant. The antisera were specific for melanoma cells, since extensive absorption of these antisera with normal human tissues was employed. Chlorambucil-antibody complexes were injected either IV or intratumorally (IT) to patients with disseminated melanoma. Some patients showed objective responses, with transient disappearance of tumor nodules after this form of immunochemotherapy. However, all the treated patients eventuelly died with extensive metastases. Unfortunately, proper controls had not been set up along with these studies. Therefore, there was no definite proof that the transient antitumor effect of chlorambucil-antibody complexes was not due to the action of drug or antibody alone, since it is possible that the dissociation of these non-covalent chlorambucil-antibody complexes may have occurred in vivo.

In another study, the therapeutic effect of the non-covalent complexes of chlorambucil and autochthonous antibody from a melanoma patient was tested [36a]. Three other patients with neuroblastoma were also treated with chlorambucil complexes of immunoglobulins from their parents. Results of the in vitro tests showed the inhibitory effect of chlorambucil-antibody complexes on tumor growth. Objective regressions of the tumors were also observed in the three neuroblastoma patients. In other studies, chlorambucil covalently linked to allogeneic antibody against neuroblastoma has been used in immunochemotherapeutic trials [19a]. A patient who had been treated with this conjugate showed objective tumor regression. Due to the lack of suitable controls in all these clinical trials, it is uncertain whether the observed antitumor effects were actually caused by the administration of drug and antibody complexes or conjugates.

B. Antibodies Specific to Tumor-Associated Fibrin as Carriers

Despite the theoretical attractiveness of the use of antitumor antibodies as carriers for chemotherapeutic agents to the tumor site, in practice, attempts to use the conventional methods for preparing the tumor-specific antibodies have met with enormous difficulties. It is not an uncommon phenomenon for the presumed antitumor antiserum, after extensive absorption with normal tissues, not to react with the immunizing tumor cells. In addition, sometimes antitumor activity was detectable in the antiserum, but the titers were too low to make it applicable for therapeutic study.

It is on the basis of these disturbing observations that a different approach has been employed in this laboratory to prepare antibodies mounted not against tumor antigens, but against the antigenic determinants of tissue components located within the tumor foci. In this regard, advantage was taken of the observation that fibrin is deposited in a variety of animal and human tumors [7, 36, 49], suggesting that fibrin may be an important tissue component that associates with tumor. We thus prepared antisera to guinea-pig fibrin by immunizating rabbits or goats with purified fibrin. Antibodies specific for the unique antigenic determinants of guinea-pig fibrin, which are distinct from the antigenic determinants shared by both fibrin and fibrinogen, were isolated by using fibrin as immunosorbant and the antibody eluates were then run on a fibrinogen-Sepharose column to remove the antibodies that cross-reacted with fibrinogen [28]. Indeed, it was shown that these purified anti-guinea-pig fibrin antibodies (AGFA) were reacted specifically with guinea-pig fibrin and not with fibrinogen in immunoelectrophoresis and double-antibody precipitation tests.

Moreover, when ^{131}I-labeled AGFAs were injected IV to strain-13 guinea-pigs bearing methylcholanthrene-induced sarcomas (MC-D) growing within a fibrin matrix, it was shown that AGFA localized in the sarcoma at considerably higher concentrations than in other organs.

The AGFAs were used to investigate two models that aimed at developing immunotherapeutic procedures for the destruction of solid tumors: (A) The first model [29] involved an indirect cell-mediated immune reaction, in which the tumor fibrin matrix was firstly coated in vivo with AGFA, and the syngeneic lymphoid cells that had been sensitized to xenogeneic immunoglobulins isotypic with AGFAs were subsequently administered to the tumor-bearing hosts. It was anticipated that the interaction between these sensitized cells and AGFAs within the tumor site would lead to a local inflammatory reaction resulting in the release of cytotoxic or chemical factor(s), which by themselves or in conjunction with other cells of the host would destroy the tumor cells in the fibrin matrix. Results showed that the growth of tumor was significantly suppressed in hosts subjected to these indirect immunologic manipulations, compared with the other animals of control groups. (B) The second model [30] explored the possibility of using AGFAs as specific carriers for the chemotherapeutic agents to the tumor site. Daunomycin was conjugated with AGFA by means of glutaraldehyde, and the resulting daunomycin-AGFA conjugates were shown to retain both the drug and the antibody activities. Moreover, multiple IT injections of these conjugates into established MC-D tumors led to significant inhibition of tumor growth and complete tumor rejection occurred in 50% of the treated animals. Systemic tumor immunity was induced in the guinea-pigs whose tumors regressed, since they were resistant to a further injection of a lethal dose of MC-D cells. The results of this study demonstrated that it was possible to destroy tumors with chemotherapeutic agents conjugated to antibodies directed against antigenic determinants of tissue components that were present on the surfaces of tumor cells.

