Macrophage activation

Potential for cancer therapy[1]

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Tumoricidal activity of activated macrophages has been clearly demonstrated by various in vitro and in vivo studies of a wide variety of animal tumor systems. Recent in vitro studies have shown that human peripheral blood monocytes can also be activated to generate similar tumoricidal activity. Significant inhibition of spontaneous lung and liver metastases in different animal tumor systems has been demonstrated with intravenous therapy of macrophage-activating agents such as muramyl dipeptide and C-reactive protein delivered in liposomes. These studies provide a rationale for a similar approach in clinical cancer therapy, particularly in an adjuvant setting. The distinct advantages of this approach include low toxicity, nonspecificity of tumor-cell killing, tumoricidal activity against drug-resistant tumor cells and cells with metastatic potential, and lack of development of tumor cells resistant to macrophage killing. Admittedly, these advantages are only theoretical at this stage, and appropriate clinical trials must be carried out to demonstrate whether they can be realized in a clinical setting.

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Conventional immunotherapy, either active or passive, has met with little or no success in cancer control, as seen from the results of the clinical trial carried out during the past two decades. It may be possible, however, that certain components of the immune system representing body defenses can be manipulated and used in conjunction with other cancer therapeutic modalities such as surgery, radiation, and chemotherapy to achieve a beneficial result. The recent creation and promotion of the Biologic Response

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Modifiers Program by the National Cancer Institute emphasize this approach. The potential use of the tumoricidal property of activated macrophages is one example of this approach and has been the subject of extensive investigations since it was first demonstrated in the early 1970s. Several excellent monographs and review articles have been published on this subject in recent years. The purpose of this article is not to review this vast literature, but rather to focus on recent, promising developments involving liposome delivery of macrophage-activating agents, which have given encouraging results in animal tumor systems.

The introduction to the concept of macrophage activation, as noted by Mackaness et al,[1] can be traced by Metchnikoff, who observed in the early 1900s the mononuclear cells from animals resistant to certain bacteria had the ability of phagocytizing and killing the same organisms. The concept was developed further by Mackaness et al[1] in the 1960s, who used the term *activated* to describe the effector mononuclear cells involved in cellular immunity to various organisms. In the early 1970s, Evans and Alexander[2,3] demonstrated that the same activated macrophages were able to destroy tumor cells. This intriguing ability of the activated macrophage to kill tumor cells has been observed by various investigators during the past decade, although the mechanisms by which the activated macrophage can distinguish between normal cells and malignant cells are not understood.

Several lines of evidence support the concept that macrophages represent one of the key body defenses against the growth and spread of neoplastic cells. Only a brief summary of this evidence will be considered here, since a detailed review of this area is beyond the scope of this article. Tumors are often infiltrated with macrophages and other mononuclear cells and the degree of this infiltrate is often inversely related to the rate of growth and spread of the respective tumor. Macrophages isolated from regressing tumors are more cytotoxic to the tumor cells than are macrophages isolated from rapidly growing tumors. In animal tumor models, adoptive transfer of macrophages has been shown to inhibit growth and metastases of these tumors. Use of nonspecific immunotherapy with agents such as Bacillus Calmette-Guerin (BCG) or *Corynebacterium parvum* has been shown to enhance the ability of recipient macrophages to inhibit tumor growth in vitro. However, a substantial number of reports have described the opposite effects of the macrophage, namely, stimulation of tumor growth, and in certain cases, a suppressor activity of macrophages blocking protective tumor immune response.

Major stimulus for using activated macrophages in a therapeutic approach came from the observation by Fidler[4] that a crude lymphokine preparation containing macrophage activating factor, when delivered in liposomes to tumor-bearing mice was effective in inhibiting and eradicating lung metastases. Subsequent studies showed that other, more specific and well-characterized agents such as muramyl dipeptide (MDP)[5] and C-reactive protein (CRP)[6] were able to produce the same effect as that of crude lymphokine preparation through macrophage activation. These recent studies have clinical relevance in that they suggest a rational approach to cancer therapy in an adjuvant setting, using macrophage activating agents with appropriate delivery systems.

