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Investigation of a rat model of osteosarcoma : immunobiology and the effects of adriamycin

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DEDICATED WITH LOVE TO:

Mary Scott, my wife and best friend, whose perseverance in the preparation of this thesis occasionally exceeded even my own, and whose constant support and encouragement made the difficult times during this investigation much less burdensome.

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ABSTRACT

An initial investigation reporting the excellent suitability of an MSV-induced osteosarcoma in rats as a model of the human disease has been confirmed and extended. Intratibial inoculation of the virus in newborn animals has reproducibly caused a localized, osteoid-producing tumor mass which is rapidly progressive and fatal (median survival = 20.0 days). The histologic nature, incidence, and distribution of metastatic disease in this tumor model are comparable in most respects to osteosarcoma in man. Particularly significant was the 89% incidence of metastatic pulmonary disease in tumor-bearing groups from 10 to 65 days of age.

Tumor-specific cellular and humoral responses were demonstrated in animal groups tested between 14 and 48 days of age. Use of an *in vitro* cytotoxicity assay demonstrated a biphasic pattern to both cellular and humoral cytotoxicity, and the theoretical correlates and implications of these kinetics have been discussed. Humoral factors consistently increased the levels of cell-mediated immunity in untreated tumor-bearing animals.

The efficacy of adriamycin chemotherapy in human osteosarcoma stimulated its use in the rat model, where its effects were twofold. The net effect of adriamycin treatment at two doses and a single schedule was beneficial due to its tumoricidal actions. Treated animals exhibited significantly retarded tumor growth and survived significantly longer than untreated tumor-bearing controls. Importantly, adriamycin treatment was not associated with suppression of tumor-specific cell-mediated immunity. In addition, however, use of adriamycin was associated with a possibly dose-related *in vivo* and *in vitro* enhancement phenomenon, as treated animals developed significantly more long bone metastases than controls, and exhibited blocking activity in their sera. It has been postulated that this enhancement was caused by adriamycin-induced antigenic shedding by the tumor, with the consequent formation of serum blocking factors.

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I. Introduction

A. The Relationship between Immunity and Malignancy

The Concept of Immune Surveillance:

In a lecture on cancer given in 1908, the brilliant German founder of immunology, Paul Ehrlich, first proposed that there exists a fundamental relationship between immunity and malignancy. In this lecture he stated:

> "The understanding of natural immunity represents the key to carcinoma... I am certain that, in the enormously complicated course of fetal and post-fetal development, aberrant cells become unusually common. Fortunately, in the majority of people, they remain completely latent thanks to the organism's positive mechanisms. If such mechanisms did not exist, one might expect carcinomas to have an enormous frequency. These cells may live in a latent state for 20, 30 or 40 years before changing to a significant tumor. This means, in light of my theory, that there has been a diminution of some vital cell activity [which] allows the rapid, parasitic growth of certain cells."[90]

Ehrlich's amazing insight was apparently not fully appreciated, however, as only one report (in 1924)[95] until the last two decades mentioned the potential significance of host immunity as a defense against neoplasia. Renewed interest in immunological studies of cancer followed the landmark experiments by Foley (1953)[112], Prehn and Main (1957)[261], and Klein (1966)[170]. These studies established the presence of tumor-associated antigens, and together with observations of cancer epidemiology, laid the foundations for a most powerful influence on cancer research -- the theory of immunological surveillance.

Although clearly suggested by Ehrlich in 1908, the theory in its modern form was formulated by Lewis Thomas in a discussion of transplantation immunity in 1959.[316] On considering the "underlying meaning of homograft rejection, as a biological phenomenon", Thomas proposed that "homograft rejection will turn out to represent a primary mechanism for natural defense against neoplasia". Stimulated by this concept, Burnet in 1963[42] first used the term "surveillance" to characterize this relationship.

Stated more precisely, the thesis of immune surveillance theory is that when aberrant cells with proliferative potential arise in the body, they carry new antigenic determinants on their cell surface. When a sufficient amount of new antigen is present, a cellular, mainly thymus-dependent immunological response is initiated which eventually eliminates the aberrant cells in essentially the same way that a homograft is destroyed.[43]

While of great clinical and heuristic importance, this theory has at its foundation a sound evolutionary explanation. Though some have casually proposed that the raison d'etre of the immune system lies in its elimination of neoplastic cells, Good and Finstad[131,132] have studied in depth the evolution of the immune response, and draw some very intriguing conclusions. In brief, they have proposed that the vertebrate capacities for malignancy and immune competence developed concomitantly in phylogeny. Both, they suggest, evolved as a function of a more basic impulse to cellular variation in higher, vertebrate forms. While the capacity for somatic mutation per se could have been of major survival advantage, "malignant adaptation" may have either

been an undesirable side effect, or teleologically, have had its own survival value in protecting ecological niches from exhaustion by groups of each vertebrate form. The immunological adaptation, in turn, progressively developed a capacity for surveillance against the threat of malignant adaptation. From this view, as stated by Burnet in his 1960 Nobel prize speech, "the faculty of immunological recognition becomes an intrinsic part of the homeostatic controls that maintain the body as a going concern".[41]

Cited as evidence for this evolutionary theory is the inability of investigators to identify malignancies in invertebrate forms; the failure of vertebrate carcinogens to induce malignancies in, or high doses of irradiation to kill, these lower forms, and the congruous inability of investigators to define invertebrate immune cells or reactions. On the other hand, both cellular immunity and definite malignancies have consistently been described in all vertebrate forms, including fishes, amphibians, reptiles, birds and mammals.

Clinical Evidence for Immunologic Surveillance:

Burnet[43] suggested that there are at least four predictions made by the theory of immunologic surveillance at the clinical level:

1. There should be an increased incidence of malignancy initiated at ages of relative immunologic incompetence.
2. Pathologic conditions associated with deficiencies of thymus-dependent immunity should be associated with a higher incidence of neoplasia.

3. Spontaneous remissions of clinically evident malignancy should occur.
4. Unselected autopsies or pathologic specimens should show larger numbers of malignant foci than could ever develop to definite tumors.

A few recent reports have elegantly reviewed the wealth of clinical evidence supporting these predictions; however, several points are noteworthy for this discussion.

First, cancer is largely a disease of the very old and the very young. The age incidence of many childhood tumors is compatible with a process initiated about the time of birth[257], a time of relative immunoincompetence. At the other extreme, consistent with thymic involution and a decrease in immunologic vigor with advanced age[12,314], malignant cells flourish. While humoral and re-call immunity (to previously experienced antigen) are adequate in the elderly[12,41,43,131, 132,257,276,314], responses to new and thymic-dependent antigens are markedly depressed. More than 95% of individuals under age 65 can be sensitized to dinitrochlorobenzene, whereas only 20-30% of patients over 65 can be so sensitized.[91] In addition, old people with malignancies do less well with these identical[189] or similar[188] immunologic procedures than do other noncancerous patients with chronic disease, or normal individuals of the same age.

Convincing evidence for the second prediction -- that pathologic conditions associated with diminished thymus-dependent immunity have an increased incidence of malignancy -- comes from two sources. The first

area includes what Good calls "experiments of nature"[131], in which immunological deficiency diseases are associated with a significantly higher incidence of malignancy. Particularly striking are the Wiskott-Aldrich and ataxia-telangiectasia syndromes, conditions of defective ontogenetic thymus-dependent lymphoid development. In these conditions, malignancy has been the cause of death in fully 13 and 10% of reported cases, respectively.[212] When all acquired and congenital immunodeficiency disorders are considered together, a very conservative estimate of the incidence of malignancy is about 5%, or about 200 times the expected rate.[203]

The second area involves malignancy associated with long-term iatrogenic immunosuppression in organ (mainly renal) transplant recipients. Immunosuppression for transplantation generally consists of azathioprine, prednisone, antilymphocyte serum and/or a variety of other methods generally intended to diminish thymus-dependent immunity. The incidence of de novo malignancies here is approximately 5.6% in patients averaging 38 years of age, or approximately 100 times the expected rate.[250] A remarkable fact reported by Penn[250] is that a wide variety of histological malignancies have been encountered. The most common lesions were cancers of the skin and lips (39%), followed by solid lymphomas (27%), carcinomas of the cervix and uterus (8%), lung (5%), and miscellaneous (21%). Perhaps even more notable is the report by Woodruff[334] that withdrawal of immunosuppressive regimens has caused regressions of tumors arising in transplant recipients. Some would hold that these data do not necessarily support immunological surveillance, but rather represent diminished host defenses against oncogenic viruses

qua infectious agents.[203] Nevertheless, the variety of malignancies observed (including, for example, lung cancer), and the occurrence of "spontaneous" tumor regressions upon withdrawal of immunosuppressives both support a role for host tumor-specific defense mechanisms.

"Spontaneous" tumor regressions have been well documented in other, non-transplant patients as well. Of 130 instances considered unequivocal by Everson[98], more than half fall into one of four categories: neuroblastoma of adrenal and related tissues, 28; hypernephroma, 21; choriocarcinoma, 13; and malignant melanoma, 12. It is pertinent that all these tumors arise either in children or in adults younger than those susceptible to the common malignant diseases.[43]

Finally, consistent with Burnet's fourth prediction, at least four types of malignant tumors (neuroblastoma[19], thyroid[222], prostatic[227], and cervical[13] carcinomas) are much more frequently diagnosed in random pathological specimens than they are found clinically in comparable populations. An unavoidable interpretation is that many accumulations of malignant cells never progress to overt malignancy, but instead are "kept in check" or destroyed by host defense mechanisms. Supporting this interpretation is the finding that the intensity of the host cellular response in specimens of a wide variety of malignancies is directly correlated with prognosis.[26,70,123,193]

Experimental Evidence:

A wealth of experimental evidence also exists to support the involvement of host defenses, especially thymus-derived lymphocytes (abbreviated "T-cells"), in surveillance and destruction of neoplastic cells. Perhaps

the best evidence is that derived from studies of tumors induced by both DNA and RNA oncogenic viruses in which ablation of thymus-dependent functions has been associated with a high incidence of malignancy. Thymectomy or anti-lymphocyte serum (ALS) treatment permits the appearance of tumors in mice and rats deliberately or naturally infected with the DNA polyoma virus.[9,10,50,323,324] Furthermore, reconstitution with tumor-sensitized thymus, spleen or lymph node cells in these immunologic cripples prevents the development of the malignancies.[10,184,290] Likewise, susceptibility of mice and rats to oncogenesis by a variety of C-type RNA viruses is significantly increased by many immunosuppressive procedures, including thymectomy[86,268,319], irradiation[302], and treatment with ALS[9,153,183,337], cortisone[286], cytoxan[100] or daunomycin.[45] While some apparently contradictory evidence exists with thymectomy in mouse leukemia virus-induced tumors[122,182,216], which require the thymic microenvironment for tumor progression[182], overwhelming evidence exists to support immune surveillance in experimental virus-induced tumors.

The interactions of virus, immunity, and malignancy are made somewhat more complex by the fact that most oncogenic viruses are <u>in themselves</u> immunosuppressive.[71] Thus, oncogenic viruses may: (1) induce neoplasia <u>per se</u>, which can then be facilitated by other forms of immunosuppression; or (2) cause immunosuppression <u>primarily</u>, making the host more susceptible to the neoplastic process.[251] In this context, it is interesting that the onset of malignancies induced by Friend leukemia virus (FLV) in mice are always preceded by the immunosuppressive effects of FLV.[71]

Carcinogenic hydrocarbons also suppress host immunity, both humoral[299,301] and cellular[180,258], while noncarcinogenic hydrocarbons do not.[299] The evidence for the effects of additional immunosuppression on chemical carcinogenesis is somewhat conflicting[203]; however, thymectomy and/or ALS treatment have usually resulted in an increased incidence of tumors, a decreased latency, or both, when animals are given chemical carcinogens.[267,335]

Other experimental approaches supporting the significance of host immunity in defense against malignancy have included: (1) the increased incidence of spontaneous tumors in deliberately immunosuppressed animals; and (2) the documented immunoincompetence of animals, such as AKR and NZB mice, which have a naturally high incidence of malignancy.[85,205]

The Demonstration of Tumor-Specific Transplantation Antigens (TSTA):

Pivotal to this entire discussion of immunological surveillance is the concept that tumors are antigenic. As early as the turn of the century the hope was held that dissimilarities between neoplastic and normal cells would enable the development of an anti-cancer vaccine, and transplantation experiments seemed to show the presence of tumor-specific antigens. The field of tumor immunology later fell into disrepute when it was recognized that these claims of tumor-specificity were based on experiments in which immunity to normal alloantigens, rather than tumor-specific antigens, had been evoked.[331]

The demonstration of TSTA awaited the development of highly inbred strains of mice which approached homozygosity so closely that individual animals could retain skin grafts from others of the same strain (syngeneic

animals). Only in syngeneic systems is it possible to analyze the conditions that cause retention or rejection of either normal or neoplastic tissues, since in any allogeneic or xenogeneic combination, tumor specificities may be masked by strain-specific or species-specific antigens.

It was under these conditions that Foley (in 1953)[112], and later Prehn and Main[261], first demonstrated TSTA in methylcholanthrene (MCA)-induced tumors of mice. Their experiments showed that otherwise progressive syngeneic MCA-induced tumors failed to grow if mice challenged with tumor cells had been pre-treated with the same tumor cells. Immunization with normal tissue, or other MCA tumors, was not protective. By preimmunization, then, these investigators showed that there were antigens unique to the tumor.

Subsequently it has been widely confirmed that specific antigenicity accompanies neoplastic transformation of most cells, whether this transformation is spontaneous[328], or effected by a chemical carcinogen[251], or oncogenic virus.[170,237,294] In addition to in vivo transplantation methods[289], in vitro methods demonstrating TSTA have included membrane immunofluorescence[214], colony inhibition tests[141], and various forms of cellular and humoral cytotoxicity assays.[37,309] These methods have convincingly demonstrated the presence of tumor-specific antigens in a wide variety of animal tumor systems as well as several malignancies in man.

As a rule, TSTA of virus-induced tumors is the same for all tumors induced by the same virus, regardless of histologic type or animal species, but differs in tumors induced by different viruses.[170,237,294] This is true for both DNA and RNA viruses. Recent evidence suggests

that polyoma virus tumors have individually unique antigens in addition to their common antigens, however.[315] The tumor-specific antigens associated with a given virus do not represent virus particles gathered at the cell surface, but rather seem to be components of the tumor cell distinct from intact virions.

Conversely, and implicit in the MCA tumor studies above, chemically-induced neoplasms produce tumor-specific antigens which do not cross react.[170,237,294] Thus, in the example cited, preimmunization with tumor cells of a different MCA-induced neoplasm did not protect against the malignant potential of the original MCA tumor challenge. Exceptions to this rule have been reported[234], but in general, TSTA's associated with carcinogens are unique for each tumor produced.

The demonstration of antigenic cross-reactivity between a tumor of unknown etiology and a second tumor of known viral causation justifies the search for a causative virus in the first tumor, but does not prove that they are induced by the same virus. First, a phenomenon known as "antigenic conversion" may have occurred, whereby a common tumor antigen has been secondarily acquired (e.g., through viral superinfection) by the neoplastic cells.[291] Second, activation of genes normally only expressed in embryonic states may have occurred, producing "carcinofetal" antigens. Three such antigens identified in man are: (1) "carcinoembryonic" antigen (CEA), associated primarily, but not exclusively, with adenocarcinomas of the digestive tract[129]; (2) α-fetoprotein, an antigen produced by hepatoma cells and some testicular neoplasms[11]; and (3) γ-fetoprotein, a fetal antigen produced by a wide variety of primarily malignant tumors.[89] Finally, genes that are normally redundant may have

been activated in the transformed cells, as is the case with the TL antigens of mouse leukemias.[32]

Mechanisms of "Escape" from Immunologic Surveillance:

A common view in the past has been that tumor-specific antigens could not possibly exist, since they would result in the immediate immunologic recognition and destruction of the cells which carried them. The existence of tumors in apparently "normal" individuals, in addition to the presence of common lymph node metastases, was taken as prima facie evidence for nonantigenicity. Yet, as discussed above, TSTA has been clearly demonstrated in almost every tumor system in which its presence has been evaluated. Why, then, do some tumors not get rejected?

At the outset, it is important to state that tumor progression or rejection is an extremely complex phenomenon which probably has no single explanation. It is perhaps useful to consider host immunity and neoplasia as independent components of resulting clinical disease, although it will be made clear that this is a somewhat artificial distinction.

In some instances, presumably independent genetic factors cause thymus-dependent immunity to be markedly diminished or absent, allowing neoplastic cells to proliferate unchecked. Good's "experiments of nature", and malignancies of the very old and the very young, fit most easily into this category.

It has already been suggested that the immunosuppressive nature of both viral and chemical oncogens may allow antigenic tumors to develop, over and above their abilities to transform cells.

Growing tumors, in themselves, may be non-specifically immunosuppressive, perhaps through elaboration of "factors" that depress immunologic reactivity.[91,148,174,192,211] Notable in this context is the significantly increased incidence of second tumors seen with Hodgekin's disease and tumors of the head and neck, malignancies often followed by non-specific immunosuppression.[211,275]

A fourth factor which may contribute to the progression of antigenic tumors in vivo is the fact that neoplasms generally develop from small foci of cells which may not provide sufficient early antigenic stimulation to elicit a vigorous immune reaction, and therefore "sneak through" host surveillance mechanisms.[237] Some evidence suggests that these spontaneous tumors are only weakly immunogenic.[133] Other experimental evidence exists that, under certain conditions, this lack of immunogenicity may be due to the coating of cells with substances (such as sialomucin) which ordinarily prevent recognition.[66,279] In still other situations, specific tolerance to the antigens of the tumor or inducing virus may occur.[223]

Once the tumor has developed, progressive growth may be unchecked or even accelerated by host-specific immune mechanisms, a phenomenon known as "enhancement". Here, TSTA is recognized by the host, but surveillance is blocked by antigen, antibody, or complexes of the two. Enhancement may be: (1) afferent, in which TSTA is blocked from stimulating immunocompetent cells of the host; (2) efferent -- either peripheral or central -- whereby blocking occurs at the "target" tumor cells or at the host's effector cells, in both cases preventing effector cells from "seeing" their targets; or (3) central, in which blocking factors regulate

or inhibit the production of tumor-specific immune mechanisms, presumably within lymphoid centers, e.g., lymph nodes and spleen.[105,147,158] Both in vivo and in vitro studies have shown that enhancement may play a significant role in the host-tumor relationship of many experimental and human malignancies. A more detailed discussion of enhancement, with special emphasis on its significance in human and experimental models of osteosarcoma, can be found in the "Discussion" section of this presentation.

Finally, there are some investigators who propose that antigenic tumors persist because of, and not it spite of, host mechanisms.[203,259,260,283] Prehn[259,260], in particular, has suggested that small amounts of host response may stimulate tumor growth, while a more intensive cellular response inhibits it. While this theory has significanct heuristic value and may be pertinent to certain tumor systems, there is currently no in vitro support for it, and it is otherwise largely untested.[259]

On the other side of the host-tumor relationship, characteristics of the neoplastic cells themselves may override the importance of host defenses. For example, intrinsically more aggressive tumors (shorter in vitro doubling time), or those that more readily "shed" antigens, may grow progressively, the latter presumably through increased blocking activity.[5] Tumor cells can also reduce their sensitivity to immunologic attack, by both selective (genotypic) and adaptive (phenotypic) mechanisms. These are the processes of "immunoselection" and "antigenic modulation", respectively.[106,238]

It is beyond the scope of this paper to comprehensively review all of the complex variables involved in the process of tumor rejection or

progression. An attempt has been made to show that many factors of both host immunity and neoplastic cells require consideration for an insight into malignancy, with the understanding that these considerations should direct future therapeutic efforts.

B. The Relationships between Chemotherapy, Immunity and Malignancy

The complicated interrelationship between oncogens, immunity, and malignancy are made even more complex by the addition of chemotherapeutic agents. Difficulties are encountered due to the fact that most cancer chemotherapeutic agents also cause immunosuppression[195,206,208], and in most cases, this effect is due to the very antiproliferative action that is at the basis of their anti-tumor activity. Anticancer drugs may inhibit both humoral and cellular responses, usually non-specifically but under certain circumstances specifically, and these effects are very dependent on drug dose and schedule, type and amount of antigen, and time and route of antigen administration.[195,282] In addition, drugs may act on neoplastic cells by a variety of means to concomitantly increase their immunogenicity or susceptibility to immune destruction.[207]

At the preclinical and clinical levels, there is substantial evidence that judiciously used anticancer drugs may act cooperatively, even synergistically, with immunological defenses directed against primary tumors.[206] It is important to define the conditions in which this synergism is most likely to occur, and to utilize this knowledge therapeutically. In many cases, however, immunosuppression by chemotherapeutic agents is unavoidable. Since most effective cytotoxic drugs may reduce

the size of a tumor markedly, but are not selective enough to kill the last tumor cells, host tumor-specific mechanisms are required to effect a cure.[206] In these cases, the exploitation of sequential chemotherapy and immunotherapy seems promising.[198] Thus, chemotherapy-induced immunosuppression may be non-specifically or specifically reversed by adjuvants such as BCG, or specific tumor-cell vaccines or transfer factor.[157,176] Before these therapeutic approaches can be successfully used, however, much more needs to be learned of the effects of given agents on both neoplastic cells and immunologic defenses.

Effects of Chemotherapy on Neoplastic Cells:

The direct tumoricidal effect of chemotherapeutic agents is the primary mechanism of their anticancer activity, and a vast literature supports the rationale for their use in different malignancies, and under different doses and schedules. That they have in certain malignancies even effected cures is indisputable[120,149,154], and these examples attest to the significance of their cytotoxic potential.

In addition, anticancer drugs have been shown to induce increases in tumor immunogenicity, and while inadvertent changes may, in part, be responsible for their therapeutic efficacy, deliberate changes have been induced to enhance host immunologic "efficiency". Basically three approaches have altered tumor immunogenicity, directed to: (1) drug-induced alterations of cell metabolism possibly leading to plasma membrane changes; (2) chemical modifications of the plasma membrane presumably providing new haptenic determinants or helper (carrier) functions; and (3) enzymatic treatments of cells leading to increased availability of

antigenic determinants in the plasma membrane.[207] Fourth, a drug may increase the immunogenicity of cells resistant to an anticancer drug through a phenomenon known as "collateral sensitivity".[159] In this discussion, the inadvertant effects of common cytotoxic agents will be emphasized, while excellent reviews of deliberate changes in tumor cell immunogenicity can be found elsewhere.[159,207]

Drugs affecting the control of gene action have altered both cell metabolism and antigenic expression in a few systems. Particularly convincing are studies with steroids used in rat hepatomas[321], and 5-bromodeoxyuridine in other experimental models.[44] Likewise, 5-fluorouracil (5-FU) treatment in vivo has caused specific changes in TSTA. In this case, however, it was demonstrated that increased tumor cell immunogenicity was due to chemical coupling of the drug to antigen or haptens on the plasma membrane.

While the clinical significance of the above effects is not known at this time, the importance of collateral sensitivity in particular malignancies has been well documented.[159,207] In one representative study[29], it was found that the immunogenicity of L1210 leukemias, as reflected by transplantability tests and survival times, was greatly increased during the development of resistance to 5-[3,3-dimethyl-1-triazene]-imidazole-4-carboxamide (DTIC). From the eighth transplant generation onward, a DTIC-resistant subline could not grow in susceptible CDF_1 (DBA/2Cr xBALB/c) mice, even after the inoculation of 10^5 cells, unless the animals were treated with immunosuppressive regimens of DTIC or cyclophosphamide. The resistant subline was more sensitive to treatment with 1,3-bis[2-chloroethyl]-1-nitrosourea (BCNU) than

parent L1210. A variety of other reports have also demonstrated that the increased chemotherapeutic sensitivity of resistant tumor sublines is dependent on the integrity of host defense mechanisms. While some reports suggest that the resistant tumor cells are more susceptible to host defenses (e.g., via changes of intrinsic growth characteristics of the cells)[343], the majority of evidence has supported the probability that resistant sublines are more immunogenic than the parent cells.[207]

Effects of Chemotherapy on the Immune Response:

In addition to chemotherapeutic cytotoxicity and changes in cellular immunogenicity, most anticancer agents are immunosuppressive in animals and man under a variety of conditions. While this immunosuppression has been successfully exploited in clinical situations such as organ transplantation and autoimmune diseases[152,208], in cancer chemotherapy it is more likely to be counterproductive.[209]

Immunosuppression by drugs is usually considered nonspecific in nature, in that it is the consequence of a pharmacological action, such as inhibition of cell proliferation, which may not uniquely affect immunological systems. Yet this is not always so; e.g., an anti-proliferative agent would affect preferentially lymphoid cells stimulated as a consequence of antigen exposure without necessarily reducing subsequent capabilities of the immune system to respond to different antigens, or eliminating non-proliferating "memory" cells.[282] Thus, a specific effect can be obtained by intrinsically nonspecific pharmacologic means. The significance of this in cancer chemotherapy will be indicated below.

As noted in preceding sections, thymus-derived "T-cells" are considered the most significant of the host immune mechanisms in defense against neoplasia. In addition, lymphoid cells derived from bursa-equivalent tissue ("B-cells") and macrophages play roles of definite significance in some tumor systems, while being of more equivocal import in others. B-cell synthesis of antibody against certain, but not all, antigens is triggered as a consequence of an interaction between certain types of ("helper") T-cells, B-cells, and macrophages. These complex, and incompletely understood, relationships between immune cells and their target tumor cells, are represented simply in Figure 1.[212]

Both B and T-cells comprise a series of individual cell types at different levels of differentiation. Unique antigenic and functional features characterizing these cell types provide the basis for a great variety of cellular responses to drugs.[282] In this context, it is likely that certain drugs exert selective effects on specific cells depending in large part on their functional and metabolic status. Known examples of this specificity include the selective inhibition of B-cell populations by asparaginase[213], and that of T-cell populations by procarbazene.[104]

The timing and schedule of drug treatment may likewise be important aspects of this cellular sensitivity and selectivity. Indeed, cytotoxic agents have been divided into groups according to whether they exert optimal immunosuppression when administered before or after antigen.[195] In general, maximum immune depression is obtained when drugs are given close to the time of antigen administration, suggesting

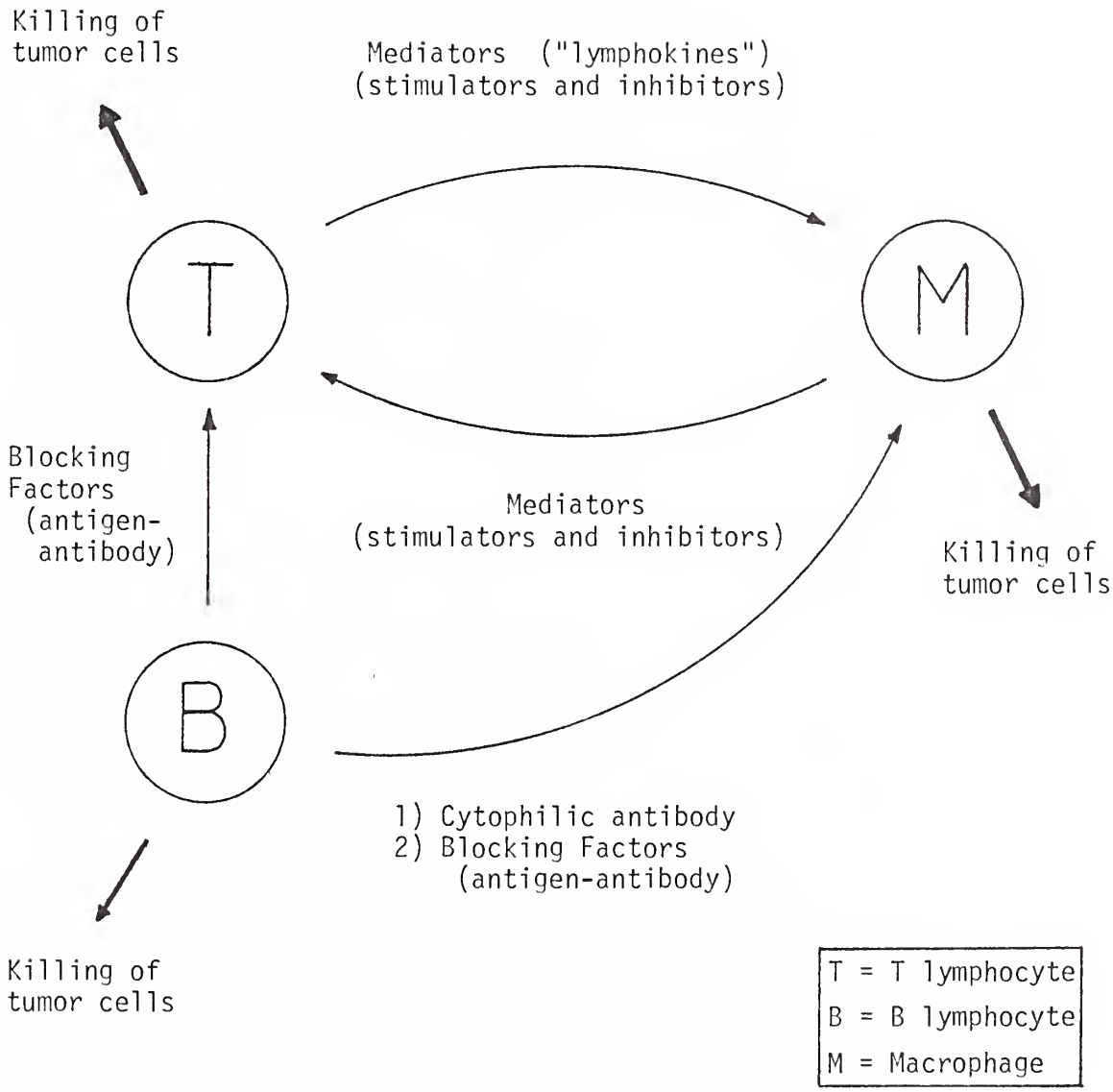


Figure 1

Interaction of lymphocytes and macrophages in tumor destruction. Adapted with permission from Mitchell (212).

that the early stages of cell differentiation and proliferation are especially susceptible to inhibition.

