V. Schirrmacher R. Schwartz-Albiez (Eds.)

Cancer Metastasis

Molecular and Cellular Biology, Host Immune Responses and Perspective for Treatment

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Preface

These are the Proceedings of the 2nd International Metastasis Congress of the Metastasis Research Society which took place in the town hall (Stadthalle) of Heidelberg, FRG, in September, 1988. This first Metastasis Congress in the FRG was organized in conjunction with the German Association of Cancer Research (SEK) of the German Cancer Society. The congress topic generated tremendous interest and attracted about 400 scientists from 22 countries. Most participants came from Europe, Israel, and the United States.

Why did we organize the Metastasis Congress? Only about 50% of all people who develop some form of cancer are curable. Despite improved patient care and increasingly innovative and effective techniques for diagnosing and treating primary cancers, the development of secondary cancer colonies, i.e., metastasis, can not be prevented and is the major cause of death. In the Federal Republic of Germany there are still as many as 160 000 cancer patients per year who succumb to their disease, often after periods of terrible suffering, and this overall figure is not improving. Partly because of the complexity of the process, basic research on metastasis has lagged behind other disciplines such as carcinogenesis and cancer genetics. Metastasis formation involves the ability of malignant cells to invade adjacent tissue and to penetrate into lymphatic or blood circulatory systems, or both, and to spread to near or distant sites to form new tumor colonies. Meanwhile, research on metastasis is receiving much attention. This congress was initiated to exchange information and to summarize where we stand in 1988 in this important, newly developing field of cancer research.

The congress was devoted to the following urgent questions: I. What are the genetic mechanisms and driving forces of tumor progression? II. How do genetic changes in malignant cells relate to programs of normal cell differentiation? III. What is the molecular basis of cancer adhesion, invasion, homing, and growth control? IV. How does the host's immune system recognize tumor cells and affect metastasis formation? V. Are there new approaches to the treatment of cancer metastases based on a biological concept?

These and other issues were dealt with in about 100 lectures and in some 200 posters which were on display during the whole congress. The first part of the Proceedings consists of the manuscripts of the invited speakers of the five main symposia. The second part contains the chairpersons' summaries of the nine poster sessions; 70 abstracts were selected for oral presentation in the respective mini-symposia.

Progress has been achieved in unraveling the diverse cellular properties of metastatic cells, including their cell surface components, enzymes, secreted components, antigens, adhesion and recognition components, chromosome markers, and hormone and growth factor receptors, as well as other properties. These findings were discussed in the context of tumor–host interactions, of influences of hormonal and immune status, and of microenvironmental factors which could influence the cancer cells phenotype via signal transduction.

The proposal that cancer metastasis depends equally on tumor and host characteristics dates back to S. Paget's famous "seed and soil hypothesis" which was formulated in 1889. The importance of the anatomical location of a growing cancer, of its surrounding microvascular systems, and of more mechanistic hemodynamic aspects was later emphasized by J. Ewing in 1928. The Metastasis Research Society has now created a scientific award for excellent research in cancer metastasis, the Paget-Ewing Award. It was a very special occasion when at this year's Metastasis Congress in Heidelberg the first award of the society was given to two other pioneers in the field, namely, Dale Rex Coman and Irwing Zeidman. These Proceedings include laudationes by Kurt Hellman, the founder of the Metastasis Research Society and of the Paget-Ewing Award, and Isaiah J. Fidler, one of Zeidman's former students.

This congress has shown that metastasis research has become a specialized field of cancer research of high scientific standing. The clinical aspects of the problems were addressed during the main congress, but we felt it important to devote a specialized satellite workshop to this problem and to provide an opportunity for intensive discussions between clinicians and basic research scientists. The main topic of this satellite workshop, which preceded the main congress, was: "Clinical Perspectives for the Treatment of Metastases". These Proceedings also include a summary of the satellite workshop and its discussions.

We like to thank a number of people and institutions for help and support: the members of the board of the Metastasis Research Society and of the SEK, the invited speakers and the chairpersons, the international scientific advisors, the local organizing committee, the congress secretariat, Zietemann GmbH, the publishers Taylor & Francis and Springer-Verlag, and all members of the Department of Cellular Immunology at the German Cancer Research Center. We owe special thanks also to a number of companies who supported the congress either directly or through participation in the industrial exposition. For financial support we also thank the German Cancer Research Center, the German Cancer Society, and the European Association of Cancer Research.

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Opening Plenary Lectures

Metastasis: The Clinical Problem

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The clinical problem is that metastasis causes most of the therapeutic failures from the common solid neoplasms. This paper will attempt first to describe the magnitude of the metastasis problem using common solid neoplasms as examples, next to focus on the problem of defining metastatic potential, including a brief discussion of recent DNA analyses in relation to prognosis, then to describe some of the characteristics of the biology of metastasis as observed by clinicians, the mechanisms of which could be hypothetically exploited in future therapies, and finally to challenge the researcher/clinician with some of the major problems in metastasis therapy.

Symptoms as Indication of an Invasive Neoplasm with Potential for Metastasis

Patients who seek medical diagnosis have a complaint. The myriad of clinical symptoms associated with solid tumors (i.e., cough, bleeding, dysphagia, pain, etc.) all indicate sufficient neoplastic invasion of the afflicted organ (i.e., bronchus, colon, esophagus, sensory nerves, etc. respectively) to create the symptom (Fig. 1). Depending on the intrinsic metastatic potential of the neoplasm metastasis may be present. The defense of "early diagnosis" is not borne out by studies of colon cancer which indicate a poorer prognosis and more advanced stages of colon carcinoma in patients with short symptom durations, of less than 3 months as compared with patients whose symptoms last longer than 3 months – 6 or twelve months (McDermott et al. 1981, Stubbs and Long 1986).

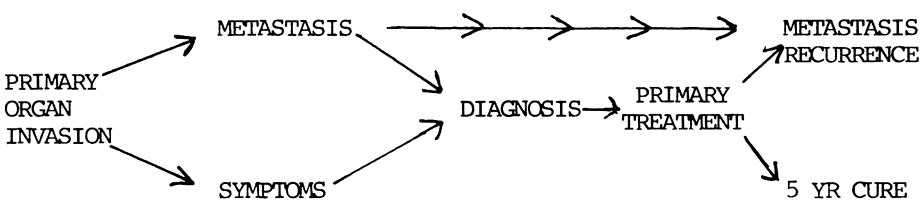

Fig. 1. Scheme of alternatives when clinical symptoms indicate potential metastasis

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Table 1. Adenocarcinoma of colon. Status of "all comers" at diagnosis; Botsford et al. (1971); McSherry et al. (1969); Moertel et al. (1969)

	%	Prognosis
Inoperable (no surgery)	7%	9.5 months
Inoperable (intra-operative)	30%	
L. M. only = 10%, local fixation, carcinomatosis, D. M. ± L. M. = 20%		
Resectable for cure	63%	45%–50%
Dukes stage A	5%–10%	80%
Dukes stage B	40%–50%	40%–60%
Dukes stage C	40%–50%	6%–25%

Magnitude of Metastasis Problem in Colorectal Cancer

Table 1 summarizes the clinical outcome in several large series of patients which include all patients presenting to the respective institutions. In 37% of over 1000 patients from these combined series macrometastasis was present as liver metastasis or peritoneal seeding (carcinomatosis) at diagnosis precluding curative treatment. Some 63% had surgical resection in absence of any detectable distant metastasis with overall cure rates of 45%–50% in resected patients. In each Dukes stage many patients succumbed to the evolution of gross metastatic disease from occult subclinical metastasis present at the time of surgery. Overall 30%–31% were cured by surgery.

Magnitude of Metastasis in Lung Cancer

As has been summarized by Minna et al. (1985; Table 2) nearly 50% of patients present with established macrometastasis and 22% with regional spread. Only 17% presented with apparently localized disease at the primary pulmonary site. The overall 5-year survival was only 8% for males and 13% for

Table 2. Overall extent of disease at time of presentation and overall 5-year survival for lung cancer (DHEW 1977)

Stage of disease in % (Cases diagnosed 1970–73)		5-Year survival in % (Cases diagnosed 1965–1969)	
		Male	Female
Local	17	28	51
Regional spread	22	10	15
Distant metastases	48	<0.1	<0.1
All stages		8	13

females. The problem of metastasis dominates the therapy of other solid neoplasms such as carcinoma of the breast, squamous cell carcinoma of the head and neck area, soft tissue sarcomas, etc.

Metastatic Potential in Clinical Cancer

The outcome of treatment for malignancies treated with curative intent is largely dependent on the intrinsic metastatic potential of the neoplasm. An accurate assessment of specific metastatic potential is critical to the physician–patient interface where individual treatments are prescribed. The spectrum of adjuvant therapies, where effective, need more precise application. Additionally better assessment of metastatic potential increases the validity of randomized clinical trials where stratification of important prognostic variables is part of study design.

Determinants of Metastatic Potential – clinically localized primary tumor

There are several determinants of metastatic potential for a primary tumor. The site or organ of origin is remarkably important even for identical histologies. Mucosal melanoma is almost always fatal, while cutaneous melanoma is often cured. Squamous cell carcinoma of the skin is relatively innocuous, while a similar histology in the lung is more often fatal. This effect of the site of origin is not conceptually dissimilar to the seed–soil hypothesis of Paget related to differential metastatic growth in distant organs. The size of the primary tumor correlates with regional metastatic potential in breast carcinoma. However, for most malignancies arising from an epithelial or luminal organ surface, the depth of cancer invasion rather than size is the important determinant of metastatic potential (bladder, melanoma, squamous cell carcinoma of the head and neck, stomach, colon, cervix, endometrium, etc.; Sugarbaker 1987).

DNA Studies and Metastatic Potential

Histologic abnormalities of stained tumor DNA contribute significantly to the pathologists' microscopic grading of a given malignancy. High-grade tumors exhibit increased metastatic potential and the converse is also observed. Automation of subjective visual grading by flow cytometry or static cytophotometry has been accomplished and many human tumors have been studied (Seckinger et al. 1988; Sugarbaker 1987). For solid tumors in which modal DNA content is nondiploid (or aneuploid) prognosis is diminished as compared with diploid tumors within each stage of a given solid neoplasm. For stage I (node negative) carcinoma of the breast a 20%–30% decrease in prognosis is reported by many authors. In stage II (node positive) aneuploidy decreases survival by 30%–40%. In colon carcinoma aneuploid tumors show 40%–60% greater recurrence or mortality rates in Dukes stage B and C. In identical stages of

nonsmall cell carcinoma of the lung 30%–50% differences are reported. Similarly, highly statistically significant differences in prognosis are found between diploid and aneuploid tumors in stage III of the ovary (Friedlander et al. 1987), stage Ib, II, and III carcinoma of the cervix (Jacobsen 1984), malignant melanoma (Kheir et al. 1986), and many other tumors.

Tumor proliferation characteristics, S-phase, and proliferation index (S, G2+m) are also of independent prognostic significance, particularly in breast carcinoma (Seckinger et al. 1988) and also in lung carcinoma (Volm et al. 1988). These important prognostic parameters provide a clinically useful prognostic enhancement of current histopathological staging systems. Evolving multiparameter flow cytometric analysis in addition to DNA indicate promise for further progress in even more accurately assessing metastatic potential of human solid tumors.

Regional Node Characteristics Predictive of Systemic Failure

Table 3 lists characteristics of lymph node metastasis which have been associated with poor treatment outcome. Ploidy characteristics(!) have been shown to add significantly in Dukes stage C carcinoma of the colon and stage II breast carcinoma as well as stage III ovarian carcinoma (Seckinger et al. 1988). However, it is to be noted that in some patients positive regional lymph nodes do not convey a negative prognosis. For instance, regional node metastases from papillary carcinoma of the thyroid has no effect on treatment outcome (Sugarbaker 1987). Also in carcinoma of the breast it was found that only approximately 37% of microscopically identified regional lymph nodes would grow to identifiable clinical status with 5-year follow-up (Sugarbaker 1987). Therefore, it is important not only to predict regional metastatic disease, but also assess growth potential.

Table 3. Regional lymph node characteristics predictive of systemic metastasis

1. Size (occult versus gross)
2. Number and location
   a) "Sentin"l versus secondary level
   b) Ipsilateral versus bilateral
3. Extent of nodal invasion
   a) Clinical
      Mobility versus "fixed" edema
   b) Histopathologic
      Pericapsular sinus versus replacement
      Extracapsular invasion
      Vascular, lymphatic, neural, soft tissue invasion
4. Biologic
   a) Historical growth rate
   b) Synchronous versus metachronous with primary
   c) "Growth potential" of occult metastases

Characteristics of Metastatic Biology in Humans

The course of uncontrolled metastasis is overall, and unfortunately, quite predictable in the clinical setting, with patient demise within an expected time frame the rule. However, exceptions to this generalization are often dramatic and, likewise, other unusual aspects of the metastatic process can be striking when observed in the clinical setting. It can be hypothesized that an understanding of the basic mechanisms of these variants of clinical metastatic biology could be exploited with therapeutic intent, and for this(!) reason some of these characteristics of metastasis have been studied extensively in the laboratory. Table 4 lists some of the salient observations recorded regarding human metastatic biology (Sugarbaker 1979; Sugarbaker et al. 1982).

Table 4. Characteristics of biology of metastasis in humans: possible therapeutic relevance of mechanisms

1. Varying metastatic potential related to clinical stage
2. Patterns of metastasis-anatomical and organ trophism, organ rejection of metastasis
3. Trauma can localize metastasis to unusual sites
4. Phenotypic heterogeneity, stability, ploidy
5. Occasional explosive metastasis after primary tumor removed
6. Dormancy/reactivation metastases

Patterns of Metastasis: Organ Selection/rejection

A review of the patterns of metastasis in human malignancies (Sugarbaker 1981) supports a dominantly mechanical mechanism with rigid anatomic definition for lymphatic metastasis and usually hematogenous metastasis to the next organ reached by venous effluent from the primary tumor. However, notable exceptions occur. Metastatic melanoma seeks the brain, nonregional subcutaneous sites, and submucosa of the intestinal tract. Ocular melanoma metastasizes essentially exlusively to the liver; adenocarcinomas of the gastrointestinal tract often find the ovary. Many other examples are present in human metastasis. Perhaps of greater potential significance is organ rejection of metastasis. Experimental studies indicate that injected cancer cells reach all perfused organs, yet a preferential organ pattern of metastasis emerges. How do organs reject viable malignant cells? Can this rejection mechanism be enhanced in antimetastatic therapies? Several hypotheses have been developed (Sugarbaker 1981). Appealing amongst these hypotheses is the embryonic homing of cells recalled in the metastatic phenotype.

Variations in the usual pattern of metastasis in the clinical setting are sometimes observed when trauma localizes metastatic disease (Derhagopian et al. 1979). Is this mechanism unique to traumatic sites? Is there a common mechanistic pathway in inflammation or wound healing and the metastatic process? What about macrophages? (Alexander et al. 1987).

Phenotypic Heterogeneity with Relatively Stable Ploidy

Consistent with observations first made in experimental model systems (Fidler 1974, Sugarbaker 1970) metastases from solid neoplasia exhibit phenotypic heterogeneity for morphology, estrogen receptor status, pigment production in melanoma, and a myriad of other phenotypic characteristics. Additionally, the metastatic phenotype has demonstrated remarkably unstable evolving chemoresistance and immunoresistance with alarming rapidity, adding to the complexity of the therapeutic problem. Can phenotypic diversity be reversed with maturational agents? Does this aspect of metastatic behavior reflect a malignant variant of uncontrolled reversion to the embryologic cellular diversification process?

In this setting of phenotypic diversity the crude genetic indicator of ploidy has remained remarkably stable within large primary tumors and during clinically detectable phases of metastatic progression. In the clinically observable phases of malignancy this DNA characteristics has been stable, with more than 90% retention of the original DNA histogram of the primary tumor (Seckinger et al. 1988).

Heterogeneity is also seen with differential host resistance to the primary tumor and metastases, suggesting the evolution of immunologic heterogeneity (Sugarbaker 1979; Sugarbaker et al. 1982).

Occasional Explosive Metastasis After Removal of Primary Tumor

Though more frequently observed in sarcomas and melanomas (but still an infrequent clinical observation), resection of the primary tumor is sometimes followed by explosive systemic metastasis. These metastatic "explosions" have led to hypotheses that a primary tumor partially regulates host systemic tumor mass, much as liver regeneration after resection proceeds only to a biologically relevant total volume. This phenomenon is experimentally demonstrable, but mechanisms remain controversial (Sugarbaker et al. 1977).

Dormancy and Reactivation of Metastatic Growth

Melanoma, breast carcinoma, renal cell carcinoma, and thyroid carcinoma, among others, not infrequently develop very slowly. Currently, theories of host immunologic control are predominantly being used to explain this observation and have fostered progress in the large field of immunotherapy. Adding to this is the occasional spontaneous regression of metastatic disease. What are the characteristics of the "cocoon" of dormant tumor cells? Are there fruitful analogies in infrequently recrudescent parasitic diseases such as malaria and filarial diseases? Are regenerating organs such as skin (wound healing) and liver regeneration subject to the same mechanism for recall or resurgent growth as are these dormant metastatic cells? Molecular biological research in the field of growth factors may provide clues to this mechanism.

Cured Systemic Metastasis

A curious, but therapeutically interesting aspect of metastasis is that a small percentage of apparently systemic metastases are "cured" by a simple surgical resection. In selected series, 20%–50% of isolated lung metastases, 20%–50% of isolated liver metastases, and some other systemic metastasis are cured by resection alone. These are remarkably good survival statistics and the question arises as to how a systemically metastasizing disease has become localized in a single organ and is still amenable to surgical cure.

In this area of molecular genetic characterization of biologic events with definition of the molecular biology affecting such observations, it is hoped that the mechanisms of these events will be identified and hopefully therapeutically exploitable for future antimetastatic therapies.

Major Problems for Metastasis Therapy and Research

Therapy of Macrometastasis – A Formidable Challenge

As discussed metastasis has occurred at the time of clinical diagnosis of symptoms. Macrometastases are a highly significant problem (defined as metastases clinically apparent or diagnosed by imaging techniques). Macrometastases, at least in terms of crude genetic studies (ploidy by flow cytometry), reflect characteristics of the primary tumor which a priori had metastatic potential. Therefore, research studies of metastasizing versus nonmetastasizing primary tumors may be more fruitful than studies of primary tumors and metastases in the autochthonous host. Therapies of macrometastases are deficient (with rare exceptions of the resectable lesion), but combined modalities reaching 4–5 logs cancer kill could be therapeutically effective if additional immunotherapies could be used to "clean up" the final residual heterogenous cell population. Neoadjuvant chemotherapy (before surgery) is currently being applied in the treatment of breast carcinoma and lung carcinoma, but the results as yet are not available.

Diagnosis, Range of Volume, Assessment of Growth Potential in Subclinical Metastasis

Subclinical disease (defined as metastatic disease below clinical recognition or detection by imaging techniques) spans a spectrum from a single cancer cell of 10 μm in diameter to ± 1 cm or 10 000 μm – a 3-log increase in diameter or a 7-log increase in the numbers of cells. If adjuvants to local regional therapies are to be devised, and, comprehensive in their effects, significant tumor volume must be eradicated. Possibly combinations of therapies could help with this problem before absolute eradicative therapies for small tumor volumes are employed.