The relevant review and discussion in this regard will be divided into four parts: (1) mechanisms of macrophage tumoricidal activity, (2) in vitro methods of measuring macrophage activation, (3) in vivo studies involving macrophage activation by liposome-encapsulated agents, and (4) rationale for clinical cancer therapy.

Mechanisms of macrophage tumoricidal activity

The capacity for tumor-directed cytotoxicity by macrophages is a well-recognized consequence of macrophage exposure to a number of activating substances such as lymphokines or microbial products. Although the exact mechanisms by which cytotoxicity occurs have not been completely defined, three basic concepts have emerged concerning macrophage killing: (1) that macrophage competence for tumor-cell killing is a transient function, (2) that binding to target cells may be involved in recognition and killing, and (3) that target-cell damage is done by secreted or released cytotoxic substances. Macrophage tumoricidal activity has been more fully described elsewhere.[7-9]

The length of time in which macrophages are competent to perform tumoricidal activity has been shown to be transient and, in some cases, irreversible.[10] Murine peritoneal macrophages exposed to lymphokine preparations demon-

strate tumoricidal activity after as little as four hours, but lose this capacity by 24 hours, although they remain fully viable.[10] One hypothesis to account for this relatively short period of tumoricidal competence proposes that an inhibitory effect is exerted by prostaglandins of the E series (PGE), which are secreted and released by activated macrophages.[11,12] Addition of PGE to macrophages together with an activating agent has been shown to inhibit subsequent development of cytolytic activity.[12] When activated macrophages were exposed to indomethacin or other cyclooxygenase inhibitors, PGE synthesis was prevented and duration of cytolytic capacity was enhanced.[12] Peritoneal macrophages have been found to secrete inhibitory concentrations of PGE into the surrounding culture medium within one hour of exposure to the activating agent—endotoxin.[12] However, cytolytic capacity was not found to be fully inhibited until 12 to 16 hours later, suggesting that PGE inhibition was a gradual process.[12] Activation by lymphokines appeared to induce cytolytic activity that was less susceptible to inhibition by PGE.[13]

Kinetic studies of macrophage activation with free or liposome-encapsulated lymphokines have shown that macrophages that have become unresponsive to free lymphokine can become activated by liposome-encapsulated material.[14] These results were interpreted as indicating that alterations in the macrophage surface may negate interaction with free lymphokine and that liposomes that are phagocytosed allow lymphokine activation by intracellular pathways. Results with liposome-encapsulated lymphokines, with respect to the transient nature of tumoricidal competence, have been similar to those with soluble lymphokines.[14,15] Tumoricidal activity has been found to decline with time, although a second exposure to free or liposome-encapsulated reagent has been shown to reestablish activity.[14] The minimum length of time required for macrophage activation with liposome-encapsulated lymphokines has been found to be four hours, which is similar to that observed with soluble reagent.[15]

The mechanism of tumor recognition by activated macrophages has not been totally characterized, although the phenomenon has been recognized for over a decade. Hibbs et al[16] reported that activated murine macrophages caused little or no destruction of normal murine fibroblasts, but were cytotoxic against the same types of fibroblasts that had been spontaneously transformed in vitro. Meltzer et al[17] examined macrophage killing against a number of sets of transformed cultured cells and their untransformed counterparts and observed in all cases that the transformed cells were sensitive to macrophage killing and that the untransformed counterpart was resistant. Other investigators[18] have confirmed the selectivity of macrophage cytotoxicity. Normal lymhocytes induced to enter blastogenesis by exposure to a mitogen have been shown to competitively inhibit macrophage killing of lymphoma cells, suggesting that macrophage recognition of tumor cells involves recognition of some property common to cells undergoing cell-cycle passage.[19] That the mitogen itself had no effect was shown by additional experiments in which interleukin 2-induced lymphoblasts similarly inhibited macrophage killing.[19] Alloantigen independence of macrophage tumoricidal activity has been well documented by experiments in which macrophages have been shown to kill tumor cells regardless of alloantigen profile.[20]

The recognition mechanism of activated macrophages may involve target-cell binding.[21] Activated macrophages have been found to bind larger numbers of neoplastic than nonneoplastic targets—a property not shared by normal, unactivated macrophages.[22] Binding of tumor target cells has been shown to be inhibitable by chelating agents or by exposure of macrophage membranes to proteolytic enzymes, and under these circumstances, cytotoxicity is inhibited as well.[22] Conversely, reagents that increase binding have been found to increase cytolytic activity.[21] Both binding and cytolysis have been completely abrogated by mechanically separating macrophages from target cells by 1.0-μm pore filters.[22]

The means by which actual cytolytic damage to target cells occurs is thought to involve cytotoxic substances released by the activated macrophage. Evidence for this hypothesis is derived from a number of studies demonstrating tumor-cell destruction by soluble materials produced spontaneously by cultured activated macrophages.[23–26] Binding to neoplastic target cells was found to enhance release of a cytolytic factor (CF) by activated macrophages.[27] This factor, which was characterized by Adams et al,[23] was found to be a neutral serine protease with molecular weight of approximately 40,000. Bovine pancreatic trypsin inhibitor blocked production of CF, but did not affect macrophage binding to

target cells, suggesting that target-cell binding and secretion of CF were separate functions of activated macrophages.[28] Kinetic studies of CF secretion by Adams and Marino[28] demonstrated that CF secretion began within 30 minutes of macrophage culture and was lost after 18 hours. Restoration of CF production could be achieved by pulsing macrophage cultures with endotoxin.[28]

Sharma et al[24] and other investigative groups[25,26] found that CF released by activated macrophages appeared to preferentially lyse neoplastic cells and to have little effect on normal, nonneoplastic target cells. Reidarson et al[29] also reported that a macrophage-produced CF with protease activity selectively bound to tumor cells.

While many of the macrophage-produced CFs appear to be proteases, activated macrophages are recognized to secrete a variety of potentially tumoricidal factors, including oxygen metabolites, interferon, and acid hydrolases. Macrophage secretory products are more fully described elsewhere.[9,30] Many of these products are not selective for tumor cells and are potentially damaging to nonneoplastic cells as well. Thus, some of the tissue changes associated with inflammatory injury may be related to the presence of toxic products released by activated macrophages. For example, lymphokines have been shown to enhance monocyte oxidation of low-density lipoprotein to an oxidized compound found to be cytotoxic to proliferating normal fibroblasts.[31] The enhanced capacity for production of oxygen metabolites by activated macrophages can be determined by using phorbol myristate acetate (PMA) to trigger oxidative metabolism.[32] Activated macrophages exposed to PMA exhibit increased secretion of hydrogen peroxide and demonstrate a tumoricidal activity that is inhibitable by catalase.[33] A variety of target cells have been shown to be susceptible to hydrogen peroxide lysis, including normal cells[33]; however, the role of hydrogen peroxide in tumoricidal activity of activated macrophages not exposed to a metabolic triggering agent has not been clarified. Monocyte tumoricidal activity has been observed in individuals with chronic granulomatous disease, although monocytes exposed to PMA did not produce hydrogen peroxide.[34] Enhanced oxidative metabolism including hydrogen peroxide production by activated macrophages mixed with tumor cells has not been demonstrated,[35] although such experiments do not rule out the possibility that oxygen metabolites may be secreted into the spaces between macrophages and bound target cells.[21]

It is possible that more than one mechanism may be involved in macrophage cytotoxicity.[9,36] Target cell susceptibility to hydrogen peroxide or cytolytic proteases has been shown to be extremely variable, and some target cells may be more sensitive to one reagent than to another.[37] In addition, synergism between the two reagents has been demonstrated. Brief exposure of target cells to a nonlytic dose of hydrogen peroxide was found to induce lysis after subsequent exposure to a nonlytic dose of cytolytic protease.[37]

Morphological and ultrastructural studies of macrophage–target-cell interactions have led to the suggestion that lysosomal components are released by macrophages and taken up by target cells.[38,39] Evidence for the existence of lysosome transfer from macrophage to target cell has been presented by the experiments of Martin et al,[40] who studied the effect of adriamycin on macrophage tumoricidal activity. Adriamycin administered intraperitoneally accumulated within mast-cell granules, which were released and then phagocytized by peritoneal macrophages. The drug could be visualized within cytoplasmic vacuoles of the macrophages that were found to be cytotoxic to target tumor cells. Analysis of target cells for the presence of adriamycin revealed localization of the drug in target cell nuclei, suggesting transfer from macrophage lysosomes.[40]

Tumor cells exposed to attack by activated cytotoxic macrophages display a series of morphological changes including vacuolation, condensation of intracellular material, rounding, membrane blebbing, and eventual fragmentation.[39,41] Activated macrophages may also induce cytostasis in target cells and under such conditions, tumor cells have been shown to lose mitochondrial oxidative phosphorylation function and to exist on glycolysis.[42]

In vitro methods of measuring macrophage activation

Tumoricidal activity

Activation of monocytes or macrophages has most frequently been determined by evaluating tumoricidal activity. A number of different assay systems have been described, and common to many is the use of radiolabeled target cells derived from cultured tumor-cell lines.[43,45] In the

first step of the general procedure, a monocyte or macrophage preparation is placed in the test container, usually a plastic microtiter plate, and cells are allowed to adhere. In studies of human monocytes, mononuclear leukocytes are first collected by Ficoll-Hypaque centrifugation and monocytes may be purified further by use of Percoll gradients[46] or centrifugal elutriation.[47] The nonadherent cells are later removed and monocyte–macrophages are exposed to an activating agent for up to 24 hours. In experimental animals, peritoneal macrophages may be activated in vivo, then removed by lavage and allowed to adhere to the test container.

In the second step of the assay, cultured target cells are incubated with a radiolabeled compound for varying periods of time until cells have incorporated the radiolabel. The third step of the assay consists of coculturing the radiolabeled target cells with the putatively activated macrophages for 6 to 72 hours. In the fourth and final step of the assay, the amount of radiolabel either in the culture medium or in surviving adherent target cells is quantitated. In the former situation, released soluble radiolabel is interpreted as indicating target-cell lysis. Release of radiolabel from target cells has been shown to occur after actual cell death.[45]

The radiolabeled compound selected influences the length of macrophage target-cell interaction. The cytoplasmic label, chromium 51 (^{51}Cr), is readily incorporated into target cells within a one-hour incubation period, but is spontaneously released, often at a high rate, and therefore, ^{51}Cr assays are seldom longer than 20 hours. Spontaneous release of ^{51}Cr is generally lower in nonadherent lymphoid tumor cells than in tumor cells, which require surface attachment for growth.[48] Among the neoplastic cell lines frequently encountered in ^{51}Cr assays of monocyte tumoricidal activity are the K562 human myeloid tumor[49] and the P388D$_1$ murine macrophage tumor line.[50]

Radiolabeled compounds that have been successfully used to label attached target cells are usually nucleotides such as tritiated thymidine or ^{125}I-iododeoxyuridine.[43] Incorporation of these DNA precursors into nuclear material by target cells requires 16- to 24-hour exposure to the label. There is little spontaneous release of DNA precursors and thus these radiolabeled compounds are suitable for long-term macrophage target-cell interactions requiring 24 to 72 hours. Macrophages mixed with radiolabeled target cells in such long-term assays have been shown not to reutilize radiolabel released from dead target cells.[43]

Results of in vitro assays for macrophage tumoricidal activity can be greatly influenced by culture medium and conditions. Exposure of macrophages to endotoxin either in vivo, as in the collection of peritoneal macrophages,[51] or in culture medium in vitro[52] has been shown to enhance tumoricidal activity. Taramelli et al[52] observed that low levels of endotoxin (between 2 and 10 ng/mL) appeared to be necessary for in vitro macrophage activation with lymphokines. Because of the critical effects of endotoxin, the amount of endogenous endotoxin in culture medium and reagents should be quantitated by a Limulus Amoebocyte Lysate assay with a sensitivity of at least 0.01 ng/mL.[52]

Cultured cells used as targets must be monitored to assure absence of mycoplasmal contamination. Mycoplasma infection has been shown to enhance macrophage tumoricidal activity.[53] Macrophage tumoricidal assays may also be affected by the concentrations and type of sera used in culture medium. High concentrations (10%) of fetal bovine serum have been found to diminish murine macrophage tumoricidal activity,[54] while human monocyte cytotoxicity has been reported to be adversely affected by fetal bovine serum.[49] Factors responsible for the varying effects of serum on macrophage cytotoxicity have not yet been identified.

The presence of soluble cytolytic products secreted by activated macrophages has been detected by using radiolabeled tumor cells and variations of the methods previously described above. Macrophages are first incubated in culture medium for ≥16 hours. The culture medium is then removed and tested for cytolytic activity against radiolabeled target cells.[23] After exposure of target cells to various dilutions of the macrophage medium for 16 to 48 hours, the amount of released radiolabel is quantitated.[23]

Oxidative metabolism

An enhanced capacity for secretion of superoxide anion or hydrogen peroxide can be used as a marker of macrophage activation.[32,55] As with the tumoricidal assays discussed earlier, macrophages of experimental animals may be analyzed for oxidative metabolism after in vitro[55] or in vivo exposure[56] to the activating agent. Human

monocytes have been found to require approximately 72 hours of incubation with an activating substance such as gamma interferon, before capacity for hydrogen peroxide secretion is enhanced.[57] In contrast, enhancement of oxidative metabolism in murine peritoneal macrophages may occur after an in vitro activation period of approximately 18 hours.[55] In order to measure macrophage oxidative metabolism, macrophages first must be triggered to produce an oxidative burst by exposure to reagents such as PMA or opsonized zymosan.[32] These compounds induce a transient burst of metabolic activity, which must be measured within hours.

Spectrophotometric methods have been used to measure superoxide anion and hydrogen peroxide. The concentration of superoxide anion can be determined by monitoring the reduction of ferricytochrome c at 550 nm,[55] or the reduction of nitroblue tetrazolium at 516 nm.[56,58] Hydrogen peroxide generation can be measured by a number of different procedures including the oxidation by horseradish peroxidase of substrates such as phenol[59] or scopoletin.[60] Each of the assays mentioned have been shown to be capable of detecting nanomole quantities of superoxide anion or hydrogen peroxide. Specificity of these assays has been determined by using the appropriate enzymes to inhibit reactivity (superoxide dismutase for superoxide anion, and catalase for hydrogen peroxide).

In vivo studies involving macrophage activation by liposome-encapsulated agents

Macrophage activation in vitro can be accomplished with various agents, many of which have been mentioned above. There are two broad groups of macrophage activating agents: one consisting of microbial cell wall components and the other consisting of lymphokines or cytokines. However, it is clear from studies in animal tumor models that not all of these are effective in vivo. Also, in some cases, the toxicity of these agents precludes in vivo administration. The delivery system for administering these agents is another important factor, and it is in this respect that the use of liposomes has generated considerable interest.

Liposomes are known to be efficient in delivering agents to the cells comprising the reticuloendothelial system, namely the macrophages. When administered intravenously, liposomes localize predominantly in organs such as the liver, spleen, and lung. The activating agents are thus delivered in a high effective concentration with the result that macrophages are activated and become tumoricidal at these sites.