While timing is particularly pertinent to experimental systems, drug schedules apply equally well to human therapeutics. In this regard, it would appear that daily treatments with certain drugs suppress more T-cell responses than humoral responses, whereas the opposite has been reported to occur with intermittent treatments.[120,209] That this may be of extreme practical importance is the evidence that in certain dispersed malignancies, such as leukemia, B-cell functions may inhibit tumor growth[213], whereas in solid tumors humoral factors may cause enhancement of growth.[104] Clearly, immunosuppression in the former instance may be deleterious, whereas it might assist tumor rejection in the latter.

In clinical situations, immunosuppression by a drug may precede its other anti-proliferative actions. In strain-2 guinea pigs, for example, established delayed hypersensitivity to tuberculin was inhibited by 2-week treatment with methotrexate at doses which did not cause leukopenia, overt lymphotoxicity, or anti-inflammatory effects.[119] That unsuspected deleterious immunosuppression may occur has also been shown in other preclinical and clinical situations.[38,73,250] The de novo occurrence of tumors in immunosuppressed transplant patients is certainly one example. Another is the earlier and increased incidence of recurrences and death in patients with bronchogenic carcinoma treated with long-term cytoxan adjuvant chemotherapy, over patients treated for shorter periods, or even left untreated.[38]

The evaluation of the interrelationships between drug effects on tumor, drug effects on immune reactions to tumor, and the anti-tumor effects of tumor-specific immune mechanisms is difficult to achieve within a given tumor-host system. Nevertheless, substantial progress has been made in the development of model systems designed to sort out the relative significance of each of these factors, and Mihich[206] has established a set of general criteria for relevant investigations. One such experiment cited by Mihich[209] will be briefly summarized.

As few as 10 leukemia L1210 cells grow progressively in DBA/2 mice and in F_1 hybrids or backcrosses derived from them. In these animals, arabinosylcytosine (AraC) was administered at 10 mg/kg i.p., once or twice daily for six days, starting 1,2,3,4 or 5 days after i.p. inoculation of 10^6 L1210 cells. If treatment was begun on days 1 or 2, only at the total daily dose of 10 mg/kg did AraC induce the expected 50% incidence of 50-day cures, whereas there were no cures seen at 20 mg/kg total daily dose. If treatment was started on day 5, only at the total daily dose of 20 mg/kg did AraC induce a 60% incidence of cures, no cures being seen at the lower daily dose. Consistent with this, total-body irradiation prevented the curative effects of AraC (at 10 mg/kg for six days from day 1) when given on days 1, 2 or 3, but not when given on days 4 or 5.

These data indicate that shortly after antigenic stimulation, in the presence of few leukemia cells, the low dose of AraC was sufficient to eradicate the leukemia without affecting the immunological response of the host. In contrast, high doses of AraC were immunosuppressive under these conditions, preventing host-dependent defenses from becoming

evident. Conversely, when treatment was started late after antigenic stimulation (in the presence of many leukemia cells), the low doses of drug were insufficient to eradicate the leukemia, whereas high doses were chemotherapeutic, being less suppressive to the now ongoing immune response. Additional support using L1210/AraC cells (a subline resistant to AraC) suggested that this subline would not regress unless rejected by cross-reacting immunity elicited by L1210 during drug treatment.

It is important to note that some evidence exists that pre-existing active immunity is usually not severely affected by anticancer drugs at therapeutic doses, and that recovery of responsiveness may occur as early as three days after the end of treatment.[120,206] In addition, reports have documented that 6-mercaptopurine, a purine anti-metabolite, may actually enhance antibody formation in certain situations.[341,342] However, as noted above, it is clear that most anticancer drugs, in addition to their tumoricidal actions, may exert immunosuppressive, and possibly "immunosynergistic" effects depending on the conditions in which they are used. As pointed out by Mihich, "the preservation of immune reactivity during treatment with anticancer drugs is undoubtedly as important a goal as is, for example, avoiding gastrointestinal or bone-marrow toxicity".[209] That such considerations are under-emphasized in clinical medicine is, in part, a reflection of the lack of available methodologies in the past to evaluate drug effects on tumor-related immune mechanisms, and the present paucity of such knowledge relative to specific drugs. In the future, cancer chemotherapeutics may be aided by the knowledge that given drugs are immunosynergistic when used

under specified conditions. Where immunosuppression is unavoidable, as noted earlier, efforts are being made at approaches directed to reversal of that immunosuppression[157,176,198] in order to maximize the host-drug attack on neoplastic cells.

C. Adriamycin

Chemistry and Mode of Action:

Adriamycin is an antibiotic of the antracycline group first isolated in 1969 from cultures of Streptomyces peucetius var. caesius.[15] Its chemical structure resembles that of daunomycin, differing only in the substitution of a hydroxyl group for a hydrogen atom in the acetyl radical of the aglycon portion of the molecule.

The biological activity of adriamycin at the cellular level is thought to be related to its ability to bind specifically with DNA by intercalation between adjacent base pairs of the double-helical structure.[84,338] By subsequently inducing stereochemical template disordering, enzymes involved in both transcription (DNA-dependent RNA-polymerase) and DNA replication (DNA-dependent DNA-polymerase) are inhibited.[326] These effects are consistent with the in vivo and cell culture data showing the inhibition of incorporation of precursors into DNA and RNA, inhibition of mitosis, and induction of chromosomal aberrations. All are thought to represent the basis of adriamycin's significant antitumor activity.[80,204,326]

Pharmacology:

The distribution and metabolism of adriamycin have been studied using tritium-labelling as well as fluorescence techniques.[16,78,336] It is rapidly cleared from the blood, and in rodents, high drug levels are maintained for a long period of time in the liver, spleen, kidney, lung, and heart.[77,336] The calculated C·x t (concentration x time) for adriamycin equivalents in all tissues has been demonstrated to be several times greater than that found for daunomycin or its metabolites.[336]

In man, adriamycin has a distribution comparable to that seen in rodents, and in both species the drug shows negligible ability to penetrate the blood-brain barrier.[20,336] Urinary excretion is minimal, with only 5% of the drug being excreted during the first five days. Adriamycin is metabolized predominantly by the liver to adriamycinol and several aglycone derivatives, and approximately half of the drug is excreted in the bile as adriamycin, another 30% being excreted as its conjugates.[21] Prolonged plasma levels of adriamycin in patients with hepatic dysfunction provide the basis of a requirement for dose deescalation in patients with impaired hepatic function.[20]

Antiviral Activity:

The antiviral activity of adriamycin has not been as extensively evaluated as that of its closely related analogue, daunomycin, but both drugs have been shown to be inhibitory against a variety of DNA and RNA bacterial and animal viruses. Inhibition of bacteriophage multiplication by daunomycin has been reported by a few investigators[244,278], and this drug protects cells from herpes simplex virus

when given before or a few hours after infection, whereas no effect was demonstrable when the drug was added four hours after infection, when the mature virions are thought to appear.[83]

Significantly, both daunomycin and adriamycin added to the incubation medium at different times before and after infection inhibit the multiplication of Moloney sarcoma virus (MSV) and the focus formation typical of this virus in mouse embryonal cells.[46] It has been demonstrated that both drugs also inhibit the reverse transcriptase activity of RNA tumor viruses.[49] The concentrations active on these processes have negligible effect on cellular proliferation.[46]

Clinical Experience:

Since 1969, well over 3,000 patients have been treated with adriamycin alone or in combination with other chemotherapeutic agents for virtually all types of malignancy.[4] Its activity is wide-ranging, being most effective against solid tumors but also active against the hematologic malignancies. Ten forms of cancer have been treated effectively with adriamycin:[99] breast cancer, soft-tissue and osteogenic sarcomas, cancers of the bladder, lung, thyroid and ovary, Wilm's tumor, neuroblastoma, Hodgekin's and non-Hodgekin's lymphomas, and acute leukemias. The particularly striking efficacy of adriamycin against osteosarcoma is described in more detail in a later section.

Tumors with encouraging, but more equivocal responses than the above, include cancers of the stomach, prostate, liver, head and neck, and multiple myeloma. The drug has apparently no role in the treatment

of adenocarcinoma of the large bowel, malignant melanoma and kidney cancer.[99] Excellent reviews of the current clinical experience with adriamycin can be found elsewhere.[4,99,232]

Animal Tumor Studies:

A substantial number of studies have shown adriamycin to be effective in delaying tumor progression and increasing survival times in a variety of transplanted and primary experimental tumors.[14,25,45,79,81,82,277] Its efficacy and toxicity vary greatly with timing, dose, schedule and route of administration.[25,45,79]

Adriamycin is highly active in inhibiting the growth of transplanted Ehrlich acsites tumors in mice, where its effect on increasing survival times was significantly greater than that of daunomycin.[14,81] Likewise, adriamycin proved superior to daunomycin in inhibiting the growth of transplanted sarcoma 180 tumors in mice.[79] These data support the higher therapeutic index and generally greater antitumor activity of adriamycin as compared to daunomycin, especially against solid tumors, in experimental systems.[79]

Of special interest is the striking efficacy of adriamycin on spontaneous mammary carcinoma of C3H mice, an autochthonous tumor highly resistant to most chemotherapeutic agents.[79] Treatment started the day after tumor implant and repeated every second day for two subsequent cycles strongly inhibited tumor growth and produced a highly significant increase in survival time. Treatment with identical doses administered on consecutive days, however, simply caused a delay in

tumor development, without increase in survival time over control animals, and, interestingly an increase in metastatic dissemination.[79,82]

Toxicity and Effect on Immunity:

In man, toxic effects induced by adriamycin are dose-related predictable, and usually reversible. The most frequent side effects are dose-limiting myelosuppression, mucositis, nausea, vomiting, and chemical phlebitis.[4]

Leukopenia is the primary hematologic toxicity, occurring in over 60% of patients, with a nadir occurring between days 10 and 14. Thrombocytopenia and anemia likewise occur during this period but their occurrence is generally less frequent and less severe. Peripheral counts usually return to normal by day 21.

Severe mucositis, which limit the administration of adriamycin, appears to be schedule-dependent.

Another unusual and dose-limiting side effect is cardiotoxicity. This may involve transient EKG abnormalities or serious irreversible drug-induced cardiomyopathy. Analyses by Le Frak et al.[187] show a frequency of non-fatal and fatal cardiomyopathy of 0.4% and 1.2%, respectively. The incidence of cardiomyopathy is dose-dependent, being markedly increased at total drug doses above 550 mg/m^2. The measurement of systolic time interval in patients approaching this dose may be a sensitive indicator of impending cardiotoxicity.[272]

There are a few reported studies in animal systems indicating that adriamycin may have immunosuppressive effects at certain doses and

schedules of administration.[45,79,161,241,281] These studies can be broken down as showing effects on either general immune function or tumor-specific host defenses.

Isetta et al.[161] reported that adriamycin significantly inhibited the titer of hemolytic and hemagglutinating antibodies in mice immunized with sheep red blood cells. These effects were dose-related and were highest soon after antigen administration. Likewise, antiviral antibodies responses were abolished by adriamycin in another animal system.[45] No data are yet available regarding the effect of adriamycin on general cell-mediated immune functions.

At least four reports to date indicate that adriamycin may inhibit tumor-specific host mechanisms, and that this inhibition may be less than that caused by daunomycin.[45,79,241,281]

As noted earlier, daily doses of adriamycin to C3H mice bearing mammary carcinomas caused no increase in average survival time (AST), and, in fact were associated with a significantly increased incidence of metastatic dissemination in these animals. That this was perhaps due to immunosuppression is the evidence for the cumulative toxicity of adriamycin when administered daily[79] as well as the data suggesting that T-cell functions may be preferentially depressed with this drug schedule.[120,209]

A study more pertinent to the present investigation involved the effects of daunomycin and adriamycin on the growth and regression of primary or transplantable murine sarcoma virus (Moloney)-induced tumors in CD-1 mice.[45] The experimental design, as discussed more fully in a

later section, is based on the observation that tumors induced by low viral titers regress spontaneously in 90% of the animals. Thus, adriamycin's chemotherapeutic activity, as well as its effects on the mechanisms of spontaneous tumor regression, can be evaluated in this system.

Treatment with daunomycin before infection inhibited spontaneous tumor regression, whereas pretreatment with adriamycin caused a delay and inhibition of tumor growth and no effect in tumor regression as compared to controls. Treatment with daunomycin after the infection (i.v. for eight consecutive days) caused a slight reduction of tumor size as compared to controls, and a high incidence of tumor recurrence. Adriamycin treatment after the infection caused a greater reduction on tumor size than with daunomycin, and tumor recurrence took place later in time.

While no in vitro tests of tumor-specific cellular or humoral immunity accompanied these data, they demonstrate that both antibiotics, in the schedules used, have tumor-related immunodepressive activity. In addition, the results suggest that adriamycin is probably less immunosuppressive and/or more tumoricidal in this system than is daunomycin.

In pursuing these possibilities, Orsini and Mihich[241] confirmed the therapeutic advantage of adriamycin in C57Bl/6J mice bearing a syngeneic EL4 lymphoma. In addition, the immunosuppressive effects of the drugs were compared in two different systems using in vitro tests measuring cellular and humoral cytotoxicity. While neither system measured responses to syngeneic tumor-specific antigens, both systems revealed the drugs to have selective effects, under certain conditions,

on cellular immunity. These immunosuppressive effects were apparently less than those caused by AraC or methotrexate in the same systems. In contrast to their expectation, no significant differences between the effects of adriamycin and daunomycin were seen in the systems used.

No studies to date have reported the effects of adriamycin on general or tumor-specific immunity in man. While some evidence exists in animals, it is not yet clear under what conditions its primary effect may be on humoral[45,161] or cellular[45,79,161] immunity. Finally, no *in vitro* data have been obtained supporting the suggestion from *in vivo* studies that adriamycin suppresses tumor-specific host immunity.

D. Osteosarcoma in Man

The term "osteogenic sarcoma" has been variously used by different authors as the generic name encompassing all primary bone tumors of mesenchymal origin, or as a specific malignant osteoid-producing entity originating in endosteal bone. This distinction is an important one, since the several entities included in the former classification have different biologic behaviors, and carry with them unique diagnostic, therapeutic, and prognostic implications. Although the more restricted use of the term will be employed herein, the literature is replete with references employing the generic usage (or more confusing modifications), thus necessitating a brief description of the various entities from which true osteosarcoma should be differentiated.

The principles outlined for the generic usage of "osteogenic sarcoma" were first described by Budd and MacDonald[39,194] and later

refined and expanded by McKenna et al.[201] Predicated on their belief that sarcomata arising in connective tissue of bone "have as their anlage the stem mesenchymal cell which may differentiate toward fibrous tissue, cartilage or bone", Budd and MacDonald subdivided osteogenic sarcoma into three primary categories: fibrosarcoma, chondrosarcoma, and osteosarcoma, respectively. McKenna et al. also included as osteogenic sarcoma those bone-producing malignancies arising in Paget's disease and areas of fibrous dysplasia, tumors induced by prior irradiation, and parosteal(juxtacortical) osteogenic sarcomata.

Defined microscopically, chondrosarcoma is a tumor in which the dominant cell type assumes a cartilaginous histopathological differentiation. Classification of these tumors according to degree of anaplasia reveals a distinct correlation with five-year survival rates, 78% of persons with lowgrade anaplastic tumors and 22% of person with highgrade malignancies living for five years.[201]

Clinically, the patient with a chondrosarcoma is likely to be in his fourth decade, and three times more likely than the patient with osteosarcoma to have his primary lesion in the pelvis, jaws, ribs, or scapulae. Although the duration of symptoms before treatment may be somewhat longer than that in osteosarcoma, presenting symptoms and laboratory findings at diagnosis are substantially very similar. Metastatic disease localizes in a likewise similar manner, although chondrosarcoma is a fundamentally less virulent malignancy than osteosarcoma, and there is much less tendency to early metastasis.[68] Amputation is the therapeutic mainstay, and five-year survivals in several series are consistently about fifty per cent.[69,201,239]

Fibrosarcoma is that malignant tumor of endosteal bone composed of predominantly interdigitating bundles of spindle-shaped fibroblastic cells, including varying amounts of collagen. It is usually subdivided according to its degree of anaplasia[162], but according to McKenna et al.[201] this grading reveals no correlation with survival. These tumors contain no osteoid; however, the dense collagen of some fibrosarcomata is so similar to osteoid as to make the differentiation from fibroblastic osteosarcoma one of great difficulty.[298]

The incidence of fibrosarcoma is approximately one-fourth that of osteosarcoma, and the typical patient is in his fourth decade.[201] The location of the primary tumor, its pattern of metastasis, as well as other clinical parameters are often similar to osteosarcoma. Fibrosarcoma generally follows a less aggressive course, however, and five-year survivals following amputation are reported as about 30 per cent.[68,201]

Perhaps the most common entity to be included in statistics pertaining to osteosarcoma is osteogenic sarcoma arising in Paget's disease.[62,63,69,200,248,308,327] This form of sarcoma may microscopically resemble classic osteosarcoma, or may be classified as a chondrosarcoma or fibrosarcoma.[200]

In addition to its pathological variation, Paget's sarcoma is clinically quite dissimilar from osteosarcoma. The median age at diagnosis of Paget's sarcoma is 60 years[201], and the site of the primary tumor is much more likely to be the ileum, humerus or skull than in osteosarcoma. Longterm survival is rare: all 11 patients of Sweetnam[308] and all 32 patients of McKenna et al.[201] were dead by five years.

Radiation-induced sarcomata are pathologically usually either osteosarcoma or fibrosarcoma.[201] Strictly defined, these are microscopically proven sarcomata having arisen in a previously normal bone five or more years after its having received at least 3000 rads irradiation.[63] Clinically, the patient population is, as expected, quite heterogeneous, antecedent irradiation being performed for a multiplicity of malignant, and some benign, conditions. Five-year survivals are somewhat better than osteosarcoma arising "de novo" in bone: data from 50 years' experience at Memorial Hospital in New York[201] suggest that after amputation, or amputation plus irradiation, five year survivals approximate 40 per cent.

Sarcomata arising in areas of fibrous dysplasia are unusual, and may pathologically be defined as chondrosarcoma, fibrosarcoma or osteosarcoma.[162] Because of their rare occurrence, they are seldom included in reports of osteosarcoma, and therefore will not be further considered here.

The last important, and often confused, entity to be differentiated from osteosarcoma is the ossifying parosteal sarcoma (juxtacortical osteogenic sarcoma).[155] Occurring predominantly during the fourth decade and more commonly in women, these bone-forming tumors develop on the surface of the bone rather than its interior. They are characterized by an indolent growth rate, "pseudoencapsulation", and apparent growth by longitudinal and circumferential extension, resulting in a large tumor mass encircling, or partially encircling the bone.[201]

In addition to age and sex difference between patients with

osteosarcoma and parosteal sarcoma, the biologic courses of the tumors are dissimilar. Parosteal sarcoma is substantially less aggressive, and metastases, when they occur, are often to the bone.[201] Cure rate is high[162]: McKenna et al. cite 82% five-year survivors, and a "theoretical curability" of 100%.[201] Nevertheless, cases of parosteal sarcoma are still often included in series of "osteogenic sarcoma"[164], and their inclusion only serves to confuse and skew statistics meant to represent responses of classic osteosarcoma to newer therapeutic modalities.

In differentiation from the above, osteosarcoma in its more restricted usage is a distinct pathological and clinical entity occurring mainly in the second and third decades of life, and rapidly progressive in most cases, with pulmonary metastases and death within two to three years from diagnosis.

Pathology:

In general, osteosarcoma may be defined as a specialized connective tissue sarcoma which elaborates neoplastic osteoid from an endosteal location during the course of its evolution. Most often, the osseous tissue arises directly from the sarcomatous connective tissue, but in rapidly growing areas it may be formed via a cartilaginous intermediary stage.[164] Histologically, osteosarcoma is remarkable for its varied appearance: its microscopic pattern varies considerably from lesion to lesion and from area to area within the same lesion, depending primarily on the age of the lesion and its degree of osteogenesis.[162] Relative to this latter feature, the tumor may be defined radiologically and

histologically as either predominantly osteolytic or sclerosing (osteoblastic) in type. In the former category there may be abundant amounts of either neoplastic cartilage or fibrous stroma, thus justifying its histologic subdivision into chondroblastic or fibroblastic osteosarcoma, respectively.[68]

Within non-ossified tissue, the cells may be predominantly anaplastic, spindle-shaped or round, the latter in lacunae suggesting cartilage cells. In ossified areas, there is relatively orderly histologic progression according to the age and virulence of the tumor. This is well described by Jaffe.[162] Initially localized to the interior of the affected bone, the originally pleomorphic tumor cells progressively spread out from one another, deposit increasing amounts of intercellular fibrillar collagenous material, and later osteoid and calcium, while the cells become progressively more "normal appearing".[249] Progressive growth of the tumor causes periosteal irritation and subperiosteal new bone formation, later distending the periosteum (causing "Codman's triangle" on radiographs), and finally disrupting and extending beyond the cortex.

Incidence:

Osteosarcoma is the most common of the primary malignant tumors of bone.[155,162] There is a definite male predominance, cited variously from 1.4:1[201] to 2:1[162]. Its peak incidence is in the second and third decades, as about 75% of all patients in any representative series are between the ages of 10 and 25 years, with few cases below age 5 and fewer still above age 40. Vigorous pursuit of pre-existing bone disease

(e.g., Paget's disease) or prior irradiation should be considered before accepting the diagnosis of de novo osteosarcoma in any person over 50 years of age.[162]

Localization:

In any large representative series, between half[68] and four-fifths[236,308] of the primary tumors are located in the long bones at the knee joint. Most of these are at the distal end of the femur, with a smaller but significant number originating in the proximal tibia. A significant number of primaries are also localized to the proximal humerous and femur, and a fair number are first diagnosed in the pelvic bones. There appear to be no absolutely privileged sites, but the spine and skull bones are rarely involved in the absence of previous irradiation or pre-existing osteitis deformans.

Clinical Manifestations:

Persistent local pain without other complaints, is the initial symptom in approximately three-fourths[236] of patients with osteosarcoma, while swelling, pathological fracture, or local warmth are presenting symptoms in a fewer number. Some patients may complain of anorexia, weight loss, and rarely, dyspnea or fever, but these are usually late symptoms. The average duration of symptoms before seeking treatment is usually three to four months.[201,307]

Of the initial laboratory determinations, only an elevated level of alkaline phosphatase is helpful diagnostically, although only approximately one-fourth of patients reveal this abnormality, and then to only

two or three times the upper limits of normal.[236] In these patients, levels usually return to normal after definitive treatment, and subsequent elevations are a sensitive index of local recurrence or metastatic disease.[162]

Radiographically, osteosarcoma presents a variable appearance, and its differential roentgen diagnosis is of little more than academic interest, in that histological confirmation is required for definitive diagnosis. Experienced radiologists can normally predict whether a bone tumor is benign or malignant in approximately 75% of cases[201], and maximum effort should be devoted to making this distinction, and to determining the best site for biopsy. "Codman's triangles", often considered as diagnostic of osteosarcoma, are encountered in both benign and malignant tumors, as well as in non-neoplastic conditions.[87] Likewise, lamellated "onion-skin" or speculated "sunburst" periosteal appearances are not pathognomonic for osteosarcoma, although they carry a high correlation with malignancy.[242]

Clinical Course:

The typical course of osteosarcoma, treated or untreated, is a rapid and relentless one, progressing to pulmonary metastases and death within two years of its diagnosis. Indeed, its course is so aggressive and predictable that Reinberg in 1960 felt justification for saying that "a case of any age that has recovered from osteogenic sarcoma has to be regarded as a diagnostic error".[339]

A comprehensive review of the literature on over 1400 patients with osteosarcoma reveals actual five-year survivals, regardless of

primary therapy (operation, radiotherapy, or a combination of the two, with or without chemotherapy), ranging from 5 to 25.5%.[61,118,236] As pointed out by Sweetnam et al.[308], however, the proportion of patients surviving five years is a relatively uninformative measure, as survival curves clearly indicate that mortality is concentrated in the first two years from starting treatment, and that thereafter the curves flatten out. This is reflected in a typical median survival time of about 16 months.[308]

A definitive prognostic and therapeutic point in the course of osteosarcoma is the onset of pulmonary metastases.[118] The average time from onset of symptoms to treatment is about four months[201], and the mean interval from starting radiotherapy, radical surgery, or a combination of the two, to pulmonary metastases, is 10 months[236,308] (median, less than five months[236]). Death, usually from respiratory failure, is imminent thereafter, the average period between the discovery of pulmonary metastases and death being consistently six to seven months[236,308] (median, six months[236]). It is assumed that probably all patients when first seen have hematogenously disseminated microscopic metastatic disease[248], and McKenna et al.[201] describe symptomatic pulmonary metastases in 10% of patients at initial presentation. Autopsy data reveal more than 95% of cases with pulmonary metastases.[201]

Only two large series have systematically evaluated their autopsied patients, and these are somewhat confused by the inclusion of some non-osteosarcoma bone tumors.[201,248] Summarized, the metastatic patterns are as follows: bone (osteosarcoma only), 14%; lymph nodes, 30-40%; pleura, 30%; pericardium, 20%; diaphragm, 18%; kidney, 12%; liver,

pancreas and dura, each 8%; adrenal, 6%; thyroid, 4%; and ovary, 2%. Local recurrences of tumor were found in 50%, and tumor venous thrombi were found in 14 per cent.

Prognostic Factors:

Much effort has been devoted to the determination of factors related to prognosis in osteosarcoma. Schwinn and McKenna[284] break down the several variables discussed into four categories: those with definite, probable, equivocal, and no discernible correlation with prognosis. The following is a brief update and modification of their findings.

Their first two factors definitely correlating with survival have already been discussed. Osteosarcoma has a significantly worse prognosis than chondrosarcoma, fibrosarcoma, or parosteal sarcoma, and a much better prognosis than Pagetoid sarcoma.

Relative to location, the rare facio-maxillary primaries have a better survival rate than osteosarcoma arising elsewhere, and, in general, those arising in the axial skeleton show worse therapeutic results than those tumors more distal to the axial skeleton.[58,201,284] Of the latter, tumors in the distal femur have greater curability than those in the tibia.[236]

Not surprisingly, a fourth definite correlate with survival was stage of disease, persons with metastases to lungs or lymph nodes faring much more poorly than those with localized diseases.

A final significant "prognostic factor" defined by Schwinn and

McKenna is therapeutic intervention -- in their series, persons treated by immediate amputation, or radiotherapy followed by amputation, survived longer than those receiving no treatment.[284]

Of the factors identified as "probably" correlated to survival, the most interesting is the duration of symptoms. This has been described in at least two previous large series[48,201] -- patients with a short interval between onset of symptoms and diagnosis have a worse prognosis than do those with a longer interval. It has been suggested that this finding supports the involvement of host immunity in those patients with a more lingering, indolent prodrome.[118]

The size of the tumor at diagnosis also probably correlates with survival, as a large tumor, other factors being equal, is a bad prognostic sign.[201,284] This, however, has recently been contested.[236]

Factors with an equivocal effect on prognosis include: (1) histological grade -- using Broder's criteria for grading the histological malignancy of the tumor, most Grade I and II tumors (least malignant appearing) may have a better prognosis, but these grades are very infrequently found.[68] Relative to histological subtype, osteoblastic, fibroblastic, and chondroblastic tumors all have similar biologic behaviors;[62,201,236] (2) sex of the patient -- some reports suggest a better prognosis for females[113,284], but this has generally not been substantiated;[118] (3) pathological fracture at the tumor site; and (4) pre- and post-operative serum alkaline phosphatase levels.

Factors reported by Schwinn and McKenna to show no correlation with survival include: (1) age and (2) antecedent trauma. The former

factor cannot be dismissed easily, however, as a recent careful study by Ohno and associates[236] found age to be the only significant prognostic variable of ten parameters studied. In this study, patients fifteen years of age or younger fared significantly worse than did the older patients.

Treatment:

As previously pointed out, actual five-year survivals for osteosarcoma, regardless of primary therapy, have varied from 5 to 25.5 per cent.[61,118,236]

The results of radical surgery, the usual form of primary therapy, have shown no change from 1946 to 1970.[196] Despite differences in pretherapeutic workup, surgical techniques and demography, a comprehensive review of 1337 patients treated surgically since 1945 has shown consistent five year survivals of approximately 20 per cent.[118] Of 438 patients evaluable at 10 years, 16% were still alive, indicating that the majority of those patients living five years appear to be "cured". The large surgical literature on osteosarcoma and its consistent findings of about one of five patients surviving five years, justifies using surgical therapy as the standard against which other therapeutic modalities must be compared.

The data for radiation therapy alone are difficult to evaluate, but they seem to indicate that irradiation is the least effective primary therapy of osteosarcoma. A fairly extensive literature suggests that the overall five-year survival after radiation therapy alone is

12.5 per cent.[118] Several factors complicate the interpretation of this response rate; patients with metastatic disease at presentation are usually considered ineligible for surgery; patients with primaries in unresectable locations (possibly secondary to Paget's disease) may have more virulent malignancies[58,201]; some patients may have had other significant medical illnesses mitigating against surgery; and finally, true tumorical doses (6000-12000 total rads) were not utilized in all patients.[118]

A combination of therapeutic modalities has enjoyed increasing and justified popularity in a variety of malignancies, and osteosarcoma is no exception. As noted previously, the onset of pulmonary metastases marks a definitive point in therapy, the majority of patients revealing clinical evidence of pulmonary disease within five to nine months of operation[68,196], and metastatic disease invariably signifying imminent death.[229,308] With this in mind, recent efforts in the treatment of osteosarcoma have been directed towards two ends: the prevention of metastatic disease after surgery, and the attack of overt pulmonary metastases.

The approaches to overt pulmonary metastases have been both surgical and chemotherapeutic. Chemotherapeutic approaches have involved both the systemic administration and local infusion of cytotoxic drugs.

Surgical resection of isolated pulmonary metastases has resulted in sporadic instances of prolonged survival, but the overall results have not been encouraging.[236] Workers in Japan have investigated the use of bronchial artery infusions of cytotoxic agents for overt metastases with encouraging, but not statistically significant results.[235]

There had been relatively few attempts at systemic chemotherapy for metastatic disease in osteosarcoma until the last few years. Nevertheless, with the two exceptions of adriamycin and methotrexate (to be discussed in the following pages), metastatic osteosarcoma has retained its reputation as a malignancy resistant to traditional chemotherapeutic agents.

Historically, the alkalating agents had been most intensively investigated, although the numbers of patients were usually small, and a variety of schedules and doses were not generally employed. Sporadic reports have documented partial responses with systemic cyclophosphamide[110,136,252,300] and phenylalanine mustard[300,303]. Overall response rates with cyclophosphamide were reported as 14.2% (4/28), and with phenylalanine mustard, 15.5 per cent (5/32).

Mitomycin C was shown to cause regression of pulmonary metastases in 1961[96,114], but the original enthusiasm for this agent dwindled by 1971[47,304]. Overall, of 76 patients treated, there were 11 responses, for a response rate of 14.4 per cent.[118]

The activity of the vinca alkaloids has been likewise disappointing, vincristine having shown no responses in 21 cases.[285,300] Other agents used with either low response rates or very small numbers to evaluate include 5-fluorouracil, hydroxyurea, procarbazine, and diethyl triazene imidazole carboxamide.[118,134,304] Little or no data are available on the use of many of the alkalating agents, antimetabolites or nitrosourea drugs.[118]

Against this background of minimally promising activity of chemotherapy

against metastatic disease are the strikingly more effective results obtained with two drugs, methotrexate and adriamycin. The former, when given in high doses and followed by citrovorum factor rescue, has caused tumor regression in four of ten patients with metastatic osteosarcoma followed from one to more than eight months.[163] At the time of publication, complete regression of pulmonary metastases had occurred in two patients, and persisted in one of these. In contrast, methotrexate alone or in conventional doses has failed to show any response.[69,313]

Finally, Cortes et al.[60] have reported objective regression of pulmonary metastases in 7 of 17 patients treated with adriamycin and followed from 4 to more than 20 months. Metastases shrinkage of over 50% was seen in five patients, and in one there was a complete remission. Although all patients treated showed signs of toxicity and two patients succumbed to adriamycin-associated cardiomyopathy, the data clearly revealed the effectiveness of adriamycin in treating metastatic disease, and stimulated research into the use of adriamycin as an adjuvant to surgery in the prevention of metastases.

Adjuvant chemotherapy is the most promising of the several methods used to approach the second, and more important problem in the treatment of osteosarcoma -- the prevention of metastatic disease. Considering that hematogenous pulmonary metastases are present in the majority of patients at the time of diagnosis[35,164], three other approaches have also been tried, including the pre-operative ligation of the veins draining the tumor site[175], prophylactic irradiation of the lungs[57,231], and immunotherapy.[296] The significant roles of tumor immunity and immunotherapy in osteosarcoma will be discussed in a later section.

While the pre-operative ligation of the veins draining the tumor site has not resulted in significantly prolonged survival or fewer post-operative metastases[35], there have been many proponents of prophylactic pulmonary irradiation in addition to amputation. These have included both pre- and post-operative radiation, at varying intervals from surgery, and with varying doses of irradiation. Most reports indicate only a slight advantage for the addition of radiation to amputation.[118] Nevertheless, at least one promising report, showing delayed onset of pulmonary metastases after pre-operative radiation plus amputation, suggests that this is one modality to be pursued further.[231]

Finally, two chemotherapeutic approaches, one using high dose methotrexate with citrovorum factor rescue, and the second using adriamycin, have shown strikingly effective activity in the prevention of pulmonary metastases. Their use as adjuvants is based on substantial experimental cytokinetic evidence that the proportion of neoplastic cells destroyed by a given treatment should be significantly greater in micrometastases than in clinically apparent metastatic disease.[281]

In the first chemotherapeutic method, high doses of methotrexate are given in conjunction with vincristine, followed two hours later by 12 intravenous doses of citrovorum factor "rescue".[164] This regimen, using increasing doses of methotrexate given every three weeks, significantly reduced the post-operative incidence of pulmonary metastases. Although only 12 of the 20 patients studied had "classic" osteosarcoma, 11 of these were free of metastases from 6 to 27 months.

In the adriamycin regimen, six three-day courses (30 mg per square

meter daily) were administered (following surgical healing) to 21 patients with "classic" osteosarcoma.[61] Of thirteen patients treated with amputation and no protocol deviation, twelve were completely free of disease at periods from one to 31 months after operation. An update of these data reported two subsequent relapses, such that 10 of the 13 patients remained disease-free at a median of 19 months post-operatively, and 3 to 34 months after the discontinuance of chemotherapy.[40] Although admittedly these data may only signify an inhibition of tumor growth and a delay in the appearance of metastases, another report supports the probability that adjuvant chemotherapy is actually preventing the occurrence of metastatic disease.[305] At least two other recent prospective, controlled studies including adriamycin in a multiple drug adjuvancy protocol have substantiated this assessment.[340,305]

Etiology and the Role of Tumor Immunity:

Although there have been several theories of causation for osteosarcoma, including antecedent trauma[162] and genetic origins[273], there is abundance evidence to suggest a viral etiology.* Before reviewing the evidence, it is pertinent to first briefly review the requirements for proof of causality and to site the limitations imposed by these requirements.

As originally posed by Koch in 1891, there are three essential conditions which must be fulfilled in order to establish a causal relationship between a suspected infectious agent and a particular disease.

* REFERENCES: 1-3,22,23,27,28,54-56,92-94,107-109,111,125,128,135,137, 138,167,172,219,225,226,254,263-265,271,273,332,333

In general terms these are as follows: (1) the micro-organism must be observed in all cases of the disease in question; (2) it must be isolated and grown in pure culture, and (3) the micro-organism so isolated should reproduce the disease in other susceptible animals. Today a fourth criterion is generally recognized -- the demonstration in the infected host of circulating humoral and/or cellular immunity specific for the organism in abnormally high concentration, or an abnormally high specific immunity to the infecting agent in a recently recovered host.[330]

Therefore, supporting evidence that viruses cause several human cancers has been gathered in several ways.[270] One approach has been to search for virus particles, viral precursors, and virus-specific nucleic acids in human tumor biopsy samples and cultured tumor cells. A second method is to experimentally induce tumors by inoculation of human tumor material or putative human oncogenic viruses. Resulting animal tumors are then examined for candidate human viruses.

Data suggesting a viral etiology for human cancer have also accumulated by monitoring the ability of a virus to transform cells in culture. These transformed cells grow indefinitely in tissue culture, lose qualities of contract inhibition, alter their surface properties, and are often capable of inducing tumors.

Finally, immunologic methods have been used to determine the presence of tumor specific antigens and anti-virus antibodies in individuals with cancer, their families and associates, and in large populations of people. Immunology has been a particularly powerful tool in establishing a viral etiology for human malignancy, in that tumors induced

by the same oncogenic virus, even in unrelated animals or when producing histologically different malignancies, share at least one common tumor-specific antigen.[170] As previously noted, this is in striking contrast to tumors induced by the same chemical carcinogen.[171,237]

In summary, then, while there is substantial evidence for a viral causation of some human malignancies, much of this evidence is indirect, depending on the host's recognition of, and response to, virus or virally determined antigens. Merely the presence of virus does not establish its causal role and yet direct proof -- to induce cancer by injection of the virus into healthy human subjects -- is morally and ethically prohibited. Nevertheless, even in bacterial non-tumor systems, the causal role of a particular micro-organism is sometimes justifiably adduced without complete fulfillment of all Koch's postulates, when the overwhelming preponderance of evidence presents a very high degree of probability.[330]

Two groups of viruses have been consistently associated with naturally occurring cancer: oncornaviruses containing single-stranded RNA, and herpesviruses composed of double-stranded DNA. Herpesviruses have been strongly linked to the causation of Burkitt's lymphoma, nasopharyngeal carcinoma, and cervical carcinoma.[269] Oncornaviruses, especially C- and B- types, possess reverse transcriptase, which enables DNA to be produced on an RNA template, reversing the usual direction of information. This DNA intermediate is then integrated into the host genome, causing neoplastic transformation by as yet unknown mechanisms.[297] B-type particles and reverse transcriptase have been associated with human breast cancer[218], and several components of C-type viruses as

well as reverse transcriptase have been isolated from patients with acute leukemia.[124] It is predominantly this latter group of oncornaviruses which have likewise been associated with human osteosarcoma, the evidence for which follows.

Electron microscopic examination of thin sections of human osteosarcomata[108,109], their derived tissue culture cells[135,226] and cell culture supernatants[107,109,264] have revealed C-type RNA particles. The incidence of these findings has varied; e.g., Gyorkey et al.[135] found nine of 12 tumor biopsies positive for the virus, whereas Morton et al.[226] could not visualize any in the specimens from six sarcoma patients, yet visualized them in tissue cultures of the same tumors. The particles were similar to the tumor viruses first described by Bernhard[24], with an outside diameter of 85-90 millimicrons, appearing singly throughout the cytoplasm or in groups within cytoplasmic vesicles, and occasionally seen in the dumbbell shape of many murine leukemia and sarcoma viruses.[226]

Cell-free extracts of human osteosarcomata, many containing C-type viral particles, when inoculated into hamsters[107,109,264,265], guinea pigs[264], and mice[107] have reproducibly produced osteogenic sarcomata in these animals. Tissue sections of these animal tumors have then been shown to contain C-type viral particles.[108]

Conversely, several studies have shown that murine sarcoma virus, strain Moloney (MSV), is capable of successfully transforming and replicating in human diploid cell lines in culture.[1-3,22,23,28,172] Similar results have also been obtained with feline leukemia and sarcoma viruses.[111,137] The results with MSV include the early and easily

reproducible transformation of some human embryo cell strains, and the interesting finding of a shift of viral tropism, once viral transformation of the human cells has occurred. Thus, relative to its transforming function, the original double tropism (murine-human) of the virus shifted to an exclusively human one, retaining its transforming effect on fresh human cell cultures with excellent reproducibility.[22]

Additional evidence for the etiology of an infectious agent is the remarkable familial incidence of osteosarcoma.[138,254,273] While originally this suggested to investigators the possibility of a genetic origin for osteosarcoma[254,273], as early as 1935 the question of an adjuvant role of an infectious agent was considered.[273] As many as four offspring in the same family have been afflicted.[138]

Finally, consistent with the intimate association of virally-induced tumors and immunological methods, there is abundant evidence to suggest the presence of a sarcoma specific antigen(s), the probability that this antigen is virus-related, and that host responses to this antigen may play a significant role in the in vivo destruction of tumors.

A variety of immunological techniques have revealed the presence of at least one antigen which is specific for sarcomas. This antigen has been detected on the cell surface[27,55,333] and in the cytoplasm[92,93,128,219,263] of malignant cells, and host response has been both cellular[54-56,125] and humoral.[27,92-94,219,225,226,263,265,271,332] Using indirect immunofluoroescence (IF), common anti-sarcoma antibodies have been found in the sera of essentially all sarcoma patients against

autochthonous or allogeneic tumor lines.[27,225,226] In addition, approximately 85% of close family members and 90% of close, unrelated associates have anti-sarcoma antibodies as determined by IF.[92,225,226] Sera from unrelated normal blood bank donors have a variable incidence of positivity, generally reported as being between zero and twenty-nine per cent.[225,226,263,265] Antibodies cytotoxic to human sarcoma cells have also been reported in patients with sarcomas.[332] That the above anti-sarcoma antibodies are specific for sarcoma cells (of various histological types) has been convincingly demonstrated -- the sera are non-reactive against a wide variety of other human cell lines.[219]

Cellular immunity in sarcoma patients is likewise sarcom-specific.[54-56,125] In one study reported by Cohen et al.[55], peripheral blood lymphocytes from 14 of 18 patients with several histological types of sarcomata were cytotoxic to an allogeneic osteosarcoma cell line, but not to fibroblasts from the same patient. Lymphocytes from melanoma patients were consistently non-reactive.

In keeping with the common antigenicity of virally-induced tumors, these immunologic data are perhaps sufficient in themselves to implicate a virus in the etiology of human osteosarcoma. To summarize these results, it may be instructive to briefly review one elegant study reported by Moore and Hughes.[219] In this study, 42 sera from 31 sarcoma patients, 17 sera from 11 patients with various carcinomata, and 34 sera from 18 non-tumor bearing control patients were tested by IF against 38 tissue culture lines, including those derived from sarcomas (16), carcinomas (7) and other tumors (2), embryonic tissue (8) and normal

adult tissues (5). In addition, IF tests were done to detect anti-nuclear antibodies (ANA) and smooth muscle antibodies (SMA), and sera were tested for HL-A antibodies by microcytotoxicity tests against a panel of 22 lymphocyte preparations.

Their results, simply put, included the following: (1) There was a significantly higher incidence of anti-sarcoma antibodies (ASA) in sera from sarcoma patients than in carcinoma sera or sera from controls; (2) sarcoma sera showed no reactivity against 21 of 22 other human cell lines, including all those of embryonic origin; (3) sarcoma sera had significantly lower incidence of ANA and HL-A reactivity; and (4) sarcoma sera had a slightly increased incidence of SMA's.

These results dramatically illustrate the specificity of the sarcoma antigens and help elucidate their nature. Failure to demonstrate HL-A reactivity, in addition to the presence of authocthonous anti-tumor activity, in the sarcoma sera strongly suggests that the antigens are not intra-cellularly expressed HL-A antigens. The lack of reactivity against cell lines recently derived from human embryonic tissue also minimizes the possibility that these antigens are of a fetal type. In addition, the immunologic specificity of the reactions renders "antigenic conversion" by exogenous virus or mycoplasma unlikely, and these conclusions were also supported by the authors' inability to isolate mycoplasma from the tissue cultures. Finally, although these may represent tissue-specific antigens which may be detected *in vitro*[202], as there was a slightly increased incidence of SMA in the sarcoma sera, a more interesting and likely possibility is that the antigens represent intracellular products specified by an endogenous virus. Although

no electron microscopic evaluation of the malignant cells was performed, these exacting immunologic data provide a convincing complement to the previously cited data supporting a viral etiology for osteosarcoma.

That the host response to osteosarcoma in man is significant for tumor destruction in vivo has been suggested by a wide variety of in vivo and in vitro correlates between the clinical progression of tumor and the hosts' immunological state. At the outset, it is important to clarify that the specific cellular and/or humoral mechanisms important for the destruction in vivo of osteosarcomata have not yet been completely defined, and that some of the data supporting these mechanisms are in conflict.

Studies of cellular immunity in patients with osteosarcoma have reported varied results, some studies indicating lymphocyte competence in all stages of disease[202,253], while others have reported apparently just the opposite.[47,322] These apparent differences are somewhat corrected when disparate methodologies are compared and clinical stage is taken into consideration. For example, whereas studies by McMaster et al.[202] and Golub et al.[130] both show normal in vitro PHA-responsiveness of lymphocytes in sarcoma patients, the latter study revealed decreased conconavalin A responsiveness in lymphocytes from these same patients. Golub et al. conclude from these data and those on patients with many other types of malignancies that con A is both a more sensitive index of lymphocyte proliferation defects and a better in vitro correlate of in vivo immunologic state in cancer patients. Thus, added credence is given to other reports of decreased lymphocyte mitogen responsiveness in sarcoma patients.

Delayed hypersensitivity reactions (DHR) to common microbial antigens and DNCB, in vivo tests of non-specific cellular immune competence, especially "recognition" competence[130], are more easily interpreted. In general, while at least one study reported normal delayed hypersensitivity reactions in sarcoma patients[253], all studies have shown significantly decreased DHR in patients with advanced disease.[91,130,253] That this may be important prognostically was shown by Eilber and Morton[91], who correlated DHR with clinical state before and after amputation. In their study, 93% of DNCB-negative patients were either inoperable or developed early post-operative recurrences, whereas 92% of DNCB-positive patients were free of disease at six months after surgery.

In vitro studies of humoral immunity have shown similar results. Cytotoxic antibodies detected in vitro are assumed to be significant for in vivo tumor destruction.[332] In addition, antibodies detected by IF in sera from sarcoma patients have been significantly depressed in patients with metastatic disease[219], and serum factors blocking cell-mediated immunity have been found in 83% of patients with progressively enlarging sarcomas.[54,56,145]

Finally, perhaps the functionally most important evidence clarifying the significance of host immunity in osteosarcoma has come from studies of immunotherapy. Essentially all the main forms of immunotherapy have been attempted, with varying amounts of vigor and with varying degrees of success; including, non-specific stimulation with BCG, passive transfers of specifically immune antibody, lymphocytes and transfer factor, and active immunization with various forms of tumor

cell vaccines.[88,190,197,296,329] Active immunization with BCG and autologous tumor cells has induced in vitro heightened immune responses against tumor associated antigens, with occasionally beneficial therapeutic results.[224] A recent clinical trial using irradiated fresh autochthonous tumor cells as a vaccine adjuvant to surgery reported four of five patients disease-free at 20-31 months post-operatively. Though of small sample size, this study has revealed significant prolongation of disease-free intervals in these patients, and underscores the other evidence supporting the substantial importance of tumor-specific host mechanisms in osteosarcoma.

E. Animal Models of Osteogenic Sarcoma

Characteristics of an Ideal Animal Model:

An ideal laboratory model for osteosarcoma, in keeping with the characteristics of the human tumor described in the previous discussion should include the following: (1) the tumor should arise "spontaneously", or be induced reproducibly by a virus; (2) it should initially be well localized for surgical ablation and/or local therapeutic manipulation; (3) its pathological nature should include osteoid-producing malignant cells; and (4) it should have a clinical course similar to the human disease, with a similar incidence and distribution of metastatic disease. A final consideration is that reproducible assays must be available for this model to permit evaluation of the immune response evoked by the tumor.

Historically, animal models of osteosarcoma have included spontaneously arising tumors[36], tumors induced by external irradiation[166] or injection of radioactive isotopes[220], and tumors caused by chemical carcinogens[53,156] or oncogenic viruses. A great variety of animal species have been used.

Spontaneously arising osteosarcomas have been observed in dogs, but only very infrequently.[36] Radiation-induced sarcomata, while very reproducible, produce only weak tumor-specific antigens, are poorly localized, and require long latency periods.[166,220] Chemical carcinogen-induced tumors have greatly enhanced knowledge of tumor immunology in animals, but most importantly lack the common antigenicity seen in virally-induced tumors, as noted previously, and therefore lack a fundamental characteristic of human osteosarcomas.

Models for osteosarcoma have included tumors induced by both DNA (SV-40)[75,76] and RNA viruses in mice, hamsters and rats. Viruses have produced tumors when inoculated by intravenous[75,76], intra-muscular[102,141,199,217,286], intraperitoneal[121,295] and intra-tibial routes.[117,160]

MSV - Mouse Systems:

By far the most extensively studied animal models are the Moloney Sarcoma Virus (MSV)-induced tumors in mice. MSV is a known oncogenic RNA virus closely realted to the murine leukemia virus (MLV) and sharing antigens with other closely related viruses of Friend and Rauscher.[52,101,150] The induced tumors are highly antigenic, although

there are different neoplastic and immunologic responses to the inoculated virus both between different strains of mice[126] and rats.[169] Thus, young BALB/c, C3H and (NZW x NZB)F_1(B/W) strains of mice all develop rapdily enlarging soft-tissue tumors, and while tumors regress in C3H mice, they grow progressively in BALB/c and B/W strains, and each strain shows unique immunologic responses.[126] The induced lesions also have been shown to vary substantially by different routes and doses of virus administration.[169]

The most thoroughly evaluated MSV model is the sarcoma induced by intramuscular inoculation of MSV into BALB/c mice. In this system, mice injected with MSV by three to four weeks from birth invariably develop tumors within approximately 10 days, which usually grow progressively and kill the animals.[102,141,199,217] Older mice injected with MSV likewise develop tumors, but these usually reach a maximum size of 10-15 mm and then regress.[102] Immunosuppression of adult mice by X-irradiation[102], ALS[185] or cortisone treatment[286], or chemotherapy with cyclophosphamide[101] or daunomycin[45], causes a significantly decreased incidence of tumor regression.

Pathologic evaluation reveals these tumors to be rhabdomyosarcomas.[217,247] The sarcomas contain and release virus as demonstrated by electron microscopy[121], the ability of cell-free tumor extracts to induce tumors <u>in vivo</u>[247], and focus formation <u>in vitro</u>.[139]

Immunologic studies in this system have been extensive, and will be discussed in greater detail later. Suffice it to say that a variety of immunological techniques have been used to demonstrate the presence

of anti-viral antibody, as well as both tumor-specific cellular and humoral immunity *in vitro* throughout the course of these tumors. Both *in vivo* and *in vitro* studies, therefore, have clearly demonstrated the significance of tumor immunity in the murine model for osteogenic sarcoma.

Relative to the ideal animal model criterial proposed above, however, the MSV-BALB/c tumor system lacks two essential components: (1) the BALB/c tumors, consistent with other MSV-induced tumors in mice[169], are myosarcomas, lacking any ability to produce osteoid; and (2) the incidence of metastases in this system is low, and metastases are not distributed in a pattern similar to the human disease. In addition, the small size of the animals makes effective surgical intervention difficult, and many animals often need to be sacrificed for sufficient serum and/or lymphocyte collection for *in vitro* immunological studies.

MSV - Rat Systems:

Factors such as these have led a few investigators to pursue an MSV-induced rat model for osteosarcoma.[51,121,160,169,295,320] Conflicting reports exist as to the oncogenicity of MSV in rats; Ting[320] states that MSV rarely produces tumors in intact newborn rats, and Kano-Tanaka *et al.*[169] report that tumor induction in rats requires doses of the virus ten thousandfold higher than those in mice. These studies reveal the importance of the route of virus administration, as Ting used intraperitoneal and Kana-Tanaka *et al.* used either intraperitoneal or subcutaneous-intramuscular virus inoculation. Ikemoto and Yamamoto[160],

however, report an 87.5% incidence of tumors when MSV is injected directly into the marrow of the tibia of newborn Wistar-Lewis rats.

One uniform finding among these studies and those of Chesterman et al.[51], Soehner et al.[295] and Fuginaga et al.[121], is that rat sarcomas induced by Moloney Sarcoma Virus, regardless of route of inoculation, are almost invariably osteogenic in nature. This is in marked contrast to the myosarcomas induced by MSV in mice.[169,217,247] Consistent with mouse models though, was the preliminary evidence that suggested significant tumor immunity in these rat models for osteogenic sarcoma. This evidence included the increased incidence of sarcomas in thymectomized rats[320], and the total inability of investigators to induce tumors in the mature animals.[51,121]

Of the rat models for osteosarcoma, that described by Ikemoto and Yamamoto seemed to be the most promising, and it motivated Friedlaender and Mitchell at Yale to pursue this system further.[117] The results of their work will be described briefly. Following intra-tibial inoculation of MSV within 24 hours of birth, 97.7% of the animals developed localized tumors defined histologically as osteogenic sarcomas. Of those rats which could be evaluated, 77.9% had progressively enlarging masses and 22.1% had regressing tumors at the time of sacrifice or shortly after death. Although a detailed incidence study of metastases was not done, metastases were frequent in progressor animals, and these were most commonly to lungs.

Tumor-specific cell-mediated cytotoxicity (CMC) was evaluated and found to be present in all animal groups tested from 19 to 88 days

of age. Since a single homogeneous tumor cell pool was not used throughout the experiments, however, changes in CMC did not necessarily reflect the changes of in vivo CMI during the course of the disease. Serum factors modifying this cell-mediated immunity (either "blocking" or "helping" factors) were also seen, and these were roughly correlated to the animals' clinical status.

Thus, an animal model has been found which very adequately fulfills the criteria previously proposed for correlation with human osteosarcoma. Intra-tibial inoculation of a virus has reproducibly produced well localized osteoid-producing tumors in newborn rats, and these tumors very readily metastasize in a pattern apparently similar to the human disease. While these data are preliminary, they suggest that further study of this model may help untangle the complex questions posed by osteosarcoma, including its relentlessly progressive nature and its alteration by immunological factors.

F. Summary Statement of the Goals of the Present Investigation

Osteosarcoma is a devastating and therapeutically resistant malignancy of young people, progressing to pulmonary metastases and death in 80% of patients within two years of its diagnosis. Abundant evidence supports a viral etiology for this tumor, including a common antigenicity for several histological types of sarcomas. Tumor-specific immune mechanisms may play a significant role in preventing the usually rapidly progressive course of osteosarcoma.

Evidence has been reviewed that suggests that immunosuppression by chemotherapeutic agents may be counterproductive and clinically significant in cancer therapy. Thus, anticancer agents are seen to be both tumoricidal and potentially immunosuppressive. Adriamycin is a relatively new anticancer drug with a broad spectrum of tumoricidal activities, but there is a paucity of data relative to its effects on tumor-specific immune mechanisms. Its tumoricidal action against osteosarcoma has been particularly striking, as it has postponed (and possibly prevented) the onset of metastatic pulmonary disease, and probably increased survival times.

An initial investigation has suggested that an MSV-induced rat osteosarcoma may be an ideal model of the human disease. Initially localized, the primary osteoid-producing tumor metastasizes and is rapidly fatal to most infected animals. Assays have been used which document the presence of tumor-specific cellular and humoral responses in tumor-bearing hosts.

With these considerations in mind, the present investigation was designed to fulfill three general goals: (1) To substantiate the extent of similarity of the rat osteosarcoma model to osteosarcoma in man, by evaluating its course, histology, and further documenting its incidence and distribution of metastatic disease; (2) to study the immunobiology of this tumor, with an emphasis on tumor-specific cellular responses throughout the course of disease; and (3) to evaluate the effects of adriamycin chemotherapy on the course and immune responses of tumor-bearing animals.

II. Materials and Methods

A. Rats

Pregnant Wistar-Lewis rats, an inbred strain, were obtained from Charles River Breeding Laboratories (Wilmington, Mass.) at 18 to 20 days' gestation. Their litters, ranging from six to fifteen pups, were inoculated with virus within 24 hours of birth. Males and females were caged together until six weeks of age, and animals were weaned at 20 days.

B. Virus

A Moloney strain of murine sarcoma virus (MSV-M), (hereafter referred to as MSV) was obtained through the courtesy of Drs. J.B. Moloney and J. Gruber of the National Cancer Institute. This was propagated in BALB/c mice and harvested at 14 days by Mr. Richard Murahata according to the method of Moloney.[215] A virus suspension was prepared as 1 gram-equivalent/ml. and stored at -70^{o}C until ready for use. After rapid thawing, the virus was immediately inoculated into the newborn rats.

C. Infection of Rats

Virus inoculation was performed according to the method originally described by Ikemoto and Yamamoto.[160] After cleansing the left hind

limb of a newborn rat with alcohol, a 27 gauge needle was inserted into the marrow cavity of the tibia through the knee joint (Figure 2). A 0.025 ml aliquot of the virus suspension in Eagle's Minimal Essential Medium (MEM, from GIBCO, Grand Island, New York) was then injected. Control animals received identical inoculations of MEM alone.

Figure 2
Technique of Virus Inoculation
See text for explanation.

D. Treatment

Treatments were performed i.p. with adriamycin hydrochloride dissolved in normal saline. Experimental groups were administered doses of 1 mg/kg/day or 2 mg/kg/day for three consecutive days. Control

animals were given comparable volumes of normal saline. Treatment was started at 10 days of age when approximately 90% of the animals in all groups had palpable tumors.

E. Clinical Evaluation of Tumor Size

Tumor growth and regression were evaluated by measuring with vernier calipers the greater of two transverse diameters of the left lower extremity. Uninfected control animals were also measured in order to discern the amount of increased diameter due to normal growth of these young animals.

F. Pathology

At the time of death or sacrifice for assays, the tumor site, regional lymph nodes, lungs, liver and spleen were grossly inspected. Portions of these tissues were preserved in 10% formaldehyde. Hard tissue (tumor and bone) was subsequently subjected to decalcification in serial baths of 5% formic acid, and all tissues were then embedded in paraffin. Tissues were stained with hematoxylin and eosin and/or Masson's trichrome preparation.

G. Radiology

At various times prior to death or at sacrifice, radiographs to illustrate bony structures were taken of both normal and tumor-bearing animals.

H. Evaluation of Cell-Mediated Immunity

Target Cells:

The primary target cells for assay of cell-mediated cytotoxicity were from a single homogeneous line of tumor cells derived from a Wistar-Lewis rat (MSV/WL) and kept in tissue culture through serial passage. Originally, a growing tumor was sterilely removed from a donor rat, minced with scissors in MEM, and filtered through several layers of fine-mesh gauze. After centrifugation of the filtrate at 900 rpm for 10 minutes, the cells were washed twice in MEM and resuspended in a Waymouth's medium (GIBCO) solution including 30% fetal calf serum (FCS), 1% each L-glutamine, penicillin and streptomycin, .15% mystatin, and buffered to a pH of 7.4 with $NaHCO_3$. After successful adherence and propagation of these original cells, the medium was changed in serial passages to an RPMI-1640 (GIBCO) solution containing 10% FCS, 1% each of penicillin and streptomycin, .10% mystatin and 1% tylocine, the latter to inhibit growth of mycoplasma organisms.

In addition to the MSV/WL cells, normal fibroblasts from Wistar-Lewis rats were obtained from explants of rat skin of uninfected animals as described above for the tumor cells, and grown in tissue culture. Tumor cells from a mouse leukemia P388D1 were also used as target cells for two experiments, and these were obtained originally from Mr. Richard Murahata who had maintained them in tissue culture.[173]

All cells in culture were maintained in 30-ml Falcon plastic tissue culture flasks (Falcon Industries, Oxnard, Calif.). Tissue cultures

were maintained in a 37°C 5% CO_2 atmosphere, and media were changed twice weekly and as necessary.

At the time of assay or serial tissue culture passage, cells were harvested by subjecting them for 10 minutes to 2.5% trypsin diluted 1:10 with a solution of phosphate buffered saline-ehtylenediamenetetraacetic acid (PBS-EDTA). After the majority of cells were floating free in solution, FCS was added in a volume equivalent to 30% of the total volume. The suspension was centrifuged at 1000 rpm for 10 minutes, and then either resuspended in medium for tissue culture, or counted, viability determined, and prepared for assay. Viability was determined by the trypan blue dye exclusion technique[31], and was consistently found to exceed 95%.

Serum:

At the time of assay, blood was obtained in one of two ways: when the animals were young (generally less than 35 days), groups of three animals were exsanguinated by jugular vein incision and their blood pooled, whereas older animals often supplied sufficient quantities through scalpel nicks made in the lateral tail veins. The blood was obtained after cleaning animal surfaces with alcohol, and following heat inactivation of the serum complement in a 56°C water bath for thirty minutes, the pooled serum was centrifuged at 2000 rpm for 20 minutes. Finally, prior to assay, sera from both tumor-bearing and control animals were further purified by filtration through 45μ millipore filters.

Lymphocytes:

Peripheral blood was used as the source of effector cells, and lymphocytes were isolated by centrifugation of diluted blood over a Ficoll-Hypaque density gradient.[33] Staining of the effector population with Wright's stain showed it consistently to be composed of 90-93% lymphocytes, 2-5% polymorphonuclear leukocytes, 3-6% monocytes, and a variable small number of red blood cells. When red blood cell contamination occasionally approached 5%, these cells were removed by treatment with a tris buffered-ammonium chloride solution.[30] Trypan blue exclusion tests on the effector population prior to assay invariably showed greater than 95% viability.

Visual Microcytotoxicity Assay:

A modification of the micro-assay of Takasugi and Klein[309] was used. Assays were always performed the same day as lymphocyte and serum isolation, and were carried out in Linbro tissue culture trays (Linbro Industries, New Haven, Conn.) with wells 16 mm in diameter.

Twenty-four hours prior to assay, each well was seeded with 200 viable target cells suspended in 0.5% of the RPMI-1640 solution previously described, and incubated at 37°C in a 5% CO_2 atmosphere. Immediately prior to assay, the solution over the adherent cells was pipetted off.

One-tenth milliliter of either normal or immune heat-inactivated and purified serum in a 1:5 solution with MEM (containing 20% FCS and

antibiotics) was then added to each well. The tissue culture tray was then incubated for one hour at 37°C.

Following incubation, 40,000 purified effector cells from tumor-bearing or control animals were added to each well in .5 ml of a solution of MEM as above. The resulting effector:target cell ratio was therefore approximately 200:1. The volume of serum in each well was then raised to .5 ml, and the trays subsequently incubated in a 37°C, 5% CO_2 atmosphere for an additional 48 hours. After removal of the solutions in each well and washing the wells once with cold MEM, the remaining cells were stained with Wright's stain and visually counted using a Nikon MS inverted phase-contrast microscope.

Each assay included wells with different combinations of components, including:

A. target cells, immune lymphocytes, immune serum
B. target cells, immune lymphocytes, normal serum
C. target cells, normal lymphocytes, normal serum
D. target cells, medium alone
E. target cells, normal lymphocytes, immune serum

Virtually all assays included wells A-D above, and many assays included the fifth well. "Target cells" most often were MSV/WL, but tests using normal fibroblasts and P388D1 cells were also performed.

Tests were always done in triplicate and reported as the mean $\pm$ SE. Results were expressed as percent specific lysis, or:

$$100 \times \left[1 - \left(\frac{\text{tumor cells remaining in experimental well}}{\text{tumor cells remaining in well with normal lymphocyte}} \right) \right]$$

Comparison of wells B and C measured cell-mediated immunity (CMI), while comparison of wells A and C indicated serum modification of CMI ("blocking" or "helping" factors). Well E tested for antibody-dependent lymphocyte-mediated cytotoxicity, while well D controlled for the presence of non-specific cytotoxicity in normal animals. Specificity of humoral and cellular immunity was tested by considering lysis of normal fibroblasts and the xenogeneic tumor line as compared to MSV/WL.

Microtoxicity Assay with Isotopic Labelling:

Several types of assays designed to measure CMI using the percent release by killed target cells of a radioactively-labelled substance were attempted. The assay employed which most closely correlated with results from the visual assay is described in the following paragraphs.

Seeding of target cells and the addition of lymphocytes and serum were accomplished in a manner identical to the visual assay. In this method, however, 1000 target cells were originally seeded, while the effector:target cell ratio was maintained at 200:1.

At the end of 48 hours' incubation, the wells were viewed under the phase-contrast microscope for the presence of lymphocytes, and subsequently flushed vigorously with a suction bulb 15 times to remove loosely-adherent effector cells. All wells were then washed and flushed three times with ice-cold MEM and the tray inverted and shaken to remove

effector cells which might incorporate the radiolabelled substance. After the last washing, wells were again viewed under the phase-contrast microscope, and it was consistently observed that only 1-2% of the effectors remained in the tray.

One milliliter of 1 μCi/ml ^{3}H-thymidine (New England Nuclear, Boston, Mass.) in MEM solution with 10% FCS, specific activity 50.7 mM, was added to each well. The tissue culture tray was then incubated in a 37°C, 5% CO_2 atmosphere for an additional 5 hours.

After incubation, wells were washed twice with ice-cold MEM as before, and the adherent cells removed by exposure to a 0.25% trypsin solution as above. Cell solutions were transferred to Falcon 2057 tubes (Falcon Industries, Oxnard, Calif.) containing 0.3 ml FCS, centrifuged at 1000 rpm for 10 minutes, and the resultant supernatant fluids discarded. Eight-tenths milliliter of a solution containing normal saline and human serum and 1.0 ml of 10% trichloroacetic acid (TCA) were then added to the tubes. Tubes were then vortexed and refrigerated overnight.

The next day, tubes were centrifuged at 1700 rpm for 10 minutes. The supernatants discarded, and to each tube was added .4 ml 100% formic acid and 3 ml absolute alcohol. The resultant solution was vortexed, poured into scintillation vials containing 10 ml of a scintillation fluid (PPO-POPOP, Mallinckrodt Chemical Works, St. Louis, Mo.), and counted for one minute at 4°C in a Packard scintillation counter.

Tests, as before, were always done in triplicate. Results were

reported as cpm $\pm$ SE, and the per cent lysis was calculated as:

$$100 \times \left[1 - \left(\frac{\text{cpm in test well}}{\text{cpm in control well}} \right) \right]$$

I. Evaluation of Humoral Immunity

Serum:

Serum for evaluation of humoral immunity was obtained at the time of assay for CMI. Undiluted serum was gathered following clotting of whole blood, and the constituent complement heat-inactivated at 56^{o}C for thirty minutes. Sera were then frozen at -70^{o}C until the time of assay, when they were quickly thawed for use.

Target Cells:

Target cells for the cytotoxic antibody assay were LSTRA tumor cells, a line antigenically cross-reactive with MSV-derived cells and used by others as targets for MSV-specific humoral immunity.[245] These cells were propagated in BALB/c mice.

LSTRA cells were collected by exposure of the mouse peritoneum and lavage and suction with a solution of Fischer's medium (GIBCO) and 10% FCS. The cells were subsequently counted and their viability determined by trypan blue exclusion. As before, where red blood cell contamination was a problem, these were removed by treatment with tris-buffered ammonium chloride.

Cytotoxic Antibody Assay:

A slight modification of the micromethod of Kaliss[168] was used to assay antibody-induced cytolysis by the release of ^{51}Cr. Two tenths milliliter of 1 mCi/ml ^{51}Cr, specific activity 51.9 mM, were added for every $5x10^6$ viable LSTRA target cells, and the resultant solution incubated in a 37°C shaking water bath for 5 hours. After incubation the cells were washed with ice-cold Fischer's medium containing 10% FCS, centrifuged at 1000 rpm for 10 minutes, and these procedures repeated for three additional washings. After viability was determined and the cells were counted, the cell concentration was adjusted to 10^6 cells/ml solution.

The assay, briefly summarized, was as follows: reagents dispensed sequentially into 0.3 ml wells of Linbro plastic trays (Linbro Chemical Co., New Haven, Conn.) included 0.05 ml each of Fischer's medium with 10% FCS, antisera which were serially titrated, radiolabelled LSTRA cells, and 1:3 dilutions of guinea pig complement. As titrations were carried out through eight wells with 0.05 ml of solution being discarded from the last well, the final volume in all reaction mixtures was 0.15 ml. Controls were wells which, in addition to targets, contained medium alone (cell control), only complement and medium (complement control), only 1 N HCl and medium (indicating maximum lysis), and normal serum instead of antiserum (normal serum control). After 10 minutes' incubation at 37°C, the trays were immediately sealed with pressure sensitive adhesive cellulose and incubated an additional 45 minutes.

Immediately following incubation, the tray was chilled for 5 min.

on ice and centrifuged for 10 min. at 800 rpm. Subsequently, .05 ml of supernatant was carefully removed and measured for radioactive content in a Picker gamma counter (Picker Nuclear, U.S.A.). Results were expressed as cpm, and a percentage of specific release was calculated.

III. Results

A. Natural Course in Untreated Animals

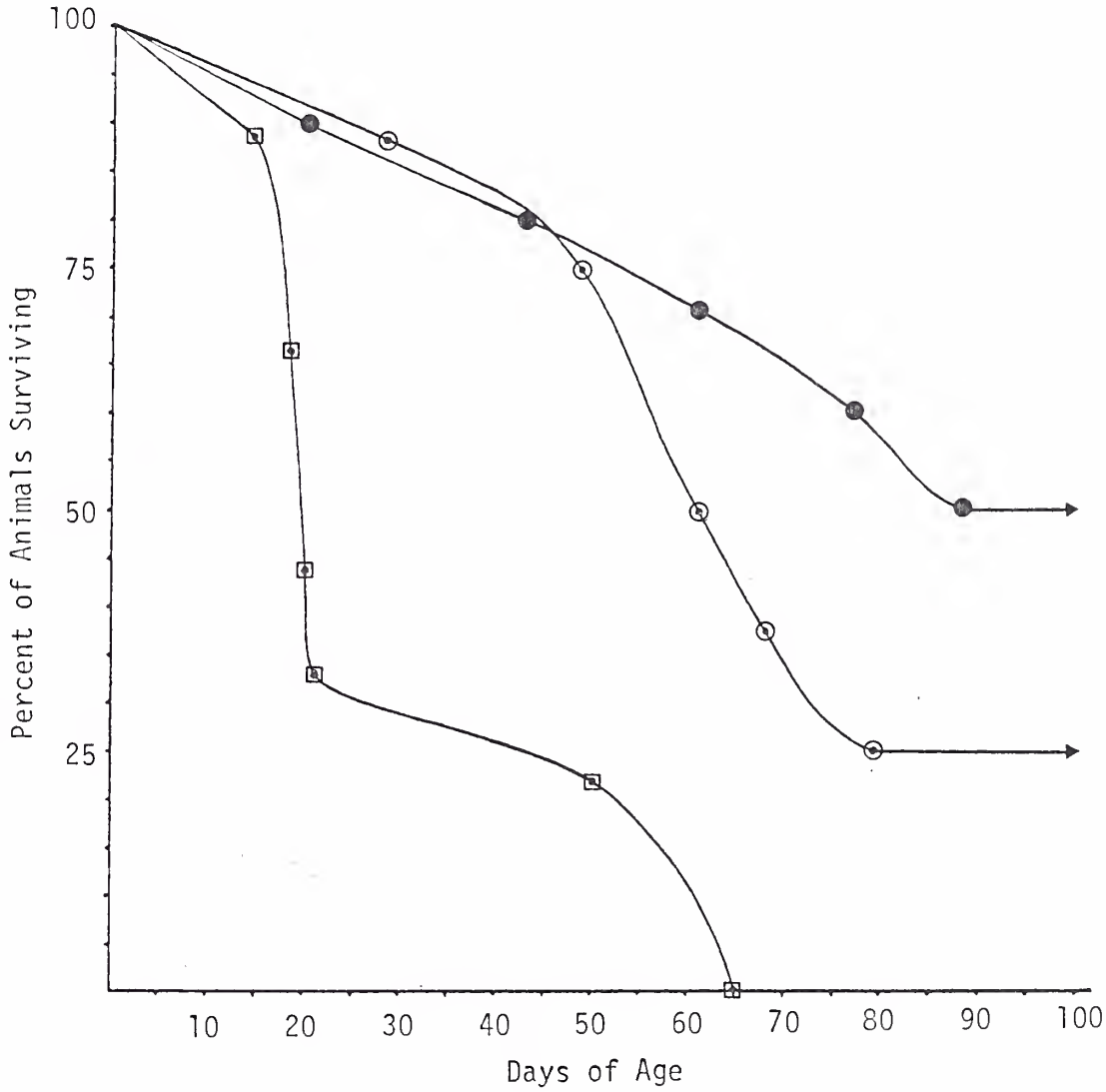
The MSV-inoculated extremity appeared grossly normal until the animals were six to ten days of age. By nine days post-inoculation, approximately 90% of the animals which would ultimately develop localized tumor masses had palpable swellings, and daily observation revealed that 96% of all infected animals became tumor-bearing.

In successfully infected rats, tumors invariably grew progressively and killed the animals. Of 22 animals, nine were not sacrificed for immunological assays, and all of these were dead by 64 days of age. Most animals died within 10-12 days of clinical tumor detection, and this is reflected in a median survival time (MST) of 20.0 days. Survival data are depicted in Figure 3.

Tumors in untreated animals enlarged progressively, and the originally well-localized hard tumor mass often extended from the tibia to involve both the femur and fibula (Figure 4). Animals surviving to 64 days thus had enormous tumor masses, and therefore no clinical "regressor" animals were seen in untreated animals. The change in tumor size over time is depicted in Figure 5.

Figure 3

Survival of Treated versus Untreated
MSV-inoculated Tumor-bearing Rats



□- Untreated tumor-bearing animals

○- Tumor-bearing animals treated with adriamycin, 2 mg/kg/day

●- Tumor-bearing animals treated with adriamycin, 1 mg/kg/day

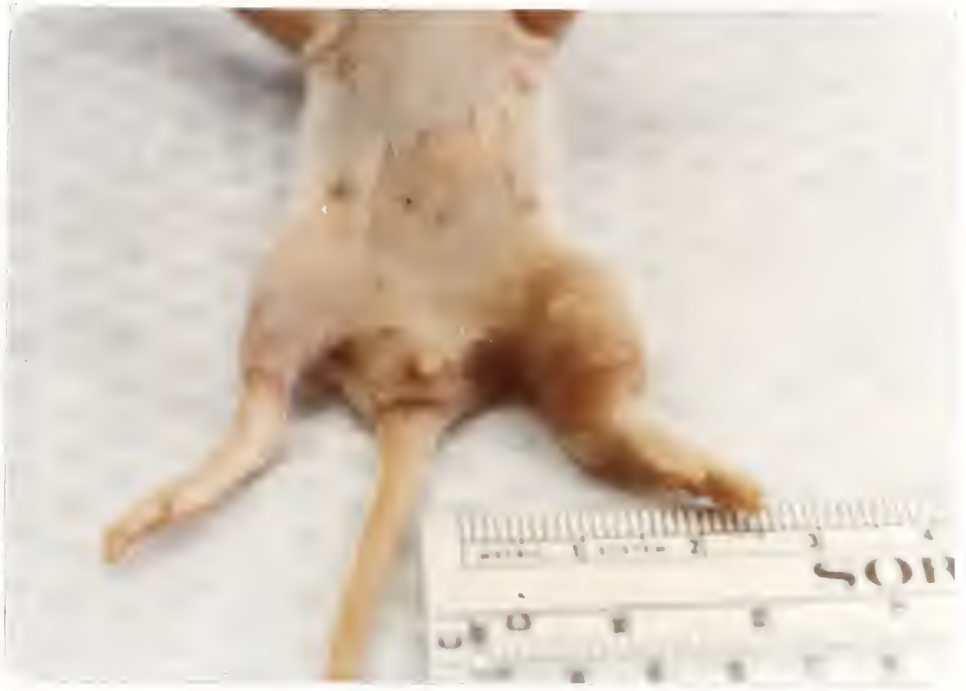


Figure 4

An untreated animal 15 days after inoculation of MSV into marrow of left tibia. Tumor at necropsy involved both left tibia and fibula.

B. Activity of Adriamycin on Tumor Progression

Survival:

Preliminary experiments were attempted using adriamycin at doses of 3 mg/kg/day and 4 mg/kg/day administered i.p. for three days beginning on day 10. These animals became sickly, failed to gain weight like the tumor-bearing control animals, and died within two weeks of

starting treatment. That these animals succumbed to drug-associated infection was supported by the lack of evidence for accelerated tumor growth and the findings of pulmonary congestion and viscous intra-peritoneal exudates at necropsy.

Of the animals treated with adriamycin at 1 mg/kg/day or 2 mg/kg/day administered i.p. on the same schedule as above, 93% had developed palpable tumor masses by two weeks of age. The incidence of tumor-bearing animals was similar in both of these experimental groups.

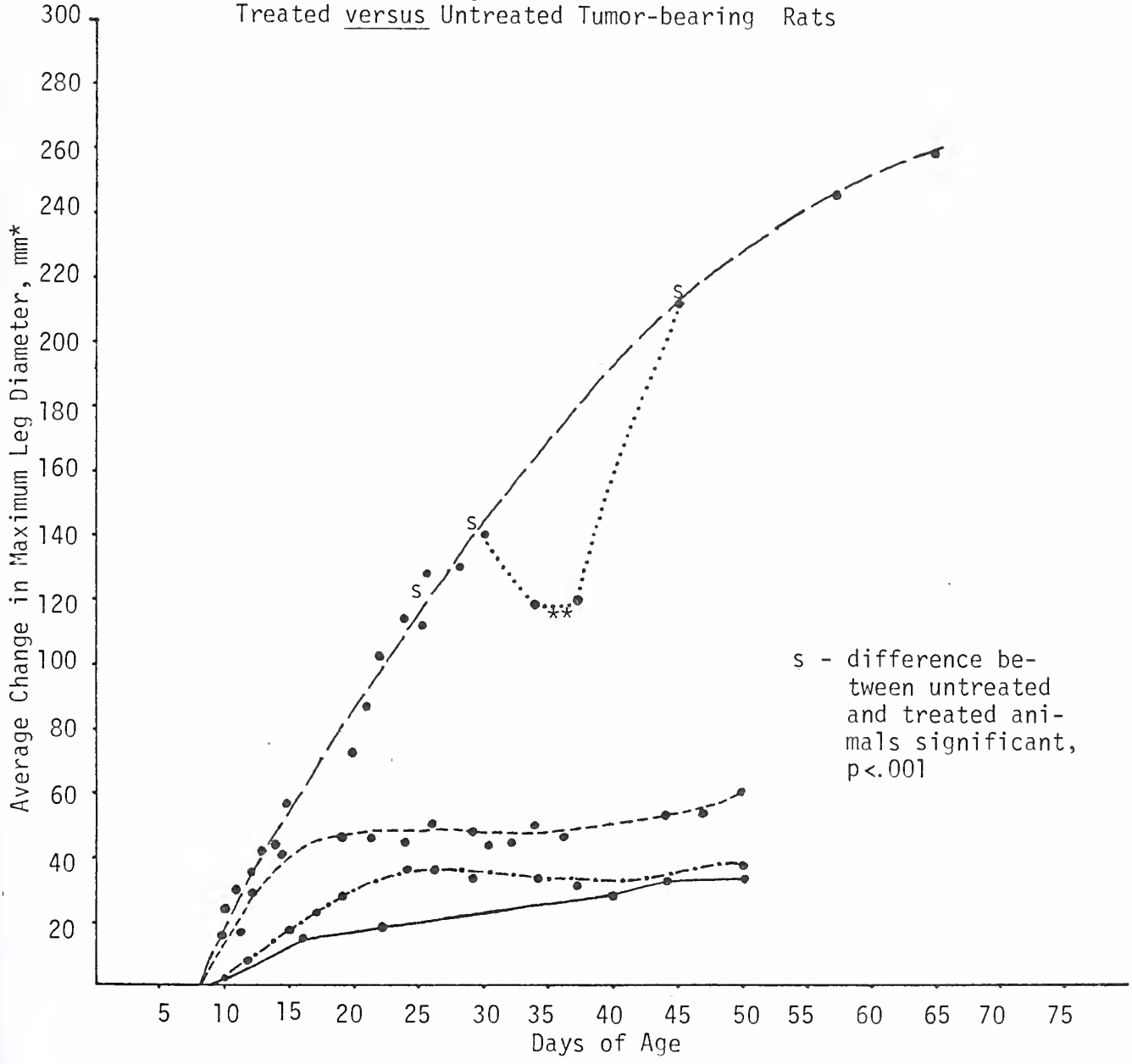
Ten successfully infected rats treated with the lower dose of adriamycin were not sacrificed for immunological assays and therefore are included in survival data. In contrast to the untreated animals, deaths in this group were distributed over several weeks. While the first animal died at 20 days post-infection, five rats survived more than 95 days. Median survival time in this group was greater than 91 days.

In the group treated with 2 mg/kg/day adriamycin a similar survival distribution was noted. Eight tumor-bearing animals survived from 28 to more than 95 days post-infection, with a small cluster of animals dying between 60 and 79 days. Median survival in this group was 63.5 days.

The composite MST for the adriamycin-treated rats was 77.5 days. When compared to the MST of 20.0 days in untreated animals, this difference is significant at the $p < .05$ level as evaluated by the "t" test for medians.

Figure 5

Change in Average Left Lower Extremity Diameter During the Course of Disease in Treated versus Untreated Tumor-bearing Rats



* - maximum transverse plane diameters, left lower extremity; corrected for minor differences in original diameters

** - when few animals were surviving in the untreated group, death of one animal with a large tumor, as on day 34, spuriously lowered average tumor diameters, which then returned (day 45) to high levels

——— - uninfected normal Wistar-Lewis rats - growth due to age changes

— — - untreated tumor-bearing animals

----- - tumor-bearing animals treated with adriamycin, 1 mg/kg/day

•—•—•— - tumor-bearing animals treated with adriamycin, 2 mg/kg/day

Tumor Growth and Regression:

In contrast to control animals, rats treated with adriamycin at either dose developed tumors that grew slowly but progressively for 15 to 20 days post-infection, and then either progressed very slowly, stabilized, or regressed. Treated animals that succumbed to their tumors had masses that were measurably smaller than those of untreated rats.

Average tumor diameters over time were compared between treated and control animals (Figure 5). At all points beyond 25 days, tumor diameters in treated rats were significantly smaller, as evaluated by the Student's t test ($p < .001$), than those of untreated animals.

Animal tumor status was classified as either "progressor" or "regressor". Regressor animals had tumors which were either diminishing in size at the time of assay, or were microscopically absent at the time of sacrifice for histologic examination.

Seven of 17 animals (41%) treated with 1 mg/kg/day adriamycin were classified as regressors. When compared to the 22 control animals by chi-square analysis, there were significantly more regressor animals in this treated group ($p < .005$).

By comparison, 18% of 22 animals treated with the higher doses of adriamycin were classified as regressors. This incidence, too, was significantly different from controls ($p < .05$). Chi-square analysis confirmed that there were significantly more regressor animals in the combined experimental groups than in the untreated group ($p < .001$).

The effects of treatment on the natural course of the disease are summarized in Table I.

TABLE I

Course of Disease in Untreated and Adriamycin-treated Animals

Group	Tumor Growth Pattern	Median Survival Time (days)	Progressors (No.)	Regressors (No.)	Regressors (%)
Untreated	Rapidly Progressive	20	22	0	0
Adr. 1 mg/kg	Slowly Progressive, regression common	91+	10	7	41
Adr. 2 mg/kg	Slowly Progressive, occasional regression	63.5	18	4	18

C. Gross Pathology

The primary tumor mass was either rubbery or hard to palpation and intimately associated with the long bones of the lower extremity. Animals surviving for longer periods generally had masses which extended proximally from the tibia to involve the femur and even invade the retroperitoneal space. In the latter cases, renal excretion was obviously impeded by ureteral obstruction by the tumor mass. The non-encapsulated portions of the tumors were whitish-gray and fleshy in appearance,and on sectioning were "gritty".

As performed by Friedlaender and Mitchell[117], control animals were inoculated intra-tibially at birth with 0.025 ml of MEM in order to rule out the possibility that mechanical injury to the bone might stimulate stem cells of the periosteum or endosteum to produce bone, and thus simulate a bone tumor. Again, there was no evidence on gross or histologic examination of control animals to suggest a significant alteration of normal bony architecture.

Opening the peritoneal cavity revealed a variety of pathological changes in progressor animals. Visibly enlarged lymph nodes, some reaching 1.5 cm in diameter, were present in approximately 10% of animals. Inguinal lymph nodes were most frequently enlarged, although the largest glands were often the inferior mesenteric or para-aortic nodes. On gross examination their appearance was similar to the primary tumor, being fleshy and rubbery or hard.

Although varying in size, both liver and spleen were grossly normal in appearance. Livers were smooth and regular in consistency without visible nodularity. Some livers were large, and on sectioning revealed the typical "nutmeg" appearance associated with passive congestion.

Spleens varied much more in size, and both small and large spleens were noted with both progressor and regressor animals. In the preliminary experiments with 3 mg/kg and 4 mg/kg adriamycin, necropsied animals invariably had very small spleens as compared to their untreated tumor-bearing controls. No such correlations between spleen size and treatment with the lower doses of adriamycin could be made.

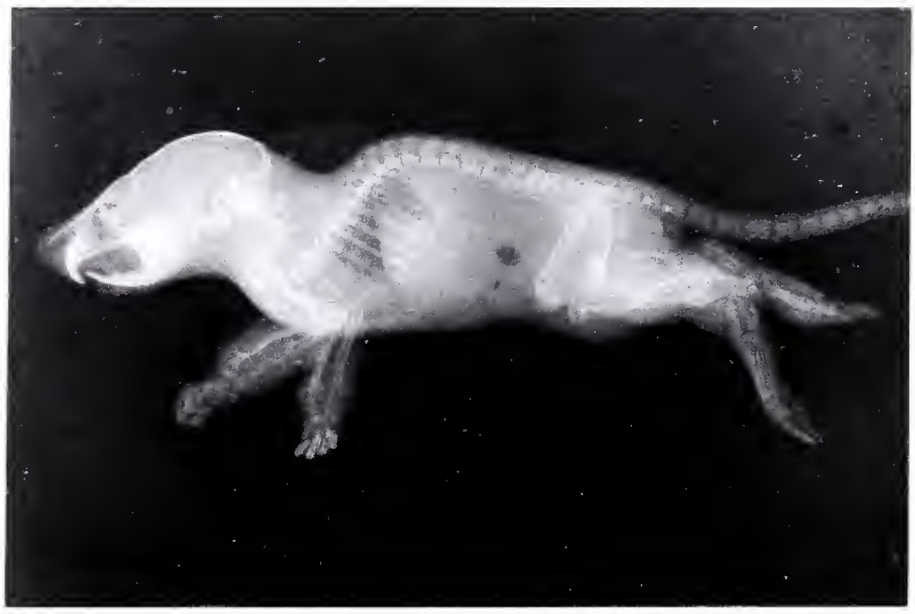
In animals with very large tumors, obstruction of both the gastrointestinal and genitourinary tracts was obvious on gross examination. Greatly dilated ureters and loops of bowel were seen, and in some cases the sections of bowel were clearly necrotic.

On opening the thoracic cavity of progressor animals, a viscous bloody effusion was occasionally seen bilaterally, which on microscopic examination contained many large and pleomorphic tumor cells.

The lungs of tumor-bearing animals revealed a spectrum of changes on gross examination. Virtually all lungs had small areas of consolidation which became more prominent when immersed in 10% formalin. At the other extreme, several animals had large, firm, whitish-gray, clearly circumscribed nodules scattered bilaterally throughout the pulmonary lobules. Approximately 30% of necropsied animals had these grossly apparent lung metastases.

All four extremities were palpated daily for evidence of long bone metastases. Together with necropsy data, a clear pattern emerged revealing the predilection of treated animals for long bone metastases. Of 22 untreated animals, there were none with osteosarcoma metastatic to other long bones. In contrast, of 39 adriamycin-treated rats, four animals had a total of five palpable tumors in other long bones. Of these, one appeared in an animal with the lower dose of adriamycin, and the remainder occurred in rats treated with adriamycin at 2 mg/kg/day. These occurred exclusively in the forearms of the treated animals, and on necropsy were found intimately associated with either the radius or ulna (Figure 6). All long bone metastases first appeared between

6a



6b

Figure 6(a-b)

X-rays of Two Adriamycin-treated Tumor-bearing Rats with Long Bone Metastases

(a) 42 day old animal treated with adriamycin, 1 mg/kg/day. Left lower extremity bears primary tumor, and right upper extremity shows destruction by metastatic lesion. (b) 26 day old animal treated with adriamycin, 2 mg/kg/day. Both upper extremities have metastatic lesions involving both radius and ulna.

21 and 29 days post-infection. On gross inspection, they were large masses identical in appearance to the primary tumors. When compared by chi-square analysis, treated animals were shown to have significantly more long bone metastases than the untreated controls ($p < .025$). Animals treated with the higher dose of adriamycin had more of these metastases than animals treated with the lower dose, although this difference was not statistically significant ($p > .05$).

Microscopic Pathology:

Organs from 27 animals of all experimental groups were examined histologically using the hematoxylin and eosin (H and E) staining technique. Most often the primary tumor, lung, liver, spleen, lymph nodes and long bones with gross tumor involvement were evaluated. In treated animals, organs from both progressor and regressor animals were examined. Except to attempt evaluation of equivalent numbers of each treatment group, and to sample animals with different tumor status, sampling for histologic examination was made on a random basis.

The primary tumor was characterized by variation from lesion to lesion and from area to area within the same lesion, typical of osteosarcoma in man as described by Jaffe[162] (Figure 7). In nonossified areas, the tissue was either chondroblastic, or more commonly, fibroblastic in nature. Cells in these areas were anaplastic, spindle-shaped, or round in appearance, the latter located in irregularly arranged lacunae suggesting neoplastic cartilage formation.

In more anaplastic areas, cells were sometimes multinucleated,

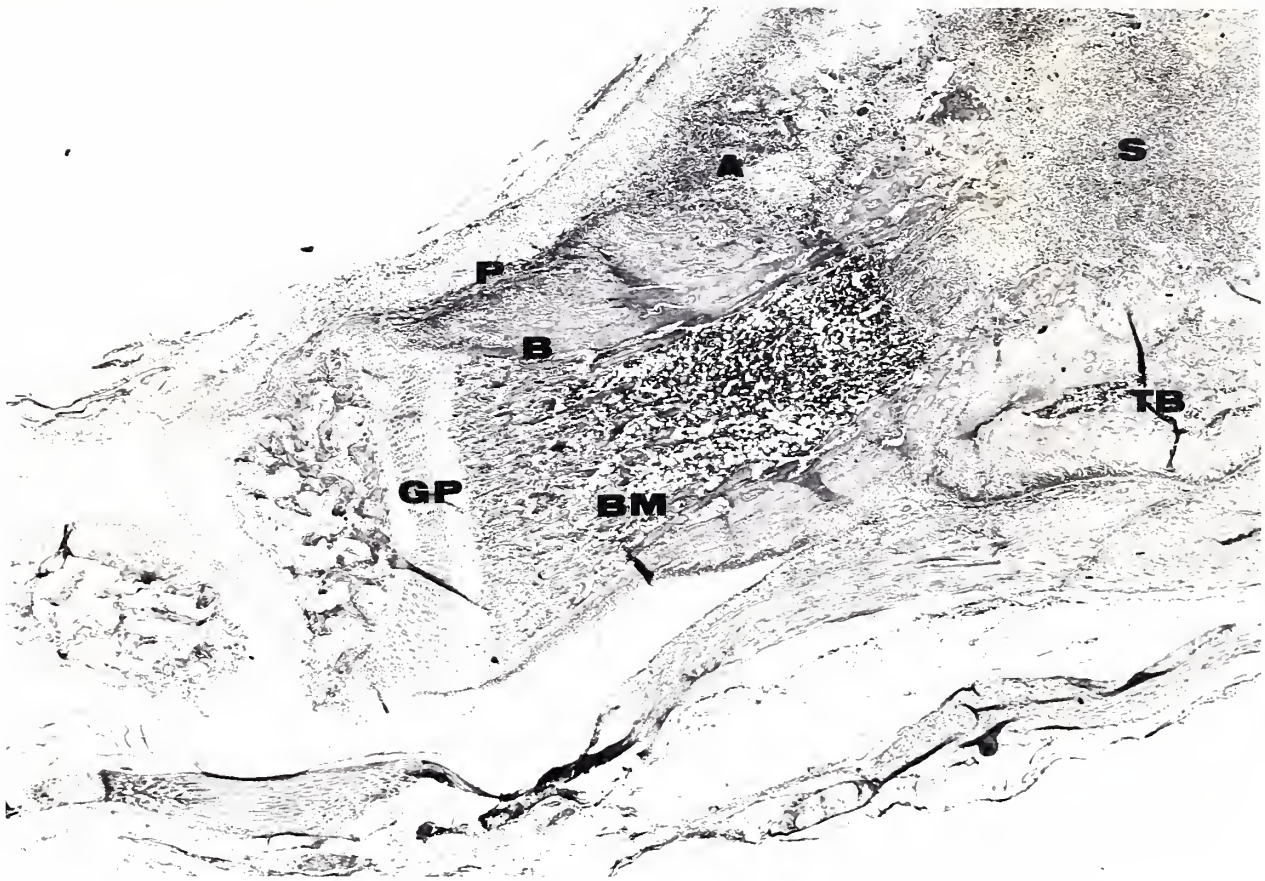
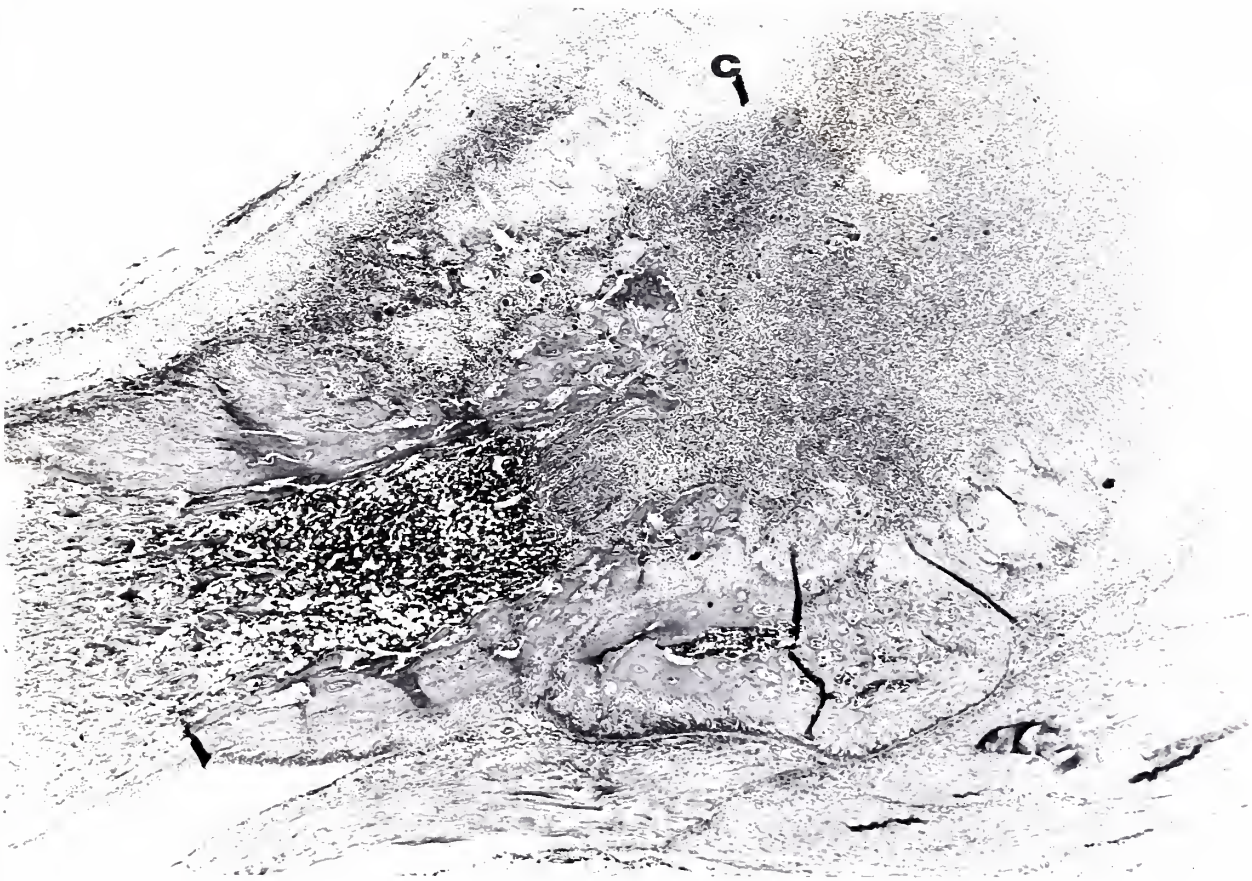


Figure 7(a-g)

Microscopic Appearance of Primary Tumors

(a) Low power view of tumor-bearing extremity revealing normal growth plate (GP) and marrow (BM). Normal bone (B) is being compressed by tumor bone (TB) and the marrow replaced by undifferentiated sarcomatous tissue (S). Highly anaplastic tissue (A) has caused periosteal elevation (P). (b) Higher power view of segment of same bone as in 7(a). Neoplastic cartilage (C) is also apparent in this photomicrograph. (c) Widely scattered spindle-shaped cells remarkably similar in appearance to tumor cells in tissue culture (Figure 11). (35X)



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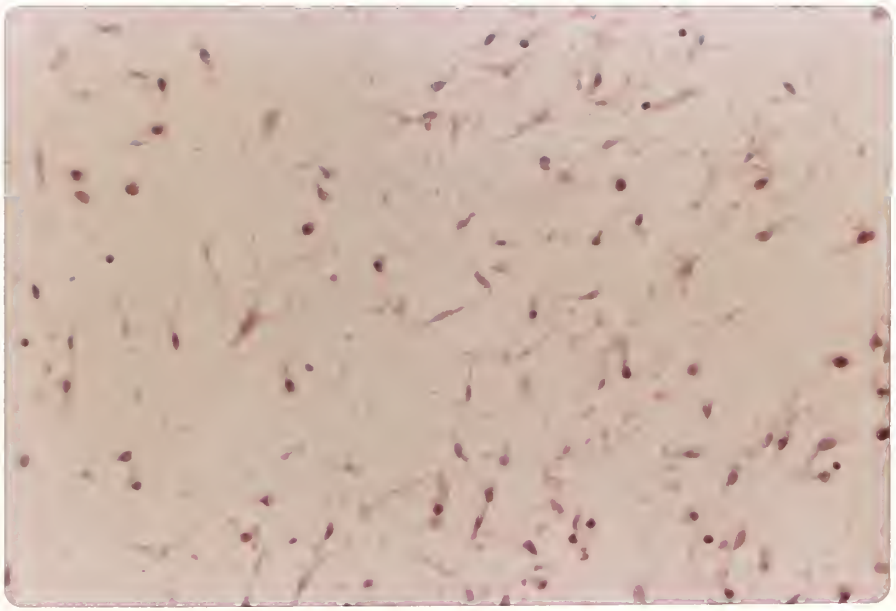

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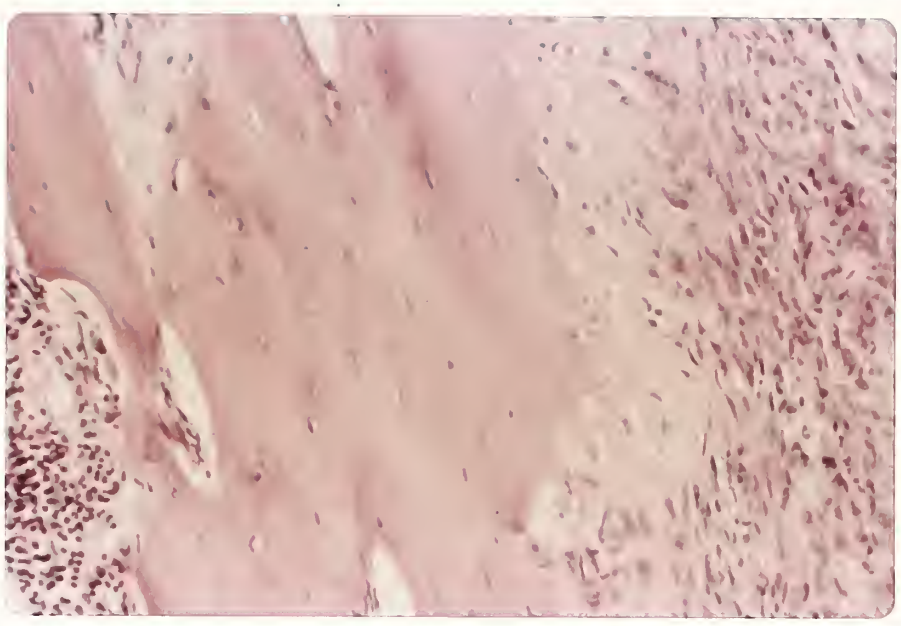

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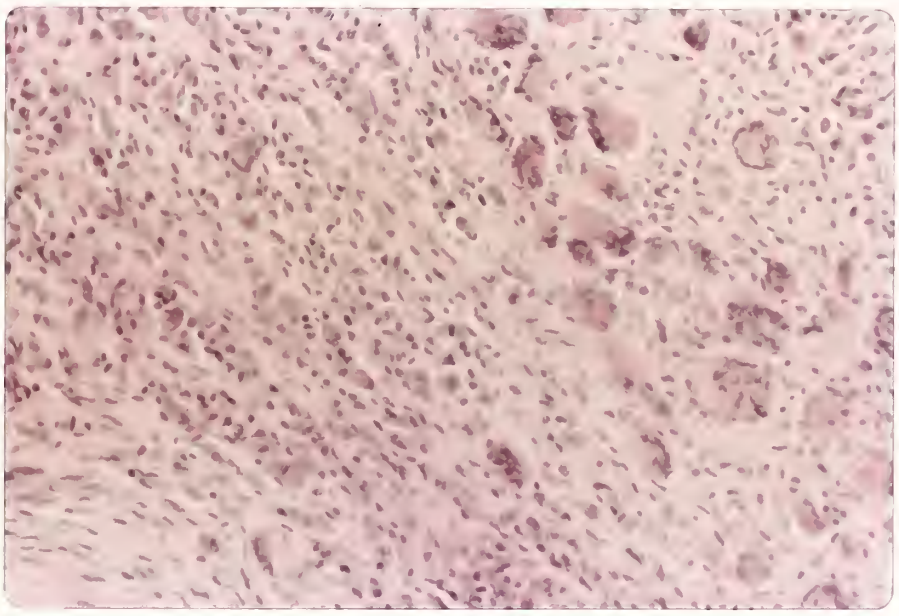
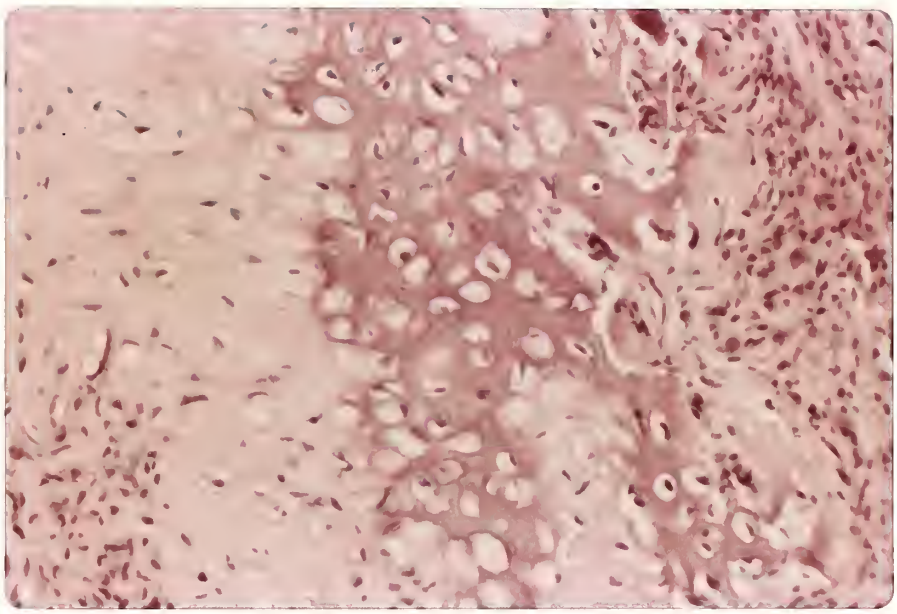


(d) Higher power view of endosteal bone compression by sarcomatous tissue. From left to right in this section are: normal bone marrow, normal endosteal bone, reactive and/or neoplastic bone, and undifferentiated sarcoma. Long axis of bone is vertical in this micrograph, periosteal elevation out of section at far right. (35X) (e) Highly anaplastic sarcomatous tissue with many multinucleated giant cells. (35X)



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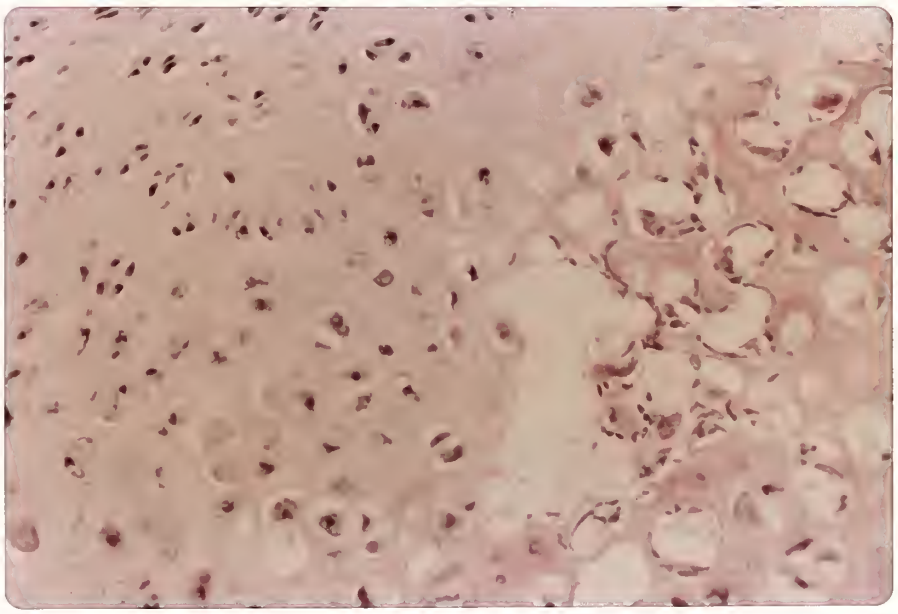


(f) Section revealing three major components of primary tumors, from left to right: neoplastic bone, neoplastic cartilage, and highly anaplastic sarcomatous tissue. (g) At left, neoplastic cartilage, with irregularly sized, shaped and arranged lacunae, and many mitotic figures. At right, poorly differentiated malignant bone. (35X)

or more often contained a single nucleus with from 1 to 3 large nucleoli. Mitoses in these areas were common.

Sections containing widely separated spindle-shaped cells generally juxtaposed areas in which the spindle cells were more densely aggregated with substantial amounts of intracellular fibrillar collagenous material. The latter dense areas were usually seen in endosteal bone, and constituted the predominant fibroblastic and undifferentiated osteosarcomatous tissue which characterized these tumors.

The presence of neoplastic osteoid in the primary tumors defines them as osteosarcomata, and malignant bone in addition to undifferentiated sarcomatous tissue was likewise a prominent component of the tumors. True tumor bone, with osteoid trabeculae and calcium deposition, was seen in its various stages[162] throughout the tissue. While early neoplastic bone was confined to an endosteal location, bone in older animals with grossly larger tumors disrupted the cortex and was even seen invading normal muscle tissue. Some sections revealed an intermediate stage in which neoplastic bone, reactive bone, and periosteal elevation were observed.

Microscopic metastases were seen in the lungs of 89% of the 27 animals in which their presence was evaluated. With equivalent numbers of each experimental group being examined, there were no differences in the frequencies of lung metastases between the groups. Micrometastases were seen in animals dying from tumor or sacrificed from 15 to 64 days of age.

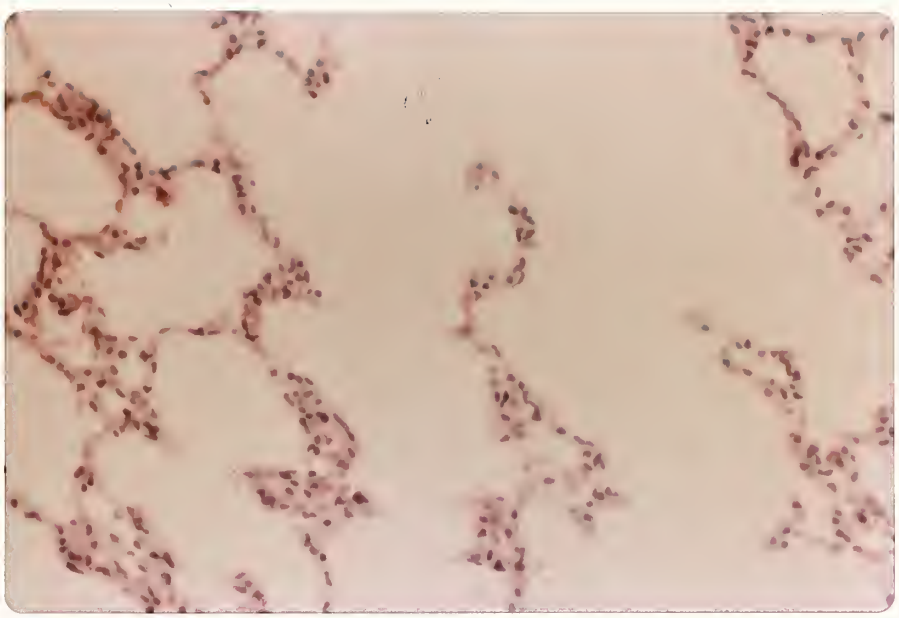
The pulmonary lesion was almost invariably a highly anaplastic, undifferentiated sarcoma, without any clear cartilage or bone-forming activity. Specimens were examined microscopically without knowledge of experimental group, and lesions were graded on a 0-4+ scale as to the presence and extent of neoplastic tissue. Metastases varied widely relative to numbers of neoplastic cells and amount of stroma. One-plus lesions contained neoplastic cells dispersed throughout the lung parenchyma, and often at pleural surfaces, consistent with the hematogenous nature of their dissemination. Four-plus lesions additionally had dense, well-circumscribed areas of sarcomatous tissue which almost completelv obscured the normal lung architecture. Typical pulmonary lesions are depicted in Figure 8.

There were several types of microscopic changes noted in the 26 liver specimens of tumor-bearing rats.* Some of these changes appeared to be merely the result of passive congestion of the livers without concomitant extensive necrosis and/or infiltration with tumor cells. These were organs which on gross sectioning showed the typical "nutmeg" liver appearance characteristic of chronic passive congestion. The predominant cellular pathology in these specimens was a centrilobular fatty change of the hepatocytes.

Other specimens showed more extensive fatty change with or without major hepatic morphologic changes and infiltrates. Where destruction and architectural changes were predominant, cellular infiltrates

* Evaluation of several liver sections was aided by the kind assistance of Dr. Joseph R. Bloomer, Department of Internal Medicine, and Dr. John A. Ogden, Department of Surgery, Yale University School of Medicine.

8a



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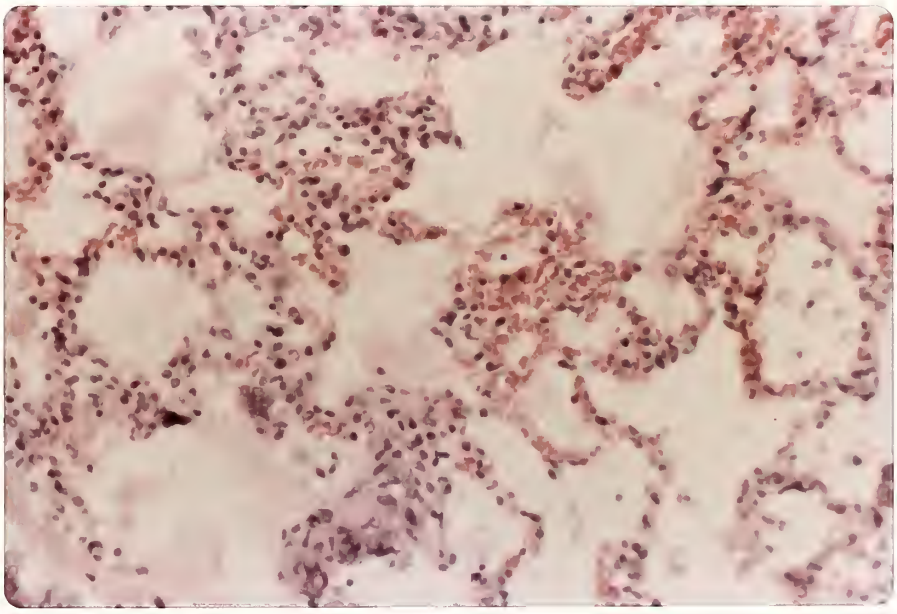
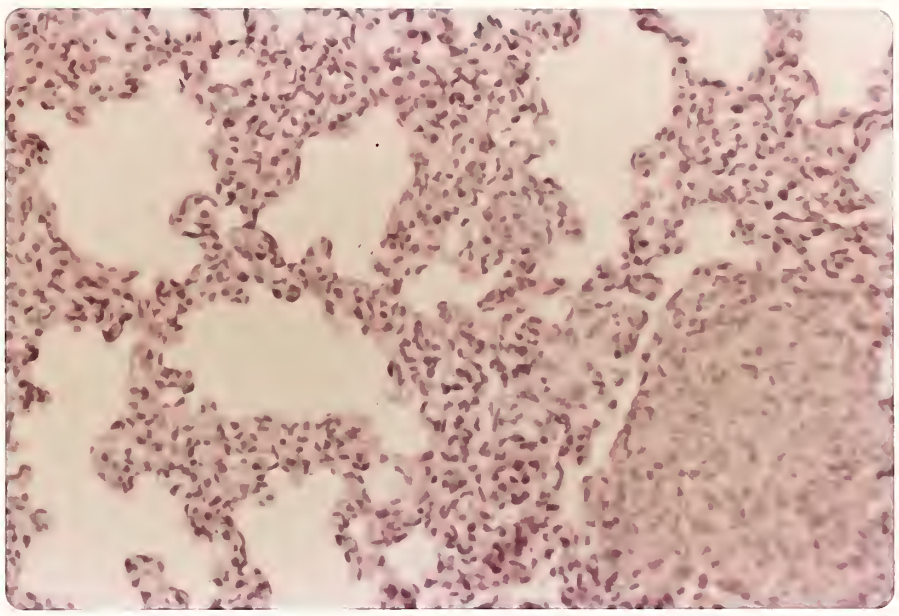


Figure 8(a-d)

Microscopic Appearance of Lung Sections

(a) Normal rat lung. Lacy alveolar walls and well aerated ducts and sacs. (b) One-plus metastatic pulmonary lesion. Alveolar walls are thickened by hematogenously disseminated pleomorphic tumor cells. Fibrinous and hemorrhagic intra-alveolar congestion. The air spaces are largely well preserved. (35X)



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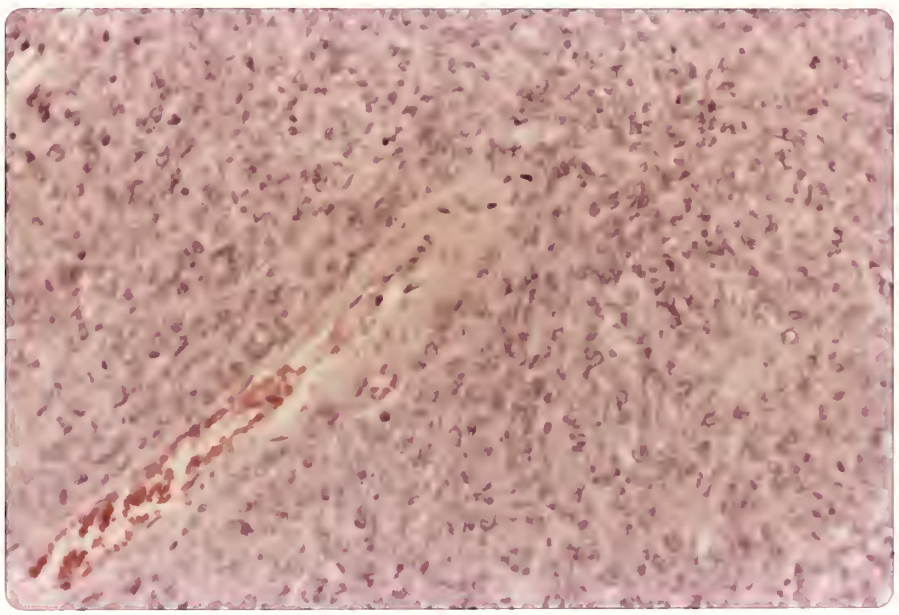
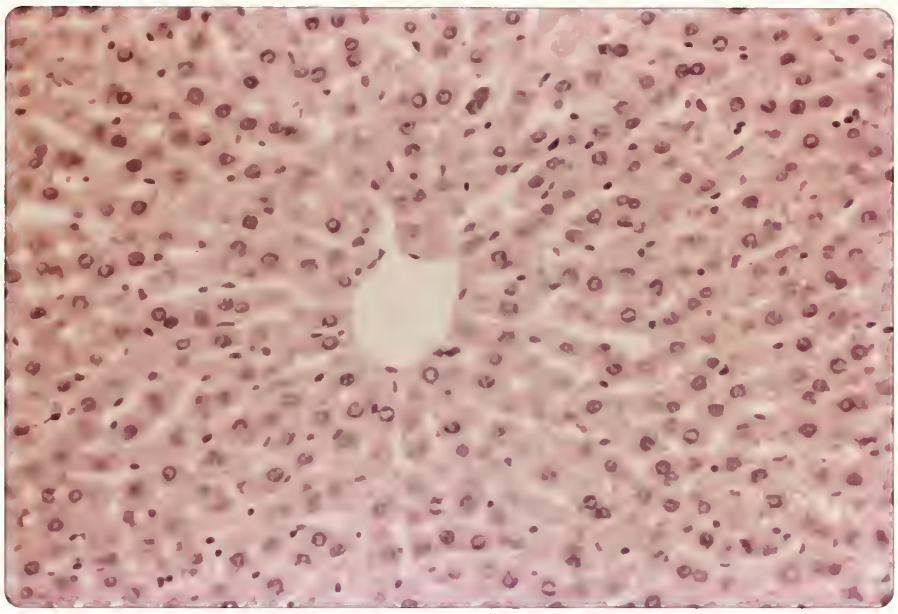


(c) Two to three-plus metastatic pulmonary lesion. Generalized alveolar wall thickening and dense aggregates of undifferentiated sarcomatous tissue are seen. Some preservation of air spaces. (d) Four-plus pulmonary metastasis. Dense sarcomatous tissue completely surrounds vessel in this section, and few open airways exist. Grossly, there were hard pulmonary nodules. This rat lived 64 days. (35X)

were common. Infiltrates included fibroblasts and more atypical, pleomorphic cells embedded in connective tissue stroma. These cells closely resembled those seen in pulmonary metastases. The lesions were occasionally well circumscribed and dense, occurring in non-portal areas, but more commonly were less organized and seen diffusely throughout a generally disorganized hepatic parenchyma.

Because infiltrates were suggestive, but not diagnostic of metastases, an attempt was made to differentiate morphologic changes secondary to inanition from those due to tumor infiltration. In addition to H and E staining, some liver sections were stained by the Masson trichrome technique, in the hope that reparative hepatic fibrosis could be distinguished from fibroblastic or undifferentiated sarcomatous infiltrates. In the sections so stained, color changes characteristic of hepatic reticulin were not seen in fibrotic areas. However, the cellular infiltrates and stroma were not entirely comparable to the metastatic patterns seen in lung, in which infiltrates were clearly malignant and similar to neoplastic changes observed in malignant bone. Thus, those hepatic sections with major structural changes and suggestive cellular infiltrates were classified as "suspicious" of containing metastatic disease, but not definitive.

Of the 26 liver specimens examined microscopically, 12 were considered suspicious for metastasis by the criteria defined above. The incidence of suspicious lesions did not vary significantly by experimental group; approximately 40-50% of both treated and untreated animals contained such hepatic infiltrates. Liver sections with probable metastatic infiltrates (containing "suspicious" lesions) are illustrated



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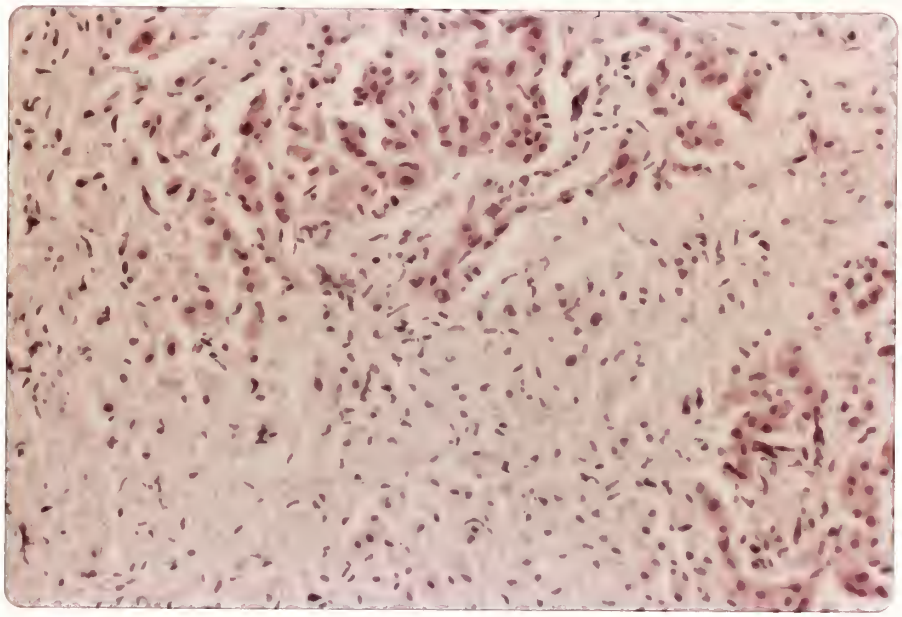
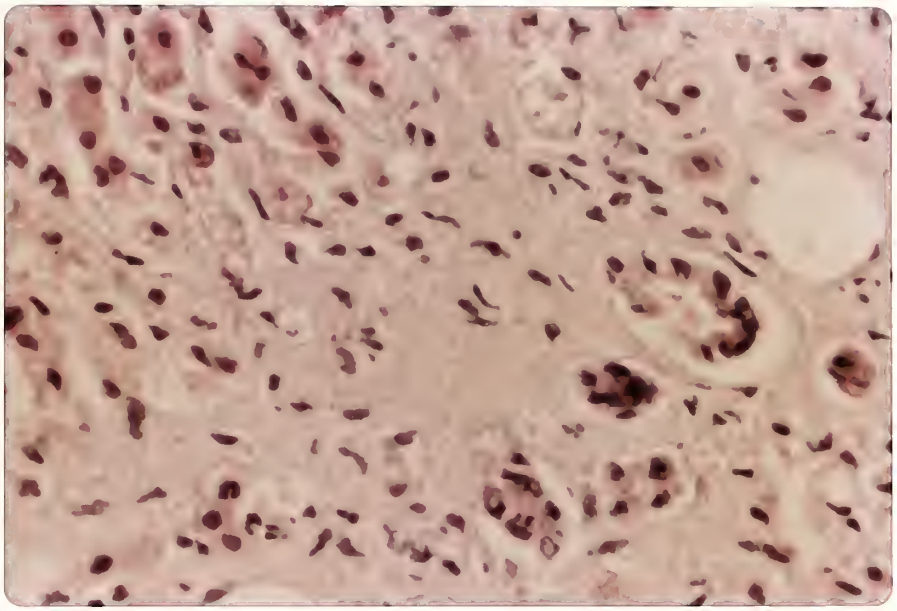


Figure 9(a-d)

Microscopic Appearance of Liver Sections

(a) Normal liver. Central vein with radiating cords and sinusoids.
(b) Typical "suspicious" hepatic lesion with diffuse destruction and pleomorphic cellular infiltrate. Few "normal" hepatocytes and little parenchymal organization is preserved. (35X)

9c



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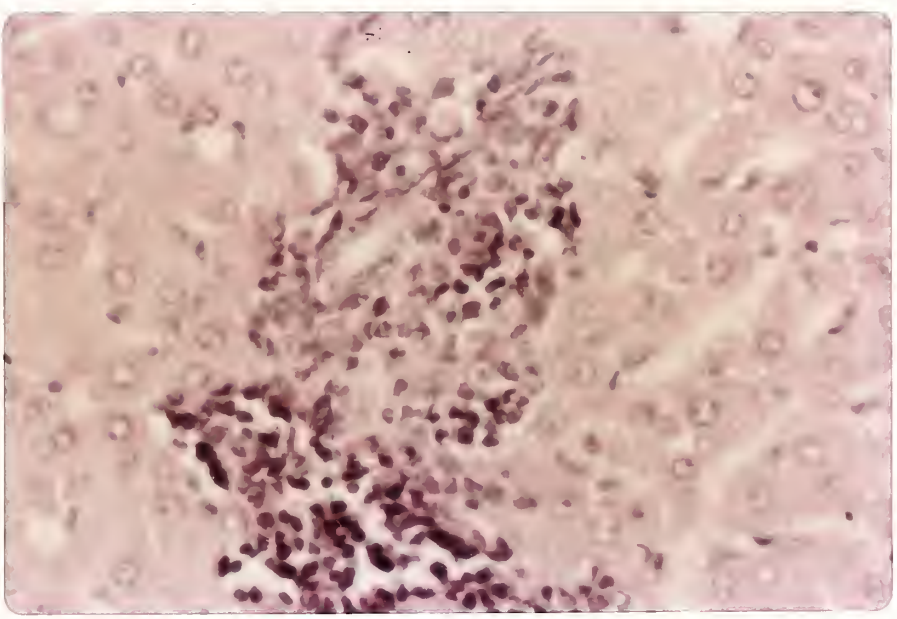


(c) Higher power view of lesion similar to that seen in 9(b). Diffuse hepatic parenchymal destruction and invasion by hyperchromatic, pleomorphic cells is evident. (70X) (d) A second type of "suspicious" hepatic lesion. Hepatic architecture is generally preserved, with several foci of well-circumscribed pleomorphic infiltrates such as this one. (70X)

in Figure 9.

Long bones which had grossly apparent metastatic disease showed typical osteosarcomatous changes on microscopic examination. As previously noted, 10% of the adriamycin-treated animals had long bone metastases, as compared to 0% of the untreated control rats.

Of 26 spleens examined microscopically, none showed convincing metastatic infiltrations. Many specimens revealed a general hypercellularity when compared to uninfected normal rat spleens, with especially prominent germinal centers.

Lymph node specimens revealed tumor infiltrations similar to that seen in metastatic lung sections, with dense fibroblastic osteosarcomatous tissue distributed in well-circumscribed lesions. Since clinically small nodes were difficult to find and remove from tumor-bearing rats, a significant bias was placed on the samples obtained. Thus, only a fairly accurate 10% figure can be sited as the incidence of lymph node metastases, and this from gross inspection of the animals at necropsy.

Table II summarizes the pathologic findings in the three experimental groups.

E. Radiographic Appearance

Radiographs taken at various intervals during the course of disease reflected the microscopic pathology of the primary tumors. The

Table II

Microscopic Pathology: Incidence and Distribution of Metastases

Group	Long Bones %	Lung %	Liver* %	Spleen	Lymph Nodes %**
Untreated	0	89	50	0	10
Adr. 1 mg/kg/day	6	88	38	0	10
Adr. 2 mg/kg/day	14	90	50	0	10

* Figures reported in this table represent probable metastases, or "suspicious" lesions. See text for explanation.

** Figures represent estimates derived from gross inspection of animals at necropsy. See text for explanation.

normal contours of the involved long bones of the lower extremity were expanded and eventually destroyed, and the tumor mass was characterized by both osteoblastic and osteolytic areas (Figure 10). Long bone metastases showed similar radiographic changes (Figure 6).

Figure 10

X-ray appearance of normal and primary tumor-bearing limbs. The normal limb contour was invariably expanded or destroyed, and lesions were radiographically blastic and/or lytic.

F. Tumor Cells in Culture

A single homogeneous line of MSV/WL tumor cells was kept in tissue culture through serial passage in RPMI medium supplemented with 10% FCS and antibiotics. At no time were fungi or typical mycoplasma intracellular inclusions seen under the inverted phase contrast microscope.

Tumor cells had a plating efficiency of approximately 50% and a doubling time of about 24 hours.[228] After adding $2x10^6$ cells to a 30 ml tissue culture flask and incubating them in a 37^oC 5% CO_2 atmosphere, cells became adherent by approximately 5 hours and confluent in three days, necessitating repeated cell passage.

There were two predominant cell types observed in tissue culture (Figure 11). The first was characterized by a large, granular and often nuclelated nucleus with scant cytoplasm. The second cell type was spindle-shaped or stellate, with long cytoplasmic processes often

Figure 11

Osteosarcoma cells from Wistar-Lewis rats in tissue culture, as viewed under the inverted phase-contrast microscope (547X). See test for explanation.

extending from one or more centrally located oval nuclei. Both cell types were adherent to plastic surfaces and appeared to replicate at equal rates.

G. Cell-Mediated Immunity and its Serum Modification in vitro as Measured by a Visual Assay

Cell-Mediated Immunity (CMI) in Untreated Animals through the Course of Disease:

Cell-mediated immunity was tested in untreated animals by a 48 hour visual microcytotoxicity assay at three to nine day intervals when animals were 10 to 48 days of age. Results are summarized in Table III and illustrated graphically in Figure 12. All assays included control wells containing lymphocytes and serum from uninfected (normal) animals, or medium alone, in addition to the MSV/WL target cells. The normal lymphocytes were virtually never cytotoxic for MSV/WL as compared to wells with medium alone, and were therefore considered the standard control wells against which specific lysis by immune lymphocytes, CMI(L), or immune lymphocytes and anti-serum, CMI(S), were compared. All experimental wells were done in triplicate, and the significance of specific lysis was evaluated by the Student's t test.

Specific lysis by lymphocytes from tumor-bearing animals was present in all animal groups tested between 14 and 48 days of age. Activity was difficult to evaluate on day 10, at which time normal serum was nonspecifically cytotoxic to target cells, presumably due to factors in the serum of animals which had recently been suckling. CMI(L) was

TABLE III

Activity of Immune Lymphocytes and Serum from Untreated Animals versus MSV/WL Cells

Age (days)	Cells in Control Wells*	Cells in Experimental Wells		CMI(L) *** %	CMI(S) *** %
		Lymphocytes Alone**	With Immune Serum		
10	192±6	-	144±15	-	25
14	511±16	505±24	409±17	1.2	20[h]
21	222±19	98±15	81±11	56[k]	64
28	437±47	341±24	159±24	22[k]	64[h]
31	211±6	193±19	153±5	9	28[h]
34	104±8	88±4	7±3	15[k]	93[h]
39	109±14	57±4	45±6	48[k]	58[h]
48	213±5	158±7	88±6	26[k]	59[h]

*- All control wells contained lymphocytes and serum from uninfected rats.

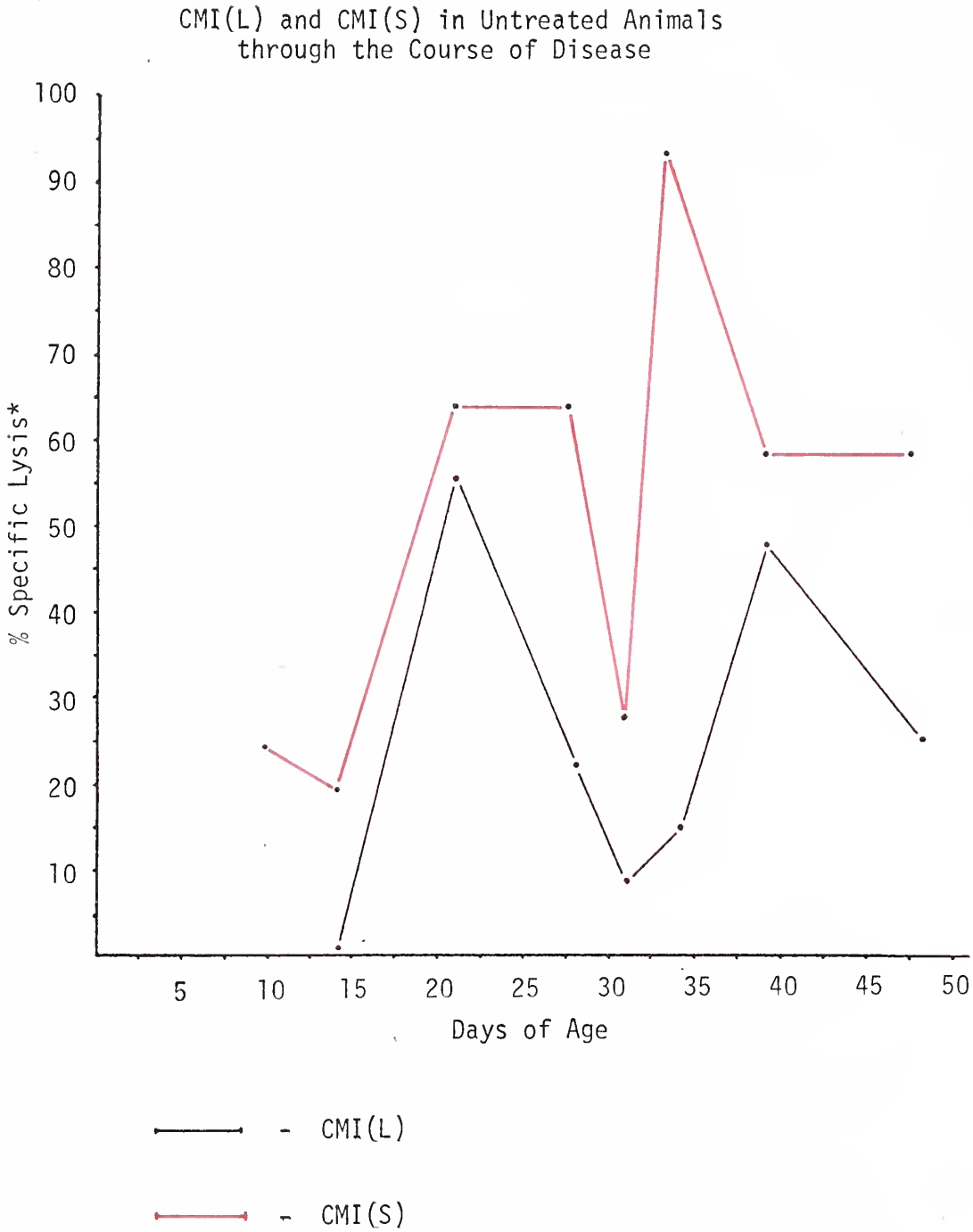
**- Wells with lymphocytes also contained MEM with 20% FCS.

***- Percent specific lysis with lymphocytes alone, CMI(L), or with immune serum in addition to lymphocytes, CMI(S), was calculated as described in text.

k- Specific cytotoxic activity significant by Student's t test ($p < .05$)

h- Significant "helper" serum activity ($p < .05$); i.e., CMI(S) CMI(L)

Figure 12



* Specific Lysis, % = $100 \times \left[1 - \left(\frac{\text{tumor cells in experimental well}}{\text{tumor cells in control well}}\right)\right]$

statistically significant ($p < .05$) on days 21, 28, 34, 39, and 48.

There appeared to be a biphasic nature to the lymphocyte response, with relatively high levels of CMI(L) on days 21 and 28, much lower levels on days 31 and 34, and a return to higher levels on days 39 and 48. Maximum levels of CMI(L) appeared on days 21 and 39, at which times there were 56 and 48% specific lysis, respectively.

Further tests of tumor-specificity in the cytotoxic response of immune lymphocytes were performed using normal rat fibroblasts and a xenogeneic mouse tumor line as target cells for the assay. At no time were lymphocytes from tumor-bearing animals cytotoxic for these cells when compared to wells containing normal rat lymphocytes.

Serum Modification of CMI(L) in Untreated Animals:

When serum obtained from tumor-bearing animals between 10 and 48 days of age was added to wells containing immune lymphocytes, specific lysis (CMI(S)) was consistently increased. CMI(S) was statistically significant ($p < .01$) in every assay, indicating the cytotoxicity of immune lymphocytes and serum compared with their normal counterparts from uninfected animals. CMI(S) generally followed the same biphasic course as CMI(L) in these untreated animals (Figure 12).

Serum factors in untreated animals invariably increased the cytotoxicity of immune lymphocytes compared with wells containing immune lymphocytes and medium alone; i.e., there were "helper" factors in these sera. When the difference between CMI(S) and CMI(L) was compared by the Student's t test, significant helper activity was found to be

present on days 14, 28, 31, 34, 39, and 48. It is noteworthy that, despite this helper activity, all untreated animals had progressively enlarging tumors at the time of assay.

Because of the possibility that antibodies in immune serum might enhance the cytotoxicity of normal lymphocytes against MSV/WL cells, wells testing this antibody-dependent cellular cytotoxicity (ADCC) were included in all assays through day 28. No cytotoxicity above that of normal lymphocytes without serum was found, and this reaction mixture was not used in subsequent assays.

CMI(L) in Animals Treated with 1 mg/kg/day Adriamycin:

Specific lysis by immune lymphocytes from animals treated with the lower dose of adriamycin was demonstrated in animal groups tested from 22 to 48 days of age. These results are summarized in Table IV and depicted graphically in Figure 13. CMI(L) was statistically significant compared with control wells ($p < .05$) on days 26, 35, and 42.

As seen in untreated animals, CMI(L) in these adriamycin-treated animals revealed a maximum of activity between 20 and 30 days of age. After a maximum specific lysis of 61% on day 26, lymphocytes in treated animals likewise showed diminishing activity by day 35. Unlike the untreated animals, however, in which CMI(L) subsequently returned to early high levels around day 40, cytotoxicity continued to diminish in this treatment group. Two possibly significant differences existed between the assays for untreated as compared to treated animals on the last two days of assay when the CMI(L) continued to decrease: (1) as

Table IV

Activity of Immune Lymphocytes and Serum from Animals Treated with 1 mg/kg/day Adriamycin versus MSV/WL cells

Age (days)	Cells in Control Wells *	Cells in Experimental Wells		CMI(L) *** %	CMI(S) *** %	Status +
		Lymphocytes Alone **	With Immune Serum			
15	140±1	144±12	104±11	-2.8	26	P
22	88±6	83±8	84±6	5.7	4.5	P
26	46±3	18±8	29±2	61	37	P
35	67±6	55±4	67±5	18	0	P
42	224±4	201±8	191±6	10	15	R
48	115±8	112±10	99±7	2.6	14	R

*- All control wells contained lymphocytes and serum from uninfected rats.

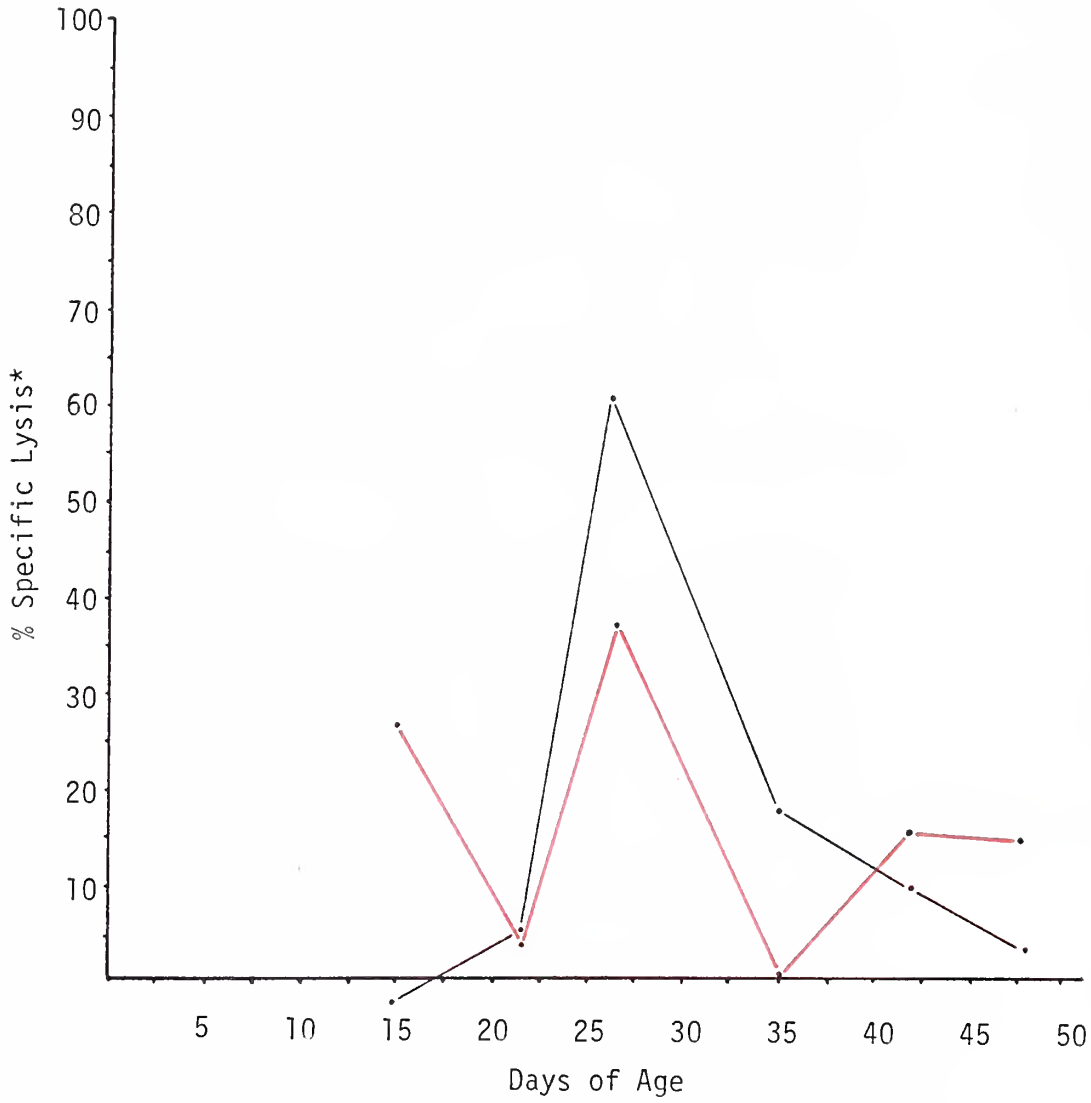
**- Wells with lymphocytes also contained MEM with 20% FCS.

***- Percent specific lysis with lymphocytes alone, CMI(L), or with immune serum in addition to lymphocytes, CMI(S), was calculated as described in text.

+- "P" refers to a progressor animal; "R" in these assays refers to an animal bearing a tumor diminishing in size.

Figure 13

CMI(L) and CMI(S) in Animals Treated with Adriamycin, 1 mg/kg/day, i.p., through the Course of Disease



——— - CMI(L)

——— - CMI(S)

$$* \text{ Specific Lysis, \%} = 100 \times \left[1 - \left(\frac{\text{tumor cells in experimental well}}{\text{tumor cells in control well}} \right) \right]$$

opposed to assays of untreated animals and the earlier assays of this treatment group in which three animals' lymphocytes and sera were pooled, the same one animal's effector cells and sera were used for the last two assays, collected from peripheral blood obtained through tail nicks; and (2) this animal, although tumor-bearing, was considered a regressor rat. The remainder of the rats assayed in this group, as well as all of the untreated animals, were demonstrated to have progressively enlarging tumors.

Serum Modification of CMI(L) in Animals Treated with the Lower Dose of Adriamycin:

Serum from animals treated with the lower dose of adriamycin had a variable effect on CMI(L) at different times during the course of disease. Similar to the effects of serum from untreated animals early in the disease course, CMI(S) on day 15 was significantly higher than CMI(L) in this treatment group ($p < .02$). This was three days after the completion of the course of treatment with adriamycin.

On days 22, 26, and 35, however, serum factors from these treated animals partially blocked the cytotoxicity of immune lymphocytes. On the latter two of these three days, blocking activity was significant ($p < .05$).

Finally, on the last two days of assay, serum factors again enhanced the cytotoxicity of reaction mixtures containing immune lymphocytes. This helping activity, however, was not statistically significant ($p < .20$).

CMI(L) in Animals Treated with 2 mg/kg/day Adriamycin:

Cytotoxicity for MSV/WL by lymphocytes from tumor-bearing animals treated with the higher dose of adriamycin was seen in all groups tested from 15 to 46 days of age. These data are summarized in Table V and illustrated graphically in Figure 14. As evaluated by the Student's t test, specific lysis was significant on days 22, 26, 40 and 46.

The course of CMI(L) in this treatment group was almost identical to that seen in the untreated animals, revealing a similar biphasic pattern. Maximum cytotoxicity occurred on days 22 and 46, each of these showing a specific lysis of 50%, and these points were symmetrically distributed about a nadir at 33 days, at which time specific lysis was 1.5%. In contrast to assays in the group treated with lower dose adriamycin, the final two days of assay in this group revealed a return to high levels of cytotoxicity.

Serum Modification of CMI(L) in Animals Treated with the Higher Dose of Adriamycin:

As seen in animals treated with adriamycin at 1 mg/kg/day, serum from animals treated with the higher dose adriamycin had a course-related variable effect on CMI(L) (Figure 14). As in both other experimental groups, CMI(S) was higher on day 15 than CMI(L) in this treatment group.

In addition, this helper serum effect converted to blocking activity on days 22, 26, 33, 40, and 46. Blocking activity was statistically

Table V

Activity of Immune Lymphocytes and Serum from Animals Treated with 2 mg/kg/day Adriamycin versus MSV/WL Cells

Age (days)	Cells in Control Wells *	Cells in Experimental Wells		CMI(L) *** %	CMI(S) *** %	Status +
		Lymphocytes Alone **	With Immune Serum			
15	140±1	138±13	116±13	1.4	17	P
22	88±6	44±12	59±14	50	33	P
26	46±3	28±1	30±7	39	35	P
33	67±6	66±8	41±14	1.5	-6.0	P
40	224±4	139±6	184±11	38	18	R
46	115±8	57±4	77±6	50	33	R

*- All control wells contained lymphocytes and serum from uninfected rats.

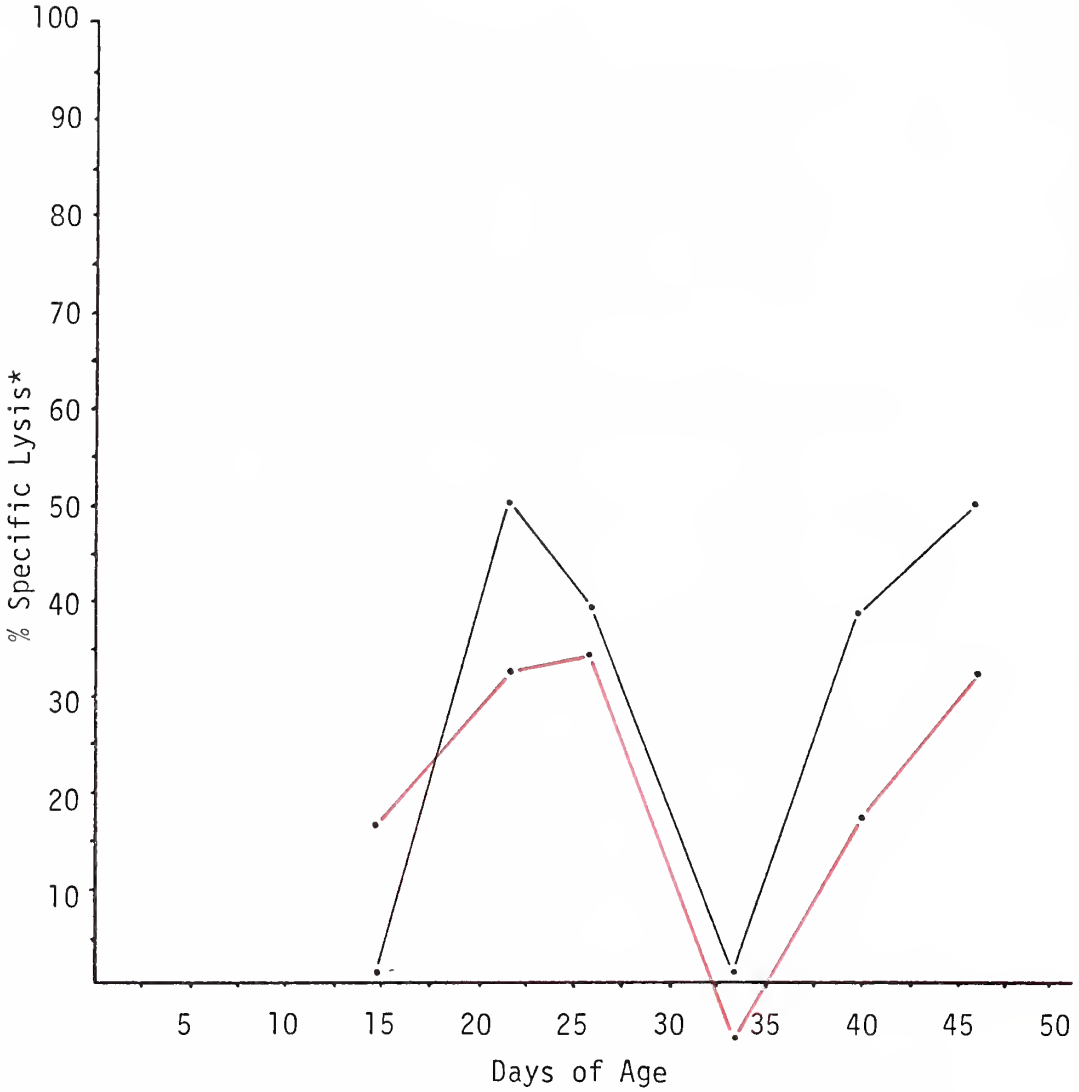
**- Wells with lymphocytes also contained MEM with 20% FCS.

***- Percent specific lysis with lymphocytes alone, CMI(L), or with immune serum in addition to lymphocytes, CMI(S), was calculated as described in text.

+- "P" refers to a progressor animal; "R" in these assays refers to an animal bearing a tumor diminishing in size.

Figure 14

CMI(L) and CMI(S) in Animals Treated with Adriamycin
2 mg/kg/day, i.p., through the Course of Disease



— - CMI(L)

— - CMI(S)

* Specific Lysis, % = $100 \times \left[1 - \left(\frac{\text{tumor cells in experimental well}}{\text{tumor cells in control well}}\right)\right]$

significant on two of these days ($p < .01$). As shown in Figure 14, throughout the course of disease, serum activity paralleled CMI(L) in its biphasic pattern.

In contrast to serum from animals in the groups treated with low dose adriamycin, serum in this treatment group continued to block CMI(L) activity on the last two days of assay. However, effector cells and sera were likewise obtained from tumor-bearing regressor animals.

Brief Summary of CMI(L) and CMI(S) in All Experimental Groups:

Specific lysis of MSV/WL by lymphocytes from animals in all experimental groups was seen from approximately two to seven weeks of age. Although all lymphocytes were tested against a single homogeneous tumor line, assays of untreated animals were performed at a different time than assays of adriamycin-treated animals, and therefore strict statistical comparison of levels of CMI(L) on particular days between the groups is not considered entirely valid. Nevertheless, levels of CMI(L) were similar in all animal groups at comparable ages, and the maximum lysis observed in untreated animals (56% on day 21) was very similar to that seen in animals treated with the lower dose of adriamycin (61% on day 26) as well as the higher dose (50% on days 22 and 46). Thus, in general, there appeared to be no significant depression of cell-mediated immunity by adriamycin at doses which caused statistically significant increases in MST and the number of regressor animals in the treated groups.

In addition, patterns of CMI(L) during the course of disease were

similar in the three experimental groups. An early peak of activity occurred in all groups around days 21-26, followed by a diminution of specific lysis by days 31-33. This was followed by a second peak of activity around days 39-40 in animals untreated or treated with the higher dose of adriamycin, whereas in animals treated with the lower dose, CMI(L) continued to fall.

In contrast to CMI(L), significant differences occurred in the patterns of CMI(S) in the three groups. Whereas only helper serum factors were present in untreated animals, early helper activity in treated animals converted to blocking activity by day 22. This persisted in animals treated with higher doses of adriamycin, while in animals treated with the lower dose, a modest reversion to helper activity was seen on days 42 and 46.

H. Cell-mediated Immunity and its Serum Modification as Measured by an Assay Using Isctopic Labelling

On every day that a visual assay was performed, a cytotoxicity assay using radiolabelled target cells was also attempted in order to find an ideal isotopic method that closely correlated to the results of the standard visual evaluation. In addition, however, visual counting of the remaining target cells is cumbersome, time-consuming, and vulnerable to subjective interpretation.[221,233] The use of a method involving isotopic release by killed target cells minimizes this subjectivity.

Several types of assay were attempted which were designed to fulfill the following criteria of an ideal isotope, which should: (1) be incorporated and permanently bound to the target cell, preferably by a known metabolic pathway; (2) be released immediately at cell death; (3) not be reutilized by the remaining target cells; and (4) be incorporated into targets in sufficient quantities to permit clear differentiation of samples from background radiation levels.[116]

Methods were used in which ^{3}H-thymidine or ^{3}H-proline were added prior to incubation of the reaction mixtures ("pre-labelling" methods) or after target-effector cell incubation was completed and effector cells removed ("post-" or "pulse-labelling" techniques). A variety of harvesting techniques to remove and isolate remaining target cells were also attempted.

The post-label assay perhaps most closely reflects the cytotoxicity and proliferation-inhibition which is characteristic of target cell measurements in the visual assay. In addition, short term "pulse-labelling" minimizes high levels of spontaneous loss of the tracer and avoids the difficulties of long-term radiation injury to the target cells occasionally seen in pre-label assays.[221]

Pulse-labelling with ^{3}H-thymidine produced results which most closely correlated with those of the visual assay in this rat model for osteosarcoma. Tritiated thymidine has been used in other experimental *in vitro* assays[116,165], and in this system effected counts approximating 0.7-1.0/MSV/WL cell/minute.

The method of harvesting remaining target cells at the end of ^{3}H-thymidine incubation was found to be crucial, and the trichloroacetic acid precipitation method (see Methods section) was found most effective. In addition, vigorous washing and removal of effector cells prior to the addition of the isotope was essential to prevent incorporation of the tracer into effector lymphocytes.

The results of a typical post-label assay with ^{3}H-thymidine and their comparison to results of the visual assay performed on the same

TABLE VI

Comparison of Visual and Post-Label Isotopic Assays of Cell-Mediated Immunity in Adriamycin-Treated Animals

Group	Age (days)	Control Wells,* cpm	Experimental Wells, cpm		Isotope Assay	Isotope Assay	Visual Assay	Visual Assay
			Lympho-cytes Alone **	With Imm. Serum	CMI(L) *** %	CMI(S) *** %	CMI(L) %	CMI(S) %
Adr. 1 mg/kg/d	48	565±17	584±7	501±18	-3.4	11	2.6	14
Adr. 2 mg/kg/d	46	565±17	302±9	367±38	47	35	50	33

*– All control wells contained lymphocytes and serum from uninfected rats.

**– Wells with lymphocytes also contained MEM with 20% FCS.

***– Percent specific lysis with lymphocytes alone, CMI(L), or with immune serum in addition to lymphocytes, CMI(S), was calculated as described in text.

day are summarized in Table VI. In brief, they show levels of CMI(L) and CMI(S) on days 45 and 47 post-infection in animals treated with 2 mg/kg and 1 mg/kg adriamycin, respectively. CMI(L) and CMI(S) consistently differed by only 2-5% between the visual and radiolabel assays. These differences are within experimental error.

I. Cytotoxic Antibody Response as Measured by a Microcytotoxicity Assay

A modification of the micromethod of Kaliss[168] using ^{51}Cr-release by killed target cells was employed to measure cytotoxic antibody responses during the course of disease. LSTRA tumor cells were used as target cells for this assay, and each cell incorporated approximately 0.15 cpm of ^{51}Cr after a 5 hour incubation at 37^{o}C. Supernatants containing released ^{51}Cr were collected after centrifugation of the target-effector cell solutions. In early assays nonspecific lysis of cells was high (approximately 25% in control wells), and this was attributed to an unnecessarily vigorous centrifugation rate. When centrifugation rates were decreased to 800 rpm for 10 minutes, lysis in control wells diminished to an acceptable 10-15% background level.

Heat-inactivated serum obtained at six times from animals nine to 41 days post-inoculation and stored at -70^{o}C showed no detectable titers of cytotoxic antibody. Sera from treated and untreated animals alike produced the same negative results. All wells were tested in duplicate and all experiments were performed twice. In addition, the micromethod detected cytotoxic antibody levels to leukemia L-1210 cells,

used as a positive control.

LSTRA cells have been effectively employed as target cells to detect cytotoxic antibody in the serum of mice bearing MSV-induced tumors.[245] However, LSTRA cells are perhaps not as sensitive to immune cytolysis as are some other MSV-related tumors used as targets for various *in vitro* assays.[181] In the present study, no attempt was made to use other target cells for this assay, and no other assays measuring antibody-mediated cytolysis were employed.

IV. Discussion

A. MSV-Induced Rat Osteosarcoma as a Model of the Human Disease

While animal models of human malignancy must always be regarded with some skepticism, experimental systems which closely resemble their human counterparts can be extremely useful in further understanding the human disease and its response to various therapeutic modalities.[117] Important characteristics requiring comparison include etiology (where this is known), histology, biological course, immunological characteristics, and response to therapy. An initial report from Drs. Friedlaender and Mitchell here at Yale suggests that the MSV-induced osteosarcoma in Wistar-Lewis rats may be one such valuable model of the human disease. A major goal of the present investigation has been to further document the extent of the resemblance of this model to human osteosarcoma.

Osteosarcoma is primarily a disease of young people, originally localized most often to the long bones of the lower extremities, and rapidly progressing to pulmonary metastases and death. Pulmonary metastases mark a definitive point in the course and therapy of this particular malignancy.

Until recently, a variety of therapeutic modalities designed to prevent pulmonary metastases or effect their regression have been entirely unsuccessful, causing no significant increase in the 20% five year survivals effected by surgery alone. Recent chemotherapeutic

approaches using adriamycin have delayed (or prevented) the appearance of pulmonary metastases post-operatively, caused a 40% incidence of metastatic regression, and most likely significantly prolonged survival.

A wealth of data exists to support a viral etiology for osteosarcoma in man, including immunological studies revealing a common antigenicity to a variety of histological types of sarcomata. Immunologic studies are now being directed to clarifying the specific components of the immune response which are responsible for tumor progression or regression in vivo and in vitro. Preliminary knowledge along these lines has already been successfully exploited in the immunotherapy of osteosarcoma in man.

Previous models of osteosarcoma have included spontaneous malignancies, and tumors induced by radiation, chemical carcinogens and oncogenic viruses in a multitude of animal species. While the spontaneous canine osteosarcoma[243] is a most valuable model system, it is expensive and, due to its rarity, is not generally available.[262] In view of the evidence for a viral etiology of the human disease, it is important that an experimental model of osteosarcoma be induced by a virus. The MSV-induced rat tumor fulfills this prerequisite.

In addition, intra-tibial inoculation of MSV has produced, with excellent reproducibility, a tumor which is initially localized to the lower extremity of the infected animal. As opposed to other MSV-induced rat osteosarcomata caused by intravenous or interperitoneal administration of the virus, the predictably localized tumor in the present system is a lesion which is accessible to observation, excision, and local radiotherapy or immunotherapy.

Like the other MSV-induced rat sarcomata, however, the lesion viewed microscopically is invariably osteogenic in nature. This is in notable contrast, as already mentioned, to the myosarcomatous nature of MSV-induced tumors in mice. Histologically, the primary tumors in rats revealed the same growth patterns and variability as their human counterparts, with different areas within the lesions showing a predominance of malignant fibroblastic, cartilaginous or osteogenic tissue. Microscopic sections and radiographs alike documented the typical periosteal elevation and cortical destruction caused by the tumors.

Another unique characteristic of the rat osteosarcoma is its propensity for metastasis. Previous experimental tumors have not readily metastasized, and their distribution of metastases have not been comparable to the human disease.[262] The preliminary experiments of Friedlaender and Mitchell documented the occurrence of metastases in the rat model, and these were most frequently observed in the lungs of infected animals.[117] These early studies, however, made no attempt to document the incidence of metastatic lesions.

That the ability to metastasize is of fundamental importance is obvious when considering the aggressive metastatic nature of osteosarcoma in man, and the pivotal role of pulmonary metastases in directing therapy. It is assumed that hematogenous micrometastases are present in most patients at the time of diagnosis, and clinically detectable pulmonary metastases are present in approximately 10% of patients before therapy is begun. Autopsy data reveal a 95% incidence of pulmonary metastases in patients succumbing to their disease.

In contrast to the initial study[117] which reported that pulmonary metastases were not evident until animals were four weeks of age, tumor-bearing rats in the present investigation had pulmonary metastases as early as 14 days post-infection. The incidence of pulmonary lesions did not vary significantly with age, as 89% of animals of all ages (either dying of tumor or sacrificed for assay) had metastatic pulmonary metastases.

Osteosarcoma in man less frequently metastasizes to other locations, including other long bones (14%), liver (8%), and lymph nodes (30-40%). In the rat model only one long bone metastasis was observed in the original study of 98 animals[115], and no bony metastases were observed in 22 untreated animals during the present experiments. Hepatic lesions considered suspicious for the presence of metastatic tumor were seen in 50% of tumor-bearing animals. Lymph nodes were grossly invaded by tumor in approximately 10% of the animals, and no metastatic lesions were observed in the 26 spleen specimens examined.

Histologically, metastases were most frequently pleomorphic, undifferentiated sarcomas, with no apparent bone or cartilage-forming activity. Early lesions in all organs were scattered diffusely throughout the parenchyma, consistent with the hematogenous nature of their dissemination; whereas more advanced metastases in lung and lymph nodes were well circumscribed and nodular.

In general then, the incidence, distribution, and histology of metastases in this rat model of osteosarcoma are comparable in most respects to the metastatic patterns seen in the human disease. The

predilection of both tumors to early pulmonary metastasis is particularly significant. This aspect of the present model gives it a notable advantage over previous animal models of osteosarcoma.

The course of disease in untreated animals was relentlessly progressive, with rapid tumor growth and death usually by 20 days of age. Although two animals survived 64 days, there were no regressor animals in the untreated group. This is in contrast to the 22% incidence of regressors reported by Friedlaender and Mitchell.[117] Two reasons can be offered to explain this apparent discrepancy in the model. First, the 22% figure is undoubtedly a moderate overestimate of the proportion of regressor animals.[116] In the earlier study, the limbs of fully 30% of the rats could not be evaluated because the animals had been cannibalized after death by the mother rat or their siblings. Progressor or regressor status was then determined on the remaining 68 animals.[116] Cannibalism occurs only in the case of a dead animal, and animals dying in the present investigation all had progressively enlarging tumors. It is suspected, therefore, that the original method of analysis minimized the proportion of rats dying with growing tumors (progressors).

Secondly, the batch of virus inoculum in the present study may have been somewhat more virulent (and perhaps unintentionally more concentrated) than the batch used by Friedlaender and Mitchell. Preliminary experiments revealed that the "aggressiveness" of the induced tumor (including frequency of progressors) was proportional to virus dose. Although identical doses were used in both studies, tumors were clinically apparent an average of three days earlier in the present investigation. Coupled with the absence of regressors in otherwise

identical groups of animals inoculated at the same age, this suggests a difference in viral potency in the two studies.

A final noteworthy general feature of the rat model of osteosarcoma is its response to therapy with adriamycin. Administered intraperitoneally after 90% of the tumors were palpable, adriamycin at doses of 1 mg/kg/day or 2 mg/kg/day for three consecutive days significantly reduced tumor size, prolonged median survival times, and increased the proportion of regressor animals. In the latter two respects, the lower dose of adriamycin appeared to be more effective than the higher dose. The sensitivity of this tumor to adriamycin chemotherapy is another characteristic shared by its human tumor counterpart.

Summarizing these data, it is clear that the MSV-induced osteosarcoma in rats bears a striking similarity to human osteosarcoma. The present study has confirmed previous work describing the morphological characteristics of the tumor and showing its progressive and metastatic course. In addition, this investigation has documented an incidence and distribution of metastatic disease comparable to that seen in man, and shown that this tumor is likewise responsive to treatment with adriamycin. The significance of the animals' immunological response to the tumor (discussed in the next section) further demonstrates the value of this model system in understanding and successfully treating osteosarcoma in man.

B. The Immunobiology of MSV-Induced Tumors

The science of tumor immunology has been greatly advanced during the past decade by the discovery in 1964[140] of Moloney leukemia virus variants, later designated murine sarcoma virus-Moloney, or MSV. Since 1964, a vast literature has arisen documenting the biology of tumors induced by this virus in mice, hamsters and rats. In particular, the attention and scrutiny of tumor immunologists to these systems have been drawn by the observation that MSV-induced tumors will often spontaneously regress -- a phenomenon unique to these systems.[102] Spontaneous regression has been shown to be dependent on the dose of the virus, the age and strain of the host, and more importantly, the status of the host immunity.[102] Both cellular and humoral immunity have been shown to be significant in effecting regression in various MSV systems, with cellular responses generally considered the more important of the two.[103,104] Precisely which cells (T-cells, B-cells, macrophages, "normal" vs. specifically immune cells) and which serum factors (cytotoxic, cytophilic, "unblocking", or "arming" antibodies) are most responsible for tumor regression appears to vary by animal species or strain,[169,181] as well as by the specific assays used.[186] In addition, the magnitude of the response of particular immune factors may vary during the course of the disease, and this time-related variation may differ in different animal strains or species.

The above variabilities notwithstanding, there is sufficient commonality to the immunobiology of the several MSV systems to warrant a well-defined comparison of one model to the next. In this context,

a brief discussion of the more intensively investigated MSV systems seems pertinent. It is hoped that this discussion will put the results of the present investigation in a more proper perspective in the otherwise overwhelming literature of MSV-induced tumors, and tumor immunology in general.

Immunobiology of MSV-Induced Tumors in Mice:

Newborn mice inoculated with MSV invariably develop tumors within 10 days which usually grow progressively and kill the animals by two weeks of age.[102,104] Older mice are equally susceptible to tumor induction by MSV,but if inoculated after 4 weeks of age will develop tumors which virtually always regress. Immunosuppression of adult mice by X-irradiation, neonatal thymectomy, ALS or cortisone treatment, or chemotherapy with cyclophosphamide or daunomycin, causes a significantly decreased incidence of tumor regression.[45,100,102,185,286]

Early reports emphasized the significance of tumor-specific cellular mechanisms in effecting tumor regression.[103,104,185] While some of these preliminary studies were lacking in the technical sophistication required to demonstrate humoral immunity, cellular responses were felt to be beneficial to tumor regression, and humoral mechanisms were thought to be unimportant. Particularly striking was the decreased incidence of adult regressors in groups which had been neonatally thymectomized[185], and the infiltration of regressor but not progressor tumors with massive numbers of mononuclear cells.[103]

Basically two differing patterns of cellular responses during the

course of disease have been reported by different investigators. Early studies by the Hellstroms[141,143] emphasized the similarities between cellular activities in progressor and regressor animals, in which lymphocyte-mediated antitumor responses remained at high levels throughout the disease course. More recent investigations have studied the time course of the cellular response in greater detail and have arrived at a different conclusion.[177,179,181,186] While using a variety of mouse strains (usually BALB/c or their F_1 hybrids with C57BL/6 strains), these reports are remarkable in their similarity to one another. In general, they have reported a maximum of cellular activity approximately two weeks after virus administration. After about three weeks, cellular cytotoxicity diminishes in both progressor and regressor animals. Most studies have indicated that progressor animals have lymphocytes that are somewhat less cytotoxic than their regressor counterparts, and that the diminution of cytotoxicity after three weeks occurs more rapidly in regressor animals. The patterns of cellular activity in both groups, however, are very similar.

Different populations of effector cells appear to be acting at various stages during the course of disease. Lamon and his associates have studied this aspect of MSV-tumor-specific immunity in great detail by a variety of immunological techniques.[177,179] Specifically, T-cell reactivity appears to be most significant before 30 days post-infection, with maximum cytotoxic levels occurring between days 10 and 20. After 30 days, however, T-cell responses are negligible, and cytotoxicity is dependent on non-T lymphocytes.

Dating back to the earliest reports of the MSV models, humoral immunity was considered to be unimportant in mediating tumor regression. Fefer et al.[103] reported that mice inoculated with Moloney sarcoma cells after incubation with immune serum developed as many tumors as mice inoculated with Moloney sarcoma cells incubated with normal serum. Pre-incubation of tumor cells with immune spleen cells, however, significantly protected the animals against tumor induction.

Furthermore, studies by the Hellstroms[141,143] showed that humoral factors could actually enhance tumor growth both in vivo and in vitro. These blocking factors were frequently found in the sera of progressor animals, while regressors most often lacked such blocking activity. Enhancement of tumor growth was first thought to be due to antibodies in the serum of progressors, and later was demonstrated to be mediated by antigen-antibody complexes.[292] Suffice it to say, at this point, that enhancement does occur, and that it has been convincingly demonstrated not only in MSV systems, but in other experimental tumor models and man as well. A more detailed evaluation of enhancement will be made in subsequent sections of this Discussion.

In addition to the blocking effects of certain serum factors, more recent investigations have demonstrated tumor-inhibitory factors in the serum of animals bearing MSV-induced tumors.[100,126,143,177,178,185,246,255,156,193,312] These factors are antibodies, as shown by immunofluorescence, neutralization, cytotoxicity and fractionation techniques. That they can be significant in vivo has been demonstrated by the studies of Pearson et al.[246], who reported that pretreatment

of recipient mice with regressor serum before tumor challenge resulted in a significantly higher incidence of regressor animals (than pretreatment with normal serum).

Tumor-inhibitory antibodies may be divided into those which are complement-dependent in their activities, and those which act independent of complement. Complement-dependent cytotoxic antibodies have been demonstrated in the sera of both MSV tumor-bearing animals[177,178,312] and osteosarcoma-bearing humans.[332] Significant titers of cytotoxic antibodies were present in 70% of regressor, and interestingly, 24% of progressor animals studied by Tamerius and Hellstrom.[312]

Complement-independent antibodies include a much larger group of globulins under a variety of names with overlapping activities. "Arming" antibodies, which aid in (normal, non-sensitized) cellular cytotoxicity, have been repeatedly found in MSV systems by a number of investigators.[177,179,255,256] It was primarily this group of antibodies which was responsible for the late B-cell activity (or, more precisely, non-T cell activity) in MSV systems referred to in the reports by Lamon et al.[177,179] Pollack and her associates[255,256] have reported such "synergistic cytotoxicity" in 75% of sera from MSV tumor-bearing animals. Significantly, serum from progressor animals armed normal lymphocytes as well as serum from regressors, and cytotoxicity was often demonstrable with sera diluted as highly as 1:1280.

Factors in the serum of regressor animals were found to "unblock" the blocking effects of progressor sera in studies by the Hellstroms.[141,143] Likewise, tumor-inhibitory macrophage "cytophilic antibodies" have been

reported in non-MSV tumor systems and man.[213,230] The relationships of "cytophilic antibodies" and "unblocking antibodies" to "arming antibodies" remain to be determined.

Blocking and "helping" activities often exist simultaneously in the same serum.[255,256,293] In addition, predominantly blocking activity may be seen in regressor animals[293], while mainly helping activity may be seen in progressors.[116,255,256,312] The net result of serum factors on *in vitro* cytotoxicity may depend on animal species[293], concentration effects[255,256,293], or the stage of disease.[177,178,255,256,293] Finally, it is clear that *in vivo* and *in vitro* tumor destruction in MSV-induced malignancies is mediated by a balance of mostly beneficial cellular, and variably beneficial serum mechanisms.

Immunobiology of the Present MSV-Induced Tumor System:

In its general immunologic features, the rat model of osteosarcoma is very similar to MSV-induced tumors in mice. Neonatally MSV-inoculated rats develop progressive tumors, and the incidence of regressor animals is inversely proportional to the dose of virus administered.[116] The present section will discuss the levels and course of cellular and humoral immunity in the untreated animals of this investigation, and relate these results to the findings in other experimental systems.

Cellular Responses - CMI(L):

There were two significant aspects of the cellular response of untreated animals to MSV/WL cells: (1) cellular cytotoxicity was found to be tumor-specific, and present in all animal groups tested from 14 to 48 days of age; and (2) the course of cellular cytotoxicity was biphasic in nature.

The first finding -- that of tumor specificity in the cytotoxic response -- was anticipated. It is characteristic of virally-induced tumors in general, and MSV-induced tumors in particular, to possess tumor-specific antigenic determinants. The fact that tumors are antigenically dissimilar from normal tissues serves as the foundation for the science of tumor immunology. Tumor antigenicity, in Ehrlich's words, "represents the key to carcinoma", and established the basis of Thomas and Burnet's theory of immune surveillance.

In the present investigation, tumor antigenicity was tested in two ways. First, lymphocytes from non-inoculated age-controlled rats were tested against MSV/WL cells and found to be unreactive. These lymphocytes were not primed to "recognize" the tumor cells as foreign. Second, lymphocytes from tumor-bearing rats were tested against syngeneic fibroblasts and xenogeneic tumor cells, and these too were unreactive. The lack of cytotoxicity against the mesenchymal control is of particular importance, in that this finding supports the evidence that tumor antigens in this system are virus-specific,rather than organ-specific.

That the pattern of cellular response to MSV/WL was biphasic was somewhat surprising. This pattern was clearly evident throughout the course in the untreated animal group as well as the group treated with the higher dose of adriamycin. It was "incomplete" in the group treated with the lower dose of adriamycin in that a final return to high cytotoxic levels was not observed. It is pertinent to this discussion that CMI(S) in the three groups followed a similar biphasic pattern.

Specifically, after very early low levels of cytotoxicity, tumor cell lysis by lymphocytes reached a maximum level around 21-26, followed by a rapid decline in activity by days 31-33. This was in turn, followed by a second peak of cellular response around days 39-40.

The very early latency and increase in cellular response is easily understood and consistent with immune reactivity in many other tumor models[177,179,181,186,240], and immune reactions in general. In these experimental systems, cytotoxic activity first appears when growing tumors are already palpable. Several models have been evaluated by Leclerc et al.[186], and this relation between tumor growth and the appearance of cytotoxic lymphocytes was found in all systems tested, whatever the latencies of the tumors (up to 65 days in late MSV tumors). This has suggested that a threshold number of tumor cells is required to trigger the immunologic response. Thereafter the kinetics of the immune reaction may vary with the biology of the specific tumor-host interaction.

Likewise, the fall of immune reactivity around days 31-33 is consistent with several other tumor models[18,240], including the MSV-induced

malignancies in mice.[177-179,181,185,186] While the specific time course differs, cellular reactivity in BALB/c mice bearing MSV-induced tumors follows a similar phasic pattern, attaining a peak of cytotoxicity at approximately 14 days, followed by a progressive decline by around three weeks. It can only be speculated that the differences in exact time sequence in the two systems reflect a difference in species and a slightly different tumor biology and course in the two hosts.

It has also been demonstrated with transplanted tumor cells in the Gross[240] and attenuated Rauscher[18] virus systems that measurable *in vitro* cellular immunity develops early after transplantation, but rapidly disappears as tumor growth proceeds. In the latter system, cellular immunity returned after surgical tumor removal.

While it is easy to cite the similarities in the phasic responses of the various models, it is much more difficult to explain why a decline in cellular reactivity should accompany tumor growth. Lamon *et al.*[179] suggest four possible explanations, which are here briefly enumerated and expanded: (1) Tumor cells may be protected from the tumoricidal effects of immune lymphocytes by blocking antibodies.[141,143] While this mechanism is possible, in this investigation only "helper" effects in the serum of untreated animals were observed, making significant antibody-mediated blocking unlikely. (2) Immune lymphocyte aggregation at the tumor site *in vivo* in growing tumors may diminish *in vitro* cellular reactivity.[7,108,210] This is certainly supported by the findings of Fefer *et al.*[100,104] of intense lymphocytic infiltrates seen in growing MSV-induced tumors. A correlate of this type

of explanation is reported by Alexander et al.[7], who have shown that a growing tumor (a primary chemically-induced sarcoma in rats) induces a specific blockade of the regional lymph nodes, preventing the dissemination of tumor-specific immunoblasts. (3) Immune lymphocytes may be inactivated by antigen overload. This explanation is particularly attractive, and is supported by numerous studies with soluble and tumor-specific antigens.[7,17,34,65,287] It has been clearly demonstrated that tumor associated antigens, in one form or another, may escape from tumor cells and become detectable in the serum of tumor-bearing animals.[318] In the study by Alexander et al.[7] just cited, the lymph node blockade was attributed to the persistent exposure of the nodes to antigen being shed by the tumor.

MSV-induced tumors are known to readily shed such antigens, as well as virus particles.[178,185] In general, with advancing tumor growth, the amount of antigen released increases rapidly.[65] Thus, in the present investigation, decreased immune reactivity (both CMI(L) and CMI(S)) is well correlated with a period of rapid tumor growth (Figures 5 and 12) in the untreated animals.

(4) Finally, Lamon et al. postulate that the viremic state itself may produce depressed lymphocyte reactivity. Since MSV-induced tumors shed infectious virus[104,185], the viremic state of the animal and the presence of virus-neutralizing antibodies may be quite variable influences on the lymphocyte reactivity to this antigen.

Only the first two parts of the CMI(L) pattern have been discussed to this point. The latency and rise in cellular reactivity are readily

explained by a "threshold" principle. The subsequent fall in reactivity *in vitro* may be explained by a variety of phenomena, including lymphocyte aggregation (either at the tumor site or in lymph nodes) and antigenic overload (by released tumor antigens or virus particles). While a combination of these events may be occurring simultaneously, evidence has been presented to support the antigen-overload hypothesis in this MSV system.

The final return to high cytotoxic levels is more difficult to explain and has no precedent in the literature. While it would be convenient to attempt an explanation representing the *reversal* of the above phenomena known to *depress* cellular reactivity, late high levels of cytotoxicity occurred in both regressor and progressor groups of animals, the latter bearing continually enlarging tumors. The reversion to elevated levels of tumoricidal activity in attenuated Rauscher virus systems[18] after tumor excision has been previously noted. In this system, however, antigen load was immediately diminished, allowing cellular responses to return to their early high levels.

Support for a similar biphasic immune response is present in studies of humoral immunity in both MSV and Gross virus-induced tumor systems.[151,178] While the biphasic pattern in the MSV tumor system appeared only in regressor animals, a clear biphasic response was seen in some progressor rats in the Gross virus system.

Considerable evidence suggests that factors operating to depress cellular reactivity also mediate humoral immune depression.[65,178] Conversely, it seems fair to assume that factors (or the absence of

same) allowing the expression of humoral immunity may likewise allow the expression of cellular responses. In this context, although a biphasic cellular response to tumor antigens is to date unreported, the comparison to similar humoral data seems unwarranted. No further explanation of the phenomenon can be offered at this time.

Although effector cells in this investigation have been referred to as lymphocytes, the effector population was in fact a more heterogeneous group of cells, including macrophages as well as T-cells and B-cells. After Ficoll-Hypaque density gradient centrifugation, the effector population was found to contain an average of 92% lymphocytes, 4% monocytes, and approximately 4% polymorphonuclear leukocytes.

Consistent with previous investigations in MSV systems[59,177,179], it is assumed that T-cells played a prominent role in the in vitro cytotoxic reactions. The role of antibody-dependent cellular cytotoxicity (ADCC) was tested in untreated animals through their first 28 days and found not to contribute to tumor cell lysis. While this is suggestive for the unimportance of ADCC in this investigation, previous studies in MSV-mouse systems have shown that ADCC does occur, but that it is not quantitatively significant until approximately 30 days post-infection.[177,179] The presence of active ADCC in the rat model of osteosarcoma after 28 days cannot therefore be ruled out. Further studies to determine the precise effector cells active during the course of disease in this system are clearly indicated.

Humoral Responses - CMI(S):

A significant finding accompanying the study of *in vitro* humoral activity was that serum factors in untreated animals were exclusively "helper" in nature; that is, throughout the course of disease in progressor animals, serum factors increased the cytotoxicity of reaction mixtures over those containing effector cells alone. This was a somewhat unexpected finding, in that the more usual pattern observed in MSV mouse tumor systems is that progressor sera block, while regressor sera increase, cellular cytotoxicity. In addition, the preliminary study by Friedlaender and Mitchell[117] in this rat model reported CMI(S) findings apparently consistent with those seen in MSV mouse systems. The present results are readily explained when the nature of CMI(S) is considered and the earlier results in this model are more carefully scrutinized.

Put simply, CMI(S) is an *in vitro* measure of the net result of factors in the serum potentially beneficial and/or deleterious to tumor cell proliferation. As noted earlier, there are several possibly different antibody populations in the serum of MSV tumor-bearing animals which may act independently or synergistically with cellular responses to kill tumor cells. Cytotoxic antibodies are complement-dependent, and therefore probably play no part in CMI(S) in the heat-inactivated serum of this investigation. This is further supported by the inability of this investigator to demonstrate the presence of cytotoxic antibodies in other *in vitro* assays. Nevertheless, several populations of complement independent antibodies, including "unblocking", "arming", and "cytophilic" antibodies are potentially significant.

The former two groups of gamma-globulins have been convincingly demonstrated in MSV tumor systems. While the inability of antibodies to arm normal lymphocytes through day 28 in this investigation might suggest the absence of significant ADCC, specific antisera may arm immune lymphocytes as well.[177,179] "Arming" antibodies may therefore have contributed to the helper effects of progressor sera in these assays.

In addition, while the relationship between "arming" and "unblocking" antibody populations remains to be resolved[255], unblocking antibodies have been convincingly demonstrated by the Hellstroms in the sera of MSV tumor-bearing mice.[141] Unblocking antibodies may also have contributed to the helper effects seen in the present study.

Macrophage cytophilic antibodies, and antibodies bound to MSV tumor cells observed by immunofluorescence techniques[178] may have been significant. While cytophilic antibodies have not been tested in MSV systems, the presence of 4% macrophages in the effector population of the present investigation makes their a priori exclusion impossible.

Whenever CMI(S) is greater than CMI(L), the quantitative activity of tumoricidal serum factors is greater than serum blocking factors. As noted previously, the correlation of tumor status with in vitro serum effects is not as clear in MSV models, or any models for that matter, as originally proposed.[141,143] Blocking factors may be observed in regressor animals[293], while predominantly helping activity may be seen in progressors.[116,255,256,312] Rates of tumor growth[177,178,255,256,293] and destruction, rather than a more absolute "tumor status"

classification, may be most important in determining CMI(S). Likewise, tumor-inhibitory or tumor-enhancing properties *in vitro* may be a result of concentration effects. Particularly pertinent in this regard are the results of Skurzak *et al.*[293], who studied MSV-induced tumors in Fischer rats. In their studies, blocking was demonstrated in a regressor animal, and, in general, high concentrations of sera were tumoricidal, whereas highly diluted sera caused blocking.

Finally, the apparent discrepancies between the earlier studies and the present investigation in the rat osteosarcoma model are minimized when the preliminary work is more carefully scrutinized. While blocking serum activity was present on days 32 and 34 post-infection in progressor animals, helper activity was clearly evident in progressor animals on days 25 and 37. In addition, blocking activity was observed in a regressor animal on day 65. It seems apparent that no broad generalizations about tumor status correlation with serum activity can be made from these data. In contrast, the seemingly paradoxical finding of helper activity in progressors was consistently observed throughout the course of untreated animals in this investigation. The specific nature of this helping activity remains to be determined.

A second significant finding accompanies studies of CMI(S) -- consistent with cellular data -- showing a biphasic humoral response. The previous findings of a biphasic humoral response in the Gross virus and MSV mouse systems have already been cited. Again, the most attractive explanation for the mid-course nadir of immune reactivity, almost precisely parallel in time to cellular depression, is antigenic

overloading. It is conceivable that all the antibody might be bound up in the form of antigen/antibody complexes[178], which may not only depress cellular reactions, but block humoral activity as well. The other explanations offered for declining cellular reactivity apply also to humoral immunity, and may have contributed to the immune kinetics observed.

The second peak in humoral activity seen in the Gross virus system[151] represented a different globulin fraction (7S) than that observed in the first peak (19S). It is possible that the two peaks of humoral activity observed in the present investigation likewise represented 19S and 7S fractions, respectively. Further studies are necessary to substantiate this possibility.

C. The Dual Effects of Adriamycin

Overview:

As discussed earlier, anticancer drugs usually have immunosuppressive effects in addition to their tumoricidal actions.[209] Immunosuppression in the course of chemotherapy for neoplastic disease may be self-defeating, compromising host defenses often required to effect eradication of the last tumor cells. It is essential to understand which drugs are immunosuppressive, under what conditions of dose and schedule they may cause such immunosuppression, and which aspects of the tumor-immune response are most affected. When the import of given immunological responses to a particular malignancy are better understood, specific drugs which avoid depression of these responses may be

employed. When immunosuppression is unavoidable by an effective tumoricidal agent, therapy in the future may be directed to non-specific or specific reversal of this effect.[157,198] Intensive research efforts are now being aimed at making this theoretical possibility a reality.

MSV tumor systems are models of human disease in which biological course and tumor-specific immune mechanisms have been increasingly well defined. This investigation has further explored the cellular and humoral responses in an MSV-induced rat model of osteosarcoma. Its consistent reproducibility makes it an excellent model to study the immunological effects in vivo and in vitro of anticancer agents.

Major goals of the present study have been to evaluate the efficacy of adriamycin in treating tumor-bearing rats in this system, and to study the effects of adriamycin on tumor-specific immune mechanisms. While adriamycin is a relatively new chemotherapeutic agent, its tumoricidal efficacy has been well documented in a wide variety of human and experimental tumors.[4] Its effectiveness in treating human osteosarcoma is particularly striking.[60,61]

The effects of adriamycin on immune functions are more poorly understood, although several studies indicate that it may depress cellular and/or humoral responses in experimental situations. One in vivo investigation in an MSV-induced mouse tumor system has evaluated the drug's action on tumor-specific immune mechanisms.[45] As reviewed earlier, this report suggested that adriamycin may be somewhat immunosuppressive, in that normally completely regressing tumors showed a fairly high incidence of recurrence. No in vitro data tested the

assumption that recurrence was related to adriamycin's immunosuppressive activity.

The following sections will discuss the results of segments of this investigation designed to evaluate the dual effects of adriamycin in a rat model of osteosarcoma.

Tumoricidal Effects of Adriamycin:

The tumoricidal effects of adriamycin were clearly apparent in animals treated with adriamycin at either 1 mg/kg/day or 2 mg/kg/day for three consecutive days begun after tumors were already palpable. Tumors grew far less rapidly in the treated animals, and average tumor diameters were much smaller in these groups than in the untreated group. After 25 days of age, this difference in tumor diameters was highly significant ($p < .001$).

Whereas untreated tumor-bearing rats were usually dead by three weeks of age, treated animals survived significantly longer (median survival in combined treatment group = 77.5 days). The lower dose of adriamycin seemed more effective in prolonging survival (MST = 91+ days) than the higher dose (MST = 63.5 days).

Finally, tumor regression was seen far more commonly in treated animals (12/39) than in untreated ones (0/22). This difference was also highly significant ($p < .001$). Comparable to survival data, the lower dose of adriamycin caused more tumor regressions (7/17, or 41%) than did the higher dose (5/22, or 18%). The difference in the proportion of regressors between treatment groups was suggestive, but not

significant ($p > .05$).

The tumoricidal efficacy of adriamycin has therefore been conclusively demonstrated by diminished tumor growth, increased survival, and increased proportions of regressor animals in the treatment groups. Adriamycin treatment at the lower dose was apparently more active than the higher dose in increasing the latter two indices of efficacy.

Effects of Adriamycin on Tumor Immunity:

The present section will discuss the following two probable effects of adriamycin on tumor-specific immune mechanisms as evidenced in the present investigation: (1) that adriamycin, at the doses and schedule used, and as tested by a particular *in vitro* method, is minimally suppressive to cellular tumor-specific immune mechanisms; and (2) that through a complex interrelation between drug, tumor, and humoral immunity, adriamycin has elicited *in vivo* and *in vitro* enhancement.

There are no published reports to date on the effects of adriamycin on *in vitro* syngeneic tumor-specific cellular immune mechanisms. An *in vitro* investigation by Orsini and Mihich[241], however, studied the effects of adriamycin, administered within 2 days before or after antigen priming, on cellular and humoral responses to sheep red blood cells and allogeneic tumor cells. While both responses were somewhat inhibited, under certain conditions adriamycin had selective effects on cellular immunity. In these respects, adriamycin was equivalent in its immunosuppressive activity to daunomycin, while being less suppressive than Ara C or methotrexate.

In contrast, the results of the present investigation show no significant cellular suppression by adriamycin administered for three days beginning on day 10 after virus inoculation. Maximum cellular cytotoxic levels in the two treated groups (61% and 50% in the lower and higher dose groups, respectively) were comparable to maximum levels observed in the untreated group (56%). In addition, the kinetics of the cellular responses were similar in the three experimental groups, revealing early peaks of activity followed by rapid decreases to low levels.

Second peaks of CMI(L) were observed in two of the three groups, while cellular responses in the group treated with the lower doses of adriamycin continued to decline. Differences in the assay of this group's cellular response (see Results section) may explain this apparent discrepancy. However, that this continued decline may have been due to delayed adriamycin related immunosuppression cannot be excluded. Both long term[4] and cumulative[79] toxicity by adriamycin is known to occur; for example, in people administered a single dose of the drug, leukopenia may not reach a nadir until 14 days later. In rodents especially, high drug levels are maintained for long periods of time in many tissues and organs, including lymphoid centers.[77,336] Nevertheless, studies in other experimental systems[161] suggest that adriamycin's immunodepressive activities are dose-related. As this seems likely, an even greater suppression of cellular activity would be expected in the group treated with the higher dose of adriamycin. This was not observed.

Why, then, the disparity between these results and those of Orsini and Mihich? First, the timing of drug administration may have been important. In the earlier study, adriamycin was administered either on the day of antigen inoculation or within two days before or after -- just at the time of lymphocyte recognition and proliferation, and an especially vulnerable period for immunosuppression. Other studies have likewise shown a predilection for immunosuppression by adriamycin which was highest soon after antigen administration.[161] Conversely, adriamycin in the present investigation was administered 10 days after virus administration, at a time when immune responses are less vulnerable to attack.[209] Second, the doses used in the present study, while therapeutically effective, may have been sufficiently low to avoid immunosuppression. Notable in this regard is the fact that slightly higher doses (3 mg/kg/day), given on the same schedule, rapidly killed the animals by two weeks of age. Finally, species and tumor differences may have been significant in the lack of cellular suppression noted with adriamycin in this sytem.

In contrast, three results of the present investigation suggest that there was a (possibly dose-related) tumor enhancement phenomenon associated with adriamycin treatment: (1) Whereas only helper activity was demonstrated in the serum of untreated tumor-bearing animals, early helper activity in the sera of both treatment groups consistently and significantly converted to blocking activity by day 22; (2) Treated animals had significantly more long bone metastases than untreated animals, and the onset of these metastases corresponded precisely in time with maximum <u>in vitro</u> blocking activity. Higher dose adriamycin-

treated animals had more long bone metastases than lower dose-treated animals; and (3) There was a (nonsignificant) longer survival and higher incidence of regressor animals in the group treated with the lower, rather than higher, doses of adriamycin. It is here postulated that these results may bear a common pathogenesis, and that this pathogenesis is a result of a complex relationship between drug, tumor, and humoral immune factors. More specifically, it is proposed that the tumoricidal effects of adriamycin may have caused, via cell lysis, the shedding of tumor antigens which, in turn, have increased the blocking activity in serum of treated animals. Concomitant direct effects on antibody populations by adriamycin may have increased the tumor enhancing properties of these sera. Direct effects include the reasonable possibility that adriamycin has depressed helper antibodies, or conversely, and unlikely, the possibility that adriamycin (comparable to 6-mercaptopurine, cited earlier[341,342]) has selectively increased blocking antibody populations.

The striking finding from in vitro assays in this investigation was that, from day 22 onward, serum factors from treated animals decreased tumor-specific cellular immunity. This blocking activity was a conversion from helper activity on day 15 in both groups, three days following treatment with adriamycin. After day 22, blocking activity persisted throughout the course in the serum of animals treated with adriamycin at 2 mg/kg/day, and reverted to (nonsignificant) helper activity on days 42 and 48 in the group treated with lower doses of adriamycin.

It is pertinent to return to a discussion of the nature of factors measured by CMI(S). CMI(S) was described earlier as the net result of factors in serum potentially beneficial and/or deleterious to *in vitro* tumor cell proliferation. Factors deleterious to tumor cell proliferation include the various cytostatic and arming antibody populations. Whenever CMI(S) is greater than CMI(L), these factors quantitatively predominate in the serum tested. If these factors alone are present in a given serum, their eradication will never cause CMI(S) to be less than CMI(L); *i.e.*, the absence of a helper effect will not cause blocking.

Thus, it is obvious that serum which causes CMI(S) to be less than CMI(L) has a predominance of "blocking factors" in it at the concentrations used in the assay. It may additionally have a shortage of "helping factors" (arming antibodies, etc.) but it must have factors which block CMI(L). Blocking *in vitro* is usually associated with *in vivo* enhancement and widespread disease.

Simple as this may seem, a voluminous literature has arisen attempting to define the nature of blocking factors, the conditions which cause their appearance, and their relation to enhancement of tumor growth *in vivo*. The phenomenon of enhancement was first noted in 1907 by Flexnor and Jobling, who proposed the presence of a "tumor-promoting factor" to explain the unanticipated growth of transplanted tumors in rodents.[105] Originally considered merely a laboratory curiosity, this "factor" has more recently become recognized as a significant *in vitro* and *in vivo* phenomenon accompanying rapid tumor growth in both experimental tumors and man. Several excellent recent articles have exhaustively

reviewed the literature on blocking factors and enhancement;[105,142,147,158] however, only a brief overview is pertinent to this discussion.

As described earlier, blocking factors may be antibody, antigen, or complexes of the two. Blocking antibodies appear to be related to the specific γG_2 subclass of immunoglobulins. Antigens involved in enhancement include the H antigens, particularly those of the H-2 locus, in mice; organ-specific antigens; and most significantly, tumor-specific antigens. Of the last group, tumor antigens causing enhancement may be autochthonous, or induced by either viral infection or chemical carcinogens.

Blocking factors may affect afferent, efferent or central processes. That is, blocking may occur at the target cell, effector cell, or central lymphoid centers.

Early studies by the Hellstroms emphasized the role of antibody which covered target tumor cells and shielded them from attack by cytotoxic cells.[141,143] To accomodate other data, "unblocking" antibody, which reversed the "blocking" antibody activity had to be evoked.[144] Later, evidence was presented that the blocking factors mediating enhancement may more frequently be antigen-antibody complexes[292] shielding either the target, or more likely, the effector cell.

Some would suggest that the concept that antibody in either manner facilitates tumor escape <u>in vivo</u> should now be abandoned.[5] Support for their position includes reports that bursectomy and the consequent impairment of antibody production did not affect tumor progression in

chickens.[64,67,191,266,317] Nevertheless, it is the prevailing opinion (shared by this investigator) that: (1) antibody mediated blocking does occur, but that it acts on effector, and not on target cells;[105,310,311] and (2) antigen-antibody complex mediated blocking is a more common phenomenon than antibody blocking alone.[147] In view of recent data[5,64,67,191,266,317], however, it is felt by this investigator that the role of antibody in causing enhancement should be de-emphasized.

In contrast, evidence is mounting that antigen-associated blocking is a more significant, and more common, event.[5,7,17,64,65,67,191,266,317] Indeed, Alexander has proposed[5] that antigenic shedding by malignant cells, as well as by embryonic tissues, may be a fundamental mechanism of their escape from immune destruction. He cites as evidence the fact that rat sarcoma cells that shed antigen rapidly metastasize much more readily than other sarcoma cells (of equivalent immunogenicity, and in the same animal strain) with a slow spontaneous release of antigen. Soluble antigens, in addition, may be poor immunogens, but may effectively inhibit both antibody and cytotoxic cells.[5]

Alexander[5] has reviewed the evidence that there are three distinct mechanisms by which antigen gains access to the circulation in a soluble form: (1) By autolysis of tumor cells; e.g., as a result of a subcutaneous inoculum of the cells. (2) As a consequence of immune attack; i.e., via lysis of tumor cells consequent to host immune reactions. (3) By spontaneous release. Some of the evidence for spontaneous shedding of tumor-associated antigens has been previously described in

this Discussion.

This investigator would suggest a fourth potentially significant mechanism of antigen release -- that caused by the tumoricidal action of a cytotoxic drug. Heretofore, cytotoxic drugs have been considered only beneficial in this regard.[212] That is, by decreasing tumor size, tumoricidal agents ultimately diminish antigen load, and, to the extent that antigen participates in blocking activity, tumoricidal agents will eventually decrease enhancement. This is logical, and in most cases is undoubtedly true.[212] However, in the process of cell lysis by the drug, consistent with the other mechanisms of soluble antigen release, an overwhelming burden of soluble antigen must surely be swept into the circulation. It is therefore postulated that tumoricidal agents may paradoxically cause two antagonistic enhancement-related effects: an increase in blocking activity when tumor growth is rapid and drug cytotoxicity is quantitatively of greatest magnitude, and a later decrease in blocking activity when tumor burden is small and/or cells have become resistant to the drug's tumoricidal effects.

Returning, at long last, to the rat model of osteosarcoma, it is proposed that the blocking activity seen in the sera of rats treated with adriamycin was secondary to antigenic shedding caused by the tumoricidal actions of adriamycin. Early in the disease course when tumor burden was small, these antigenic shedding effects of adriamycin were apparently quantitatively less than the tumoricidal effects of serum seen in both treated and untreated animals at about two weeks of age, when CMI(S) was consistently greater than CMI(L). While untreated

animal tumors grew rapidly, however, at about three weeks of age a plateau occurred in the graph of treated animal tumor diameters (Figure 5). This corresponds precisely in time not only with blocking activity in vitro, but with the onset of long bone metastases in tumor-bearing treated rats as well. In addition, blocking activity persisted in animals treated with the higher doses of adriamycin (with presumably greater and more persistent tumoricidal activity), while it reverted to helper activity in the group treated with the lower doses (consistent with persistent later helper activity in untreated animals). The seemingly greater survival time and higher incidence of regressor animals in the group treated with the lower dose (vs. higher dose) of adriamycin is also consistent with the antigen shedding hypothesis.

The significantly higher frequency of long bone metastases in treated animals is particularly fascinating. Again, it was seen that animals treated with the higher dose (vs. lower dose) of adriamycin developed a greater number of long bone metastases. Interestingly, the finding of increased metastatic disease associated with cytotoxic drug treatment has been reported on several occasions in both experimental tumors and man.[38,72,73] Deodhar[72,73] noted a significant enhancement of metastases in a mouse sarcoma tumor model in animals treated with prednisone, cyclophosphamide, Ara C, or asparaginase. With asparaginase, metastatic enhancement was noted with doses that were considerably smaller than those required to produce immunosuppression.[73] Even more important are the results of consecutive day adriamycin treatment on tumor growth and metastases in the spontaneous mammary carcinoma model in C3H mice noted in the Introduction.[79,82]

Whereas alternate day treatment strongly inhibited tumor growth, consecutive day treatment increased the incidence of metastatic disease over untreated controls, and did not prolong survival.

Antigenic shedding caused by drug treatment may also be clinically significant in man. Pertinent in this regard is the report (cited earlier) demonstrating earlier and more recurrences, as well as decreased survival, in patients with resected bronchogenic carcinoma treated with cyclophosphamide chemotherapy, over patients left untreated.

Other mechanisms are possible to explain the results of the present investigation: (1) Adriamycin may have suppressed humoral immunity in addition to causing antigenic shedding. The ability of adriamycin to depress antibody production has already been cited. That humoral immunity may be significant in preventing the dissemination of disease has been demonstrated by Alexander.[6] After cannulating the thoracic duct and draining lymph from sarcoma-bearing rats, he returned only lymphoid cells to the animals (retaining the removed immunoglobulins) and found a significantly increased incidence of metastases over control animals. While antibody suppression may have played a role in metastatic enhancement in the present model, diminution of CMI(S) to blocking levels can only be explained by the presence of additional blocking factors -- possibly released tumor antigens. (2) Adriamycin treatment may have caused a selective increase in blocking antibodies. This explanation seems unlikely. The fact that adriamycin suppresses humoral immunity, and the decreasing likelihood that antibody-mediated blocking is a very significant event, both force one to reject this possibility.

(3) Adriamycin may have caused cellular immunosuppression which was undetectable in the *in vitro* assay used in the present investigation. This explanation seems reasonable, and is consistent with other studies demonstrating that minimal immunosuppression by mechanisms other than cytotoxic drugs (ALS treatment, sub-lethal irradiation) may enhance metastasis formation.[72,74,306] Again, additional mechanisms are needed to explain the blocking activity seen in the sera of treated animals. (4) Finally, and similar to the above explanation, minimal immunosuppression by adriamycin *per se* may have enhanced tumor formation. This refers to the "immunostimulation" theory of Prehn[259,260], which suggests that "a little immunity may be good for a tumor (but that a lot is bad)".[259] This hypothesis, as previously noted, has gained little support, and therefore is considered an unlikely mechanism to explain the results of the present investigation.

In summary, a study of the effects of adriamycin on tumor-specific immune mechanisms has resulted in two significant conclusions: (1) Adriamycin was minimally suppressive to cellular responses at doses and a schedule which retarded tumor growth and prolonged survival times; and (2) adriamycin use was associated with *in vivo* and *in vitro* enhancement. It has been proposed that cytotoxic drugs may have a dual effect on the phenomenon of enhancement relative to their actions against tumor cells and consequent antigen load. It has been postulated that adriamycin treatment in the present study has caused, via its tumoricidal effects and antigenic shedding, an increase in serum blocking activity and a higher incidence of long bone metastatic disease. These enhancement phenomena were possibly dose-related. That

the net effect of adriamycin treatment was beneficial, however, is obvious, as tumors in treated animals progressed more slowly and regressed more frequently than those in untreated animals. Treated animals survived significantly longer.

It is clear that our understanding of the complicated interrelationships between cytotoxic drugs, tumor-immune mechanisms, and neoplastic cells is far from complete. Critical evaluation of appropriate experimental tumor systems may aid in the understanding of these relationships, so that host defenses against malignancy can be exploited to the fullest degree possible, and so that chemotherapeutic drugs may be used in the most rational manner.

V. Conclusion

The present investigation was designed to achieve three general goals: (1) To document the extent of similarity of the MSV-induced rat osteosarcoma model to osteosarcoma in man; (2) to evaluate the immunobiology of this tumor-host system, with an emphasis on the kinetics of tumor-specific immune responses;and (3) to evaluate the effects of adriamycin chemotherapy on the course and immune reactivity of tumor-bearing animals.

This study substantiated the initial report of Friedlaender and Mitchell suggesting the excellence of the rat osteosarcoma system as a model of the human disease. The tumor was osteoid-producing, initially localized to a long bone of an extremity, and rapidly fatal. The present investigation extended the study of the nature, incidence and distribution of metastatic dissemination. Pulmonary metastases, which are an integral aspect of the human disease, were documented in 89% of necropsied animals of all ages. The tumor metastasized to a lesser degree to the liver, lymph nodes, and other long bones.

Tumor-specific cellular and humoral responses were present in all animal groups tested from 14 to 48 days of age. Both CMI(L) and CMI(S) exhibited a biphasic course, with an early peak of cytotoxicity around days 21-26, followed by a rapid decline by days 31-33. This nadir was, in turn followed by a second peak of immune reactivity around days 39-40. Serum factors in untreated animals were consistently

"helper" in cytotoxic function.

Adriamycin treatment at two doses was effective in retarding tumor growth and prolonging median survival times. Significantly, its use was not associated with suppression of tumor-specific cell-mediated immunity. In addition, however, its use was associated with a possibly dose-related in vivo and in vitro enhancement phenomenon, as treated animals developed significantly more long bone metastases than controls and exhibited blocking activity in their sera. It is postulated that this enhancement was caused by adriamycin-induced antigenic shedding by the tumor, with the consequent formation of serum blocking factors.

The rat osteosarcoma system warrants further critical evaluation as an excellent model of a human malignancy. The present investigation has posed several new questions. It is important to know the nature of the effector cells involved in CMI(L), the antibody populations responsible for the paradoxical helper activity observed in the sera of progressor animals, and the cause for the biphasic immune kinetics. More precise definition of these questions and others will undoubtedly increase the utility of this system, which has already enhanced our understanding of the intricate relationships between drug, tumor, and host immunity.

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