In summary the clinical problem of metastasis is a formidable challenge. Clinical metastasis encompasses a broad spectrum of malignant disease more complex to this observer's perspective than all the experimental systems of tumor metastasis as yet described. Macrometastasis and subclinical metastasis cause most clinical failures in cancer treatment.

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The Nude Mouse Model for Studies of Human Cancer Metastasis

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Introduction

The progressive growth of metastases represents the lethal event in the clinical course of most neoplastic diseases. While primary tumors can be resected or treated locally, systemic treatment for disseminated disease has not produced satisfactory results. Several reasons can be cited for this failure. First, at the time of diagnosis and surgical resection of primary tumors, metastasis may have already occurred, but as lesions too small to be detected. Second, the location of many metastases limits our ability to selectively deliver therapeutic agents to them without damaging normal tissues. Third, the heterogeneous nature of malignant neoplasms leads to the rapid development of metastases that are resistant to conventional therapies (Poste 1986; Fidler and Balch 1987; Nicolson 1987).

By the time of diagnosis, human neoplasms can contain multiple populations of cells with differences in such properties as growth rate, antigenic and immunogenic status, cell-surface receptors and products, response to cytotoxic agents, invasion, and metastatic potential. Recent data from our laboratory and many others indicate that metastases can arise from the nonrandom spread of specialized subpopulations of cells that preexist within the primary tumor (Fidler and Kripke 1977), that metastases can be clonal in their origin (Talmadge et al. 1982), and that different metastases can originate from different progenitor cells (Talmadge et al. 1982) or even from a single surviving cell (Fidler and Talmadge 1986). These data partially explain the clinical observations that, even within the same patient, different metastases often exhibit different susceptibilities to chemotherapy and radiotherapy.

The majority of the above-mentioned studies have been carried out with rodent tumor systems. Whether or not human tumors are heterogeneous for invasion and metastasis remained unanswered. Animal models have proved to be invaluable for the elucidation of various host factors and tumor properties involved in the pathogenesis of metastasis. The use of such models could enhance our knowledge of the biology of metastasis and thus contribute to the development of new approaches to the therapy of metastasis. However, adequate animal models for in vivo studies of human neoplasia in general, and of metastasis in particular, have been lacking. The discovery that human xenografts can be heterotransplanted into athymic T-cell-deficient nude mouse

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(Rygaard and Povlsen 1969) has encouraged in vivo investigations of human cancer metastasis. However, most human tumors transplanted into nude mice by intravenous, intraperitoneal, subcutaneous, or intramuscular routes rarely produce metastasis, regardless of their clinical behavior in patients (Sharkey and Fogh 1984; Fidler 1986). Some of the failures may have been caused by the high levels of natural killer (NK) cell activity in nude mice (Naito et al. 1987), a level that has been associated with inhibition of cancer metastasis in mouse systems (Hanna 1982). As a consequence, we have used NK-cell-deficient, 3-week-old mice to study the metastatic behavior of human tumors and in so doing demonstrated that metastatic formation by human cancer cells is dependent upon their route of inoculation into nude mice. In general, orthotopic implantation of tumor cells (i.e., implantation into organs involved in the original anatomical environment of the tumor) was the major factor determining whether human melanoma (Kozlowski et al. 1984a), human colon carcinomas (Giavazzi et al. 1986a, b; Morikawa et al. 1988a, b), prostatic cancer (Kozlowski et al. 1984b), and human renal cell cancer (Naito et al. 1986, 1987) would produce metastasis in nude mice.

The Use of Nude Mice to Ascertain the Malignant Potential of Human Colon and Rectal Carcinomas

The choice of postoperative therapy of human colorectal cancer (HCC) depends on whether the cancer produced metastasis prior to its surgical excision. Since histopathological examinations do not produce unequivocal evidence for metastatic potential, we wished to determine whether the nude mouse could be used as a model to determine the malignant potential of individual HCC (Giavazzi et al. 1986a, b; Jessup et al. 1988). To do so, human tumor cells from primary neoplasms and from metastases were injected into different organ sites of nude mice. The implantation of HCC cells into the subcutis or musculature of nude mice produced only local tumors regardless of the malignant potential in the patients. We implanted cells from more than 70 different HCC into the subcutis of more than 700 nude mice, and in only one mouse did we find macroscopic evidence of visceral metastases at the time of autopsy. Moreover, growth of HCC in the hind thigh with or without amputation of the local tumor also did not produce frequent metastases. Only in a very few mice did we find lung metastases at 6 months after tumor implantation, resection, and local tumor recurrence.

These results prompted us to question the validity of implanting HCC into the subcutis or muscularis of nude mice. Although implantation at these sites was relatively easy to accomplish, its relevance to the biology of colorectal carcinoma appeared to be questionable. A potential solution was suggested from earlier studies by Kozlowski et al. (1984b) who injected human tumor cells into the spleen of mice and thus produced extensive liver and lung metastases. We confirmed and extended these results by the use of tumor cells directly isolated from surgical specimens of HCC. The implantation of the HCC cells into the spleen of nude mice allowed us to distinguish between cells

isolated from primary neoplasms with low malignant potential and cells isolated from liver metastases. Specifically, by days 30–40 after injection of cells isolated from patients' metastases, the nude mice became moribund because their livers had been replaced by neoplastic growth. These growths were ascertained to be HCC by morphologic, isozyme, and karyotypic analyses. No gross liver metastases were found in nude mice killed 40 days after the intrasplenic injection of cells from primary Dukes' stage B carcinomas. However, if nude mice injected with primary colon carcinoma cells were allowed to survive for up to 90 days, few liver metastases were found. Thus, if the assay was terminated after 30–40 days, qualitative differences between metastatic and nonmetastatic cells were observed.

We next compared the malignant potentials of different HCC cells isolated from a primary tumor, from a lymph node metastasis, and from hepatic metastases. Again, all the cells were implanted into the spleens of athymic nude mice. Cells isolated from the liver metastases produced rapid and consistent growth of the human cells in the liver. Cells derived from a primary colon cancer produced tumors in the spleen of injected nude mice, but exhibited a lower malignant potential as measured by the ability to produce liver tumor foci. The human origin of all tumors growing in the spleens and livers of injected mice was repeatedly ascertained by both isoenzyme and karyotype analyses. Collectively, the results suggest that the implantation of malignant HCC cells into the spleen of athymic nude mice can produce tumor growths in the liver, an organ most often involved by metastasis from these neoplasms.

The Use of Nude Mice to Demonstrate the Metastatic Heterogeneity of HCC

By the time many colorectal cancers are diagnosed and surgically excised, micrometastases are already present in lymph nodes and the liver (August et al. 1984; Weiss 1985). The prognosis of a patient with colorectal cancer metastasis is generally poor because no effective systemic therapy is currently available (August et al. 1984). In order to develop new therapeutic approaches for this disease, the metastatic heterogeneity of HCC must be studied. Specifically it is necessary to know whether human colorectal carcinomas are heterogeneous for metastatic properties and whether the nude mouse model can be used to isolate highly metastatic subpopulations of human colorectal cells. To do so, we obtained a surgical specimen of a Dukes' stage B_2 primary colon carcinoma and enzymatically dissociated it (Morikawa et al. 1988a). Cells derived by this procedure were directly established in culture or were injected into the subcutis, cecum, or spleen of nude mice. Progressively growing tumors were excised and dissociated, and lines were established in culture. Subsequent to implantation into the cecum or spleen of nude mice, cells from all four lines produced only a few liver tumor foci. HCC cells from the few liver metastases were expanded in culture and then injected into the spleen of nude mice to provide a source for further cycles of selection. With each successive in vivo selection cycle, the metastatic ability of the isolated-propagated cells increased. Four cycles of selection yielded cell lines with a very high metastatic efficiency

in nude mice. Differenccs in spontaneous metastatic capability between cells of the primary nonselected lines and cells of the successively selected lines were also found for tumors growing in the wall of the cecum. Nonselected cell lines produced visible mesenteric lymph node metastases in 50% of the injected mice and no hepatic metastases. Cells isolated from hepatic tumor foci (following implantation into the spleen) exhibited increased metastasis to regional lymph nodes and to the liver (Morikawa et al. 1988a).

The metastatic potential of the HCC cells in the nude mice was determined by two different assays. The first involved the implantation of cells into the spleen and the production of liver tumor foci (experimental metastasis). The second assay measured the ability of HCC cells to produce lymph node and liver metastases subsequent to implantation into the wall of the cecum (spontaneous metastasis). The term experimental metastases refers to tumor colonies produced after the i.v. injection of cells. Although these tumor cells do not go through the initial steps of metastasis (separation from primary neoplasm and invasion into blood vessels), all the subsequent steps in the metastatic process must occur for metastases to become established. Elimination of the initial steps of the process introduces the possibility that noninvasive tumor cells could form metastases when injected i.v., but might be unable to metastasize spontaneously when implanted s.c. However, studies have shown that this is not the case. A good correlation between the ability of tumor cells to produce metastases after i.v. (experimental) and s.c. (spontaneous) implantation has been reported for many heterogeneous murine tumor systems (Talmadge and Fidler 1982; Fidler and Poste 1985) and this was also the case for the HCC system. In general, there was good agreement in the results of the two assays: a cell line highly metastatic to the liver after intrasplenic injection was also highly metastatic to the mesenteric lymph nodes and liver after intracecal injection.

The nude mouse model has been used to select metastatic cells from heterogeneous nonselected murine neoplasms (Pollack and Fidler 1982). Our present results confirm these findings and show that the nude mouse can be used to isolate and expand metastatic subpopulation of cells from human neoplasms. The isolation of HCC cells with enhanced metastatic ability should be a useful model for studying tumor and host interaction that control metastasis.

The Nude Mouse Model for Studies of the Biology of HCC Metastasis

In the next set of studies, we determined whether the biological and metastatic behavior of tumor cells isolated from fresh surgical specimens of HCC are influenced by the isolation method and the organ site of implantation and growth in nude mice (Morikawa et al. 1988b). Cells of parental Dukes' stage B_2 HCC were injected into the spleen or cecum of nude mice to produce experimental and spontaneous hepatic metastases, respectively. HCC lesions were harvested from livers of nude mice and established as individual cell lines in culture. The selection cycle for cells implanted into the spleen was repeated four times. Cells of the parental and the selected variants were injected into nude mice by different routes: intravenously and subcutaneously and into the

cecum and into the spleen. Subsequent to implantation into the spleen, all cell lines were shown to be tumorigenic. Cells from the selected lines produced a significantly higher number of experimental liver metastasis than the parental cells. However, subsequent to injection into the cecum, only cells of the line selected for spontaneous metastases produced spontaneous liver metastases. These results demonstrate that the orthotopic implantation of HCC cells into the appropriate organ environment can be used for the efficient isolation of metastatic subpopulations of HCC cells.

The production of cancer metastasis by human tumor cells in nude mice can be enhanced by implantation into anatomically relevant organs (Kyriazis et al. 1978; Fidler 1986). Thus, the implantation of melanoma cells into the skin (Kozlowski et al. 1984a; Kerbel et al. 1984), HCC into the spleen or cecum (Giavazzi et al. 1986a, b; Bresalier et al. 1987; Morikawa et al. 1988a, b), renal carcinoma cells into the kidney (Naito et al. 1986, 1987), pancreatic carcinoma into the spleen or pancreas (Vezeridis et al. 1985), bladder cancer into the bladder (Ahlering et al. 1987), lung cancer into the lung (McLemore et al. 1987), and prostatic carcinoma into the spleen (Kozlowski et al. 1984b) has been associated with higher rates of tumorigenicity and, in many instances, distant spread. These data as well as others suggest that if nude mice are to serve as useful models of invasion and metastasis by human tumor cells the cells must be implanted orthotopically, i.e., into organs involved in the original anatomical environment of the tumor. Thus, the implantation of cells from HCC cell lines into the colon of the nude mice was associated with invasive and metastatic behavior. In contrast, the intrasplenic injection of HCC cells was most advantageous for determining the ability of these cells to grow in the liver.

Conclusion

The heterogeneous nature of metastatic human neoplasms can now be studied under defined conditions in healthy athymic nude mice. The neoplasms must be free of mouse pathogens, and the mice must be kept in specific-pathogen-free conditions. Careful consideration must be given to the intimate tumor–host relationship for each tumor system studied because the metastatic potential of human neoplasms can vary with the site of implantation. The healthy young nude mouse can be a useful in vivo model for ascertaining the metastatic potential of human neoplasms, for selecting and maintaining cell variants of high metastatic potential from heterogeneous human tumors, and for studying therapeutic agents directed against metastatic cells proliferating in visceral organs.

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Symposium I:
Genetic Control of Tumor Progression

*Tumor Cells Genetically Tagged with Transferred DNA Markers Reveal Evidence for Clonal Dominance of Primary Tumors by Metastatic Subpopulations**

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Introduction

Despite the evidence that neoplasms begin with the oncogenic transformation of a single normal cell they virtually always become genotypically and phenotypically heterogeneous by the time they are clinically detectable (Nowell 1976). This is thought to occur through the continuous emergence of new variant subclones as a result of random or induced genetic mutations combined with selection of the more aggressive of these new subpopulations (Nowell 1976). As a result, tumor progression can be viewed as a Darwinian-like microevolutionary process. There is evidence that variant cell subpopulations having competence for metastasis or manifesting certain kinds of drug resistance arise as a result of this process (Nowell 1976). With respect to the metastatic phenotype, a prediction of this model is that cells recovered from metastases would on average manifest a greater metastatic ability than tumor cells recovered from the corresponding primary tumor. This prediction was fulfilled, as first reported by Fidler (1973). As a result of this pioneering study it has become routine to compare cells of primary tumors and their metastases as a means of determining the cellular factors which govern expression of metastatic behavior.

Unfortunately the conclusion that metastases represent a stable subpopulation of cells within the primary tumor has been vigorously challenged by a large number of investigators, usually on the basis of their failure to select variant subpopulations having greater metastatic behavior from a variety of primary tumors. This work has been reviewed by Weiss (1986) and Hill et al. (1986). As a result, there still exists considerable controversy and confusion about the nature of the metastatic phenotype and how best to study it. Thus, Hill et al. (1986) and Ling et al. (1985) have proposed that although metastatic cells are genetic mutants, the phenotype is highly unstable, i.e., it is prone to rapid

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reversion under nonselective conditions – the so-called "dynamic heterogeneity" model of metastasis.

With the benefit of hindsight it is easy to understand that establishing the nature of the genetic or lineage relationship of primary tumors and metastases requires a more direct approach than simply evaluating their relative metastatic propensities upon retransplantation. An example of such an approach would be a method in which metastatic and nonmetastatic cell subpopulations could be "labeled" so that their individual fates could be traced during the course of primary tumor growth and progression. We have devised such a procedure, which we summarize here along with the main findings. It has helped uncover the genetic basis of tumor progression and metastasis. This work has been published in several recent papers (Kerbel et al. 1987; Korczak et al. 1988; Waghorne et al. 1988) and a detailed review (Kerbel et al. 1988). Therefore only the main findings will be summarized, in brief, in this paper.

Random Integrations of Transferred DNA Can Be Used to Generate Large Numbers of Uniquely Tagged Tumor Cell Clones

It is well known that DNA molecules transferred into eukaryotic cells by various types of transfer techniques integrate into the host genome in a random manner. As described by Waghorne et al. (1988) and Price (1987) this fact can be exploited as a convenient way of genetically marking cell populations. Examples are abundant, especially with respect to the use of retrovirus vectors as a means of genetically tagging cells to study cell lineages during early embryonic development, in the nervous system and in hematopoiesis (Price 1987). Prior to 1987, this type of technology had yet to be applied to tumor cell populations in any systematic way. Studies from our laboratory (Kerbel et al. 1987) and by Talmadge and Zbar (1987) represent the first such attempts to do so.

With respect to our own initial studies (Kerbel et al. 1987) we found that calcium phosphate mediated transfection of a mouse mammary adenocarcinoma called SP1 with the plasmid pSV$_2$*neo* resulted in the acquisition of spontaneous metastatic potential by a significant proportion (almost 20%) of the transfectants (Kerbel et al. 1987). In one experiment we pooled about 50–100 individual pSV$_2$*neo* transfectants and grew the cells to mass culture. The pooled population was then injected subcutaneously into a number of individual CBA/J mice. As expected, when the pooled population was analyzed by Southern blotting (using pSV$_2$*neo* as a probe) and BamHI as the restriction enzyme (which cutsondes within pSV$_2$*neo*) a faint, diffuse smear was observed. This is, of course, reflective of the presence of a large number of individual (and therefore uniquely tagged) transfectants, any of which was not present in a high enough proportion (i.e., >10%) of the cell population so as to be able to visualize its distinctive "genetic signature" (i.e., restriction pattern) of hybridizing bands. However, if the pooled population was cloned, each clone was found to manifest two distinctly different bands and each clone manifested a distinctive restriction pattern. This reflects the random integration of a single

copy of the pSV$_2$*neo* plasmid in the transfectants; we found little evidence for integration of multiple head-to-tail random assay repeats of the plasmid.

When primary tumors and spontaneous lung tumors were removed from several mice and analyzed we were surprised to find they consisted of the progeny of the *same* single clone (which we called "*neo*5"). If other clones were present, they could not have comprised more than 5%–10% of the cells. Thus it appeared that the primary tumors were effectively overgrown or "dominated" by the progeny of a single clone; moreover, this clone was competent for spontaneous metastatic spread (Kerbel et al. 1987). It would thus appear that metastatic SP1 cells had a selective advantage for growth *in the primary tumor,* as well as for dissemination from the primary tumor and establishment of new growths at distant sites such as the lungs.

We next performed additional experiments to confirm the validity of this finding (Waghorne et al. 1988). For example, we found that if an excess of nontagged, nonmetastatic SP1 cells was mixed with tagged *neo*5 cells and the mixtures were injected into CBA/J mice, the *neo*5 cells manifested a pronounced growth advantage in the primary tumor. We calculated the growth enrichment to be about five- to ten fold over a 4–6 week period (Waghorne et al. 1988). We performed a similar mixing experiment using an independent genetically tagged metastatic clone of SP1 called "C1". It had been obtained by transfection of SP1 cells with an activated human H-*ras* gene (Waghorne et al. 1987, 1988). Once again we found a significant growth enrichment for the metastatic clone at the primary site, which in this case was calculated to be about 20–50-fold (Waghorne et al. 1988).

The growth advantage of the metastatic clones in the primary tumor was observed regardless of the site of inoculation. Thus, if the cells were injected into an "orthotopic" site (the mammary fat pads) as opposed to an "ectopic" site (the subcutis) the same results were obtained (Waghorne et al. 1988). This is important since it is known that the tumorigenic and metastatic properties of various types of tumors can be enhanced if injected into an orthotopic site (Fidler 1986). Since this has been shown for breast cancer (e.g., Miller 1981) it could be argued that the overgrowth of a single mammary adenocarcinoma clone after subcutaneous injection may be an artifact due to an inability of most clones to grow in a "foreign" ectopic site.