Studies with "lymphokine" and muramyl dipeptide (MDP)

The first successful application of liposomal therapy to inhibition of lung metastases was reported by Fidler in 1980.[4] Mice of two different strains, bearing spontaneously metastasizing melanomas, were treated with liposomes containing "lymphokines," a crude supernatant of concanavalin A-stimulated spleen cells, and a significant inhibition of metastases was observed. Treatment with this liposome-lymphokine preparation was also found to be effective in inhibiting lung metastases in another mouse tumor model involving a malignant fibrosarcoma.[6] One obvious drawback of this crude lymphokine preparation was that it contained a wide variety of active biological factors and the active component responsible for the biological effect was not identified. In these therapy models, the respective tumors were first established by subcutaneous injection of tumor cells in the hind footpad and the tumors were allowed to grow to a stage where lung metastases were known to have occurred. At that point, the primary tumors were removed by amputation of the tumor-bearing limb and intravenous treatments with liposomal agents were started. The efficacy of the treatment was then evaluated at a later date by studying the treated and appropriate control mice with respect to survival and presence of lung metastases. Treated animals showed a substantial benefit with respect to both of these parameters. In subsequent studies, Fidler et al[61] demonstrated that the biological effect of crude lymphokine could be reproduced with MDP, a well-characterized structural component of mycobacterial cell wall. More recently, Fidler and Schroit[62] have shown that when both lymphokine and MDP were encapsulated in liposomes simultaneously, and then administered for therapy of spontaneous lung metastases, a synergistic effect could be observed on reduction of these metastases. In vitro studies with these agents were also shown to activate macrophages and generate tumoricidal activity.[14,63] The methodology for preparation of various liposome-encapsulated agents has been discussed in detail in the articles mentioned. The liposome composition in these studies involved phosphatidylcholine

and phosphatidylserine, and the latter was found to be essential for localization in the lungs.[64] The evidence that eradication of metastases was mediated by activation of host macrophages was derived from three types of experiments. When macrophage-activating agents were delivered in liposomes that were not efficiently localized in the lung, little or no inhibition of metastases was observed. Secondly, eradication of metastases was not observed when tumor-bearing animals were treated with agents that impaired macrophage function, e.g., silica and carrageenan. Thirdly, adoptive transfer of macrophages activated in vitro with liposome-encapsulated agents into mice bearing lung metastases also showed significant inhibition.[65] In further studies, Fidler[66] showed that generation of tumoricidal activity and alveolar macrophages by liposomes containing MDP is a thymus-independent process. This conclusion was based on the observations that macrophages could be activated to become tumoricidal in the absence of active T cells such as that induced by thymectomy and whole body irradiation and as tested in congenitally athymic nude mice.

In vivo studies with C-reactive protein (CRP)

During the past several years, research in our laboratories has focused on study of lung and liver metastases in different mouse tumor models. Initially, we were able to confirm Fidler's observation on the inhibitory effect of crude lymphokine preparation delivered in liposomes on lung metastases using a malignant fibrosarcoma (T241 tumor) in C57 black mice.[67] A search for a well-characterized, pure agent that would substitute for the crude lymphokine in these studies led to our observation that human CRP can function in this capacity.[6]

CRP was first described by Tillet and Francis[68] as a serum protein capable of precipitating pneumococcal capsular (C) polysaccharide, and hence it was termed CRP. Human CRP is present in normal serum in trace amounts (<1 mg/dL); however, the serum levels increase markedly (15 to 25 mg/dL) in a wide variety of clinical conditions characterized by acute inflammation. CRP is different from other acute phase reactants such as transferrin, ceruloplasmin, fibrinogen, alpha-2-macroglobulin, and others in that its rise in serum level is more marked (100- to 1,000-fold) and it occurs much earlier than that observed for other acute phase reactants. Recently, CRP was purified to homogeneity, and its structure is now well characterized physicochemically.[69,70] CRP is known to be present in a wide range of animal species, both vertebrates and invertebrates, and also in certain species not known to possess immunoglobulins or the conventional immune system.[71,72] Also, CRP molecules of various animal species have been demonstrated to possess considerable homology with human CRP. Thus, the excellent preservation of this molecule in the animal kingdom suggests that it must have an important biological function. However, despite these recent advances, the pathophysiological role of this molecule remains a mystery. The possible function of CRP as an immune modulator has received some attention on the basis of interactions of this molecule with C1q component of the complement system[73] and with lymphocytes and monocytes, particularly those bearing the Fc receptor.[74,76] Our studies now suggest that another important function of CRP may be that of macrophage activation.

Our studies, in vivo and in vitro, have involved three different metastasizing mouse tumor systems, namely, T241 fibrosarcoma, B16/BL6 melanoma, and MCA-38 colon carcinoma, all syngeneic tumors in C57 black mice. In the first two tumor models, an appropriate number of tumor cells were implanted as a subcutaneous injection in the left hind footpad and the respective tumor was allowed to grow to a stage where spontaneous metastases were known to occur in the lungs. At that point, the tumor-bearing foot was removed by simple, below-knee amputation, and intravenous treatments with liposomes containing CRP or appropriate control agents were started after removal of the tumor-bearing limb. Treatments were given three times per week for two weeks and at the end of the experimental period, the lung metastases were evaluated both in terms of number and size of the tumor nodules. Under the experimental conditions employed in these studies, approximately 35% to 40% of the animals treated with CRP were completely free of metastases as compared with 0% to 2% for the various control groups.[6] There was also a marked improvement in survival of the treated animals as compared with that for the control groups. In these studies, human CRP was purified to homogeneity from pathological serous fluids by procedures previously described, and the purity of the final preparation was documented by sodium dodecyl sulfate-polyacrylamide gel electrophore-

sis (SDS-PAGE). Also, it was shown to be free of any immunoglobulins, complement components, or of any activity attributable to interferons or interleukins. The CRP preparation was also shown to be free of significant endotoxin activity. Later, in vitro studies[77] showed that the effect of CRP could be attributed to macrophage activation as demonstrated by rapid uptake of CRP-containing liposomes by peritoneal macrophages, and subsequent activation of these macrophages, as demonstrated by chemiluminescence, production of superoxide anion, and generation of tumoricidal activity against various tumor-cell lines. In these studies, liposomal CRP was shown to be 100- to 1,000-fold more active than free CRP.

In the MCA-38 colon carcinoma model,[78] tumor cells were implanted in the wall of the cecum and treatments with liposomes containing CRP or crude lymphokine were started at varying times after the primary tumor was established. In this model, however, the tumor-bearing cecum was not resected and the effects of various treatments were evaluated in the presence of a growing primary tumor in the cecum. The treated animals showed significantly better survival and fewer liver metastases as compared with those in the untreated controls and those treated with liposomes containing medium only. Thus, these studies clearly showed that the liposomal treatment was also effective in controlling liver metastases from a primary colonic tumor.

These studies with CRP may have clinical relevance since the lung and liver represent the two most common sites of metastases by various malignant human tumors. Additional considerations include the nontoxicity of CRP and also its human origin. More recently, we have shown that human CRP can activate human peripheral blood monocytes to develop tumoricidal activity as studied with human astrocytoma and human renal-cell carcinoma cells.[79] Thus, our studies show that CRP has great potential as yet another "biologic response modifier" of value in cancer therapy.