C. Mechanisms of Action

There has still been no systematic study on the mechanisms by which the drug-antibody conjugates exert their cytotoxic effect on tumor cells. However, several possible mechanisms have been suggested [22, 37]. The first possibility is that once the conjugates have reached the target environment, the bond(s) that linked the drug and the antibody molecules must be cleaved by certain degradative enzymes. The free drug thus released might

enter the interior of the tumor cell by diffusion or endocytosis [1], and it then finally reacts with its target. Indeed, proteinases and lisosomal enzymes have been detected on the surface of tumor cells [49a].

Another possibility is that the intact drug-antibody conjugate may enter the tumor cell by pinocytosis and that the drug molecules are dissociated from the conjugate after it has been digested by certain intracellular enzymes, such as lysosomes. Alternatively, the antibody-linked drug may not necessarily have to be split off from the conjugate to exert its cytotoxic effect on tumor cells, since evidence has been presented [22, 30] that the antibody-linked drugs retain their pharmacological activity in vitro. However, whether this mechanism can also operate in vivo remains to be studied.

When the conjugates are the results of coupling drug with antifibrin antibodies, their mechanism of action can be expected to be very similar to that of the drug-antitumor antibody conjugates. Presumably, both antibodies would only serve as carriers for the chemotherapeutic agents and no effective cytotoxicity would be expected to be associated with them. In this context, the mechanism for the synergistic or additive cytotoxic effects of drug and antitumor antibodies to tumor cells, administered either separately or as a mixture, may be different from that of covalently bonded conjugates, since in the former case the contribution of antitumor antibodies to the killing of tumor cells is quite apparent.

Finally, it is plausible that the rejection of tumor grafts by animals after treatment with drug-antibody conjugates may be caused by tumor-specific immunity, which could be elicited by the dead tumor cells as a result of the drug action. Results obtained in our laboratory [30] and in Flechner's [12] have demonstrated that after rejection of a lethal dose of tumor cells in mice resulted from the treatment of drug-antibody conjugate or complex, the surviving animals were able to reject a second lethal dose of tumor cell inocula. In this connection, several investigators have also shown that tumor-specific immunity can be detected in mice that have survived treatment of their tumor with chemotherapy [1, 37].

D. Discussion and Conclusions

An important factor in success with drug-antibody conjugates in the treatment of cancer is the immunological specificity of the antitumor antibodies. Evidence accumulated to date has unequivocally proved that most of the tumor cells are immunogenic in either syngeneic or xenogeneic hosts. The problem is that this immune reaction to tumor antigen is usually weak, especially with respect to humoral immunity.

Many physical, chemical, and immunological methods have been utilized to modify tumor cells in attempts to increase the immunogenicity of tumor antigens on tumor cell surfaces. For example, tumor cells that had been treated either by physical manipulations such as freezing and thawing or lyophilization and homogenization or by chemical coupling to foreign proteins such as γ globulin, have been used in immunization [38]. In addition, immunization of the hosts with tumor cells that had been either treated with neuraminidase or other chemical agents [45] or coated with antibodies against the homologous normal tissue antigens has also been tried [46]. Other workers attempted [31] to render the hosts tolerant to normal tissue antigens by neonatal injection of this tissue, and then immunized the adult hosts with tumor cells. However, none of these modified techniques showed any advantage over the conventional method of immunization with tumor cells only for obtaining antitumor antibodies.

Recently, a lymphocyte hybridoma technique has been developed, which shows great promise for the production of a large amount of monospecific antitumor antibodies. It was originally reported by Kohler and Milstein [26] that fusion of mouse myeloma cells with antibody-producing cells can give rise to a hybrid cell population. After proper clonal selection from this hybrid cell population, some clones of cells have been shown to produce monospecific antibodies. These antibody-producing cell clones can be maintained as tumor cell lines either in cultures or in animals. Thus, this approach provides a continuing source of monospecific antibodies, and it has been used for the production of antibodies specific to different kinds of antigens, including human melanoma antigens [27].

Alternatively, antifibrin antibodies can be used as specific carriers for targeting drug to tumor. As has been mentioned before, it is relatively easier to prepare a large quantity of antifibrin antibodies than to prepare antitumor antibodies. However, the strategy of using a drug-antifibrin antibody conjugate for tumor cell destruction is applicable only to tumors that are associated with fibrin matrix.

For the chemotherapeutic agents to be able to conjugate with antibody carriers, they must possess certain chemically reactive groups that can be utilized for linking to the antibody. For example, most drugs used for coupling purposes have reactive groups such as $-NH_2$, $-COOH$, $-OH$, etc., which can be readily employed for coupling to antibody by appropriate cross-linking agents. If the reactive group on the drug is not available or is undesirable for coupling, some additional reactive groups can be either introduced or modified from the molecules of drugs by appropriate chemical manipulations, provided that this would not affect the pharmacological activity of the drugs [2, 9]. The choice of func-

tional group on the drug to be linked to antibody must be taken into consideration. If a functional group is essential for the cytotoxic activity of the drug, the utilization of this group for coupling should be avoided, since theoretically this could greatly reduce the activity of drug. On the other hand, it would be interesting to see whether some functional groups on a drug might still retain their pharmacological activity after they have been linked to antibody.

Another requirement for successful targeting of drug by the drug-antibody conjugate is that it should retain its toxic activity after coupling to antibody. There are studies showing that the covalent linkage of drug and antibody could reduce the toxicity of drug in the conjugate. It may be argued that this reduction of drug toxicity has an advantage, since it also reduces the nonspecific toxicity of the drug to normal tissues. Ideally, however, the retention of highly active drug in the conjugate with tumor specificity is desirable, so that the maximal or a highest therapeutic index can be readily achieved.

Although higher ligand substitution of antibody by drug molecules is desirable, in practice the number of drug molecules that are allowed to become attached to antibodies is quite limited, because extensive substitution usually leads to a loss of antibody activity in the conjugate.

Despite the risk of losing some of the drug and antibody activities, covalent linkage between drug and antibody is preferable, since this should provide a more stable drug-antibody conjugate, which will not be easily split off before it reaches the target. The advantage of a non-covalent complex of drug and antibody is its tendency to preserve the activities of both drugs and antibody, since this complex is prepared by the mild condition of mixing, without the involvement of drastic chemical manipulation. However, there is solid evidence that the drug-antibody complex is dissociated in vivo, and that the antibody from this complex does not serve as a homing carrier for the drug to the target. Therefore, the mode of action of this non-covalent drug-antibody complex for inducing tumor regression may be different from that of the covalent drug-antibody conjugate. Further study is necessary to elucidate this mechanism.

Finally, for consideration of intracellular penetration by drug-antibody conjugate, a modified technique for linking the drug and antibody, which would render the drug more easily able to enter the interior of tumor cells, needs to be explored. One such technique involves the entrapment of drug in liposomes [18], lipid bilayers that are readily incorporated into the cells by either endocytosis or fusion, and the coupling of this drug-tagged liposome with specific antibody as a homing carrier. Thus, the antibody will direct the liposome-entrapped drug to the target, followed by intracellular uptake of this liposome-entrapped drug and subsequent liberation of the drug from liposome.

In conclusion, the prospects for the use of drug-antibody conjugates or complexes for eradication of a small residual tumor load are promising, since the dream of obtaining monospecific antitumor antibodies in large quantity has become a reality with the recent development of the lymphocyte hybridoma technique. It remains to be seen whether the pharmaceutical chemists will be able to manipulate the molecular structure of the drug and/or antibody molecules to devise optimal conditions for coupling the drug and antibody so that both the antibody specificity and the drug toxicity can be fully retained. Thus, the interdisciplinary approaches combining pharmacology and immunology deserve further development and evaluation.

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