THE USE OF ANTI-FIBRIN ANTIBODIES FOR THE DESTRUCTION OF TUMOR CELLS

by

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ABSTRACT

Antibodies specific for the unique antigenic determinants of guinea pig fibrin, which are distinct from the antigenic determinants shared by both fibrinogen and fibrin, were isolated with appropriate immunosorbents from antisera produced in rabbits and goats by immunization with fibrin. The specificity of the purified anti-guinea pig fibrin antibodies (AGFA) was demonstrated by immunelectrophoresis and by the double antibody precipitation method using ^{131}I-labelled fibrinogen and antibodies to rabbit anti-goat IgG. The ^{131}I-labelled AGFA were injected i.v. into inbred Sewall Wright strain 13 guinea pigs carrying the transplantable methylcholanthrene induced sarcoma (MC-D) growing within a fibrin matrix and were shown to be localized in the tumor tissue at considerably higher concentration than in other organs.

Next, the transplantable MC-D sarcoma in strain 13 guinea pigs was used to test the hypothesis that tumor cells growing within a fibrin matrix could be destroyed by an immunologically specific strategy involving an indirect cell-mediated immune reaction. The experimental design consisted of two steps: (i) in vivo fixation of AGFA on the fibrin matrix enmeshing the tumor cells and (ii) the reaction between AGFA fixed to the fibrin matrix and lymphoid cells from syngeneic animals which had been sensitized to xenogeneic immunoglobulins isotypic with AGFA. Indeed, using ^{51}Cr-labelled lymphoid cells, evidence was obtained for the localization of these sensitized lymphoid cells within the fibrin lattice when the latter was coated by AGFA. Moreover, significant tumor growth suppression ($P < 0.01$) was achieved in guinea pigs which had received intravenously rabbit or goat AGFA and subcutaneously lymphoid cells from syngeneic guinea pigs sensitized to a state of cell-mediated immunity to rabbit or goat IgG. On the other hand, the administration of the antibodies or of the sensitized cells alone did not affect the growth of the

tumor. Preliminary results suggest that peritoneal exudate cells may play an important role for the success of the strategy for tumor cell destruction.

Finally, the possibility of using AGFA as specific carriers for cytotoxic drugs to tumor nodules was tested. Daunomycin was coupled with the aid of glutaraldehyde to goat AGFA. The resulting daunomycin-antibody conjugates inhibited cellular RNA synthesis and induced cell death in vitro of a MC-D sarcoma of strain 13 guinea pigs. The cytotoxic capacity of the conjugate was not significantly different from that of free daunomycin. The specific localization of daunomycin-antibody conjugates within the fibrin matrix enmeshing the tumor tissue was demonstrated by indirect immunofluorescence with FITC-conjugated rabbit antibodies to goat γ-globulins. Multiple injections of daunomycin-antibody conjugates intratumorally in vivo, into well established MC-D tumors, led to significant tumor growth retardation and complete tumor rejection occurred in 50% of the guinea pigs. Moreover, systemic tumor immunity was induced in the guinea pigs so cured, as demonstrated by the fact that these animals were resistant to a further lethal dose of MC-D tumor cells.

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An Overview of Tumor Immunology

Chapter I

AN OVERVIEW OF TUMOR IMMUNOLOGY

TUMOR ANTIGENS

At the beginning of this century, experiments performed in outbred animals have shown that the growth of transplanted animal tumors could be prevented by immunization of recipients with the same tumors. However, it was soon realized that the rejection of tumor grafts in these experiments was due mainly to the sensitization of the recipients to alloantigens present in the original tumor inocula. Nevertheless, these studies led to the search for a unique antigen which can only be found in tumor cells and should not be present in normal tissues.

According to the cellular distribution, tumor antigens can be distinguished in two categories: those that form part of the cell surface and those that do not. There is ample evidence to indicate that only tumor antigens that belong to the former category can elicit humoral and/or cell-mediated immune responses, which, in many occasions, lead to tumor rejection. Tumor antigens have been detected on cells of tumors induced by either chemical carcinogens or oncogenic viruses.

Chemically-Induced Tumors

Using a methylcholanthrene (MCA)-induced sarcoma in mice, it was demonstrated that syngeneic mice immunized by intradermal inoculation of MCA-induced tumor cells rejected the subsequent grafts of the same tumor (Gross, 1943). Unfortunately, this result cannot exclude the possibility that the tumor may have mutated during repeated transplantation since the histocompatibility within the experimental mice had not been checked.

More critical demonstration of the specific antigenicity of tumor cells was performed by Foley (1953). Using the MCA-induced sarcoma in inbred C3H mice, he demonstrated that after excision of these tumors by ligation the host could be rendered resistant to subsequent challenge with the same tumors. Later, Prehn and Main (1957) substantiated this observation with additional controls, including immunization of the host with normal tissues. This did not render the host with immune resistance to the tumor grafts; moreover, skin grafts from primary donor were accepted by the immune host. This excluded the possibility that tumor rejection was due to either tissue entigens or isoantigens and was thus tumor specific.

Tumor antigens of different tumors, induced in the same strain of animals by identical carcinogens, were shown to be individually specific, since no crossreactions were observed among different tumors of similar morphology (Klein et al., 1960; Old et al., 1962). However, there is also evidence supporting the view that crossreacting tumor antigens exist in tumors induced by either similar or different carcinogens (Reiner and Southam, 1969; Takeda, 1969; Holmes et al., 1971). One of the proposed mechanisms for the appearance of these crossreacting antigens is that the chemical carcinogen activates the oncogenic viruses which in turn induce neoplastic transformation of cells, giving rise to virus-induced type specific crossreacting antigens on their surfaces. Another mechanism, which is based on the studies of Reiner and Southam (1969), invokes the possibility that chemical carcinogens may induce several sets of tumor antigens on the surface of tumor cells, some having a crossreacting determinants which elict only weak immune responses which are difficult to detect.

Virus-Induced Tumors

Tumor antigens have been demonstrated in tumor cells induced by DNA and RNA viruses. Thus, it has been shown that mice after infected with polyoma virus became resistant to a cell transplant of a tumor induced by DNA polyoma. Since attenuated virus or passively transferred antibodies to virus had no such effect, it was suggested that polyoma-specific antigens were induced by the virus in the infected cells of mice (Habel, 1961; Sjogren, 1961). Two classes of antigens are induced by DNA viruses: (i) cell surface antigens which elicit tumor transplantation immunity, demonstrable by the induction of resistance to tumor grafts following immunization with the homologous DNA virus-induced tumor cells; (ii) T- or neoantigens which are intra-cellular and specific to the induced viruses.

Tumor antigens of RNA virus-induced leukemia cells were demonstrated by the presence of specific anti-leukemia antibody from the sera of mice which had been immunized with the leukemia cells (Old et al., 1963).

Recently spontaneous tumors have been shown to carry tumor antigens by the demonstration of retardation of tumor growth in hosts which had been immunized with the same tumor cells. Antigens responsible for the inhibition of tumor growth in immunized hosts have been shown to be tumor specific and not related to the tumor virus (Morton et al., 1969).

It has been recognized for some time that tumors induced by one type of virus show common crossreacting antigens, even if the tumors are of different morphology or are induced in different species. However, recent evidence has been adduced to show that mammary tumor virus (MTV)-induced carcinomas have individually specific as well as common antigens (Morton et al., 1969; Vaage, 1968). This finding is similar to that observed in

chemically-induced tumors which may indicate that both types of tumor cells could have individually specific as well as crossreacting antigens.

Tumor Associated Embryonic Antigens

Interest in studying the relationship between embryonic tissues and neoplastic transformation was first raised by the report of Schöne (1906) who showed that mice after injection with embryonic but not adult tissue could reject a tumor transplant. Although the exact mechanism underlying this observation is still not clear, the presence of embryonic antigens in tumor tissues was unequivocally proved by the demonstration of α-fetoproteins in the serum of hepatoma bearing mice (Abelev, 1963) and carcinoembryonic antigens in adenocarcinomas of the human digestive tract (Gold and Freedman, 1965a, 1965b).

Embryonic antigens are usually referred to as macromolecules which are found in embryonic as well as tumor tissues, and are demonstrable to be immunogenic either in syngeneic or xenogeneic (after proper absorption) hosts. It is currently postulated that the production of this macromolecule during the neoplastic transformation is due to the activation of silent genes normally expressed only in the embryo.

Embryonic antigens have been shown in many tumors of experimental animals induced by either chemical carcinogens (Brawn, 1970) or viruses (Coggin et al., 1970). Immunization of the animals with embryonic tissues sometimes protects the hosts from subsequent tumor challenge (Coggin et al., 1971; Ting et al., 1973). However, the relationship of antigenic specificity between embryonic and tumor tissues is still unclear.

HUMAN TUMOR ANTIGENS

Tumor Specific Antigens

Using several sensitive *in vitro* methods such as immunofluorescence (Klein et al., 1966), complement fixation (Eilber and Morton, 1970) and colony inhibition (Hellstrom et al., 1970), antigens on tumor cells which induce specific immune responses have been demonstrated in a variety of human tumors. For example, a high percentage of patients with Burkitt's lymphoma (Klein et al., 1966) and melanoma (Lewis et al., 1969) have antibodies to the surface antigens of tumor cells, detectable by immunofluorescence after the tumor had regressed.

Tumor antigens in human tumors have been also detected with other methods such as lymphocyte transformation (Vanky et al., 1971) and skin tests (Oren and Herberman, 1971). However, the specificity of these reactions is still not established.

Using the techniques of mixed hemadsorption and immune adherence, autologous antibodies to the surface antigens on the patients' own melanoma cells have been demonstrated. It appears that there are at least three kinds of surface antigens on melanoma cells: a) unique melanoma-specific antigens which are only found on autologous melanoma cells; b) common melanoma-specific antigens detected on melanoma cells of different patients, and not on other kinds of tumor cells; c) some antigens which have also been found on normal human cells and nucleated cells of some animals (Shiku et al., 1976a;1977). The presence of these complex antigens on human tumor cells adds to difficulty of distinguishing which antigen is unique to the neoplastic transformation of cells.

Tumor Associated Embryonic Antigens

In the search for tumor specific antigens of human tumors, it was found that a component from embryonic tissues could crossreact with antiserum against tumor antigens. It is apparent from this finding that there is a common antigen expressed on both embryonic and tumor tissues. Currently two types of embryonic antigens, namely, carcinoembryonic antigens (CEA) and α-fetoprotein (AFP), are being studied widely.

(a) Carcinoembryonic Antigens

CEA has been detected in carcinomas of the human digestive system and of the embryonic digestive organs. Anti-CEA antisera, prepared in rabbits and absorbed with normal colon tissues, were shown to contain antibodies which specifically reacted with CEA extracts but not with normal colon tissue extracts (Gold and Freedman, 1965a, 1965b).

Sensitive radioimmunoassays were developed to detect the circulating CEA levels in the serum of cancer patients (Thomson et al., 1969; Hansen et al., 1971; Berczi et al., 1976). The levels of circulating CEA in patients with colonic carcinomas was persistently higher than in normal individuals. Therefore, radioimmunoassays for CEA are useful for the diagnosis and more importantly for the prognostic assessment of colonic cancer. However, it ought to be stressed that increased levels of CEA have been also detected in other types of cancer, in non-malignant diseases and in heavy smokers (Hansen et al., 1974). The exact causes for these findings are still unknown.

(b) α-Fetoprotein

This antigen was first demonstrated in the sera of mice bearing hepatomas and was since found in serum of normal embryos (Abelev, 1963). Subsequently, AFP was also found in serum of patients with malignant

hepatomas (Tatarinov, 1964). AFP does not appear to be immunogenic in the same species, but anti-AFP antibodies can be readily obtained by immunization of another species. Clinically, AFP has been used in diagnostic tests for hepatomas and hepatitis.

IMMUNE REACTIONS TO TUMORS

Evidence from *in vivo* and *in vitro* studies has demonstrated that a host is capable of mounting specific immune response to antigenic tumor cells. Thus, normal syngeneic mice could be immunized to tumor antigens by excision of growing tumors, so that subsequent tumor cell isografts were rejected (Prehn and Main, 1957). Also, sarcomas induced in adult mice by murine sarcoma virus (MSV) sometimes regress due to specific immune reactions (Fefer et al., 1968).

However, inspite of the host's ability to mount immune reactions against the tumor cells, they continuously grow and eventually kill the host. This fact suggests that effective immune defence reactions against tumor cells may not be operating in the tumor-bearing host. In the following sections, the nature of humoral and cell-mediated immune reactions to tumor antigens, as well as their possible roles in tumor rejection will be examined.

EFFECTS OF ANTIBODIES ON TUMOR GROWTH

Cytotoxic Effects on Tumor Cells

(a) Complement-Dependent Cytotoxic Antibodies

It has been demonstrated with different tumor systems that cytotoxic antibodies can be produced in syngeneic animals by immunization with tumor

cells. For example, mice immunized with homogenates from lymphoma cells produced anti-lymphoma antibodies detectable by the complement-dependent cytotoxic test (Klein and Klein, 1964). Cytotoxic antibodies against sarcoma cells have been demonstrated in the IgM fraction of an antiserum produced in syngeneic mice immunized with MCA-induced sarcomas (Bloom and Hildemann, 1970). Similarly, in studies carried out in this laboratory (Dalton et al., 1976) it has been shown that antibodies produced in response to immunization with lymphoma L1117 cells in syngeneic A/J mice belonged primarily to the IgM class.

Antibodies cytotoxic to autologous tumor cells have been detected in the sera of patients whose tumors were surgically removed (Lewis et al., 1969; Morton, 1971). Cytotoxic antibody activity resided mainly in the IgM fraction of the serum immunoglobulins. In some patients whose tumors were completely removed, this antibody activity could be elevated by the injection of irradiated autologous tumor cells. This observation indicated that hosts were capable of producing specific cytotoxic antibodies against tumor cells once the tumor load was reduced.

(b) Antibody-Dependent Cellular Cytotoxicity (ADCC)

Sera obtained from animals immunized with tumor cells (De Landazuri et al., 1974a) or from hosts after tumor regression (Harada et al., 1972) have been shown to confer specific cytotoxicity onto non-immune lymphocytes against the tumor cells which were used as targets in vitro. Similar antibody activity had been reported earlier in allogeneic and xenogeneic graft systems (McLennan et al., 1969).

It was shown that antibodies of either IgG or IgM class with an intact Fc fragment were able to mediate this type of cytotoxic reaction (Basten et al., 1972; Lamon et al., 1977). This reaction does not require complement. The effector cells which mediate ADCC reaction have Fc recep-

tors (Pape et al., 1977), and have characteristics of bone-marrow derived (B) cells. However, there is no evidence to suggest that these cells are antibody-forming cells; therefore, these cells may be neither thymus (T) nor B cells and may probably be 'null' cells (Allison, 1974). The specificity of the ADCC reaction seems dependent on the binding of antibody onto the target tumor cells and not onto the lymphocytes, since ADCC activity can be completely abolished by absorption of the sera with the specific tumor cells. Also, the attacking lymphocytes, obtained from xenogeneic donors have been shown to react with antibody-coated tumor cells (Wunderlich et al., 1975).

(c) Synergistic Cellular Cytotoxicity by Immune Serum

Immune sera obtained from animals which had been immunized with syngeneic tumor cells (De Landazuri et al., 1974b) or infected with MSV (Skurzak et al., 1972) were capable of enhancing the cytotoxic reactivity of immune lymphocytes to tumor cells in vitro (Hellstrom et al., 1971).

In a virus-induced tumor system, it was found that non-T lymphocytes were the effector cells and the cellular component of this synergistic effect was nonspecific so that lymphoid cells sensitized to a chemically-induced tumor were also effective (De Landazuri et al., 1974b).

The role of these factors in the in vivo suppression of tumor growth remains to be elucidated.

Interference with Cell-Mediated Immune Reactions to Tumors

Early in vivo experiments showed that transfer of small amounts of heterologous or isologous anti-tumor serum into tumor-bearing animals could enhance tumor growth (Kaliss, 1958). It has been demonstrated in vitro that sera from tumor-bearing animals or cancer patients could block

the specific cytotoxic reactions of lymphocytes to tumor cells. It was suggested that the cause of this blocking phenomena was due to a 'blocking' factor in the sera of tumor-bearing hosts which can block the immune reaction of cytotoxic lymphocytes against the tumor cells (Hellstrom et al., 1969).

The nature of the 'blocking' factor in sera of tumor-bearing hosts has not been clearly defined. In the case of the MSV-induced sarcoma in mice, this 'blocking' factor could be absorbed out with the corresponding sarcoma cells, or with goat anti-mouse IgG, and it was shown by gel filtration to possess characteristies of IgG. Therefore, it was assumed that this 'blocking' factor was IgG antibody. In subsequent studies, however, antigen-antibody complexes (Sjogren et al., 1971) or soluble antigen alone (Brawn, 1971) have been implicated as factors responsible for the observed blocking phenomena.

More recent data have further complicated this interpretation. Thus, 'blocking' factors can be absorbed and eluted out from immunosorbent columns made by coupling to Sepharose immunoglobulin fractions of sera from mice immunized with the tumor. These 'blocking' factors bind to ConA-Sepharose indicating that they may belong to some types of serum glycoproteins. They can be identified as polypeptides smaller than conventional immunoglobulin (M.W. = 56,000). All these findings forced the authors to revise their orginal claim that 'blocking' factors were 'blocking' antibodies. The concept that 'blocking' factors may represent a kind of immunosuppressive molecules produced by the T cells of tumor-bearing hosts has been recently proposed (Nepom et al., 1977). This view may thus confirm the earlier demonstration in this laboratory that specific immunosuppressor T cells were present in tumor-bearing mice and that soluble suppressor factors were isolated from these T cells (Fujimoto et al., 1976a, 1976b; Greene et al., 1977).

If the 'blocking' factor were an anti-tumor antibody, it would be rather difficult to explain why in other studies anti-tumor antibodies were shown to be cytotoxic for tumor cells (see previous section). However, it is possible that this is an anti-idiotypic antibody, i.e. and anti-receptor antibody (Rowley et al., 1973; Wight and Binz, 1977) which may block the receptor sites on immune lymphocytes thus preventing them from reacting with tumor cells. A preliminary study carried out in this laboratory showed that antisera raised against the receptor of the immune lymphocytes to 1509a sarcoma indeed affected the growth of this tumor in vivo (Lee et al. unpublished data).

In a recent model proposed by Gorczynski et al. (1974), it was suggested that the immune reaction of T lymphocytes can be blocked by antigen-antibody complexes both specifically and non-specifically. Thus, in the specific blocking, the antigen reacts with a T lymphocyte receptor and the antibody then binds to this antigen. In the non-specific blocking, the antigen-antibody complex binds to T lymphocyte via the Fc receptor of the latter. This model may provide one way to explain the 'blocking' mechanisms. However, it is difficult to visualize that the former reactions of T lymphocyte receptors with antigen resulted in blocking instead of proliferation of this sensitized T lymphocyte, which is a common finding in in vitro culture of sensitized lymphocytes with antigen.

It should be borne in mind that these blocking phenomena were observed by in vitro experiments, and there is still no definite proof of an equivalent situation existing in vivo. In addition, the loss of immune cytolytic function of T lymphocytes after interaction with antigen or antigen-antibody complex may be brought about by other mechanisms, such as the induction of receptor modulation or shedding from the cell surface.

Antigenic Modulation

Another phenomenon related to the effect of antibody on tumor cells is antigenic modulation, by which the density of tumor cell surface antigens can be altered after reaction with specific antibodies. A classical example is the loss of the TL(thymic leukemia)-antigen on lymphoma cells after exposure to anti-TL antibody (Old et al., 1968). This reduction in antigen distribution may enable tumor cells to escape immune destruction. Evidence has been obtained in this laboratory to the effect that ascites fluid or serum from tumor-bearing guinea pigs was capable of inducing resistance of tumor cells to cytolysis mediated by antibody and complement (Abe et al., 1977). To explain these results, it was proposed that antibody-antigen complexes in the ascites fluid or serum of tumor-bearing guinea pigs was responsible for inducing the change of antigen density on the surface ot tumor cells, which provided a route for the tumor cells to escape from the immune destruction mediated by the cytotoxic antibodies. Possibly this type of antigenic modulation could also cause structural changes of tumor antigens which induce the production of suppressor T cells (Kirkwood and Gershon, 1974).

CELL-MEDIATED IMMUNE REACTIONS TO TUMORS

In Vivo Demonstration of Immune Cells to Tumor Antigens

Animals can be made highly immune to antigens of some tumors, e.g. MCA-induced sarcoma in guinea pigs and mice, either by excision of the tumors following repeated challenges with tumor cells, or by multiple injections of irradiated or mitomycin C treated tumor cells in Freund's complete adjuvant (FCA). These immune animals will reject even a supra-lethal dose of tumor cells.