Further experiments demonstrated the considerable potential of using this approach to study kinetic aspects of clonal evolution and selection during primary tumor growth. We refer to time course analyses in which animals were injected with a mixture of large numbers of independent genetically tagged pSV$_2$*neo* transfected tumor cells and the resultant primary tumors removed at various periods thereafter (Waghorne et al. 1988). These experiments demonstrated that the clonal dominance of the *neo*5 metastatic clone could effectively take place over a period of 4–5 weeks.

Genetic Tagging of Tumor Cells Using Retrovirus Vectors

We have also successfully employed retrovirus vector mediated gene transfer as a means to genetically tagging SP1 cells (Korczak et al. 1988). The advantage of this technique stems from the fact that it is a highly efficient method of transferring genes, (e.g., up to 100% of cells in other systems can be successfully infected in some cases) so that exceptionally large numbers of genetically tagged cells can be obtained in a single-step selection. Indeed, in one experiment we pooled up to 10^4 uniquely tagged SP1 cells after infection with a retrovirus vector called $\triangle p \triangle eMoTN$ (Korczak et al. 1988). Even so, we still found that the resultant primary tumors obtained 6 weeks after s.c. injection of this mixture were effectively comprised of the progeny of just one or two clones. These results demonstrated the astonishing degree of selection that can take place when cultured tumor cells are injected into animals and raises the specter of excessive tumor "growth inefficiency" in vivo. Weiss (1986) has for some time put forward persuasive evidence and arguments for the concept of "metastatic inefficiency". This refers to the concept that only a fraction of the cells which detach from a primary tumor and gain access to the bloodstream or lymphatics ultimately form distant metastases. Our results suggest the same type of inefficiency may be true for growth at the primary site as well, at least if one uses cultured tumor cells for injection into animals. In any case, our results indicate the enormous potential that plasmid transfected or retrovirus vector infected genetically tagged tumor cell populations have for exploring the nature and extent of selection pressures on tumor growth in vivo or in vitro.

Possible Mechanisms of Clonal Dominance of Primary Tumors by Metastatic Subpopulations

An obvious question our results raise is the mechanism by which metastatic clones are so easily able to achieve "clonal dominance" in primary tumors. An equally obvious answer would be that they simply have a shorter doubling time. Thus, given enough time (i.e., enough population doublings) they would eventually overtake the primary tumor. If this was so, however, we would expect to see shorter lag times and faster rates of tumor growth in vivo of metastatic clones compared to their nonmetastatic counterparts. In fact we could not detect any such difference (Waghorne et al. 1988). It may be that differential doubling times are only achieved when metastatic and nonmetastatic clones grow in the presence of one another. Thus, it is theoretically possible that the presence of the nonmetastatic component somehow stimulates the division of the metastatic cell component. Conversely, the metastatic cells may inhibit the division of the nonmetastatic cells. This type of interclonal or cell–cell interaction, of which there are many examples in tumor progression literature (Heppner et al. 1983; Poste and Greig 1982), could arise through differential release and response to growth factors such as transforming (TGF)α, TGFβ, growth factor (PDEF) etc. This is currently under investigation in our laboratory.

Before concluding this section we would draw attention to the fact that there are a number of reports in the literature which suggest that clonal dominance of primary tumors by metastatic cell subclones can take place during the growth and progression of some human neoplasms. The most notable of these are the studies of Johnson, Riethmüller and their colleagues (e.g., Holzmann et al. 1987). They have defined, using monoclonal antibodies, two different antigens (P3.58 or I-CAM-1, and MUC-18) which are associated with advanced melanoma. Thus, the antigens are *not* detected on normal melanocytes, dysplastic nevi melanocytes, or radial growth phase (early) primary melanomas. They *are,* however, detected on metastatic melanomas and advanced vertical growth phase (late) primary melanomas. The results are consistent with the notion that some selective advantage enables subpopulations early in primary lesions that have metastatic competence to eventually overgrow their less or nonmalignant counterparts. Similar conclusions have been reached by Herlyn and his collaborators after studying human malignant melanoma (Herlyn et al. 1985; Kath et al., submitted for publication). These observations are entirely consistent with the model of clonal dominance we have described here.

Implications of the Results for Studies in Tumor Biology and Metastasis

Because primary tumors can be overgrown by initially cryptic subpopulations of metastatically competent tumor cells, it is obvious that any genotypic or phenotypic comparison of primary tumors and their metastases may, or may not, reveal any differences depending upon the stage at which the primary tumor is removed and analyzed. Thus, highly advanced primary lesions – such as "thick" (>3 mm) vertical growth phase (VGP) melanomas – may be identical in almost every respect to distant melanoma metastases. In contrast, less advanced lesions – RGP melanomas or "thin" VGP lesions – may be quite phenotypically distinct from metastases. From this it is also easy to see why in some cases it may be impossible to select more aggressively metastatic variants from primary tumors (Talmadge and Fidler 1982). However, failure to do so, or to detect any differences between primary tumors and their metastases does not justify a dismissal of the concept of the selective nature of the metastatic phenotype – as some investigators have concluded (e.g., Vaage 1988).

The results, and considerations, also highlight one obvious source of biological variability when a large number of independent primary tumors of the same histological origin are analyzed. If a particular parameter under study is related to the metastatic phenotype, then the results of an analysis of a given primary tumor may be affected by the extent to which it is clonally dominated by metastatically competent cells. Hopefully an awareness of such a possibility will be taken into account in future studies and will serve to bring about greater consistency in the experimental results of such analyses. This view also highlights the need to develop comparative staging systems for experimental animals tumors, of which few, if any, presently exist.

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*Oncogenes and Tumor Progression: State of the Art**

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Are Classical or Specific Oncogenes Implicated in Malignancy?

Among tumors, benign and malignant ones can be discerned. The difference between both types is of the utmost importance for prognosis and therapy. Malignant tumors invade the surrounding tissues, lymph and blood vessels. Ultimately, they may become metastatic when they colonize distant sites. There exists circumstantial evidence that invasiveness and metastatic capability represent steps during natural tumor progression, apart from immortalization and tumorigenicity per se (Mareel and Van Roy 1986). Therefore, it might be expected that besides "classical" oncogenes (Weinberg 1985; Bishop 1987), separate "oncogenes" – so-called invasion or metastasis genes – play a role in the acquisition of full malignancy. Recently, DNA from malignant human tumors was transfected into a recipient tumor cell with no or low inherent metastatic ability (Glenn et al. 1988; McMenamin et al. 1988; Radler-Pohl et al. 1988) in hopes of defining, isolating, and characterizing malignancy genes as well as classical oncogenes. Alternatively, cells expressing classical oncogenes are very often found to be fully malignant.

Correlating Oncogene Action with Tumor Progression

Several approaches have been used in order to demonstrate functions of classical or new oncogenes in the invasion-metastasis cascade. One corresponds to the analysis of oncogene activation and expression in benign versus malignant forms of the "same" cancer type. This approach can be followed both with natural human tumors and with experimental animal tumors. A second approach is the direct assessment of the effects of transfected oncogenes on the malignant properties of recipient cells, preferably in a quantitative or conditional way. A third one is the fundamental exploration of the activities of defined oncogene products and the attempt to integrate this knowledge into mechanistic models of tumor progression.

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Oncogenes in Early and Progressed Tumors

Doubtless, oncogenes exist in activated or amplified form in diverse human tumors, though it is less evident that any of these oncogenes is causally or even prognostically related to progressed stages of human disease, as indicated by several contradictory reports. Alternatively, several animal models exist for two-stage chemical carcinogenesis of, for instance, skin, breast, and liver. Mostly *ras* oncogenes were found to be mutated in a carcinogen-specific way, pointing at early, direct effects of the chemical on the oncogene (Barbacid 1986). Even in the multitude of benign lesions, from which several would never progress, the *ras* oncogene was activated (e.g., Balmain et al. 1984; Brown et al. 1986; Wiseman et al. 1986; Leon et al. 1988). Natural murine benign tumors also were found to carry activated *ras* oncogenes (Reynolds et al. 1986). In B16 melanoma variants with low and high metastatic potential, expression of Ki-*ras* was not found to be changed (Kris et al. 1985). On the other hand, Ki-*ras* was activated in metastasis of a mouse T lymphoma, while it was not in the primary tumor (Vousden and Marshall 1984). Even more interestingly, progression of benign skin papilloma was claimed to be correlated with the activated *ras* oncogene becoming either amplified or homozygous (Quintanilla et al. 1986): a remarkable observation, although not found in all cases of chemical-induced skin malignancy (e.g., Harper et al. 1987).

Malignancy of Oncogene-Transfected Fibroblastic Cells

For the study of malignancy of oncogene-transfected fibroblastic cells the *ras* oncogene has often been introduced in NIH3T3 fibroblastic cells. The usual outcome has been a marked stimulation of artificial metastasis after introduction and expression of an activated v- or c-*ras* oncogene (Bondy et al. 1985; Muschel et al. 1985; Thorgeirsson et al. 1985). However, *ras*-induced spontaneous metastasis, metastasis induced by normal proto-*ras*, the quantitative correlation between *ras*-expression and metastasis, metastasis by occasional 3T3 variants or by nononcogene transfectants are all points of debate (Greig et al. 1985; Egan et al. 1987a; Bradley et al. 1986; Hill et al. 1988).

Variants on the 3T3-*ras* theme are other fibroblastic cell lines like C3H10T1/2, 208F, CCL39, etc., and oncogenes like *scr, sis, fps, mos,* etc., with essentially similar results (e.g., Egan et al. 1987a, b; Spandidos and Anderson 1987; Chadwick and Lagarde 1988). From these reports we do not know if the recipient cells or their spontaneous transformants formed any benign or malignant tumors during long observation periods. Nevertheless, it is generally agreed upon that NIH3T3 and similar cells are preneoplastic and at the border of malignant conversion. We found that NIH3T3 cells were invasive in vitro (Van Roy et al. 1986). Established fibroblastic cells like Rat1, Rat2 and FR3T3 formed invasive and metastatic tumors with long latency periods (Van Roy et al. 1986; Coopman et al. 1989; Reynolds et al. 1987). Artificial metastasis by Rat2 cells was as efficient as by *ras*-oncogene-transfected Rat2 cells (same

number of lung colonies), leading to death in 6 months and in 1 month, respectively (Gao et al. 1987). This implies that:

a) complete clearance of i.v. injected cells in the lungs is by no means as fast as often supposed;
b) malignant conversion in vivo does occur.

The question therefore remains whether the *ras* oncogene product confers de novo malignancy on the recipient cells or merely speeds up their intrinsic tendency of progression by, for instance, promoting ectopic growth. The finding of several increased cellular activities besides proliferation (e.g., protease secretion, enhanced motility, etc.; see below) after *ras*-oncogene transfection might favor the former theory. In contrast, increased growth potential and autonomy of *ras*-transfectants has been described several times as well (see below also).

Experiments with cultures of primary, mostly embryonic, fibroblast-like cells are more unequivocal. Cultures transfected with cooperating oncogenes like *myc* + *ras* invariably yield highly invasive, metastatic tumors, while the recipient cells are nontumorigenic even upon very long observation (Muschel et al. 1985; Van Roy et al. 1986; Garbisa et al. 1987; Mareel et al. 1988a; Storer et al. 1988). Under selected conditions, a single *ras* oncogene can simultaneously immortalize diploid fibroblastic cells and make them tumorigenic, invasive, and metastatic (Spandidos and Wilkie 1984; Mareel and Van Roy 1986; Pozzatti et al. 1986; Muschel et al. 1986; Storer et al. 1988). The mechanistic details remain unknown as they seem to be at variance with the multistage model of tumor progression. The fact that supertransfection of *ras*-transformed rat embryo cells with the E1a gene of adenovirus 2 does not affect tumorigenicity, but completely abolishes (artificial) metastatic potential points at other mechanisms than mere growth promotion by *ras* (Pozzatti et al. 1988); yet, growth of the primary tumor does not necessarily imply growth at an ectopic site. Alternatively, a remarkable correlation was reported between metastasis and type-IV collagenase secretion for these various cell-oncogene combinations (Garbisa et al. 1987). Apparently, the embryonic and the fibroblastic nature of this cell system together create a genetic environment that allows development of full malignant potency by the *ras* oncogene.

Malignancy of Other Oncogene-Transfected Cell Types

Three types of nonfibroblastic cell systems were challenged with exogenous oncogenes: nontumorigenic cells, cells forming benign tumors, and cells forming invasive tumors but with no or low metastatic ability.

Primary secretory cells of the rabbit mammary gland belong to the first type. They become tumorigenic, apparently invasive, but nonmetastatic after coinjection of SV40 DNA and the activated *ras* oncogene (Garcia et al. 1986). The established, epithelioid cell line C127 derived from murine mammary gland is tumorigenic, but is not metastatic after v-*ras* introduction and overexpression

either (Muschel et al. 1985). Still, invasiveness in vitro of C127 cells was considerably enhanced by introduction of papilloma or polyoma virus genes (Mareel et al. 1988a). In contrast, immortalized rat mesothelial cells 4/4RM4 became simultaneously tumorigenic, highly invasive, and metastatic after transfection with the *ras* oncogene (Vleminckx et al. 1988). Preneoplastic hamster epidermal cells became tumorigenic with short latency as well as invasive after introduction of the activated *ras* oncogene (Storer et al. 1986). Even primary human bronchial epithelial cells were reported to become fully tumorigenic, invasive, and metastatic after introduction of a single v-Ha-*ras* gene (Yoakum et al. 1985). Yet, it is doubtful that the latter situation arose without other concomitant cellular changes as a "crisis" was observed in the transfectants as well as the generation of aneuploidy. This is in line with the results of Rhim et al. (1985) who were unable to transform primary human epidermal keratinocytes by Ki-v-*ras* without previous immortalization. Once established, the cells became susceptible to *ras*-induced malignancy.

Cell cultures of the second type with the ability to form fully benign tumors in vivo seem to be rare. Nevertheless, such systems look promising for the study of malignancy as they allow the subtraction of mere tumorigenic effects of activated (onco)genes. The NMuMG cell line was originally derived from normal murine mammary gland (Owens et al. 1974). These cells, if low in passage number, form benign cystadenomas in vivo and are noninvasive in vitro (Mareel et al. 1988a). After introduction of the *ras* oncogene, invasiveness is acquired both in vitro and in vivo, and metastases can be observed readily (Hynes et al. 1985; Mareel et al. 1988a). However, parental NMuMG become eventually malignant after longer observation periods (F. M. Van Roy and M. M. Mareel, to be published). Therefore, the original population was subcloned. The various isolates differed in morphotype, uvomorulin and keratin expression, growth potential in vivo, but during up to 1 year of observation remained either nontumorigenic or tumorigenic but noninvasive. Transfection of these diverse subclones with the *ras* oncogene invariably yielded fully malignant derivatives, pointing at a clear-cut role for *ras* in tumor progression by this particular cell line (Vleminckx et al. 1988; F. M. Van Roy and M. M. Mareel, to be published). It is doubtful that the T lymphoma BW5147, which is noninvasive in vitro and nonmetastatic in vivo, can be considered a bona fide benign cell type, but in this case, too, *ras* oncogene transfection, in a dose-dependent way, converts it to the fully malignant phenotype (Collard et al. 1987).

Finally, oncogenes were introduced in tumorigenic cells of the third type. The latter were locally invasive but scored poorly in either experimental metastasis or in both experimental and spontaneous metastasis. These cell lines included murine bladder carcinoma, murine mamma carcinoma, and human prostate carcinoma. The final outcome after transfection with *ras* or p53 oncogene is generally a rise in spontaneous metastasis (Vousden et al. 1986; Waghorne et al. 1987; Kozlowski et al. 1988; Nicolson et al. 1988; Pohl et al. 1988). However, as might be expected for recipients which have reached that stage of malignancy, transfections with nononcogene genes regularly produced metastasis as well.

Oncogene Activities During Malignancy

The p21 *ras* oncogene product shows homology with the signal-transducing G proteins (Masters and Bourne 1986). This might be the basis for the plethora of activities that has been allocated to this protein in multiple cell systems. Of particular importance for the possible role of *ras* in tumor progression are the following correlates of *ras* oncogene expression : secretion of type-IV collagenase (Thorgeirsson et al. 1985; Garbisa et al. 1987); secretion of cathepsin L (Denhardt et al. 1987); increased cell motility (Varani et al. 1986; Bar-Sagi and Feramisco 1986; Liotta et al. 1986); loss of intercellular communication (Nicolson et al. 1988); increased cellular growth autonomy allowing ectopic growth (e.g., Stern et al. 1986; Zhan and Goldfarb 1986; Chadwick and Lagarde 1988). All these activities might be the direct effect of the *ras* protein, but they might also be the consequence of more general *ras*-induced phenomena, such as changes in membrane glycosylation (Santer et al. 1984; Collard et al. 1985; Dennis et al. 1987), in lipid metabolism (e.g., Bar-Sagi and Feramisco 1986; Wolfman and Macara 1987; Hancock et al. 1988), and in overall cellular gene expression (Owen and Ostrowski 1987).

How oncogenes other than *ras* influence tumor progression is even more speculative. In case of *fps*-enhanced metastasis a decrease of growth factor requirement has been invoked (Sadowski et al. 1988). A number of other oncogenes, but not all, might induce or enhance malignancy by using metabolic pathways at least partly shared by the *ras* oncogene (Egan et al. 1987b). Low expression of the class I major histocompatibility complex (MHC) might favor survival of circulating and colonizing metastatic cells. Apparently, c-*fos* and c-*myc* (proto-)oncogenes play antagonistic roles by upregulating and down regulating, respectively, the MHC, which in turn showed an inverse correlation with metastasis (Kushtai et al. 1988; Versteeg et al. 1988).

Conclusions and Perspectives

A lot of effort has already been invested in order to delineate the exact role of oncogenes in tumor progression and malignancy. As every new day a new oncogene and its new host environment is being discovered, much remains to be explored. What is most striking so far is the almost ubiquitous appearance of activated *ras* oncogene in the invasive/metastatic cell systems analyzed. This is probably mainly due to experimental convenience. Yet, the lesson is that this quite potent oncogene can play an apparently pivotal role during progression of at least some cell systems. There exist exceptions and their list is expected to grow steadily. For instance, we found in the MDCK and NMuMG cell systems good correlation of intrinsic malignancy with reduced uvomorulin and keratin expression in both the absence or presence of *ras* oncogene expression (F. M. Van Roy and M. M. Mareel, to be published). It is clear and not that surprising that other characters are in the play and that the details of this play can vary considerably. Even by applying the most sensitive measurement techniques, the number of malignant tumors bearing *ras* oncogenes turns out to be only a

fraction of the total (Bos 1988). Tumors vary considerably in their overall features. Thus, it is likely that variety exists also in the spectrum of cooperating genes these tumors can offer to a particular oncogene, as well as in the spectrum of (onco)genes they need in an activated form in order to become malignant.

Future experiments could involve even more cell types as well as oncogene types in order to understand better the mechanism of malignancy. They should focus also on the diverse activities described for $p21^{ras}$ and other oncogene products. These oncogenes are powerful tools, but their pleistropic action, even if conditionally expressed, hampers accurate analysis of causal relationships. Therefore, trying alternative approaches seems worthwhile. These include comparison with situations in which malignancy is transiently suppressed or induced by nononcogenic means (e.g., Mareel et al. 1988b); comparison with situations in which only part of the full oncogene potential is either blocked or activated (e.g., by tissue- and cell-specific factors, in selected somatic hybrids, differentiated cells, transgenic animals, etc.); and detailed analysis of the individual oncogene-effected activities, again, in an oncogene-free context (e.g., by cloning and conditional expression of the appropriate effector or inhibitor genes).

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Genetic Analysis of Invasive and Metastatic Capacity in T Lymphoma Cells

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Introduction

The most important characteristic of malignant tumors is their tendency to infiltrate normal tissues and their ability to metastasize. Metastasis formation is a multistep process that probably requires a complex interplay of a large and heterogeneous group of genes, including genes involved in cellular resistance to immunorejection and genes controlling adhesion and invasive potential of cells. At present little is known about the genetic control of invasion and metastasis and the role of oncogenes in these processes. In our studies we have employed mouse BW5147 T lymphoma cells to analyze the genetic basis of invasion and metastasis of tumor cells by means of transfection experiments and somatic cell fusion studies.

BW5147 T Lymphoma

The BW5147 is an immature T lymphoma cell line spontaneously arisen in an AKR mouse. These tumorigenic cells are noninvasive *in vitro* when assayed on monolayers of hepatocytes or fibroblasts and nonmetastatic *in vivo* after tail-vein injection in syngeneic AKR mice. By different means invasive potential can be induced in these cells and a correlation has been established between invasiveness in vitro and metastatic potential in vivo (Roos et al. 1985; Collard et al. 1986).

Oncogene Transfection

It has been shown that transfection of the activated human c-Ha-*ras* oncogene enhanced spontaneous metastasis formation in tumorigenic, but low metastatic mouse mammary carcinoma cells (Vousden et al. 1986) and rat prostatic tumor cells (Treiger and Isaacs 1988). We have demonstrated that transfection of the activated c-Ha-*ras* gene in BW5147 T lymphoma cells gives rise to invasive and metastatic transfectants (Collard et al. 1987a). The induced level of both invasive and metastatic potential in the transfected BW5147 cells correlated well with the level of expression and amplification of the exogeneous *ras* gene.

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BW5147 cells transfected with nuclear oncogenes, like adenovirus E1A or c-*myc,* were noninvasive and nonmetastatic.

These data and many transfection experiments employing normal NIH3T3 cells indicate that in particular the *ras* oncogene may be involved in the expression of the metastatic phenotype of tumor cells (for review see Collard et al. 1988). It is therefore surprising that increased expression or mutational activation of this oncogene has not been detected more consistently in metastatic human tumors in vivo. It remains possible that the effect of the *ras* gene has no relevance to clinical metastasis but is an artifact caused by the inappropriate high expression of a transfected human oncogene in rodent cells. However, even if *ras* does not play a direct role in metastasis of spontaneous tumors, its ability to mimic the activities of as yet unknown genes provides us with an experimental tool to define properties essential for metastasis formation.

DNA Hypomethylation

It has become evident that methylation of cytosine to 5-methylcytosine is important in regard to the differential regulation of gene expression (Cedar 1988). This has been substantiated by the fact that demethylation of DNA as obtained by replacement of cytosine with 5-azacytidine (5-azaC) may result in the expression of otherwise silent genes (Olsson and Forchhammer 1984). In order to investigate whether demethylation of DNA is able to induce invasive and metastatic potential in the BW5147 cell line, these cells were treated for 48 h with low nonmutagenic concentrations (0.2 μg/ml, 1.0 μg/ml, and 2.0 μg/ml) of 5-azaC. Cells were allowed to recover for 72 h and subsequently selected on monolayers of rat embryo fibroblasts for invasive subpopulations as described by Collard et al. (1987b). Only the cultures treated with 0.2 μg/ml 5-azaC yielded invasive subpopulations of cells after five selection rounds. Subclones of these cultures showed different invasive potential (Table 1) and were further analyzed. Five highly invasive clones were cultured for prolonged time (up to 3 months) and showed stable expression of the invasive phenotype. Preliminary results indicate that these invasive cells also produce metastases after tail-vein injection in syngeneic AKR mice. These data indicate that 5-azaC is able to induce invasive potential in the BW5147 T lymphoma cells, presumably by demethylation and thus activation of one or more silent genes.

Table 1. Invasive potential of subclones of 5-Aza-C-treated BW5147 cells after five selection cycles for invasive cells

Clone number / percentage of invasive cells in vitro			
1./ 10%	[a] 6./ 25%	11./ 15%	16./ 10%
2./ 5%	7./ 15%	12./ 15%	17./ 5%
3./ 20%	8./ 10%	13./ 20%	[a] 18./ 40%
4./ 5%	[a] 9./ 30%	14./ 15%	19./ < 1%
5./ < 1%	[a] 10./ 50%	15./ 10%	[a] 20./ 30%

[a] Indicated clones were further analyzed, see text

Mouse-Human T Cell Hybrids

In vivo or in vitro fusion of closely related lymphoid tumor cells and normal host cells resulted in many cases in the emergence of highly metastatic tumor variants (DeBaetselier et al. 1984). We have demonstrated that mouse T cell hybridomas generated by in vitro fusion of BW5147 T lymphoma cells and activated mouse T cells acquired high invasive potential in vitro. All highly invasive T cell hybrids produced widespread metastases upon i.v. injection in syngeneic mice, in contrast to the BW5147 parental cell line (Roos et al. 1985). Based on the results obtained with the intraspecies mouse T cell hybrids, we hypothesized that normal human-activated T lymphocytes or human leukemia T cells, or both, might contain active genes responsible for invasiveness. To demonstrate the existence of such a gene (or genes) and to reveal their chromosomal localization, interspecies human-mouse T cell hybrids were generated by fusion of BW5147 mouse cells with either phytohemagglutinin (PHA)-stimulated normal human T lymphocytes or human acute lymphoblastic leukemic T cells.

The human-mouse T cell hybrids gradually lost invasiveness as a result of chromosome segregation. Cytogenetic analysis of the hybrids revealed a 100% concordance between invasive capacity and the presence of human chromosome 7. Invasive hybrids metastasized after tail-vein injection in nude mice in contrast to noninvasive hybrids and the parental BW5147 cells (Collard et al. 1987b). By using monolayers of rat embryo fibroblasts, we were able to select for the invasive cell populations. Subcloning and repeated rounds of selection yielded invasive hybrids that had retained human chromosome 7 only. Subclones of these cells showed loss of invasive potential in the cells which had segregated this last human chromosome, and thus confirmed that the invasive potential depended on the presence of human chromosome 7.

In summary, our data indicate that one or more genes residing on human chromosome 7 is necessary, and sufficient, for both the establishment and maintenance of the invasive and metastatic potential of the interspecies T cell hybrids.

Human Chromosome 7 and Invasion and Metastasis

In order to identify the DNA sequences on human chromosome 7 responsible for invasion and metastasis, the expression of several proto-oncogenes and genes involved in growth control and T cell functions located on human chromosome 7 was analyzed. The expression of *c-ERB-B1,* encoding the epidermal growth factor-receptor (EGF-R), and the gene coding for the A subchain of the platelet-derived growth factor (PDGF) was analyzed by Northern blot analysis. In addition, the expression of genes like *c-met,* encoding a protein with the structure of a growth factor receptor, and *c-ral,* with strong homology to the *ras* gene family was studied. None of the above-mentioned genes was expressed in our invasive human-mouse hybrids (not shown). Furthermore, we transfected the BW5147 cells with a cloned PDGF-A cDNA (kindly provide

by Tucker Collins 1987). Although both PDGF-A-specific DNA and mRNA were present in the transfected cells, no invasive transfectant was obtained.

Since the BW5147 cell line is an immature T cell lymphoma which does not express a functional T cell receptor (TCR), the human TCR β and/or γ chains, both encoded on chromosome 7, could create an interspecies α-β or δ-γ heterodimer T cell receptor. In such a manner full maturation of BW5147 could result in cells with invasive potential. However no mRNA of either TCR chain could be detected in the invasive human-mouse hybrids, nor any functional TCR using an anti-T3-specific antibody in fluorescence-activated cell sorter (FACS) analysis.

Characterization of the Invasive Lymphoma Cells

In addition to genes located on human chromosome 7 we analyzed several other characteristics of both the invasive and noninvasive hybrids that could be related to their invasive and metastatic capacity. We found that both cell types were insensitive to natural killer (NK) cell activity. Furthermore, no difference in H2 antigen expression could be detected. Similarly the expression of differentiation markers like Lyt-1 and Ly 6.2 was not associated with expression of the invasive phenotype in our BW5147 derivatives.

In collaboration with Dr. Israel Vlodavsky we analyzed the capacity of the invasive hybrids to degrade extracellular matrix (ECM) material formed by bovine aortic endothelial cells (Naparstek et al. 1984). Neither the noninvasive BW5147 cell line nor the invasive hybrids were able to degrade ECM material. In addition no urokinase- or tissue-type plasminogen activator activity could be detected in the conditioned medium of either cell types. So, thus far we have found no differences in properties associated with metastatic capacity between invasive and noninvasive T cell hybrids.

Approaches to Identify Invasion-Relevant Genes on Chromosome 7

In order to identify and isolate the invasion inducing-DNA sequences located on human chromosome 7 we currently employ different techniques. By DNA transfection, with genomic DNA derived from an invasive hybrid cell line harboring only human chromosome 7, we try to transfer the invasion relevant DNA sequences into noninvasive BW5147 cells. Subsequent selection for a cotransfected selectable marker and, after that, for the invasive phenotype should result in invasive transfectants with a reduced amount of human chromosome 7-specific DNA sequences. Repeated transfection rounds should allow isolation of the human DNA sequences responsible for the induction of the invasive phenotype. Although thousands of transfectants were screened, clonally as well as in mass selections, up to this point no invasive transfectants could be isolated. We are currently continuing this type of transfection experiment although we are well aware that success with this powerful technique depends on the size of the relevant gene. In addition we do not know whether

one single or more genes are involved in the induction of the invasive phenotype in our T cell hybrids. In the latter case the transfection approach is hardly feasible.

Therefore we are also analyzing a cDNA library from activated human T cells cloned in an eukaryotic expression vector (kindly provided by DNAX, Palo Alto, Ca. USA). Since activated T cells are inherently invasive and were used to generate our interspecies hybrids with the murine BW5147 T cell lymphoma cell line, this library should contain the human chromosome 7-derived cDNAs from transcripts that induce the invasive machinery of our T cell hybrids. The library is currently analyzed using labeled cDNA from an invasive hybrid with only human chromosome 7 as probe; cDNA from BW5147 is used as a negative control. Clones that are expressed in the invasive hybrids and not in BW5147 are further analyzed for invasion specificity and will be finally transfected into BW5147 cells. Induction of the invasive machinery in BW5147 should prove the isolation of the relevant gene.

Conclusion

We are employing a system in which the induction of invasive capacity in vitro correlates highly with the formation of metastases in vivo. So far, no cell characteristics like increased protease activity or the capacity to degrade extracellular matrix, properties implicated in invasion and metastasis in other tumor systems, seem involved in the acquisition of invasive capacity of our BW5147 derivatives. Although we have no proof that the mechanisms inducing invasion and metastasis in our *ras* transfectants, T cell hybrids and 5 azaC-treated cells are similar, we anticipate that careful analysis and comparison of the different invasive cells will allow us to identify the crucial gene(s), and gene product(s), involved in invasion and metastasis of T lymphoma cells.

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Transforming Growth Factor-Beta: Possible Roles in Carcinogenesis

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Introduction

Transforming growth factor "-beta" (TGF-β) is a multifunctional regulator of cell growth and differentiation which exhibits context dependent biological effects. Thus, *in vitro,* TGF-β can stimulate or inhibit proliferation, promote or block differentiation, and modulate cellular function depending on the cell type and cell environment (Roberts et al. 1988a; Sporn and Roberts 1988). Structurally, the active form of TGF-β is a disulfide crosslinked homodimer with a molecular weight of 25 kDa in the unreduced form, with each monomer being 112 amino acids. TGF-β is synthesized as a 390 amino acid precursor of which the mature peptide comprises the carboxyl terminal sequence (Derynck et al. 1985). This precursor sequence is very important for proper folding and disulfide bond formation of TGF-β to ultimately generate a biologically active molecule.

Through cDNA cloning it is now known that the sequence of mature TGF-β is highly conserved, suggesting that it has an important biological function. The human, bovine, porcine and simian sequences are identical and there is a difference of one amino acid in the murine sequence (Roberts et al. 1988a). TGF-β was first identified as a factor secreted by sarcoma virus-transformed mouse fibroblasts that could confer the malignant phenotype on cells as assayed by promotion of anchorage independent growth (Roberts et al. 1981). However, further work has shown that TGF-β is produced by virtually all cells in culture. *In vivo* TGF-β mRNA is expressed in a variety of normal tissues, such as heart, liver, and kidney, suggesting it plays a role in growth homeostasis (Thompson et al. 1988). Since the most abundant sources of TGF-β in the body are platelets (Assoian et al. 1983) and bone (Seyedin et al. 1985), it appears that TGF-β also plays a role in wound healing and tissue remodeling.

Regulation of TGF-β Action

As discussed above, TGF-β is expressed in many tissues and, similarly, receptors for TGF-β have been found on every normal cell type examined to date (Wakefield et al. 1987). This raises the question of how the action of this potent bioeffector might be regulated. Recent work has shown that TGF-β is secreted by platelets and by cells in culture in a biologically inactive latent form (Miya-

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zano et al. 1988; Wakefield et al. 1988). This form cannot bind to the TGF-β receptor, nor is it immunoprecipitable by antibodies to TGF-β. Biological activity can be conferred on the latent form by treatment *in vitro* with acid, alkali, urea, and sodium dodecyl sulfate (SDS) (Pircher et al. 1986). Recent work showing that the proteases plasmin and cathepsin D can activate latent TGF-β (Lyons et al. 1988) suggests that activation *in vivo* could involve controlled proteolysis.

The latent form of TGF-β secreted by platelets consists of the mature TGF-β homodimer (25 kDa) noncovalently associated with a homodimer of the remainder of the TGF-β precursor sequence (75 kDa) which in turn is disulfide linked to a third protein (135 kDa). The sequence of this third component is reported to contain epidermal growth factor-like repeats (Miyazano et al. 1988). The latent form of TGF-β secreted by the A549 lung carcinoma cell line is similar to the platelet complex (Wakefield et al. 1988). Several possible functions of the latent form include:

a) enhancing the stability of TGF-β which has a very short half-life when the mature form is injected *in vivo,*
b) restricting the actions of TGF-β to cells and tissues which are capable of activating the latent form, and
c) establishing an extracellular pool of inactive TGF-β which can be activated when needed.

Control of Cell Growth

Even though TGF-β was originally described as a growth stimulatory factor for fibroblasts, it is now known that TGF-β can be either growth stimulatory or inhibitory for mesenchymal cells *in vitro* depending upon the growth conditions (monolayer vs soft agar), other growth factors present, and the cell type (Roberts et al. 1988b). For example, TGF-β stimulates the growth of osteoblasts in monolayer culture, but inhibits the monolayer growth of normal rat kidney cells. In contrast, TGF-β generally inhibits epithelial cell growth *in vitro.* Thus, it has been shown to inhibit the growth of liver, lung, intestine, and kidney epithelial cells and keratinocytes (references cited in Sporn and Roberts 1988). Many tumor cell lines are also growth inhibited when active TGF-β is added to the cultures. Since these tumor cells are usually producing TGF-β, sometimes at higher levels than their nontransformed counterparts (Anzano et al. 1985), one would expect their growth to be inhibited in an autocrine manner. Loss of growth regulation by interruption of the autocrine growth inhibitory effects of TGF-β is thought to play a role in malignant transformation as discussed below.

Possible Roles in Carcinogenesis

Figure 1 shows a variety of means by which TGF-β may be involved in the pathogenesis of malignant disease. The autocrine effects deal with the actions

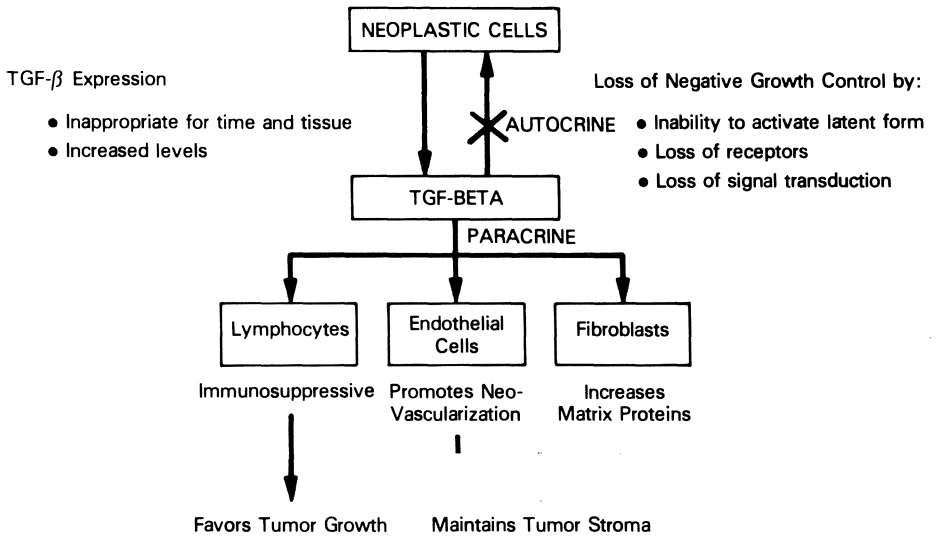

Fig. 1. Possible involvement of TGF-β in the pathogenesis of malignant disease. TGF-β is produced by neoplastic cells which may have lost their ability to be growth inhibited by it in an autocrine fashion. Furthermore, tumor growth may be supported by the ability of TGF-β to act on other cells in a paracrine manner

of TGF-β to control the growth of the neoplastic cells which produce it. The paracrine effects of TGF-β on other cell types may modulate development of the tumor stroma.

Autocrine action

A number of defects may be responsible for the loss of function of growth inhibitory peptides which will cause uncontrolled growth. Under normal circumstances, a growth inhibitory peptide will be synthesized by the cell, and then processed by and secreted from the cell. The secreted growth inhibitor will bind to its receptor on the cell surface and activate a signal transduction pathway which will elicit a growth inhibitory response. Tumor cells expressing alterations at each of these points in the TGF-β negative autocrine loop are shown in Table 1. Thus, cell growth appears to be regulated by a combination of positive and negative effectors, and any change in the negative part of this balanced system may give rise to uncontrolled proliferation and contribute to carcinogenesis.

Paracrine actions

Actions of TGF-β on nonneoplastic cells can also stimulate tumor growth indirectly through enhancement of the development of supporting tumor

Table 1. Transformed cells with possible lesions in TGF-β negative autocrine loop

	Cell type	Lesion
LY-2	Breast carcinoma[1]	No TGF-β secretion
A2380	Pancreatic carcinoma[2]	
A549	Lung carcinoma[3]	Inability to activate latent TGF-β
SCC-25	Squamous cell carcinoma[4]	No TGF-β receptors
Y79, RB13, RB18 RB20, RB22, RB24	Retinoblastomas[5]	
CALU 1, HUT 282 SW900	Lung carcinoma[6]	Post-receptor defect
HUT 460	Lung carcinoma[7]	Growth stimulated by TGF-β

[1] Knabbe et al., unpublished. C Knabbe
[2] Wakefield et al., unpublished. L. M. Wakefield
[3] Wakefield et al. (1988)
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stroma (Fig. 1) by processes similar to those that occur in formation of the granulation tissue of the healing wound. However, while the acute release of TGF-β and other growth factors from platelets at the site of a wound initiates the healing response consisting of inflammation, neovascularization, and fibrosis, tumors provide a continuous source of growth factors that perpetuate the normally self-limiting healing response and support growth of the tumor stroma (Roberts et al. 1988b).

In the immune system TGF-β is strongly suppressive (Mulé et al. 1988 and references therein). It inhibits the proliferation of both T and B lymphocytes and blocks antibody secretion by B cells. TGF-β also depresses the activity of natural killer cells and the generation of lymphokine activated killer cells and cytotoxic T cells. The identification of a TGF-β as the principal immunosuppressive agent in patients with glioblastoma suggest that TGF-β can decrease immune surveillance of the tumor and thereby indirectly favor tumor growth (Wrann et al. 1987).

TGF-β also affects the processes of angiogenesis and neovascularization. *In vivo* TGF-β injection induces the growth of new blood vessels (Roberts et al. 1986), even though TGF-β inhibits the monolayer growth of endothelial cells *in*

vitro. Recently, Madri et al. (1988) have shown that in three-dimensional cultures, TGF-β induces organization of endothelial cells into tubelike structures. Thus, the effects of TGF-β on angiogenesis *in vivo* may result from its ability to organize tubule formation, along with its ability to activate monocytes to release other growth factors which are mitogenic for endothelial cells.

Perhaps one of the most important actions of TGF-β is its ability to increase accumulation of extracellular matrix by several mechanisms. While TGF-β is chemotactic for fibroblasts (Postlethwaite et al. 1987), it also causes them to produce more extracellular matrix proteins, including types I, III and V collagen and fibronectin (Ignotz and Massagué 1986; Roberts et al. 1986). TGF-β also increases the production of proteoglycans by epithelial cells (Bassols and Massagué 1988). Recently, Ignotz and Massagué (1987) have shown that TGF-β can control matrix protein biology by increasing the synthesis of the receptor for fibronectin, and possibly other integrins as well. TGF-β enhances matrix production not only by increasing production of matrix proteins, but also by inhibiting matrix degradation. This is accomplished both by decreasing secretion of serine proteases such as plasminogen activator, as well as by increasing secretion of protease inhibitors such as plasminogen activator inhibitor (Laiho et al. 1986).

Conclusions

It is apparent from this brief review that TGF-β has a wide spectrum of biological effects. Because it controls such important processes, it has very exciting potential therapeutic uses, perhaps as an immunosuppressive agent or in wound healing. Since the uncontrolled growth of certain epithelial cell cancers may result, in part, from an aberration in the normal autocrine growth inhibitory response of the cells to TGF-β, administration of active TGF-β may restore growth control. However, modes of delivering a localized dose of TGF-β which will remain active *in vivo* have yet to be investigated. An understanding of the mechanism of action of TGF-β, along with the factors that control its biological activities, will aid in development of TGF-β as a therapeutic agent.

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Altered Gene Expression in Tumor Metastasis: The nm23 Gene

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Tumor metastasis is a complex process involving tumor cell invasion, locomotion, intravasation and extravasation of the circulatory system, angiogenisis, colony formation, and avoidance of host immunological responses. Two premises have guided our investigation into the genetic influences on tumor invasion and metastasis. First, if the metastatic process is regulated, at least in part, by the activation and deactivation of specific genes, then the multiplicity of cell functions in metastasis dictates that many genes are involved. Second, the biochemical nature of molecules regulating and executing each of the tumor cell functions in metastatis is incompletely understood. Because of the tedious purification process, it is likely that many metastasis regulatory and effector compounds and the genes encoding them are presently unknown. Based on these premises, we initiated differential colony hybridization experiments to identify genes associated with the tumor invasion and metastatic process. This technique identifies genes either activated or deactivated between tumor cells of low and high metastatic potential. It can therefore identify metastasis-related genes in advance of conventional biochemical purification and DNA cloning. This paper describes the identification and characterization of one such gene, *nm23*.

Identification of the nm23 Gene

The selection of an optimal model system is critical to the identification of genes differentially regulated in metastasis. We utilized seven cell lines derived from a single K-1735 murine melanoma (Fidler et al. 1981; Fidler 1984; Kalebic et al. 1988) that exhibit significant differences in both spontaneous and experimental metastatic potential (Fig. 1). This system offers several important advantages for differential colony hybridization:

a) the tumor cell lines are closely related, which optimizes the chance that differentially expressed genes will relate to metastasis and not differentiation, basic metabolic differences, etc.;
b) the use of homogeneous cell lines, as opposed to resected tumors, ensures that the differentially expressed genes identified will not be metastasis-unrelated genes present in various normal cells contaminating the tumors; and

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c) the K-1735 cell lines do not differ significantly in their susceptibility to host T lymphocyte, macrophage, or natural killer cell cytotoxicity (Steeg et al. 1988a). Thus, the different metastatic potentials of the K-1735 tumor cell lines, and differentially expressed genes derived from them, are probably related to the intrinsic aggressiveness of the tumor cells.

A 40 000 component cDNA library was constructured by G/C tailing K-1735 cDNAs into the *Pst*I site of pBR322. Duplicate filters of the library were hybridized to ^{32}P-labeled mRNAs from a low metastatic (clone 19) and a high metastatic (Tk-Eve) K-1735 cell line. Of 24 cDNA clones identified that were differentially expressed between these two K-1735 cell lines, the biology of one clone, pNM23, has been extremely interesting. Expression of the *nm23* gene has been consistently down-regulated in tumor cells of high metastatic potential in four experimental models of tumor metastasis.

K-1735 Murine Melanomas

The *nm23* RNA levels of seven K-1735 melanoma cell lines were determined by Northern blot hybridization (Steeg et al. 1988a). *nm23* RNA levels in the low metastatic K-1735 clone 16 and clone 19 cell lines are ten fold higher than in five related, high metastatic cell lines (Fig. 1). In situ hybridization experiments indicate that virtually all tumor cells express high *nm23* RNA levels, as opposed to a subpopulation of cells (Steeg et al. 1988a).

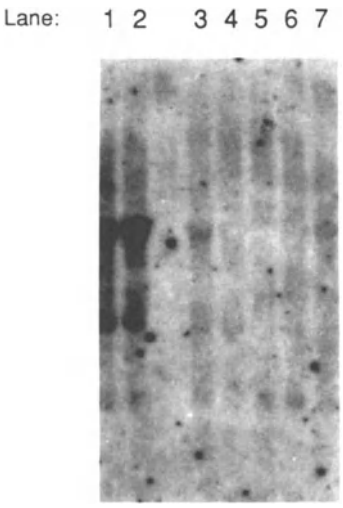
Oncogene-Transfected Rat Embryo Fibroblasts

To confirm that the pNM23 cDNA clone was associated with the tumor metastatic process, *nm23* RNA levels were determined in additional experimental metastasis systems. *nm23* RNA levels in three low metastatic, c-Ha-*ras* + adenovirus 2 *E1a* cotransfected rat embryo fibroblast (REF) lines were two- to eightfold higher than in three control, high metastatic c-Ha-*ras*-transfected REF lines (Steeg et al. 1988b). Again, in situ hybridization experiments indicated that the high *nm23* RNA levels observed in the low metastatic REF *ras*+*E1a*-cotransfected line were due to relatively uniform expression by each tumor cell. In vitro translations of RNAs from the oncogene-transfected REF lines indicated that several proteins were consistently differentially expressed between the low and high metastatic cell lines (Steeg et al. 1988b).

Nitrosomethylurea Induced Rat Mammary Tumors

Nonmetastatic nitrosomethylurea (NMU)-induced rat mammary tumor contained *nm23* RNA levels an average of 1.7-fold higher than did metastatic primary NMU-induced tumors, and 3.2-fold higher than did pulmonary metastases of NMU-induced tumors (Steeg et al. 1988a). The observation that *nm23*

RNA levels exhibited only a two- to threefold difference between the various NMU tumors may be the result of contaminating normal stromal cells, lymphocytes, and endothelial cells, which all express relatively high *nm23* RNA levels.



Lane	K-1735 Cell Line	Median (Range) Pulmonary Metastases/Mouse: Experimental	Spontaneous
1	clone 16	6 (0-10)	0 (0)
2	clone 19	0 (0-1)	0.5 (0-1)
3	M2	113 (72-225)	N.D.
4	M4	63 (29-258)	47 (34-49)
5	TK	258 (204-303)	46 (21-70)
6	TK-Eve	143 (100-255)	20 (0-57)
7	TK-Liver	189 (102-209)	14 (5-28)

Fig. 1. Northern hybridization of pNM23 cDNA insert to K-1735 murine melanoma cell line RNAs. Total cellular RNA from each K-1735 cell line was hybridized to the *Pst*I insert of pNM23 (Steeg et al. 1988a). To determine spontaneous metastatic potential, 5×10^4 cells of each K-1735 line were injected subcutaneously along the backs of C3H/HeN$^-$ mice. Seven weeks postinjection, the animals were killed and gross pulmonary metastases were quantitated ($P < 0.05$, Mann-Whitney test). For experimental metastasis experiments, 2×10^4 cells of each K-1735 line were injected intraveoulsy into the tail veins of C3H/HeN$^-$ mice and gross pulmonary metastases quantitated 2 weeks postinjection ($P < 0.05$ by Mann-Whitney). ND = not done

Mouse Mammary Tumor Virus Induced Mammary Tumors

Balb/c mice carrying the C3H strain of mouse mammary tumor virus (MMTV) develop mammary tumors of relatively high metastatic potential, while Balb/c mice carrying the RIII strain of MMTV develop low metastatic mammary tumors (Basolo et al. 1987). Total cellular RNA was extracted from C3H and RIII strains primary breast tumors and their *nm23* RNA levels determined by Northern blot hybridization (Fig. 2). As shown, the low metastatic RIII tumor contained higher *nm23* RNA levels than did the C3H tumor. In vitro translations of these RNAs are shown in Fig. 2. Several protein bands are differentially expressed between the low and high metastatic MMTV tumors. Of these, bands at 41.5 and 100 kDa, expressed to a greater extent by the high metastatic tumor, are also expressed to a greater extent by the high metastatic *ras*-transfected REF lines (Steeg et al. 1988b). Thus, the metastatic process may involve a consistent series of changes in the expression of specific genes.

Loss of Gene Expression in Metastasis

Our data indicate that in four experimental metastasis model systems, *nm23* RNA levels declined in tumor cells of high metastatic potential compared with control non- or low metastatic tumor cells. Metastasis is therefore accompanied by a loss of expression of a specific gene. Whether changes in *nm23* gene expression accompany or cause lower metastatic behavior will be determined by transfection experiments. The DNA sequence of a 700-bp pNM23-1 cDNA

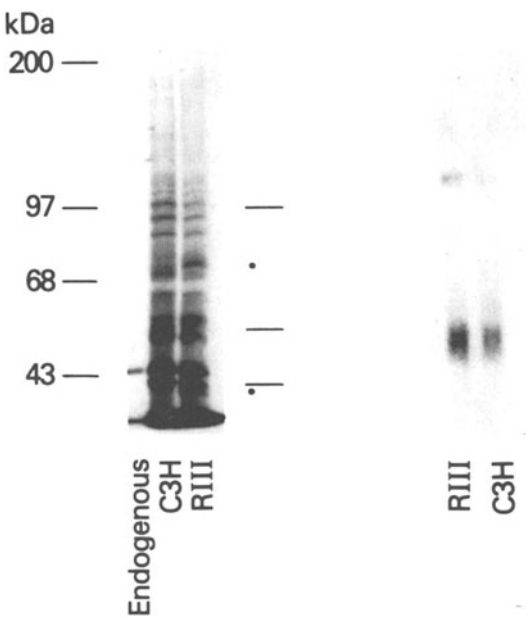


Fig. 2a, b. Analysis of mouse mammary tumor virus (MMTV)-induced primary breast tumor RNAs. Primary breast tumors from Balb/c mice carrying either the RIII (low metastatic potential) or C3H (high metastatic potential) strains of MMTV were resected and total cellular RNA extracted. **a** In vitro translation. Total cellular RNA (1 μg) from MMTV-induced mammary tumors were translated in vitro in a rabbit reticulocyte lysate. Also shown is an *endogenous* translation, where no RNA was added. The translation products were electrophoresed in a 7% sodium dodecyl sulfate-polyacrylamide gel, a fluorograph of which is shown. Bands noted by *solid circles* are present to a greater extent in low metastatic potential *RIII* tumors; bands indicated by *lines* are present to a greater extent in high metastatic potential *C3H* tumors. **b** Northern hybridization. MMTV-induced mammary tumor RNAs were hybridized to a 600-bp Bam HI – Pst I restriction fragment of pNM23-1 cDNA clone

insert was novel compared with Genebank sequences (Steeg et al. 1988a). The cDNA insert contains an open reading frame from its 5' end to double stop codons at position 498. Within this reading frame, three potential initiating methionines are found, of which one is surrounded by optimal translation initiation sequences and would encode a protein of approximately 16.7 kDa. This putative *nm23* gene product contains no sequences indicative of a membrane or secreted protein and may be an intracellular protein. Characterization of the *nm23* protein, and its function in normal and metastasizing tumor cells will proceed by raising antisera to predicted *nm23* peptides.

Our data and the data of other laboratories suggest that inhibitory genes exist for the metastatic process. It has been demonstrated that transfection of the ras oncogene into some "normal" cells induces both the tumorigenesis and metastatic processes (Thorgeirsson et al. 1985; Muschel et al. 1985). Of the four metastasis systems described herein, three (K-1735, NMU, oncogene-transfected REF) have *ras* gene expression in both the non- or low metastatic and high metastatic tumor cells (Pozzatti et al. 1986, and data not shown). Despite the presence of a metastasis-inducing gene in each of these systems, certain tumors or lines remain non- or low metastatic, suggesting the presence of inhibitors. Indeed, in the oncogene-transfected REF system, the exogenously added *E1a* gene directly inhibited metastatic potential (Pozzatti et al. 1986). The *nm23* gene represents the first cellular nonimmunologically related gene whose expression correlates with the inhibition of metastatic behavior.

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Symposium II:
Metastatic Phenotype and Cell Differentiation

In Vivo Observations on Tumor Invasion

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Tumor invasion in vivo is a very complex and dynamic process which depends upon a variety of interactions between heterogeneous tumor cell populations and the different cellular and extracellular components of the host tissue. This situation cannot be reproduced even in the most sophisticated in vitro system. On the other hand, there is at present no method available for a direct and dynamic observation of tumor invasion deep in the nontransparent living tissue, so that all evidence has to be derived from indirect observations made on the basis of static morphology. But all these limitations do not deny the potential value of in vivo investigations in following the natural course of tumor invasion. The present article discusses the major mechanisms of tumor invasion based on in vivo observations in various animal and human tumors with special emphasis on tumor differentiation.

Role of Tumor Differentiation in Tumor Invasion

According to present concepts (Fidler et al. 1978; Sherbet 1982; Pauli et al. 1983), tumor invasion is thought to be the result of the combined action of three basic mechanisms:

a) active tumor cell locomotion,
b) enzymatic degradation of the host tissue matrix, and
c) tumor cell proliferation.

It is quite surprising to find that the influence of tumor differentiation on the invasion process has attracted very little attention in this concept, although all tumor grading systems in human pathology are based on the experience that poorly differentiated tumors possess a higher invasiveness and worse prognosis than the differentiated tumors of the same tissue of origin. This could also be confirmed by some experimental investigations which showed that there is an inverse relationship between the grade of tumor differentiation and the metastatic capacity of a particular tumor (Barnett and Eccles 1984). Following this hypothesis it could also be shown that the dissociation of organized tumor cell complexes into isolated tumor cells (Fig. 1 a) is a constant and striking feature at the invasion front of some experimental tumors (Gabbert et al. 1985; Carr et al. 1986). The emergence of these isolated tumor cells is the result of a block in

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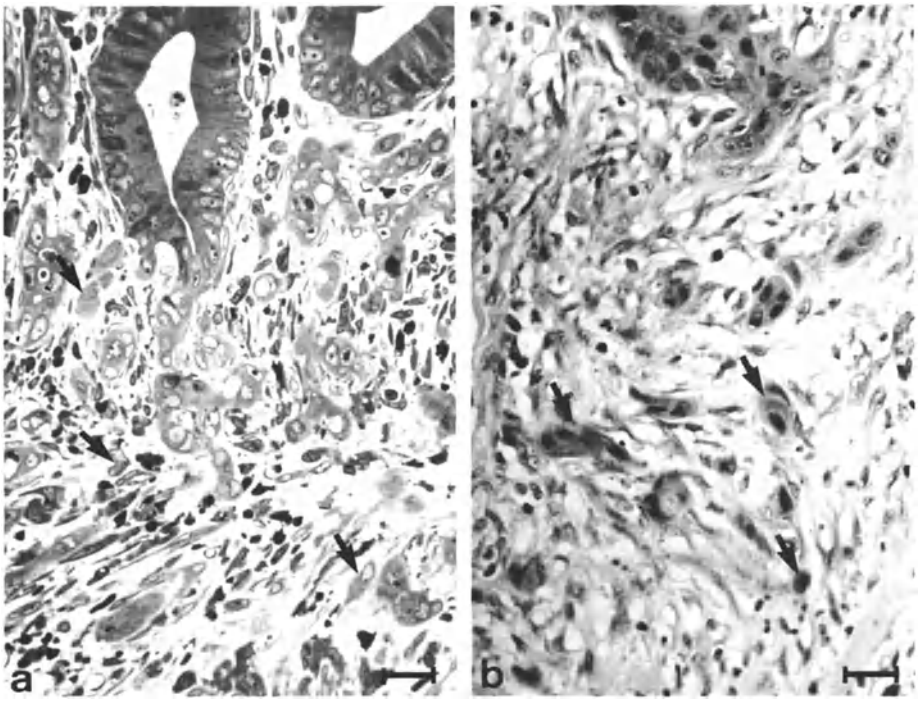

Fig. 1 a, b. Block of differentiation leading to dissociation of the organized tumor cell complexes into isolated tumor cells and small tumor cell aggregates *(arrows)* at the invasion front of a colonic carcinoma of the rat (**a**) and of a human squamous cell carcinoma of the skin. **a** and **b** *scale bar,* 20 μm. (Fig. 1 a from Gabbert et al. 1985)

tumor cell differentiation leading to a uniform, morphologically undifferentiated tumor cell type. This cell type, which is also found at the invasion front of human carcinomas (Fig. 1 b) has lost most of the morphological characteristics typical of noninvading tumor cells in the tumor center (e.g., loss of cell junctions, loss of basement membrane). Concerning the mechanisms of tumor invasion, this block of differentiation represents a crucial prerequisite for tumor cell mobilization and, hence, for active tumor cell locomotion.

Active Tumor Cell Locomotion

It is generally accepted that tumor cells possess the ability for active locomotion (Sträuli and Haemmerli 1984). The in vivo evidence, however, remains rather circumstantial, due to the lack of transparency of most tissues. Nevertheless, there are many morphological observations strongly suggesting active tumor cell locomotion in vivo, too. Thus, isolated tumor cells can be found far away from the main bulk of the primary tumor showing pseudopodium-like

cytoplasmic cell protrusions, often combined with an extreme deformation of the cell shape. These morphological equivalents of active locomotion can best be shown when tumor cells enter blood or lymphatic vessels (Carr et al. 1975). Furthermore, actin filaments can be demonstrated in the cytoplasms of invading tumor cells. However, no increase in the amount of actin could be found in the invading tumor cells when compared with the noninvading tumor cells of the tumor center (Gabbert et al. 1985). Therefore, it may be concluded that the most important prerequisite for active locomotion is not reinforcement of the actin-microfilament system, but rather dissociation, i.e., mobilization of the organized tumor cell complexes into isolated tumor cells.

Enzymatic Degradation

Wherever tumor invasion takes place, the actively migrating tumor cells are confronted with the extracellular matrix of the host tissue. Reports on raised activities of proteolytic enzymes in various tumors have led to the concept that invasion of the host tissue may require an enzymatic degradation of the host interstitial matrix (Woolley 1984; Liotta 1986; Pauli and Knudson 1988). Thus, a positive correlation between proteolytic activity in vitro and the metastatic potential in vivo could be demonstrated for some tumors (Liotta et al. 1980; Tarin et al. 1982). However, to what extent proteolytic processes actually take place in vivo is still controversial (Irimura et al. 1987), and direct evidence for enzymatic action in vivo is still missing due to the fact that changes in the viscoelastic interstitial matrix are not traceable by presently available morphological methods. Despite this lack of direct evidence, it seems reasonable to assume that an enzymatic loosening of the host interstitial matrix would ease tumor cell invasion. In our experience, however, the following mechanism may contribute even more to the loosening of the interstitial matrix.

Interstitial Edema

The fact that interstitial edema develops in the host tissue adjacent to tumors has already been known for a long time (Sylven 1967). Nevertheless, the importance of this phenomenon for the invasion process has been rather underestimated. However, the interstitial edema specifically may enable the tumor cells to invade the host tissue in large numbers, possibly without colliding to a noteworthy degree with local host tissue structures. This view is based on observations of human and animal tumors, where extensive interstitial edema was found preceding the invasion front and forcing apart host tissue cells and fibers (Gabbert 1985). As shown in Fig. 2 the edema is maintained by a continuous fluid convection directed from the tumor to the host tissue. This fluid convection (Jain 1987) not only contributes to the interstitial edema, but may also provide some additional passive transport for the actively migrating tumor cells, guiding them preferentially in the direction of lymphatic vessels. It has been shown that in mammary tumors these convective streams can reach a speed of up to 25 μm/s (Reinhold 1971).

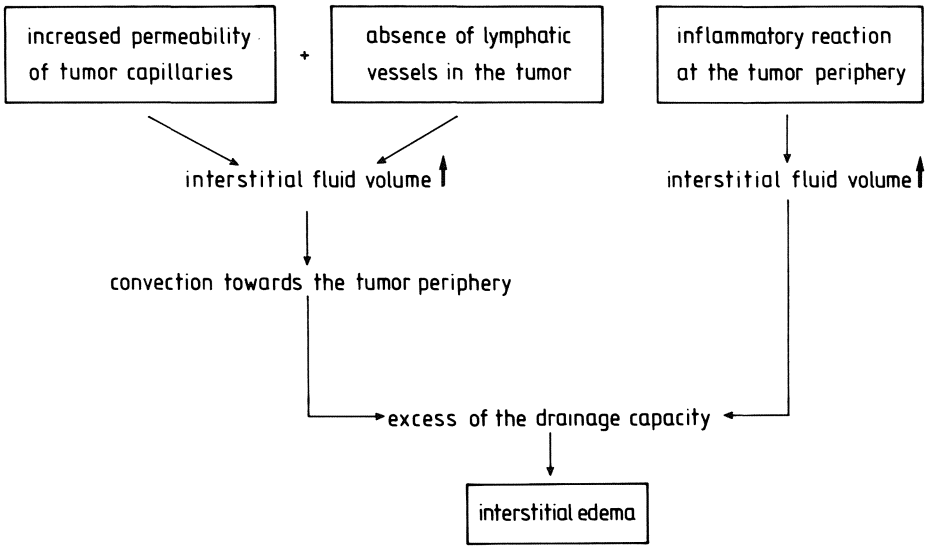

Fig. 2. Pathogenesis of the interstitial edema present in the host tissue adjacent to the tumor invasion front. (From Gabbert 1985)

Tumor Cell Proliferation

The debate about the role of tumor cell proliferation in tumor invasion is still controversial, and there are a number of well-documented in vivo observations which limit the importance of growth pressure as a major mechanism in tumor invasion. Thus, isolated tumor cells are often found far away from the primary tumor, not connected to other tumor cells which could exert pressure on them. Furthermore, no increased proliferation activity could be found at the invasion front of different carcinoma types when compared with the tumor center (Broyn 1975; Rubio 1978). Finally, tumor invasion can be demonstrated even in the presence of agents that completely inhibit tumor cell proliferation (Mareel and de Mets 1984). Two aspects should nevertheless be considered: (a) Tumor cell proliferation increases the number of potentially invasive tumor cells, and (b) tumor cell proliferation completes the invasion process in so far as the invading tumor cells are still able to proliferate, in this way giving rise to focally expanding tumor cell nests in the host tissue.

Host Tissue Destruction

The vanishing of host tissue in the immediate vicinity of invading tumors has for a long time been thought to be caused by proteolytic enzymes. Many electron microscopic investigations have, however, failed to detect any signs of enzymatic degradation, even when intimate contact between tumor and host

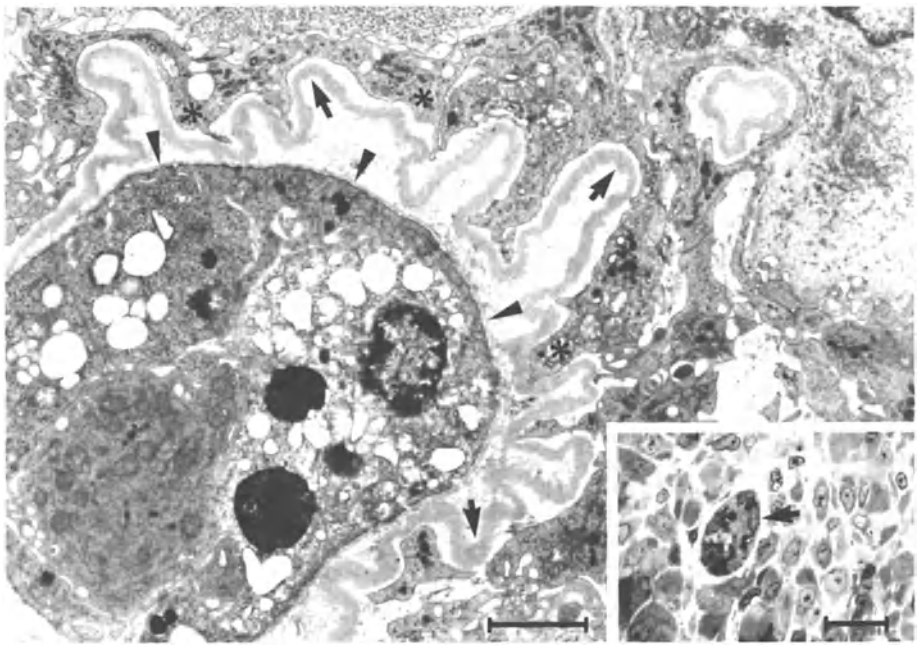

Fig. 3. Electron microscopic picture of a highly atrophic tubulus of the kidney. Note the detached, but still continuous basement membrane *(arrows)* despite the close contact with tumor cell protrusions *(stars)* and the delicate, newly synthesized basement membrane beneath the old one *(arrowheads). Inset,* corresponding light microscopic picture with an atrophic tubulus *(arrow)* embedded between rhabdomyosarcoma cells. *Scale bar,* 2 μm; *Scale bar inset,* 50 μm

tissue cells was reported (Carr et al. 1976; Roos et al. 1978). Furthermore, it could be shown that the confrontation of host tissue with invading tumor cells does not result in the immediate destruction of the host tissue (Gabbert et al. 1987; Dingemans and Mooi 1987), but in a transitory state of coexistence which gradually proceeds to progressive host tissue atrophy (Fig. 3). This process of progressive atrophy is considered to be caused mainly by the increasing pressure and the competitive withdrawal of oxygen and nutrients by the invading and proliferating tumor cells. Morphological changes suggesting any enzymatic breakdown of host tissue cells by tumor cells were not observed in this investigation.

Concept for the Mechanism of Tumor Invasion In Vivo

The first and essential step of tumor invasion is a block of tumor differentiation at the invasion front resulting in tumor cell dissociation. This process mobilizes the tumor cells and enables them to invade the host tissue by active locomotion

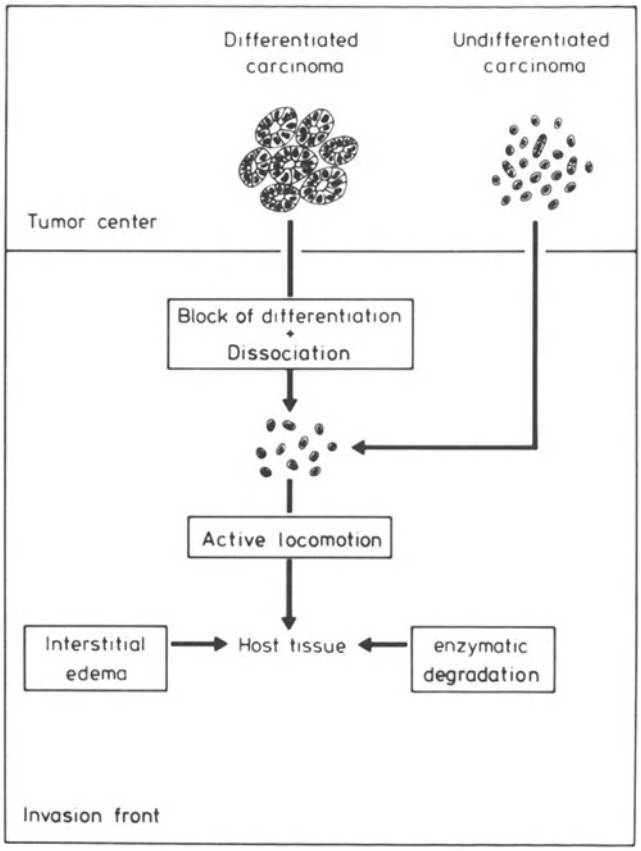

Fig. 4. Concept for the mechanism of tumor invasion in vivo

(Fig. 4). Interstitial edema and enzymatic degradation of the host tissue matrix may promote this process. The grade of tumor differentiation is important in so far as the tumor cells in undifferentiated tumors already primarily possess a more or less mobilized status. This fact, however, provides a crucial advantage for the undifferentiated tumors and may explain their high invasiveness and poor prognosis when compared with differentiated tumors.

Tumor cell proliferation completes the invasion process in so far as the invading tumor cells are still able to proliferate, leading in this way to expanding tumor cell nests in the host tissue. The overall course of tumor invasion can therefore be seen as a repetitive cycle (Suh and Weiss 1984) of active tumor cell locomotion followed by focal proliferation in the host tissue with the final result of host tissue destruction.

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Metastatic Phenotype and Cell Differentiation in Melanoma Cells

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Altered control of cellular proliferation and altered differentiation represent two important pathways determining the phenotypic properties of tumour cells. Indeed, induction of differentiation as a means of reducing tumour proliferative capability and directing the development of benign from malignant cells has long held promise as an alternative to conventional chemotherapy as a therapeutic approach to neoplastic diseases (Pierce 1967). The induction of differentiation as a means of reducing malignancy and improving survival has been studied most extensively using haematological tumours, particularly the myeloid and erythroid leukaemias of mice, but the phenomenon can be duplicated in some common solid neoplasms (Bennett et al. 1986).

Murine melanomas (which grow well in tissue culture, produce melanin as a visible marker of cytodifferentiation and metastasize in syngeneic mice) would seem to provide attractive model systems well suited to the analysis of the effects of differentiation on tumour developments. In spite of these potential advantages very little work has been done on the relationship between melanoma differentiation and metastatic behaviour. At a more basic level almost no information is available on the molecular changes which accompany induction of differentiation in cells of melanocyte origin. We have begun to try to address both these questions in our laboratory. Moreover, since histological studies on clinical material have documented six discrete stages of tumour progression culminating in metastasis (Clark et al. 1984) we have tried to establish murine lines which serve as counterparts to some of "these different stages and to compare their responses to" differentiation-inducing agents.

Recently we isolated a diploid melanocyte line, Melan-a from the epidermis of neonatal C57 mice (Bennett et al. 1987). Continuous cultivation of the Melan-a line in Dulbecco's modification of Eagle's medium (DEM), plus 10% foetal calf serum, at pH 7.4 led to the emergence of the Mel-ab subline. Compared with the Melan-a cells the Mel-ab cells are more epithelioid and less dendritic in appearance, have greater evidence of pigmentation, grow slightly faster in tissue culture and have a median chromosome number of 37. However, in spite of these changes from the more normal Melan-a line, Mel-ab cells will not proliferate in vitro in the absence of activators of protein kinase C (e.g. 12-0-tetradecanoylphorbol-13-acetate (TPA), mezerein or teleocidin) and are non-tumorigenic in athymic and syngeneic mice.

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Transfection of these cells with the v-Ha-*ras* gene under the transcriptional control of the MoMuLV long-terminal repeats (LTR) released recipient cells from in vitro growth requirements and caused their transformation to the tumorigenic state (Wilson et al. 1988). LTR *ras* transformants gave rise to rapidly growing tumours after injection into the s.c. site of athymic and syngeneic mice, though the i.v. injection of these cells failed to lead to grossly evident lung tumour nodules in similar animals within the same time period (R. E. Wilson and I. R. Hart, unpublished observations). A striking change in differentiation status, as assessed by melanin formation, was apparent both in vitro and in vivo in that the transformed cells failed to show any obvious pigmentation. However, any simple correlation between induction of tumorigenicity and reduction in differentiation status was made unlikely by results obtained in comparable experiments in which Mel-ab cells were transfected with the polyoma middle T (PyMT) oncogene (Dooley et al. 1988). In these studies uptake of PyMT-expressing constructs after calcium-phosphate transfection resulted in Mel-ab cells becoming independent of the requirement for TPA and acquiring the ability to form progressively growing tumours in recipient mice. While these appear superficially to be very similar to those achieved with v-Ha-*ras* the tumours produced as a consequence of PyMT transformation were distinctly different at both the gross and histological level, being black in appearance and producing substantial amounts of melanin (Dooley et al. 1988).

These experiments, in which melanoma cell lines have been derived by transfection of Mel-ab cells with known oncogenes, mean that we now have available a series of stable cell lines of melanocyte origin, all of which are syngeneic to C57BL mice, which show a spectrum of neoplastic behaviour ranging from the nontumorigenic Mel-ab cells, through the benign but non-metastatic v-Ha-*ras* and PyMT transformants to the metastatic B16 melanoma of spontaneous origin. Within this range of cell lines no correlation between melanin production, as an indicator of differentiation status, and in vivo behaviour can be established. Thus both the non-tumorigenic Mel-ab cells and the malignant B16 cells may appear highly pigmented under in vivo and in vitro conditions, while oncogene-dependent benign transfectants may or may not produce melanin.

Melanin production, which is a consequence of the oxidative conversion of tyrosine, appears to be in part controlled by cAMP so that those agents which raise intracellular levels of this cyclic nucleotide generally bring about an increase in cytodifferentiation as evidenced by morphological changes, increased pigmentation and a concomitant decrease in proliferation (Johnson and Pastan 1972; Kreider et al. 1973). We have examined the response of our series of melanocyte lines to two agents, cholera toxin and melanocyte-stimulating hormone (MSH), which elevate intracellular cAMP levels by activating adenylate cyclase. Table 1 illustrates the proliferative response of various representative cell lines to the provision of $10^{-9}M$ cholera toxin or $10^{-9}M$ MSH as determined by incorporation of tritiated thymidine into acid-precipitable material at different time points. Over a series of experiments it was evident that the different cell lines consistently showed the same variable pattern of response

Table 1. Cellular proliferation in various lines of melanocyte origin in response to cAMP elevation

Cell line/ treatment	[3H]-Thymidine incorporation (cpm) 24 h	48 h	72 h	96 h
Mel-ab				
1. Control	7,219[a]	12,182	37,702	103,061
2. Cholera toxin	7,557	14,291	45,611	148,971
3. MSH	7,139	12,562	34,530	90,762
LTR*ras*.2				
1. Control	33,460	83,250	194,503	181,434
2. Cholera toxin	40,649	101,885	221,303	189,510
3. MSH	41,077	105,942	199,765	165,492
PyMt 1.1				
1. Control	1,013	13,808	33,106	90,649
2. Cholera toxin	3,612	34,303	78,302	194,918
3. MSH	1,262	16,266	35,480	94,898
B16				
1. Control	36,289	104,944	192,984	146,521
2. Cholera toxin	40,855	97,414	126,572	11,441
3. MSH	33,926	80,324	131,531	27,281

[a] Values given are means of triplicate samples; standard deviation $\leq 10\%$

documented in Table 1. That is, Mel-ab cells responded to the presence of cholera toxin with increased mitogenesis but manifested little change in the presence of MSH, oncogene-transfected derivatives of this cell line, such as LTR*ras*.2 or PyMT 1.1, either showed little or no response to both MSH and cholera toxin or responded to cholera toxin with increased proliferation. In contrast, B16 cells maintained in the presence of these two substances for longer than 48 h showed a marked reduction in proliferative capacity, which corresponded with accumulation of cells in the Go/G_1 phase of the cell cyle as monitored by fluorescence-activated cell sorter (FACS) analysis. Interestingly, the presence of both cholera toxin and MSH caused substantial increases in pigmentation in Mel-ab, PyMT 1.1 and B16 cells, but not LTR*ras*.2 cells. Thus pigmentation and proliferation are not co-ordinately linked throughout cells of the melanocyte series; induced pigmentation and a decrease in proliferation occurs in B16 cells, while induced pigmentation and an increase in proliferation occurs in Mel-ab cells and PyMT transformants.

In order to study changes in gene expression evoked in response to addition of either MSH or cholera toxin, total RNA was extracted from the various cell lines at specific times after addition of these agents and analysed by Northern blotting. Because of their known association with changes in proliferation and differentiation of eukaryotic cells we have looked specifically at the kinetics of expression of c-*fos* (Varmus 1987), Egr-1 (Sukhatme et al. 1988), which is a zinc finger-encoding gene identical to Krox-24 (Lemaire et al. 1988) and which

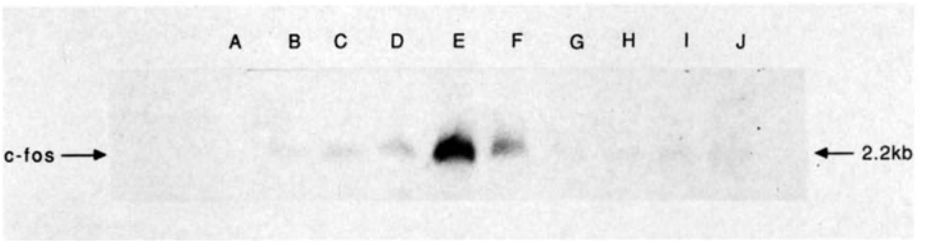

Fig. 1. Induction of c.fos mRNA in exponentially growing LTR *ras*.2 cells by treatment with $10^{-9}M$ cholera toxin. Northern blot analysis of mRNA collected before treatment *(A)* and 15 min, 30 min, 60 min, 90 min, 4 h, 18 h, 24 h, 48 h and 72 h *(B, C, D, E, F, G, H, I, J,* respectively) after addition of cholera toxin. Blot was subsequently stripped and reported with GAD_{28} to quantitative RNA per lane

may encode transcription factors (Egr-1/Krox-24 is closely related to NGF1-A which was activated by nerve growth factor (NGF) in rat PC12 pheochromocytoma cells (Milbrandt 1987), providing additional rationale for examining kinetics in melanocyte cells), gro (Anisowicz et al. 1987), which encodes the mitogenic polypeptide melanoma growth stimulatory activity (Richmond et al. 1988), and the CDC2 M (Lee et al. 1988) homologue of the yeast cdc2+ gene.

The most dramatic changes in terms of induction of steady state mRNA levels were observed with c-*fos*. Treatment of all the melanocyte cell lines with both $10^{-9}M$ MSH and cholera toxin resulted in a rapid and transient accumulation of c-*fos* transcripts. Neither the transformed or non-transformed nature of the cell line or the subsequent mitogenic or inhibitory proliferative response affected this response to any detectable degree; this is illustrated in Fig. 1. Here treatment of LTR*ras*.2 cells with cholera toxin led to accumulation of c-*fos* transcripts which reached a maximum at 90 min after stimulation and had returned to background levels by 18 h post-initiation of treatment. Additional studies with NGF, which is not a mitogen for melanocyte cells, have shown that as has been found in rat PC12 pheochromocytoma cells (Milbrandt 1986) there is also a rapid induction of c-*fos* mRNA. Since NGF works through the cAMP pathway (Cremins et al. 1986) it appears that changes in cyclic nucleotide levels in cells of the melanocyte lineage play a direct role in the induction of c-*fos* expression. It has been reported that cAMP induces c-*fos* expression in macrophages (Bravo et al. 1987) and thyroid cells (Tramontano et al. 1986), suggesting that while phospholipids may prove to be universally involved in regulation of this gene the cAMP pathway may be cell type specific.

B16 melanoma cells responded to both MSH and cholera toxin with similar changes in pigmentation, dendrification, proliferation capacity, cell-cycle distribution and detectable changes in gene expression. It was therefore somewhat surprising to note the marked differences these two treatments had on the subsequent metastatic capacity of B16 cells (Table 2). Over numerous experiments cholera toxin pretreatment consistently reduced the lung-colonizing capacity of B16 cells, whereas MSH gave a marked and significant increase in lung nodule-forming ability (Bennett et al. 1986). It is clear that the lack of

Table 2. Effects of cholera toxin and *N*-MSH on experimental metastatic capacity of B16 cells

Cell treatment	Median	No. lung nodules (range)
Control	57	30– 86
10^{-9}M Cholera toxin	4	4– 12[a]
10^{-9}M MSH	132	90–206[a]

[a] Significantly different from control values by Mann-Whitney U test, $P \leq 0.001$

concordance between malignant behaviour and the differentiated phenotype, which is evident from the comparision of the various cell lines, also is apparent when different agents, thought to act via the same second messenger system, are used to induce what appears to be the same degree of cytodifferentiation. Since cAMP can regulate transcriptional control via cAMP response elements (CREs) found in the 5′-flanking region of cAMP-responsive genes which bind a 43-kDa nuclear protein (Dean et al. 1988; Montminy and Bilezigjian 1987), it may be that differences in the synthesis or phosphorylation of such a nuclear protein, as a consequence of the two different treatments, are responsible for differences in transcriptional activation of genes which affect the metastatic phenotype. Such a possibility currently is under investigation in our laboratory.

At the present time little is known about the regulation and control of melanocyte differentiation, growth and transformation, partly because of previous difficulties in the cultivation of this fastidious cell type. The derivation of a series of lines of varying tumorigenic capacity from a single precursor non-tumorigenic melanocyte line (Mel-ab) will prove to be of value in elucidating such regulatory mechanisms, while with the existence of a melanoma of spontaneous origin (B16) from the same mouse strain, it will prove possible to determine how regulatory events alter with tumour progression. Early results suggest that simple generalisations are unlikely to be drawn and that the situation probably is complex, but further studies should resolve some of these seeming paradoxes and provide valuable information on the biology of this cell type.

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Symposium III:

Organotropism: Adhesion, Invasion, Angiogenesis, Homing, Growth Factors

*Adhesive, Invasive, and Growth Properties of Organ-Specific Metastatic Cells**

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Introduction

The selective metastatic colonization of certain organ sites has intrigued pathologists since the early 1800s. Paget (1889) observed quite different patterns of organ colonization in breast cancers and microorganism infections and proposed that metastatic development was a consequence of particular or specialized tumor cells (seeds) finding a suitable environment (soil) in order to develop and grow. Although several tumor cell and host mechanisms have been proposed to explain preferential organ colonization by metastatic cells, most investigators feel that malignant cells simultaneously use a variety of different tumor and host properties to spread to specific organ sites (Sugarbaker 1981; Hart 1982; Nicolson 1982; 1988a, b; Kiernan and Longenecker 1983; Nicolson and Poste 1983; Schirrmacher 1985). This has been most convincingly demonstrated using animal metastatic models, where low and high metastatic potential cells that colonize particular organs are available (reviewed in Nicolson 1988a, b).

Organ-Specific Metastasis of Human Malignancies

Many cancers often possess their own patterns of distant metastatic colonization. This occurs irrespective of the first organ encountered by circulating cancer cells (Sugarbaker 1981; Hart 1982; Nicolson 1982, 1988a, b; Kiernan and Longenecker 1983; Nicolson and Poste 1983; Schirrmacher 1985). Although some organs and tissues have a very small fraction of the blood supply and vasculature, such as brain, adrenals and bone, they are often involved with metastatic deposits from particular cancers. Similarly, certain tissues in man, such as heart, muscle, skin, kidney, intestine and spleen, collectively account for the majority of the total blood output of the heart but are only sporadically colonized by malignant cells (Nicolson, 1988b).

In breast cancer several clinical studies, beginning with Paget's (1889) classic observations, have documented that metastasis is not a random phenomenon.

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Cancer Metastasis

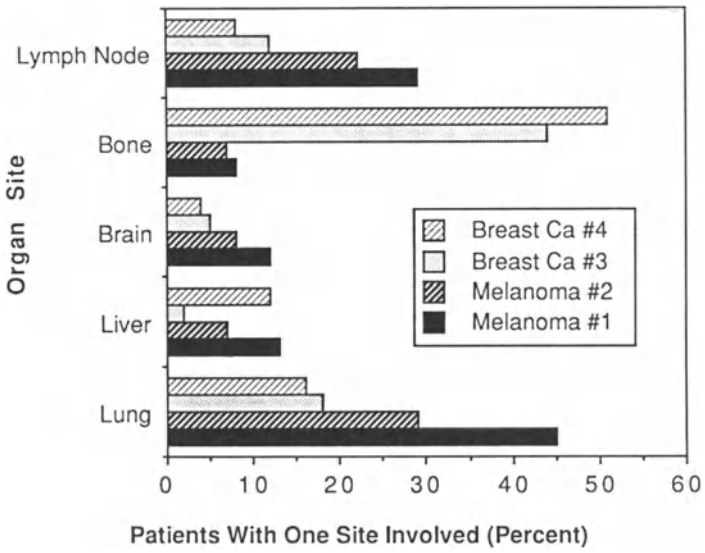

Fig. 1. Sites of initial distant metastases in malignant melanoma and breast cancer patients. (From Nicolson 1988b)

When the first evidence of metastasis is discovered, often only one organ site (bone > lung > liver > brain) is involved (Fig. 1). Although lung is the expected secondary site for breast cancers, if only anatomical-mechanical considerations dictate colonization site, lung is initially involved by breast cancer in only a fraction of patients. Interestingly, when malignant melanoma and breast cancer are compared for their initial sites of distant metastases, a different pattern emerges (Fig. 1).

As malignancies progress to the terminal stage, however, multiple site colonization is the most common finding. Even in cancers that spread initially to only one site, as the cancer progresses, more sites usually become involved. This suggests that the secondary tumors change and acquire the ability to colonize multiple sites. Although in highly progressed malignancies, such as malignant melanoma and breast cancer, some patients die of metastases exclusively in one organ. For example, some patients with malignant melanoma die with metastases only in the CNS, without evidence of a primary tumor (Alsslen et al. 1987).

Use of Metastatic Models in Studying Organ-Specific Metastasis

To determine whether "seed-soil" principles dictate organ colonization patterns, investigators have implanted neonatal organ grafts into ectopic sites and then used the transplanted organ grafts as target sites for tumor cell blood-

borne metastasis (Sugarbaker et al. 1971). After intravenous injection of lung-colonizing murine melanoma cells into mice bearing ectopic grafts, Hart and Fidler (1980) noted that the B16 cells colonized both lung and the subcutaneous lung grafts, even though colonization of the ectopic lung grafts required that some of the B16 cells arrest in and escape from initial capillary lodgment in the lungs and recirculate to the subcutaneous lung grafts. In control mice bearing ectopic kidney grafts, tumor colonies were only found in the lung and not in the ectopic kidney grafts. These experiments demonstrated that anatomical-mechanical concepts alone cannot explain the colonization of lung by B16 cells.

Properties important in the metastatic process have been studied by selecting animal tumor cell variants for enhanced metastatic properties and comparing these with the unselected parental cell populations of lower metastatic potential. Such selections have yielded more metastatic cell populations in about one half the attempts. There are probably a number of reasons for success or failure to select more metastatic cells, including tumor cell stability and heterogeneity, suitability of the selection pressure(s), and the size of the metastatic cell fraction in the starting tumor cell population (reviews: Nicolson 1988a, b). Initially metastatic animal tumor models were developed by sequential selection for organ colonization after intravenous injection of singly suspended tumor cells (Fidler 1973). Using this strategy variant murine B16 melanoma sublines have been selected for enhanced colonization of lung (Fidler 1973), brain (Brunson et al. 1978; Miner et al. 1982), ovary (Brunson and Nicolson 1979), or liver (Tao et al. 1979). Although most selected B16 melanoma variant lines are not very metastatic when implanted at subcutaneous sites, they show similar experimental metastatic properties and organ specificity when injected intravenously or intra-arterially (Fidler and Nicolson 1976). More recently, metastatic models have been developed by sequential selection for enhanced spontaneous metastatic properties. For example, a rat mammary adenocarcinoma system was selected for spontaneous metastasis from the mammary fat pads of syngeneic rats to obtain lines and eventually cell clones of increased spontaneous metastatic potential to lymph nodes and lung (Neri et al. 1982).

Metastatic tumor models have also been established by implantation of human tumor cells into suitable nonhuman recipients. These have usually been immune-deprived animals, such as athymic nude mice (Fidler 1986). One of the problem in assessing these metastatic models has been the in vivo assays used for assessing metastasis. Intravenous injection of tumor cells, the experimental metastasis assay, clearly measures only the terminal steps of metastasis, whereas the subcutaneous injection of tumor cells and measurement of spontaneous metastasis, although more time consuming, is certainly a more appropriate assay for the overall metastatic process. However, the subcutaneous implantation assays are not appropriate for many tumor systems because of artificial dissemination of tumor cells into the lymphatics after subcutaneous, intrafootpad, or intramuscular injection. In the development of metastatic tumor models there has been an increasing tendency to use the tissue of primary tumor origin as the preferred implantation site (Fidler 1986). Some metastatic human tumors rarely metastasize in nude mice, unless they are implanted in the appropriate tissue.

Tumor Cell Adhesion Mechanisms in Organ-Specific Metastasis

That metastases form in many tumor systems is not a fortuitous event due to the passive filtration and mechanical arrest of tumor cells in the first capillary system encountered. In the classic experiments of Zeidman and Buss (1952) circulating tumor cells or their multicell emboli were not always arrested in the first capillary bed, and these cells could be deformed and released to recirculate to other organs. Cell adhesion events are thought to play an important role in tumor metastasis. During blood-borne transit malignant cells can undergo homotypic adhesion and heterotypic adhesion with platelets, lymphocytes, monocytes, and soluble blood components (reviewed in Nicolson 1988a, b). The formation of larger multicell tumor emboli should increase nonspecific lodgment of tumor cells in the first organ encountered, usually the lungs, and this has been shown to be the case experimentally. Properties that favor formation of multicell tumor emboli in the blood would not be expected to favor organ-specific metastasis formation.

The adhesion of circulating malignant cells to specific microvessel endothelial cells is an important event in determining organ-specific metastasis (reviews: Nicolson 1988a, b). With the recent development of microvessel endothelial cells from various organs these interactions have been studied in detail. For example, Auerbach et al. (1987) found that the adhesive interactions of malignant cells with organ-derived microvessel, but not large vessel, endothelial cells correlated with their origin, metastatic properties, and organ preference of metastasis. Malignant hepatoma and glioma cell lines preferentially adhered to the microvessel endothelial cells of the organ of tumor origin, while metastatic teratomas and hepatomas preferentially attached to microvessel endothelial cells of the target organ for metastasis. In general terms, a high rate of tumor cell–endothelial cell adhesion seems to correlate with high metastatic properties (Korach et al. 1986). B16 melanoma sublines that colonize lung at high efficiencies had significantly higher rates of adherence to murine lung microvessel endothelial cells, and B16 cells that colonize brain show higher rates of adhesion to murine brain microvessel endothelial cells (Nicolson 1988a). In contrast, high liver-colonizing sublines of the murine RAW117 large cell lymphoma system adhered at higher rates to murine liver sinusoidal endothelial cells, while only the lung-colonizing subline showed a high rate of adhesion to lung microvessel endothelial cells (Nicolson et al. 1989). Some of the organ endothelial cell surface components important in tumor cell–endothelial cell interactions have been identified by solubilizing endothelial cell surface membranes in detergent solutions and performing polyacrylamide gel electrophoresis, followed by Western transfer of the isolated cell surface components onto nitrocellulose. By using radiolabeled, liver-colonizing lymphoma cells as the indicator cell, five glycoproteins ($M_r \sim 48.000$, ~ 32.000, ~ 30.000, ~ 25.000 and ~ 20.000) were identified by their binding to murine hepatic sinusoidal endothelial cell surface components. The RAW117 cells bound, as expected, to the Western transfers on the basis of their metastatic potentials (H10 >> P) and organ preference of metastasis (murine hepatic sinusoidal > murine lung microvessel >> bovine aortic endothelial cells). Such organ differences seen i

tumor cell–endothelial cell adhesive properties are probably due to quantitative, not qualitative, differences in the expression of particular microvessel endothelial cell surface components (Nicolson 1988a).

The differential adhesion of organ-colonizing tumor cells to the appropriate microvessel endothelial cell monolayer suggests that microvessel endothelial cells display organ-specific determinants. In fact, microvessel endothelial cells in vivo and in vitro express organ-specific patterns of cell surface glycoproteins (Belloni and Nicolson 1988). Using lactoperoxidase immobilized on small latex beads the endothelial cell surface glycoproteins were differentially labeled in situ in perfused animals, indicating that they are expressed on the luminal surfaces of microvessels in specific organs. Some of the tumor cell surface components involved in endothelial cell interactions have also been identified by antibodies that block tumor cell–endothelial cell interactions in vitro and experimental metastasis formation in vivo. Shearman et al. (1980) used a monoclonal antibody (MAb) made against a specific cell-surface antigen to inhibit liver colonization of chicken lymphoma cells. This antibody reacted with a trisaccharide structure on the lymphoma cell surface. McGuire et al. (1984) used antibodies made against mouse fetal liver cells and cross-reactive with adult RAW117 large cell lymphoma cells to inhibit blood-borne liver colonization in vivo and block liver cell adhesion in vitro.

A variety of data implicate a role of cell surface glycoconjugates and endogenous lectins in important steps of the metastatic process. Specific changes in glycoconjugates on malignant cells are closely associated with metastasis formation in some metastatic systems, and tumor cell metastatic and endothelial cell-binding properties can be affected by metabolic alterations in tumor cell surface oligosaccharides (review: Nicolson 1988b). To demonstrate a role for endogenous lectins in metastasis Mermomsky et al. (1986) used a MAb directed against the endogenous galactoside-specific lectin of murine melanomas and fibrosarcomas. Binding of this MAb inhibited homotypic adhesion of melanoma and fibrosarcoma cells and reduced (up to 90%) the numbers of lung tumor colonies formed in experimental metastasis assays.

Tumor cells can apparently use normal cell-surface adhesion mechanisms during blood-borne metastasis. Since normal lymphocytes have the capacity to attach to vascular endothelium and extravasate under the appropriate conditions, it seems reasonable that malignant lymphoid and other tumor cells might use the same or similar mechanisms during endothelial cell attachment and extravasation (Gallatin et al. 1986). Using a panel of MAb directed against various normal lymphocyte antigens that block lymphocyte binding to human endothelial cells, Mentzer et al. (1986) discovered that the lymphocyte function-associated antigen-1 (LFA-1) was involved in lymphocyte-endothelial cell binding. The LFA-1 molecule is an "integrin" class cell-surface glycoprotein composed of an α subunit of $M_r \sim 180.000$ and a β subunit of $M_r \sim 95.000$. Roos and Roossien (1987) recently demonstrated that the integrin class LFA-1 molecule is also involved in T lymphoma binding to hepatocytes. Anti-LFA-1 inhibited metastatic T lymphoma- and T hybridoma-hepatocyte cell adhesion and subsequent invasion.

No one tumor cell surface adhesion component or "homing" receptor appears to be completely responsible for the specificity of tumor cell–endothelial cell adhesion. The fact that a MAb against one cell-surface adhesion component can dramatically inhibit in vivo organ localization of some tumor and normal blood cells cannot be taken as convincing evidence that only one cell adhesion system is responsible for organ-specific localization. Although blocking one of several adhesion systems may be sufficient to significantly inhibit high-avidity endothelial cell attachment, multiple adhesion molecules may be necessary to overcome the circulatory shear forces encountered in vivo while providing for organ specificity of attachment. There is circumstantial evidence for parallel, multiple adhesion mechanisms in tumor cell–endothelial cell interactions. Such molecules include: integrin-like adhesion molecules, cell adhesion CAMs, proteoglycans, endogenous lectins, glycolipids, and other molecules (reviewed in Nicolson 1988a, b).

Malignant cells can also bind to the subendothelial basement membrane (Sindelar et al. 1975). This process has been examined using cultured monolayers of endothelial cells. Since the exposed subendothelial matrix is usually, but not always, a much better adhesive substrate for tumor cells than the endothelial cell surface (Kramer et al. 1980), there is a net movement of the malignant cells to the subendothelial matrix. Not all metastatic cells use this pathway of endothelial cell and basement membrane invasion. Some malignant cells arrest in the microcirculation and grow expansively until they rupture the vessel wall (Kawaguchi et al. 1983; Lapis et al. 1988). Like the adhesion of metastatic cells to microvessel endothelial cells, the adhesion of malignant cells to basement membranes appears to involve multiple, parallel adhesion mechanisms. Several basement membrane components have been identified as tumor cell adhesion molecules: fibronectin, laminin, type IV collagen, heparan sulfate proteoglycan, and vitronectin (reviewed in Nicolson 1988a, b).

The role of particular basement membrane components in malignant cell-basement membrane interactions have been shown by:

a) direct cell binding to the isolated basement membrane components and their effect on experimental metastasis,
b) inhibition of cell binding to basement membranes or subendothelial matrix by specific antibodies and their effect on experimental metastasis, and
c) inhibition of adhesion and experimental metastasis by specific polypeptide fragments derived from basement membrane components.

For example, specific antibodies to (basement membrane) adhesion components have been used to block cell adhesion and metastasis (Nicolson et al. 1981). Vollmers and Birchmeier (1983) used a MAb against B16 melanoma cell surfaces successfully to block B16 cell adhesion to tissue culture surfaces and inhibit blood-borne experimental metastasis. This MAb also blocked binding of B16 cells to subendothelial matrix and attachment to laminin (Vollmers et al. 1984), and the receptor was identified as a laminin-binding component. Alternatively, peptides that inhibit tumor cell receptors for basement membrane components have been used. For example, fibronectin (GRGDS; Pierschbacher and Ruoslahti 1984) and laminin (YIGSR; Iwamoto et al. 1987) cell

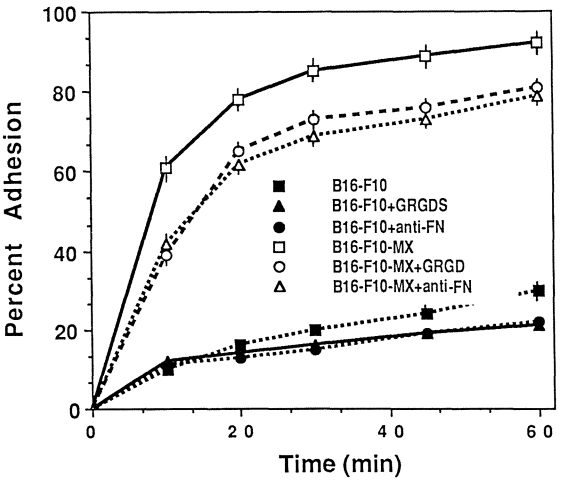

Fig. 2. Kinetics of adhesion of B16-F10 melanoma cells to microvessel endothelial cells and subendothelial matrix (MX) in the absence or presence of high concentrations of the fibronectin inhibitory peptide (GRGDS) or antibodies against fibronectin (anti-FN)

attachment peptides have been identified, and high concentrations of these peptides (Iwamoto et al. 1987; Humphreys et al. 1986) interfere with cell adhesion and inhibit experimental metastasis. However, peptides such as GRGDS do not completely block adhesion of malignant cells to subendothelial matrix. As shown in Fig. 2, addition of GRGDS to B16 melanoma cells inhibits adhesion to matrix only about 30%, suggesting that B16 cells have other adhesion systems that, in addition to binding fibronectin, are responsible for the attachment of the malignant cells to basement membranes.

Differences in the adhesive components of the basement membranes of each organ may also be involved in determining the organ specificity of metastasis (Lichtner et al. 1988). Malignant cells that have higher relative affinities for laminin over fibronectin, such as certain mammary carcinoma cells, tend to metastasize predominantly to lung, while those that have a lower relative affinity for laminin and higher relative affinity for fibronectin, such as many lymphoma cells, tend to metastasize to liver. Laminin is a prominent component of lung basement membranes, but it is much less prominent in the sinusoids and small blood vessels of liver (Foidant et al. 1980).

Tumor cell–parenchymal cell adhesion is also important in the organ-specificity of metastasis. Cell adhesion studies have been conducted with malignant cells and target and nontarget organ parenchymal cells. These studies suggest that in many tumor systems highly metastatic cells adhere at greater rates or more extensively to target than to nontarget organ parenchymal cells (reviewed in Nicolson 1988a, b). Kamenov and Longenecker (1985) studied the organ distribution and growth of murine leukemia lines and a lymphoma line in syngeneic mice before and after fusion with SP2/0 myeloma cells. The leukemia lines, but not the lymphoma line, colonized bone marrow in vivo, and the hybridomas showed high rates of adherence to bone marrow cells in vitro, high levels of bone marrow retention in vivo, and rapid overgrowth of bone marrow. In contrast, neither the SP2/0 parent line nor the seven other hybridomas displayed significant bone marrow cell adherence in vitro or bone marrow

retention and growth in vivo. Different liver-metastasizing tumor systems apparently use different liver adhesion mechanisms to achieve successful liver colonization. Roos et al. (1988) and Middlekoop et al. (1982, 1985) have examined the liver adhesion systems used by murine lymphoma and mammary carcinoma cells. Murine lymphoma T-cell hybridoma cells were inhibited in their adhesion to murine hepatocytes by antibodies to LFA-1 antigen. Additional adhesion systems were implicated, however, by the use of rabbit antisera prepared against whole cells or plasma membranes (Roos et al. 1988).

Tumor Cell Invasion Mechanisms in Organ-Specific Metastasis

The selective invasive properties of malignant cells can be another step in the metastatic process in which organ specificity could be determined. The invasive behaviors of malignant cells involve a number of properties: cell adhesion, motility, destruction of host tissues, and growth (Hart 1981; Mareel 1983). Malignant cells that have the correct set of these properties could selectively invade certain host tissues, but not others, resulting in nonrandom invasion of tumor cells into particular tissue and organ compartments. Although loose connective tissues and bone are readily invaded by most malignant tumors, cartilage, aorta, cornea, lens, and other tissues are relatively resistant to tumor invasion (Pauli et al. 1983). The resistance of certain tissues to tumor invasion is thought to be due to tissue structural properties as well as to tissue substances that can directly inhibit tumor cell invasion. In cartilage, an anti-invasive factor (AIF) has been isolated that can inhibit the degradative enzymes of invading tumor cells. Malignant tumors can also make use of normal host homeostatic mechanisms during their invasion of resistant tissue structures. For example, malignant cells can stimulate surrounding mast cells, fibrobasts, and other host cells to secrete degradative enzymes that could aid tumor cell invasion.

Differences in the attachment and invasion of small pieces of syngeneic organ tissues have been demonstrated in vitro using selected tumor cell variants (Nicolson et al. 1985). High lung-colonizing B16 cells bound to and invaded mouse lung tissue at significantly higher rates than the other B16 lines, whereas high ovary-colonizing cells bound to and invaded ovary tissue at significantly higher rates than the other B16 sublines. Similar evidence for organ specificity of attachment and invasion of target organ tissue has been obtained with murine lymphoma cells (Nicolson et al. 1989). These data and additional results obtained with rat mammary adenocarcinoma cell clones of varying spontaneous metastatic potentials (Nicolson 1988) indicate that highly metastatic organ-preferring tumor cells attach to and invade target organ tissues at higher rates than to nontarget organ tissues.

Tissues, such as syngeneic bladder, have been used to select invasive variants, or to monitor the invasive behaviors of bladder carcinoma cells. As a model of bladder cancer invasion, Ahlering et al. (1987) developed several human bladder transitional cell carcinoma cell lines. The low-grade, noninvasive lines failed to invade the bladder wall, but the high-grade, invasive lines

invaded bladder after intravesical inoculation into nude mice. Many of the invasive substrates used for tumor cell invasion, such as amnion, lens, chorioallantoic membrane, and others (reviewed in Hart 1981; Mareel 1983) may be inappropriate for studying organ preference of invasion. Reconstituted basement membranes, such as Matrigel (Kleinman et al. 1986), might be useful for such studies if they are made from the appropriate organ tissue. The invasion of malignant cells into their target tissues is probably determined by several mechanisms:

a) selective target tissue adhesion,
b) selective destruction of target tissue,
c) selective and directed chemotaxis mediated by tissue-specific chemotatic and/or haptotactic factors, or more likely, a combination of these mechanisms.

Directed cell movement stimulated by soluble (chemotactic) or insoluble (haptotactic) factors appears to be important in malignant cell invasion. Chemotactic or haptotactic properties are apparently mediated by a variety of different factors derived from tumor as well as normal tissues, including small proteolytic fragments of collagens, complement components, and extracellular matrix constituents (review: Varani 1982). Orr et al. (1981) examined the responses of fibrosarcoma cells to the C5a chemotactic peptide and found that highly metastatic cells responded well in dose-response experiments to the peptides, while nonmetastatic cells were unresponsive. Intact molecules or fragments derived from extracellular matrix components, such as fibronectin, laminin, and collagen, could be important sources of tumor chemotactic or haptotactic factors. These could stimulate the directed movements of malignant cells into various tissues, and the directed motility of tumor cells driven by immobilized factors (haptotaxis) may be important in directing the penetration of malignant cells through basement membranes. For example, Lacovara et al. (1984) found that immobilized fibronectin stimulated directed migration of melanoma cells, suggesting that this molecule could function as a haptotactic signal in basement membranes and tissue extracellular matrix. Laminin and its fragments also stimulate haptotaxis (Graff et al. 1987), and antilaminin receptor blocked laminin-mediated attachment and haptotaxis of human melanoma cells on laminin- but not on fibronectin-coated surfaces (Wewer et al. 1987).

Organ-derived chemotactic factors have been identified and these may be involved in target organ invasion by malignant cells. Hujanen and Terranova (1985) reported the activities of chemotactic factors derived from bone, brain, liver, and lung tissue. They found that brain-colonizing melanoma cells responded best to brain-derived factors, lung-colonizing fibrosarcoma cells to lung-derived factors and liver-colonizing monocytic tumor cells to liver-derived factors. They also used a reconstituted basement membrane like matrix to measure invasion and found that human breast adenocarcinoma cells responded to extracts of bone and brain, possible targets for metastatic colonization in this tumor system, by invading across the reconstituted matrix, whereas these cells were not stimulated by lung or liver extracts. Bresalier et al. (1987) found that highly metastatic liver-selected tumor lines were significantly more responsive to liver than lung or brain factors in chemotaxis assays. Such tissue chemotactic

factors may be involved in the differential tissue invasion of organ-preferring metastatic cells.

Tumor cells can also make their own autocrine motility factors that stimulate the random (chemokinetic) movements of tumor cells. Liotta et al. (1986) and Atnip et al. (1987) have isolated what appears to be similar autocrine motility factors of $M_r \sim 53.000$ from metastatic tumor cells. The factor stimulated random movements of cells, but it was not a chemoattractant for normal neutophils. Highly metastatic mammary adenocarcinoma cells synthesize large amounts of autocrine motility factor, and the factor stimulated chemokinetic movements of low metastatic clones that fail to synthesize autocrine motility factor (Atnip et al. 1987). Similar to the different, multiple adhesion systems that are probably involved in organ-specific metastasis, different, multiple organ tissue invasion mechanisms are also likely to be involved.

Tumor Cell Growth Mechanisms in Organ Specific Metastasis

In some malignant cells overexpression of oncogenes that encode autocrine growth factors or their receptors may lead to enhanced organ growth properties. For example, overexpression of the *neu* or *erbB*-2 oncogene (Venter et al. 1987) caused by gene amplification (Venter et al. 1987) or other genomic changes (Kraus et al. 1987) correlates with poor prognosis and malignant progression of breast cancer (Slamon et al. 1987). The *neu*/*erbB*-2 oncogene product is a growth factor receptor similar to, but distinct from the receptor for EGF. This receptor might respond to an autocrine growth factor, or alternatively it could be constitutively active.

Malignant cells at different sites could also be regulated by their responses to microenvironmental growth signals in certain organs. That such paracrine growth factors might be involved in differential growth regulation of organ-colonizing tumor cells has been demonstrated. Horak et al. (1986) used secreted medium from organ cultures and found that the patterns of attachment to substratum, survival, and growth of a series of spontaneous mouse mammary tumors approximated their known metastatic distributions in vivo (lung > ovary > kidney > other organs). The soluble organ factors were found to be nondialyzable and labile and were released into the medium from the organ tissue fragments. Similarly, Naito et al. (1987) found that lung-conditioned medium stimulated the growth of lung-colonizing melanoma cells, but it also stimulated, albeit to a lesser degree, the growth of liver-colonizing tumor cells. Using low and high metastatic mouse colon adenocarcinoma cells Tsuruo et al. (1986) found that growth of both cell lines was stimulated by a lung extract; however, the more metastatic cell line was differentially stimulated at lower extract concentrations.

Using lung- and ovary-colonizing B16 melanoma sublines, we have found that cell growth in serum-limited medium was differentially stimulated by nondialyzable factors from target organ tissue. High lung-colonizing melanoma cells were growth stimulated by lung-conditioned medium and high ovary-colonizing melanoma cells were growth stimulated by ovary-conditioned

medium significantly more than the other organ-conditioned media tested (Nicolson and Dulski 1986). Exclusively lung-metastasizing rat mammary adenocarcinoma cells were growth stimulated by both lung- and liver-conditioned medium (Fig. 3). The highly metastatic MTLn3 cells, however, were growth stimulated at significantly higher rates than the low metastatic MTC cells (Nicolson 1988).

The response of malignant cells to organ paracrine growth factors does not solely determine whether metastasis occurs to that organ. Although liver-selected lymphoma cells were growth stimulated by lung tissue-conditioned medium (Nicolson 1987), they failed to colonize lung in vivo. However, these cells adhered poorly to syngeneic lung microvessel endothelial cells and may not have had the chance to respond to lung growth factors in vivo (Nicolson et al. 1989). Kahan (1987) has also found that target organ growth stimulation by

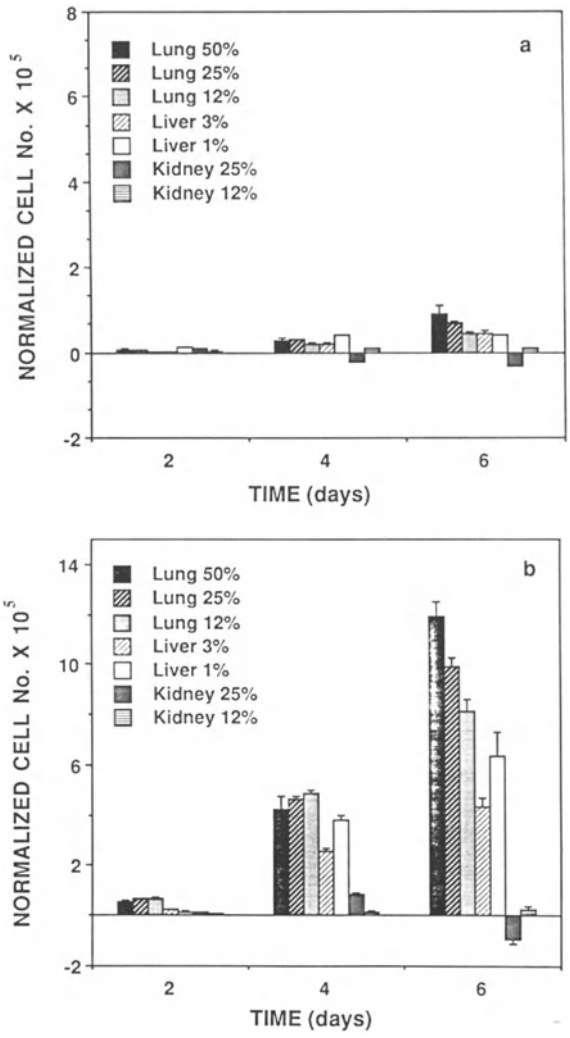

Fig. 3a, b. The effects of various concentrations of rat organ tissue-conditioned medium on the survival and growth of rat 13762NF mammary carcinoma cell clones of **a** low (MTC) and **b** high (MTLn3) spontaneous metastatic potential. (From Nicolson 1988)

soluble factors does not explain metastatic organ colonization of murine embryonal carcinoma cells.

Only a few organ-derived growth factors have been identified and isolated. Szanuiawska et al. (1985) recently reported that lung tissue-conditioned medium contains both cell growth-stimulating molecules of $M_r \sim$ 50.000–70.000 and growth-inhibitory molecules of $M_r \sim$ 12.000–20.000 and $\sim$ 3.000–5000. One of the most potent metastatic cell growth-stimulating factors has now been purified to homogeneity from lung-conditioned medium in our laboratory using a five-step purification procedure. This $M_r \sim$ 67.000 glycoprotein lacks the known properties of other characterized growth factors and so it may be representative of a new class of organ-specific paracrine growth factors (Cavanaugh and Nicolson 1989). A different lung (paracrine) growth factor has been isolated and partially purified by Yamori et al. (1988). They found that murine lung tissue-homogenates stimulated the growth of four highly metastatic cell clones of murine colon carcinoma cells, but only one of four poorly metastatic cell clones were growth stimulated. The activity was trypsin and heat labile and was partially purified by gel chromatography. The active component was eluted in the M_r range of 90.000 to 120.000.

Conclusions

Explaining organ specificity of metastasis based solely on one properity, such as organ growth stimulatory and inhibitory factors, is a gross oversimplification. Organ preference is best explained at the level of endothelial and parenchymal cell adhesion, tissue and basement membrane invasion, and organ and tissue growth stimulatory and inhibitory signals (Nicolson 1988a, b). Malignant cells appear to require a number of properties to be successful metastasizing cells, and different tumor systems appear to respond to multiple, normal factors that are expressed at various concentrations in organs and are probably important in maintaining normal organ cell homeostasis. These factors are provided by either the organ stroma, endothelial, or parenchymal cells, in soluble or insoluble forms.

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Importance of Cell Surface Carbohydrates in Tumor Cell Metastasis

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Introduction

Monoclonal antibodies raised against tumor-associated antigens often recognize aberrant cell-surface carbohydrate sequences associated with glycoproteins and glycolipids. Many of these aberrant structures are also expressed at specific times during normal embryogenesis and show a limited tissue distribution in the adult organism (reviewed by Feizi 1985; