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The Immunotherapy of Cancer

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I. Introduction

IN recent years the possibility that the immune response might be exploited in the treatment of malignancy has received considerable experimental study. The justification for this work comes from a number of important fundamental observations in tumor immunology: 1) Most, possibly all, malignant tumors in man and experimental animals contain antigens that are largely or entirely absent from normal adult tissue. Some of these antigens represent a re-expression of proteins present in the fetus. Others can be demonstrated only in neoplastic cells. Some are common to tumors of the same histological type from different individuals. Others are apparently unique and immunological cross reactivity cannot be demonstrated. 2) The development of humoral and cellular immunity can be shown under normal conditions of tumor growth *in vivo*, both in man and experimental animals and the immune profile can be correlated with the clinical status of the tumor. 3) In animals immunological resistance to tumor growth can be produced by active or passive immunization. In man there is an increased incidence of tumors in individuals with immunological deficiency diseases or on immunosuppressive drugs and tumors with a favorable prognosis frequently show histological features suggesting an especially active immune response.

Taken together these observations suggest an important role of the immune apparatus in resistance to tumor growth and indicate that attempts to increase immune resistance might be useful in tumor therapy. This possibility is attractive because the biochemistry of tumor cells has not proved to be sufficiently distinctive to permit the design of truly specific cytotoxic agents. Moreover, provided conditions are optimal the immune response has the capacity to kill 100% of tumor cells, whereas radiotherapy and chemotherapy act by single hit kinetics and the same treatment dose is required for each 10-fold decrease of the tumor cell population, regardless of whether the population is large or small (19). But before the immunity can be properly exploited for tumor resistance its limitations must be understood and ways found to amplify the response.

II. Limitations in Immune Resistance

Even though it is possible to obtain immune resistance to tumors, the level of resistance is severely limited. In order to demonstrate inhibition of tumor growth in syngeneic animals, a carefully designed experimental protocol in which limited numbers of tumor cells are used is required. In transfer studies in syngeneic mice, for example, resistance cannot be demonstrated if the number of transplanted tumor cells exceeds 10^4 to 10^7, the maximal number depending on the tumor (1). Obviously experiments with such a limited number of cells say very little about the level of immune responsiveness required to reject an established tumor. The capacity of the immune system in man, in terms of the numbers of cells that can be eliminated, is likely to be considerably higher but it is doubtful that an effective response can be mounted when large amounts of tumor are present. Regardless of what approach is taken to immunotherapy, the amount of viable tumor tissue will have to be reduced to a minimum before

attempting treatment. It is also clear that when a tumor has reached a clinically significant size immune resistance to tumor growth has already partially failed despite the theoretical capacity of the host to reject the tumor. The question is why and what can be done about it. Possible factors in failure of immune resistance to tumor growh will now be considered. It is necessary first, however, to briefly review the individual segments of the normal immune response.

III. Normal Components of the Immune Response

A. *The Afferent Arm*

The immune response can be divided into afferent and efferent arms. The afferent arm is the antigen recognition portion in which antigens localize in peripheral lymphoid tissues (lymph nodes and spleen), interact with macrophages and antigen sensitive T (thymus derived) and B (bone marrow derived) lymphocytes, and induce lymphocyte proliferation (fig. 1). With most tumors this reaction takes place primarily in regional nodes. As a result of the proliferation there is a great increase in number of cells with specificity for activating antigen. Responding T cells give rise to immunoblasts and ultimately to immunologically committed small lymphocytes which serve as effector cells in T cell mediated cellular immunity and carry T cell memory. Responding B cells form B immunoblasts which in turn give rise to plasma cells, the major source of antibody, and long lived B lymphocytes, which carry immunological memory for antibody producing cells.

B. *The Efferent Arm*

In the efferent (effector) phase of the response sensitized cells and antibody interact with target cells producing cytotoxic effects. In order to exert their effector function cells, and antibody must leave the node, reach the target tissue, and establish contact with individual target cells (fig. 2). In regional nodes during active phases of the response, T and B immunoblasts enter the efferent lymph, reach the blood stream and become disseminated to various tissues of the body, resulting in generalized sensitization. Antibodies, sensitized small lymphocytes and plasma cells reach the circulation by the same channel. Antibodies reach the target tissue by transversing the capillary wall or by active biosynthesis by B lymphocytes which have become localized in the target area. Antibodies produce tissue damage by combining with tumor cells in the

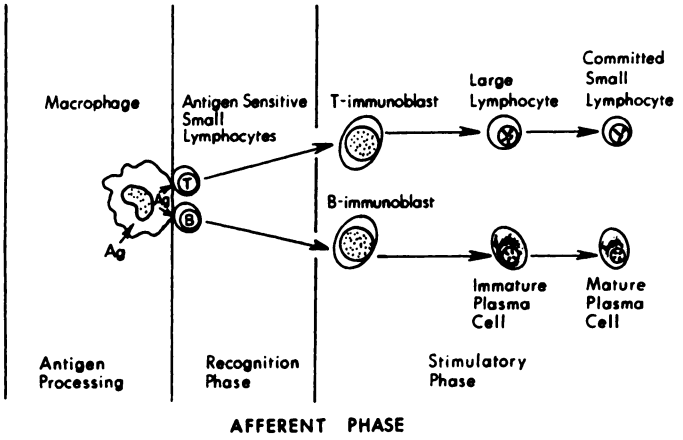


FIG. 1. The afferent arm of the immune response. Ag is antigen. T and B cells are thymus-derived and bone marrow-derived lymphocytes, respectively. (Modified from ref. 31, C. W. Parker and J. D. Vavra: Immunosuppression. *In* Progress in Hematology, vol. VI, ed. by E. Brown and C. V. Moore, pp. 1-81, Grune & Stratton, New York, 1969.)

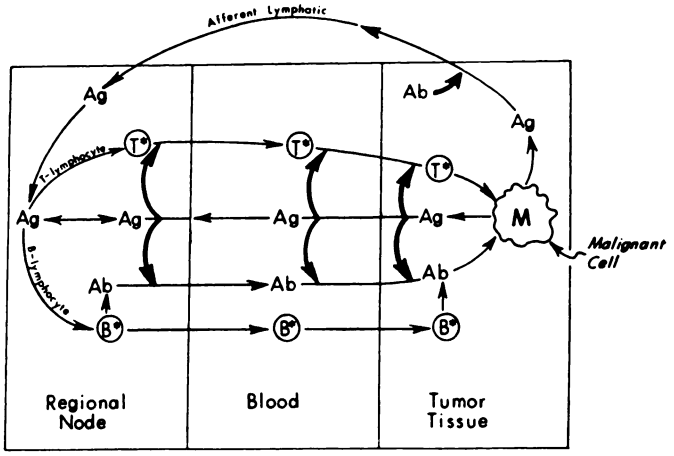
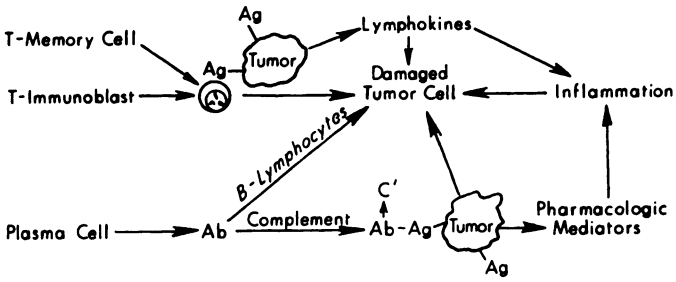


FIG. 2. Transport of antibody (Ab) and sensitized T and B cells (designated by an asterisk) from responding lymph node to primary tumor. Transport is blocked by free antigen (Ag) and antigen-antibody complexes.



EFFERENT PHASE

FIG. 3. The efferent arm of the immune response. Ag is antigen; Ab is antibody; C′ is complement. (Modified from ref. 31, C. W. Parker, and J. D. Vavra: Immunosuppression. *In* Progress in Hematology, vol. VI, pp. 1–81, ed. by E. Brown and C. V. Moore, Grune & Stratton, New York, 1969.)

presence of complement (fig. 3). The reaction with complement also releases chemotactic factors which stimulate local inflammation and activate macrophages. *In vitro*, antibodies also have the capability to prime unsensitized B cells to exert direct cytolytic activity (16) but the importance of this mechanism in the whole animal is not certain. The molecular basis for T cell function is not fully understood. It seems clear that T cells must be physically present in the target tissue in order to exert their function. When sensitized T cells come in contact with tumor cells they attach and produce direct damage. They also give off soluble factors (lymphokines) which amplify the response. The lymphokines are a poorly characterized mixture of macromolecules with cytotoxicity for non-lymphocytic cells, B cell stimulating activity and an ability to promote macrophage accumulation and activation. Activated phagocytic cells in turn act as nonspecific adjuncts in tumor cell destruction. Thus immune resistance can be viewed as a cooperative enterprise involving specific and non-specific elements of the lymphoid system, each magnifying the reaction. Interference with any of the multiple processes

involved is likely to reduce the overall effectiveness of the response.

IV. Possible Problems in the Afferent Arm

Many tumors contain limited amounts of tumor specific antigen restricting the magnitude of the immune response even under optimal circumstances. Under conditions of weak antigenic stimulation, previous immunological conditioning and genetic and anatomic factors influencing immune responsiveness can play a critical role in determining the level of immunological resistance that is achieved.

A. Properties of the Tumor Antigen and How It Is Presented to the Host

The site at which tumor growth originates is undoubtedly an important variable in determining the ultimate fate of a tumor. It has long been known that when foreign tumors are implanted in the cheek pouches of hamsters, they are likely to thrive whereas when the same tumor is implanted in subcutaneous tissue elsewhere, it is promptly rejected. The term, immunologically privileged site, has been used to describe areas in which transplanted tumors have an increased likelihood of survival. It is not entirely certain whether the survival advantage is operative in the afferent or efferent arm of the immune response. Quite possibly the functional status and anatomic arrangement of the lymphatic vessels and regional nodes draining a tumor could play a critical role in the early development of an effective immune response. If limited amounts of tumor antigen are released, a failure to localize the antigen in regional lymphoid tissue would dilute the antigen and limit immune stimulation.

Little is known about tumor antigens and how they are metabolized. No doubt this varies with the tumor, its localization, and the type of antigen it produces. With tumors induced by oncogenic deoxyribonucleic acid (DNA) viruses, the quantities of antigen released from the tumor in the early stages of growth are small and this may be an important factor in a failure to obtain immunological rejection (18). From empirical immunization studies with soluble and particulate purified protein antigens, it is clear that the route of antigen injection, the amount given and the form in which it is administered can influence the quantity, affinity, and immunoglobulin class distribution of the antibody which is formed. The magnitude of the cellular immune response is also altered by these variables. Since antitumor antibodies may exert a blocking effect on cell mediated tumor destruction (see below), the quantitative relationships between humoral and cellular immunity in the early stages of tumor growth may be critical in determining the outcome.

B. Host Responsiveness

Genetic influences on immune responsiveness are well recognized and are particularly important for relatively simple macromolecules such as the synthetic polypeptides. There is considerable evidence to indicate that immune response (Ir) genes are closely linked to major histocompatibility antigen genes and that the influence is expressed largely or entirely through T cells. Even where an antigen has the necessary structural complexity and foreignness to produce an immune response on a regular basis, there are important genetic influences on antibody concentration, affinity, and immunoglobulin class distribution as well as on the level of cellular immunity. In tumors induced by polyoma virus in inbred mice, resistant strains have been shown to develop cell mediated immunity to virus-induced tumor antigens at an earlier age than susceptible strains, suggesting a critical role of immune responsiveness in tumor rejection in these animals (2a).

Factors other than genetic ones may also have an important influence on immunological responsiveness. Many tumor antigens are present in normal fetal tissue and to a much more limited extent in adult tissue, creating the possibility of partial immunological tolerance. If partial tolerance is present at the time a tumor appears, it will qualita-

tively or quantitatively alter the immune response to the tumor. The influence of fetal tolerance on immune resistance is evident from studies in mice in tumors induced by vertically transmitted oncogenic viruses (38). Mice with very early exposure to non-cytopathogenic oncogenic ribonucleic acid (RNA) viruses develop little or no immune resistance to tumor growth, even after repeated attempts at immunization.

The fact that there is an increased incidence of tumors in animals and human beings with impaired cellular immunity (immunological deficiency states, thymectomy, immunosuppressive therapy) would suggest that the immune system has a surveillance function in preventing the growth of spontaneously occurring tumors. However, the extent to which decreased immune responsiveness contributes to the establishment of tumor in the usual patient with cancer is not altogether clear. Since the overall incidence of malignancy is higher in older people, there has been considerable discussion of the possible role of age dependent changes in immune responsiveness on tumor formation. There are scattered reports of decreased cellular and humoral immunity with increasing age, but the data are not completely convincing and more studies are needed, particularly in man. Many patients with widespread malignancy have evidence for depressed cellular immunity, both to tumor specific and to unrelated antigens (25). But during periods of clinical remission, or before the tumor has become widespread, the available techniques for the evaluation of immunological reactivity do not, as a rule, reveal alterations in immune reactivity. It seems probable, therefore, that the impairment seen in severely ill patients is as much a result as a cause of the unfavorable clinical course.

V. Possible Problems in the Efferent Arm

A. The Delivery Problem

Sensitized lymphocytes and antibodies act locally on metastatic tumor cells in regional nodes, or penetrate through efferent lymphocytes into the blood stream where they play a role in the prevention of metastatic tumor growth. They also migrate through capillary walls and enter the area of the primary tumor. Considering the different anatomic and biochemical environment in regional nodes, blood, parenchymal organs, and the primary tumor, the immunological modality that is most effective in preventing tumor growth in one area may be unimportant elsewhere. Immunological resistance is more likely to be effective in prevention of metastatic spread of tumors than it is in arresting primary tumor growth. Even under optimal circumstances, delivery of sensitized cells into the tumor area is probably an inefficient process and if sensitized lymphocytes do not accumulate, there will not be a sufficient stimulus to macrophage infiltration to produce a local inflammatory response. Penetration of specific antibody into the tumor may also be a problem since as a rule the most effective cytotoxic antibodies are in the IgM class and IgM antibodies are largely restricted to the intravascular compartment and lymphatic fluid. Even for IgG antibody it is entirely possible that penetration of B lymphocytes into the tumor tissue area is limited and local antibody synthesis is a more effective mechanism for delivering antibody than translocation of antibody from the blood. Problems in penetration may explain the survival advantage of tumor cells in immunological privileged sites such as the hamster cheek pouch. Hershey and MacLennon (16) recently have suggested another form of tumor sequestration in which viable leukemia cells are ingested and survive in rodent macrophages presumably because they are protected from immunological damage. However, under the usual transfer conditions *in vivo* these cells increase rather than decrease resistance to tumor growth (2).

1. Circulating Tumor Antigens. The delivery problem is compounded when circulating antigen is present. Many tumors release substantial amounts of antigen into the blood and lymph. The antigen may be in the form of solubilized protein, membrane fragments, or intact cells. Tumor cells can be

demonstrated in the peripheral venous blood of about 30% of patients with advanced carcinoma of the colon and when mesenteric venous blood is studied an even higher percentage is obtained (39). Large numbers of circulating tumor cells have also been demonstrated in the peripheral blood of patients with carcinoma of the breast and lung. Increased levels of circulating carcinoembryonic antigen (CEA) (undefined as to whether it is on intact cells or in a solubilized form) are demonstrable in about 60% of all patients with adenocarcinoma of the colon and in almost 100% of patients with extensive metastatic disease (35, 42). The concentration of CEA in the serum of patients with colonic carcinoma can be as high as 500 ng/ml (35). If it is assumed that CEA is evenly distributed in extracellular fluid there would be milligram amounts of circulating CEA in some individuals. Moreover, part of the circulating antigen may already be complexed to antibody and therefore undetectable by immunoassay. The presence of relatively large amounts of circulating tumor antigen is not unique to carcinoma of the colon. Large quantities of α-fetoprotein have been demonstrated in the serum of patients with hepatoma (26), the only other human tumor antigen for which extensive quantitative immunochemical measurements have been made.

Antigen can interact with sensitized lymphocytes in the responding node, in the circulation or in the interstitial fluid of primary tumor (fig. 2). At each level the effect of antigen is to block the immune response. In responding nodes large amounts of antigen appear to prevent the emigration of sensitized lymphocytes (2). In rats with sizeable local tumors the thoracic duct lymph does not contain the expected number of lymphoblasts even though the nodes draining the tumor are undergoing intensive immunological stimulation. The primary tumor is involved in some way in the inhibition of lymphoblast migration as demonstrated by the rapid appearance of lymphoblasts in thoracic duct fluid when the primary tumor is removed. Alexander and Hall (2) have speculated that large amounts of tumor antigen paralyze sensitized lymphoblasts, preventing them from leaving the node and becoming available for systemic control of tumor growth. They feel that impaired dissemination of sensitized lymphoid cells is an important factor in restricting host resistance to tumors.

Antigen in the blood will also have access to circulating antibodies and sensitized lymphocytes before the primary tumor will. In the case of circulating antibody this leads to the formation of soluble immune complexes, and diversion of antibody away from the target tissue into blood vessel walls and phagocytes. Possible hypersensitivity phenomena such as nephritis, cutaneous rashes, and manifestations of autoimmunity are well recognized complications of malignancy and it seems highly probable that circulating immune complexes are involved. The fate of a sensitized lymphocyte encountering large amounts of soluble antigen in the circulation is less certain but from studies of delayed hypersensitivity and contact skin sensitivity in guinea pigs it would not be surprising if hyposensitization occurred. Sensitized guinea pigs given injections intravenously with milligram amounts of specific antigen fail to show the expected delayed inflammatory response to skin on subsequent local challenge (6). The loss of skin reactivity is temporary, lasting just a few days, but it seems likely that with continued exposure to antigen, as would be the case with a widespread tumor, persistent hyposensitization would occur. Whether the diminished responsiveness represents some form of sterile lymphocyte activation, antigen excess inhibition, or redistribution of lymphocytes is uncertain.

Continuing massive exposure to antigen may also explain the non-specific depression of immunity that occurs in individuals with widespread tumors. In the sensitized guinea pig model large amounts of soluble antigen produce a non-specific as well as specific depression of immunological reactivity (6). It is tempting to speculate that non-specific

products of a continuing systemic response, possibly lymphokines released from responding lymphocytes, are responsible for the altered immune reactivity.

2. Antibodies and Antigen-antibody Complexes. Another mechanism which might interfere with the host response is the formation of blocking antibodies (*e.g.*, antibodies that produce enhancement of tumor growth). Three apparently distinct mechanisms by which an antibody might promote tumor growth have been identified or suspected:

1) An enhancing effect of antibodies on tumor growth *in vivo* has been demonstrated in experiments in which antisera was given prior to the grafting of a tumor and caused a delay in graft rejection (10). The inhibition of tumor growth was obtained with antisera from animals immunized with dead tumor cells and tumor cell extracts as well as with sera from animals with viable tumors. Originally there was speculation that the transferred antibody might cover up antigen on the tumor cell protecting it from the host's efferent immune response. However, it has not been possible to demonstrate that large amounts of antisera can prolong tumor survival indefinitely in animals with established tumors (10). It, therefore, seems more likely that the blocking action of antiserum is primarily in the afferent arm of the host response to prevent immunization.

2) A blocking factor capable of interfering with cellular immune resistance to tumor cells has been obtained from the sera of human beings and animals with progressively growing tumors (37). Most of the work dealing with the inhibitor has involved a colony inhibition system in which lymphocytes from donors with tumors can be shown to interfere with the growth of autochthonous or allogeneic tumor cells of the same histological type (homologous tumor cells). When the serum inhibitor is present sensitized lymphocytes fail to interfere with target cell growth. The ability of the serum to diminish target cell damage is specific in that tumor growth is promoted only in the homologous system and the inhibitor can be adsorbed by homologous but not heterologous tumor. Specific absorption by tumor would imply that the inhibitor contains antibody but the immunoglobulin class and immunochemical specificity of the putative antibody are not known and the possibility that it is partially degraded is not excluded. The inhibitor also appears to contain antigen, since it is bound by sensitized lymphocytes. Presumably it is this capacity to specifically adhere to sensitized lymphocytes which leads to lymphocyte inactivation and if inactivated lymphocyte are thoroughly washed they regain the ability to interfere with tumor cell growth. In view of the ability of the inhibitor to combine both with homologous tumor cells and sensitized lymphocytes it is presumed to be a special form of antigen-antibody complex.

3) There has also been speculation that a non-cytotoxic antibody might act as a stimulus to resting tumor cells, increasing the overall rate of tumor cell growth (34). This suggestion is based in part on the observation that antibodies to lymphocyte surface antigens (histocompatibility and immunoglobulin determinants) promote lymphocyte transformation. However, because lymphocytes are specialized cells whose normal function is to respond to cell surface stimulation by antigen the relevance of the lymphocyte model to antibody enhancement of tumor cell growth is uncertain. Recently Shearer *et al.* (36) have demonstrated that trinitrophenylated (TNP) HeLa cells exposed to anti-TNP antisera in tissue culture take up increased amounts of ^{125}I iododeoxyuridine. However, in subsequent studies similar effects have not been observed with antisera to tumor antigens, and even in the TNP system it would appear the effect is due primarily to an increased uptake of iododeoxyuridine into the cell rather than an increase in cell number. The lack of convincing effects of antibody on tumor cell replication *in vitro* is an argument against the immunostimulation concept although certainly more studies are indicated.

VI. Resistance of the Tumor Cell to Immune Destruction

Another critical factor in determining the influence of the immune response of tumor cell growth are the properties of the tumor cell itself.

1. Distribution of Tumor Specific Antigen. Nearly all of the evidence in regard to both cell and antibody mediated cytotoxicity indicates that the immunologically specific component of the reaction takes place at the cell surface. This seems axiomatic in the case of lymphocyte mediated cytotoxicity but it is almost certainly also true for the cell damaging effects of antibody. An interesting exception is a tumor inhibiting effect of antithymidine antibodies recently described by Liebeskind *et al.* (21). The antibodies involved apparently penetrate into the tumor cell by pinocytosis and once inside act to reduce the intracellular thymidine pool or interfere with essential polynucleotide function. The applicability of the antithymidine antibody to the immunotherapy of malignancy is uncertain because the concentrations of antibody required are high and selectivity for tumor cells is based solely on the relatively high rate of pinocytosis in the malignant cells studied. Nonetheless these observations clearly indicate that antibodies with specificity for intracellular constituents cannot be totally ignored. It may be significant that individuals with melanoma who have a favorable clinical course frequently have antibodies to antigens in the cytoplasm of the tumor cell (20). A possible explanation is that when immune resistance is effective, tumor cells are destroyed and release interior antigens which give rise to anticytoplasmic antibodes. In accord with this possibility anticytoplasmic antibodies are seen usually in association with cytotoxic antibodies to tumor surface membrane antigens. Conceivably antibodies to interior antigens directly contribute to immune resistance by penetrating into cells already damaged by a cytotoxic reaction at the cell surface and preventing their recovery. Moreover, even if the cells were already dead, the interaction of antibody with cytoplasmic antigen and complement in the region of the tumor would lead to the release of chemotactic factors and amplification of the local inflammatory response. It is, therefore, possible that antibodies to surface membrane and cytoplasmic antigens act synergistically in promoting tumor cell destruction (35).

Focusing now on cell surface antigens another important consideration is the precise localization of the tumor antigen. In some tumors, the antigen is partially masked by glycopeptides and the immunological reactivity of the cells can be increased by digestion with neuraminidase, which removes sialic acid residues from the cell surface (35a, 36a). Other antigens such as CEA are in the glycocalyx and not part of the cell membrane proper (12). It is uncertain whether tumor antigens in this location predispose the cells to immunological damage to the same extent as antigens incorporated on the cell membrane. Quite possibly the exterior localization of the CEA may reduce the deposition of complement components on the plasma membrane, minimizing cell damage. Unfortunately, appropriate studies with anti-CEA antibody and human colonic tumor cells are not yet available. The involvement of CEA in cellular immune resistance to colonic tumor cell growth is also not adequately evaluated.

2. Surface Antigen Density. It is possible to draw a distinction between the influence of surface antigen density on susceptibility to complement mediated cytotoxicity and susceptibility to sensitized lymphocytes. In general, tumors with a high surface antigen density can be destroyed by cytotoxic antibody whereas tumors with a low antigen density are resistant (19). The variation in cell susceptibility is due to differences in the absolute quantity of cytotoxic antibody (and ultimately the amount of complement) which can be fixed to the cell. Indeed, when cytotoxic antisera are diluted they sometimes protect rather than injure the cell. The requirement for substantial levels of antibody binding in order to obtain cell destruction

may seem surprising since studies with sensitized sheep cells indicate that a cell can be destroyed if complement produces a single defect (one hit) on the cell surface. However, erythrocytes differ from nucleated tumor cells in being unable to repair their external cell membrane and this may be an important factor in their marked susceptibility to lysis (38). Moreover, while the production of a single hit theoretically requires only one IgM antibody molecule, the ability of IgM to penetrate into primary tumor areas must be assumed to be limited. IgG molecules penetrate more readily but the production of complement damage requires two IgG molecules in close juxtaposition which is a decisive limitation in tumor cells with a low density of tumor specific antigen (10).

The extent to which complement fixing antibodies contribute to host resistance in human tumors is not fully established. There is an overall correlation between high titers of serum cytotoxic antibody and a favorable clinical course with Burkett's lymphoma, osteogenic sarcoma, and melanoma (19, 25). With many human tumors cytotoxic antibodies are not demonstrable, even when sera are obtained at a favorable time after extirpation of the primary tumor. In experimental tumor systems with the exception of certain leukemias and lymphomas and one virus-induced mouse sarcoma, antisera are usually not cytotoxic and the passive transfer of antibodies does not confer resistance (19). As predicted, the exceptions are tumors with a high surface antigen density.

Transfer studies in animals with tumors that have a low tumor specific antigen density indicate that immune resistance can almost always be obtained with sensitized cells although, as already indicated, the magnitude of the resistance is limited. The effectiveness of sensitized cells in this situation can be ascribed to the unique biological properties of sensitized lymphocytes as killer cells. Under optimal conditions a single activated, specifically bound lymphocyte (presumably a T cell) is capable of destroying a foreign cell. Obviously in a target cell system that is operating this efficiently, the antigen density on the tumor cell need not be critical. Yet there is little doubt from tissue culture studies that when the antigenic difference between the sensitized lymphocyte and the target cell is small, cytolysis is diminished, often to 50% or less, even at high lymphocyte to target cell ratios (14). It may be that when the antigenic dissimilarity is minimal (as would appear to be the case in most human tumor systems) the affinity of the interaction between the sensitized cell and the target cell is low and lymphocyte activation is inefficient. A low frequency of sensitized cells in the total lymphoid cell population reducing effective cell-cell interaction or variation in the susceptibility of the tumor cell to cytolysis during different stages of the cell cycle (13) may also be involved. Whatever the explanation, with weak antigenic differences, large numbers of lymphocytes from sensitized animals often fail to produce 100% tumor cell lysis, even with target cell monolayers in which contact can theoretically be established with every cell. In solid tumors where there is the additional problem of delivering the sensitized cell to the target area, it is not surprising that cellular immunity is often less than fully effective.

3. Antigenic Modulation and Tumor Cell Selection. Recent observations indicate that when immunoglobulin-containing lymphoid cells interact with an anti-immunoglobulin antibody there is an alteration in their surface antigen distribution with collection of antigenic molecules at one pole of the cell (capping), followed by internalization (41). Similar changes in lymphocyte membrane protein distribution occur with phytoagglutinins and antibodies to other surface antigens. The change in the localization pattern is presumed to be caused by perturbation of the lymphocyte surface by a cross-linking agent since univalent antibodies do not produce this effect. Capping has been observed in fibroblastic cell lines (7) suggesting that the phenomenon is general and likely to be a property of malignant cells. The extent to which modulation of this type might occur

during the reaction of antibodies or sensitized lymphocytes with tumor cells is uncertain but it could be important in determining susceptibility to further injury. Faanes *et al.* (9) have found that the ability of antibody to block lymphocyte mediated cytotoxicity with allogeneic mastocytoma cells is maximal early and decreases over a period of several hours. The change in cell susceptibility with time does not appear to be due to the elimination of antibody, raising the possibility that antigen redistribution is involved.

A more important mechanism for altering antigenic density may be immunoselection. When tumor cells are grown in tissue culture in the presence of cytotoxic agents it is frequently possible to select resistant cells. Presumably cells with variable degrees of drug resistance are arising by somatic mutation in the tumor cell population and have a survival advantage under restrictive growth conditions. There is evidence to indicate that under conditions of partial immunological suppression of cell growth *in vivo* in immunized animals, a similar selection can occur, leading to a population of relatively resistant cells (19). Such selection may be on the basis of lower surface antigen density or some other property of the cell rendering it less susceptible to immune elimination. Even under conditions of repeated passage through resistant animals, however, some tumor specific antigen always remains. Moreover, in tumors with a low specific antigen content from the very beginning, it is difficult to demonstrate any change in antigen content with continued passage. Thus it is mainly in cells that are especially susceptible to immune elimination that immunoselection is an important mechanism.

4. Structural and Biochemical Properties of the Tumor Cell. The structural and biochemical properties of a tumor cell also influence its resistance to immunological injury. Mouse mastocytoma cells are used frequently as target cells in studies of cell mediated immunity because of their marked susceptibility to cell mediated lysis. The vulnerability of mastocytoma cells to injury presumably reflects the marked fragility of mast cells in general. Other properties of tumor cells involved in resistance to immune-destruction have not been identified. It is not inconceivable that tumors might secrete products that interfere with macrophage, lymphocyte, or complement activation. The fibrinolysin recently described in tumor cell cultures by Ossowski *et al.* (27) should be studied for these capabilities.

5. Growth Characteristics of the Tumor. Highly malignant tumors may proliferate so rapidly they outdistance the immune response, even though immune destruction is taking place. Under such conditions increased amounts of antigen are likely to be released, helping protect the tumor. The pattern of tumor growth will also determine the percentage of cells in different phases of the cell cycle with ancillary effects on immune resistance. Recent evidence indicates that the density of surface tumor antigen is greatest in G_1 and that cells are especially susceptible to cytotoxic antibodies at that time (5). There is one report that resting tumor cells are less susceptible to lymphotoxins (13) which, if verified, could be an important clue to why tumor cell populations vary so markedly in their resistance to sensitized lymphocytes.

VII. General Considerations in Immunotherapy

If immunotherapy proves to be an important adjunct in the control of cancer it seems likely that its most effective application will ultimately be based on a careful evaluation of the host-tumor relationship in the individual to be treated: the level of cellular and humoral immunity; the antigenic and metabolic properties of the tumor; the total quantity of tumor; and the amount of circulating antigen. Unfortunately our present methods of analysis do not have the necessary sophistication to permit the development of *ad hoc* regimens fitted to the problem at hand. Initial investigational efforts, therefore, should focus on the immunochemical characterization of tumor antigens

and therapeutic manipulations that would have broad applicability. Limitations imposed by circulating tumor antigen, low tumor antigen density, and partial immunological tolerance and an inability to deliver lymphoid elements to the tumor are likely to be common to many tumors. If ways can be found to circumvent these these difficulties with one tumor they are likely to have applicability to other tumors. Approaches currently being tried include conventional active or passive immunotherapy and more speculative but exciting concepts such as circulating antigen removal or the use of antibody toxin conjugates. Since multiple factors are involved in the failure of immune resistance and the growth stage of the tumor cell may influence its susceptibility to injury, it seems almost certain that a combination of therapeutic measures will be more effective rather than any single measure alone. Regardless of what approach is taken, preliminary efforts to reduce the total tumor load as much as possible by non-immunological means will be needed to increase the likelihood of success.

VIII. Conventional Approaches to Immunotherapy

A. Active Immunization

Amplification of the immune response has been attempted by immunization with tumor cells or partially purified tumor antigens in adjuvant. Immunization with tumor is simplified if there is cross reactivity between tumor cells from different individuals and autochthonous tumor cells are not needed. Specific immunization usually is carried out in multiple peripheral sites to maximize the amount of lymphoid tissue stimulated. Peripheral immunization avoids the problem of paralysis of responding lymphoid elements that is sometimes present in nodes regional to a tumor (see above). As a rule the clinical response to active immunization has been disappointing. Mathé *et al.* (23) have observed objective improvement in more than 50% of leukemic patients given irradiated autologous lymphocytes in combination with BCG. But other investigators have failed to obtain such impressive results and the combination of immunization with chemotherapy has failed to yield better results than either method alone. Despite the relative lack of success with direct immunization further attempts in conjunction with measures to control circulating antigen levels are strongly indicated. Since partial immunological tolerance may limit the response it may be desirable to use chemically altered tumor antigens in some of these studies. It is well established that immune tolerance to soluble proteins can be broken by immunization with the same protein, substituted with hapten. Immunization with chemically or enzymatically modified tumor cells has been used previously in an attempt to control cancer growth with limited success but this approach has not been combined with other measures designed to maximize therapeutive effectiveness. Another advantage of using chemically altered tumor cells has to do with a difference in antigen recognition mechanisms between T and B lymphocytes. It has been learned recently that T cell recognition is less susceptible than antibody recognition to chemical manipulation of the antigen (28). We have immunized guinea pigs with acetoacetylated CEA (AA-CEA) and found that the antibodies that are formed react poorly with unaltered CEA (3). In the same animals (*e.g.*, immunized with AA-CEA) CEA produced a marked delayed hypersensitivity response, indicating that cellular recognition is intact despite the difference in immunizing and skin test antigens. Thus, AA-CEA produces marked cellular immunity to unmodified CEA in face of a very limited humoral antibody response to the same antigen. The selectivity of the immunization is of considerable interest because one of the objections to tumor antigen immunization is the possibility that blocking antibody levels might be inadvertently increased, conceivably doing more harm than good. It would appear that by giving an appropriately altered antigen this risk may be minimized or avoided altogether.

B. Non-specific Amplification of Immunity

Adjuvants such as BCG and *Corynebacterium parvum* have been used in an effort to increase non-specific resistance to tumors (40). The experience has been similar to that with specific immunization in that some therapeutic benefit has been obtained but the results are not necessarily superior to what is accomplished by chemotherapy. There is currently controversy as to whether all of the BCG preparations that have been used have comparable adjuvant activity. Possibly more favorable overall results will be obtained when preparations are better standardized. The mode of action of BCG and other adjuvants in resistance to malignancy is not well understood. BCG is known to activate macrophages and otherwise promote inflammation but it can also increase specific cellular and humoral immunity.

Another way of promoting inflammation in lymphoid tissues is by the induction of a graft *versus* host reaction (2b). The recent observations of Lim and Good[1] on the beneficial effect of allogeneic leukocytes in lepromatous leprosy indicate the possible usefulness of this approach in the treatment of malignancy. The presumed role of inflammation in the resolution of cutaneous tumors in areas repeatedly exposed to contact skin sensitizers is discussed below.

The possibility of pharmacological amplification of immune resistance by hormonal agents, purified lymphokines and microbial products also deserves exploration. It has been reported recently that the effect of sensitized cells in target cell systems is increased by cholinergic agents (40a) but studies on the effect of such therapy *in vivo* have not been carried out.

C. Passive Immunization with Conventional Antibody

Attempts to use antibodies as specific cytotoxic reagents for tumors date back to 1895 to the experiments of Hericourt and Richet (15). Much of the work has been poorly controlled and lacking in objective criteria for improvement. Convincing reports of success are so few that optimism in regard to the potential usefulness of this approach is not warranted. Nonetheless, in animal leukemias, antisera clearly transfer resistance and its is possible that by using procedures for maximal antigen removal in combination with partially purified cytotoxic or cytophilic antibodies success might be achieved in human tumors as well. Passive immunization with cytotoxic animal antibodies of high affinity, purified to remove blocking antibody activity, would be of particular interest. Since animal antibodies are foreign proteins they are capable of producing antiglobulin antibodies leading to serum sickness and a reduced antibody half-life. However, recent studies indicate that when foreign γ-globulins are ultracentrifuged so as to remove polymeric protein the monomeric fraction may be tolerogenic rather than immunogenic, suggesting that serum sickness problem can be controlled partially.

[1] R. Good: Personal communication.

D. Transfusion of Sensitized Allogeneic or Xenogeneic Lymphocytes

Sensitized allogeneic or xenogeneic lymphocytes are capable of reacting against tumor cells in foreign hosts but major problems can arise because of host *versus* graft or graft *versus* host responses, particularly if repeated injections are given. Not surprisingly the experience with allogeneic transfers has been disappointing. In four studies involving more than 200 subjects objective evidence of improvement has been obtained in less than 20% of individuals (40). The limited success achieved may be due as much to non-specific stimulation of host responsiveness by allogeneic cells as to transmission of specific cellular immunity.

IX. New Approaches to Immunotherapy

A. Selective Removal of Blocking Antigen and Antigen-antibody Complexes

Circulating tumor antigen may well represent the major obstacle in the immunotherapy of cancer. Even assuming attempts to increase the level of cellular or humoral immunity to the tumor are successful, if circu-

lating antigen is not removed it will interfere with the practical yield of immune resistance that is achieved. Circulating tumor antigen concentrations can be controlled partially by surgical extirpation of as much of the tumor as possible. Cytotoxic agents and irradiation may also decrease the number of tumor cells and the level of tumor antigen production although there is a danger that active immunological processes would also be inhibited, which would be partially self defeating. Once the tumor antigen load has been decreased further control might be accomplished by selective removal of tumor antigens and antibodies by exchange transfusion, plasmapheresis or solid phase immunoabsorbents. Recent observations from this laboratory indicate that it is feasible to attach protein to plastic tubing and use the tubing as an immunoadsorbent *in vivo* (22). Nylon tubing was derivitized with human serum albumin (HSA) and ovalbumin (OA) (fig. 4). The HSA and OA derived nylon had

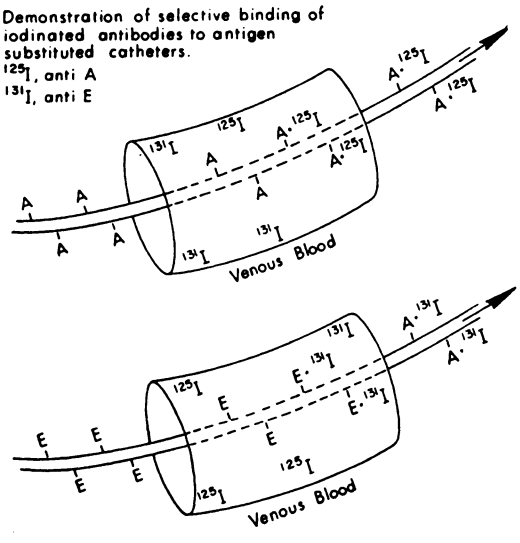


FIG. 4. Solid phase immunoadsorption of protein *in vivo*. Catheter A contains human serum albumin; catheter E contains egg albumin (ovalbumin). The two catheters are implanted in the inferior vena cava of dogs previously injected with ^{125}I-labeled antihuman albumin (anti-A) and ^{131}I-labeled anti-egg albumin (anti-E). The A catheter selectively binds anti-A antibody, the E-catheter selectively binds anti-E antibody.

selective reactivity for rabbit anti-HSA and rabbit anti-OA antibodies respectively as demonstrated in experiments *in vitro* with ^{125}I- and ^{131}I-labeled antibodies. Selective reactivity with the appropriate iodinated antibody could also be shown *in vivo* in dogs with HSA and OA catheters implanted in the inferior vena cava (fig. 4). From a kinetic analysis of the *in vivo* adsorption data it was calculated that in a reel arrangement in which the nylon catheter would be cycled slowly through the inferior vena cava milligram amounts of antibody could be removed in a 24-hr period. The indwelling catheter approach is readily adaptable to the removal of antigen and antigen-antibody complexes, hopefully on a semicontinuous basis. The removal of circulating antibody from tumor patients also merits consideration although the role of antibody in tumor homeostasis presently is uncertain and this may not be necessary or desirable. Moreover, from the studies of Uhr (43) in which antibody was removed with an extracorporeal technique, antibody depletion might be associated with a marked rebound in antibody synthesis, limiting the effectiveness of the procedure. It should be emphasized that work with solid phase immunoadsorbents *in vivo* is at a preliminary stage. Even with further development the limited availability of human tumor antigens places a temporary restriction on using this approach in human cancer patients. But with recently developed techniques for obtaining tumor antigens from growing tumor cells in tissue culture this should ultimately be less of a problem. The possibility is not yet excluded that the nylon immunoadsorbent would also deplete sensitized cells. It seems likely that if this proves to be a problem, the design of catheters with surface properties that would permit absorption only if antibody and not sensitized cells might be possible. Non-specific sticking of formed elements of the blood to the catheter is another potential difficulty but has been essentially absent with the nylon catheters currently under evaluation.

TABLE 1

*Amplification of antibody cytotoxicity by direct or indirect toxins**

A. *Direct toxins*		
Diphtheria		
Phospholipase A		
B-amanitin		
Ricin		
Radioactive iodine (or another radioactive molecule)		
Nitrogen mustard		
Streptonigran		
B. *Indirect toxins*		
Substrate	Enzyme	Toxic product
Azonitrogen mustard	Azo reductase	Active mustard
Glucose, I^-	Glucose oxidase Lactoperoxidase	Iodinated cell membrane
Glucose, arsphenamine	Glucose oxidase Horseradish peroxidase	Toxic arsenical
Cycasin	B-glucosidase	Alkylating agent
Allyl alcohol	Alcohol dehydrogenase	Acrolein

* Modified from C. W. Parker: Immunologically directed cell destruction *In* Immunologic Intervention, pp. 196-204, ed. by J. W. Uhr and M. Landy, Academic Press, New York, 1971.

B. Antibody-toxin Conjugates (Educated Cytotoxins)

Another possible method of improving selective tumor immunotherapy is to attach highly potent toxins onto tumor specific antibodies providing "educated cytotoxins" in which the antibody gives selectivity to the toxin and the toxin increases the killing potential of the antibody. This approach is particularly attractive for tumor cells where antigen density is too low to provide for direct complement mediated cytotoxicity. The idea of utilizing toxin containing antibodies was visualized shortly after the turn of the century by Ehrlich (8) and has received sporadic attention since with only limited success. Feeling that what was needed was a systematic examination of a variety of possible methods of increasing antibody toxicity, several years ago Philpott, Aach, and Parker undertook a detailed study of this approach (29). Some of the possible methods for achieving amplification of antibody toxicity considered by us and others are given in table 1. These include direct toxins such as diphtheria toxin, nitrogen mustard, radioactive iodine, and asparginase. The possible potential value of conjugating direct toxins to antibody is already evident from studies with diphtheria toxin (24, 32) nitrogen mustard (in this case reversibly bound to antibody) (11), and radioactive iodine. Our group has been especially interested in the possible usefulness of indirect toxins, particularly enzymes capable of catalytically converting protoxins to toxins in the region of the tumor cell (29) (table 1).[2] Our most extensive studies have been carried out with antibodies conjugated to glucose-oxidase, an enzyme with a high turnover number which converts glucose to gluconic acid generating hydrogen peroxide.[2] The H_2O_2 activates lactoperoxidase and, in the presence of I^-, cells can be catalytically iodinated (fig. 5). Studies in tissue culture indicate that when the level of cell iodination is sufficiently high the cells die. In the complete system antibodies conjugated to glucose-oxidase are capable of killing cells at very low concentrations—levels at which the unconjugated antibody produces little or no complement dependent cytotoxicity. The cytotoxicity is selective as can be demonstrated in cell mixing studies in which cells

[2] G. W. Philpott, W. T. Shearer, R. J. Bower and C. W. Parker: Selective cytotoxicity of hapten substituted cells with an antibody enzyme conjugate. J. Immunol., in press.

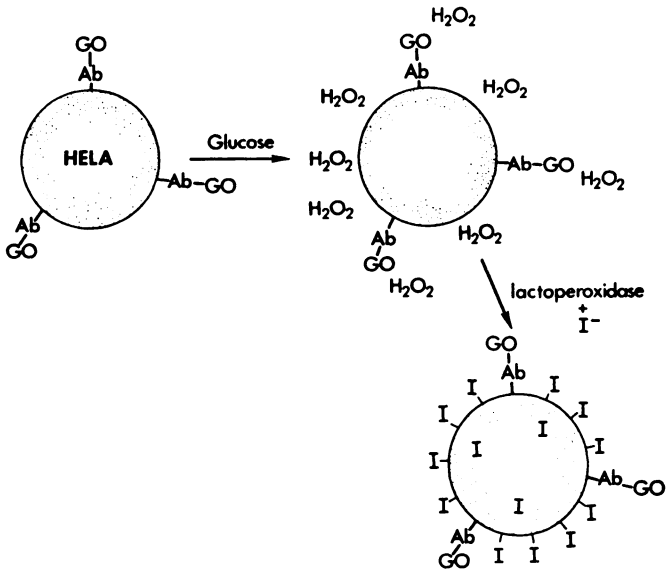


FIG. 5. Enzymatic iodination of tumor cells. The antibody-glucose oxidase conjugate attaches to tumor cells, and in the presence of glucose generates H_2O_2. The H_2O_2 activates lactoperoxidase, permitting the introduction of iodine molecules onto the cell. (Taken from ref. 30, C. W. Parker, R. J. Bower, R. D. Aach and G. W. Philpott: The immunologic therapy of cancer. *In* Proceedings of the Fifth International Congress on Pharmacology, S. Karger, Basel, in press.)

not containing the antigen are present but not killed. Potent cytocidal effects also have been obtained with an antibody-alcohol dehydrogenase-acrolein system and with an antibody-glucose oxidase-horseradish peroxidase-arsphenamine system, indicating the generality of this approach. It is now necessary to evaluate effects on tumor growth *in vivo* and in order to obtain maximal effectiveness it may be necessary to improve penetration by reducing the molecular size of the conjugates. This can be accomplished by utilizing Fab or Fv antibody fragments in the conjugation reaction, choosing enzymes of minimal molecular size and designing the conjugation procedure so as to minimize the formation of high molecular weight polymers.

Whole animal studies with the enzymatic iodination system will be of particular interest because the reaction introduces large numbers of iodine molecules onto the cell membrane, altering the antigenic character of the cell. We plan to immunize tumor bearing animals with iodinated tumor cells prior to the injection of antibody-glucose oxidase conjugates, lactoperoxidase and I^-. If the enzyme-mediated iodination reaction is successful, the altered tumor cells should be much more susceptible to immune elimination. We are also interested in using antibody-enzyme conjugates to enzymatically trap circulating toxins in the tumor, magnifying the level of toxicity. Attempts to trap additional cytotoxic molecules in tumors might also involve the use of second antibody (an antibody-toxin conjugate specific for immunoglobulin determinants on the anti-tumor antibody). All these systems ideally would be carried out in conjunction with methods for rapidly clearing antibody-toxin molecules that have not localized in the tumor. This would permit the use of large quantities of toxic-antibodies without undue systemic toxicity.

C. Efforts at Improving Delivery of Immune Reactants

The simplest approaches to increasing the delivery of specific and non-specific elements of the immune response into the tumor areas are regional perfusion techniques, employing

high concentrations of sensitized cells or antibody, or the introduction of an inflammatory stimulus directly into the tumor. The dramatic response of malignant cutaneous tumors which have been repeatedly exposed to cutaneous sensitizers (17) is ample evidence of what can be accomplished if an adequate inflammatory stimulus is provided. It is possible that tumor bound antibody-enzyme conjugates could be used to generate chemical sensitizers locally in the tumor. However, good localization and high enzymatic selectivity would be needed to avoid the liberation of substantial amounts of sensitizer systemically. The possibility that specific immunological inflammation might be magnified by interfering with inhibitors of the complement system also deserves consideration. Other interesting approaches relate to the use of agents which alter lymphocyte distribution such as the various chemotactic factors or lymphocytosis-producing bacterial fractions. The deliberate use of IgE antitumor antibody may also be contemplated as a means of releasing agents which increase vascular permeability in the area of the tumor. In this connection it is interesting that malignant tumors are said to occur with decreased frequency in individuals with allergy (44).

D. Possible Synergistic and Antagonistic Interactions between Cytotoxic Agents and Immunity

Interrelationships between the immune system and cytotoxic drugs merit careful study. It is recognized that toxic drugs interfere with immune resistance but synergism is also possible. It would be of interest to determine what would happen if cells with plasma membranes damaged by complement were exposed to cytotoxic drugs which do not normally enter cells such as the nitrogen mustard glucuronides. Even if this form of synergism cannot be demonstrated obviously cytotoxic drugs will be needed frequently to control tumor growth to the point that effective immune resistance is at least a theoretical possibility. More studies are needed as to how this can best be achieved without impairment of immune reactivity. Cytotoxic agents which selectively inhibit antibody producing cells also can be used if this seems desirable.

E. Administration of RNA Extracts from Sensitized Lymphocytes

Other approaches to increasing tumor immunity include the possibility of using homologous or heterologous RNA extracts or transfer factor in an attempt to stimulate the immune response of the host (33). This might involve the use of lymphocyte extracts from patients who have recovered from the tumor or heterologous RNA from lymphocytes of immunized animals. The use of a foreign species would have the advantage of permitting the deliberate production of a high degree of immunity in the animals used as the source of sensitized cells. The possible usefulness of heterologous RNA is suggested by the recent studies indicating that RNA extracted from sensitized guinea pig lymphocytes transfer tumor immunity to mouse lymphocytes (33). The future of this approach is uncertain since the immunity achieved to date has been modest and there is no evidence that the degree of immune resistance can exceed that obtained by active immunization *per se*. Nonetheless the possible usefulness of RNA extracts deserves thorough exploration.

F. Attempts at Activating Non-sensitized Lymphocytes

Another approach to obtaining more effective cellular immunity involves attempts to non-specifically activate lymphocytes with non-specific mitogens such as concanavalin A and phytohemagglutinin (PHA) (4). It is known that when lymphocytes are activated by mitogens, they secrete toxic substances and interfere with cellular growth. Tumor cells cultured in the presence of unsensitized lymphocytes and PHA show evidence of cytotoxicity. The details of how the cell damage is produced are not fully elucidated. The mechanism appears to differ from

what occurs when sensitized cells attack target tumor cells. Application of this approach *in vivo* presents serious problems because of the uncertainty that lymphocytes binding the mitogen would reach the tumor and the lack of specificity of the lymphocyte for tumor specific antigens. A better approach may be to attempt to passively sensitize the lymphocyte with antibody. We are currently attempting to sensitize normal T lymphocytes with antitumor antibody bound to non-mitogenic fragments of concanavalin A but the data are too preliminary to warrant discussion at this time.

IX. General Conclusions

At best attempts to increase immune resistance to established tumors have met with limited success and immunotherapy cannot be considered to be an accomplished goal. However, some of the problems in maximizing immune resistance have been recognized only recently and if these difficulties can be solved better results can be anticipated. Bold and imaginative approaches are needed but it is difficult to predict which ones are most likely to be successful. Certainly, the prospects are sufficiently good to warrant continued intensive research in this area. My own prediction would be that immunotherapy will ultimately prove to be an effective means of control of tumor growth, at least with many tumors, but that the accomplishment of this objective will not come easily.

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