Rationale for clinical cancer therapy

Recent studies by Kleinerman et al[80] have shown that peripheral blood human monocytes can be activated in vitro by free and liposome-encapsulated human lymphokines to generate tumoricidal activity. Similarly, Sone and Tsubura[81] demonstrated that human alveolar macrophages can be activated in vitro to generate tumoricidal activity by liposome-encapsulated MDP. Similar activation of human peripheral blood monocytes has also been demonstrated with liposomes containing human gamma interferon.[82] As mentioned earlier,[79] CRP was also found to generate tumoricidal activity in human blood monocytes. These observations provide a firm rationale for attempting clinical trials with appropriate delivery of macrophage-activating agents in the treatment of human cancer. Liposomes need not be the only delivery system for such agents, and consideration should be given to other approaches such as the use of autologous red blood cells for such delivery. High-yield entrapment of various proteins and enzymes into red blood cells for such delivery has been reported previously.[83]

Tumor-cell killing by activated macrophages is known to be nonspecific.[84] A wide variety of tumor cells, including syngeneic, allogeneic, and even xenogeneic, can be killed by this approach. Also, the tumor-cell killing is independent of the particular germ-cell origin of the tumor. Thus, killing of tumor cells by CRP-activated macrophages was effective against different tumors, namely fibrosarcoma, melanoma, and colon carcinoma representing malignant tumors of the three basic germ layers: mesoderm, neuroectoderm, and entoderm, respectively. This nonspecificity, if it can be translated in a clinical setting, would offer a distinct advantage.

It is generally accepted that macrophage-activated killing is directed primarily towards tumor target cells and that normal host cells are preserved in the process. This again would be a distinct advantage in the clinical setting considering major drawbacks of the present therapeutic modalities, particularly chemotherapy and radiation, which include toxicity and damage to normal host cells.

The ability of activated macrophages to kill drug-resistant tumor cells has not been investigated in great detail with respect to a wide variety of chemotherapeutic drugs currently in use; however, preliminary studies with selected mouse tumor-cell lines indicate that activated macrophages can kill drug-sensitive and drug-resistant tumor cells with equal facility.[85] Clinically, this would represent another desirable feature.

Lack of development of tumor cells resistant to macrophage killing would be another advantage of this therapy. Both in vivo and in vitro studies have shown that most tumor cells develop little or no resistance to killing by activated mac-

rophages.[85–87] Thus, tumor cells harvested from metastases were found to be fully susceptible to killing by activated macrophages, indicating that progressive growth of the metastatic lesions did not result in development of tumor-cell variants resistant to killing.[88]

The most serious clinical consequences of cancer are due to the metastatic property of the malignant cell. Control of metastases, therefore, remains the most important and, admittedly, still the most difficult goal of cancer therapy. In this regard, possible approaches using macrophage activation deserve serious consideration, since activated macrophages appear to destroy malignant cells, both metastasizing and nonmetastasizing, with equal facility. It is generally accepted that the process of metastasis is not random, but rather a highly selective one. In other words, not every tumor cell in a given tumor has the same potential for metastases, but the ability is limited to a few aggressive, specially endowed cells. Therapeutic methods that can affect the attack and destruction of these cells should significantly benefit our efforts in the clinical control of cancer. Experimental studies with agents such as MDP and CRP have shown that both lung and liver metastases can be effectively inhibited even when therapy with these agents is started after the metastases are established. This therapy may be particularly helpful in an adjuvant setting in patients with cancer who, at the time of primary resection of their tumors, are known to be at high risk for the presence of clinically undetectable micrometastatic disease. Such malignancies include colorectal, melanoma, renal cell, breast cancer, and many others. For example, in colorectal cancer it is estimated that, at the time of first clinical detection of such cancers, approximately 25% to 30% of these patients have micrometastatic disease, which is not detectable by current diagnostic modalities. It is in this clinical setting of micrometastases that therapy with macrophage activating agents may have potential value. Recent studies have shown that the antitumor effects of interferon gamma and tumor necrosis factor may, in part, involve the macrophage, and the possibility that combined therapy with one or more of these agents would produce an additive or even synergistic effect has not yet been investigated.

Thus, strong and persuasive arguments can be developed in favor of macrophage-mediated tumor-cell killing as one of the strategies to be considered for cancer therapy in man. The major limitation, as with any other modality, of course, is the size of the tumor burden. This approach is therefore best considered in the adjuvant setting.

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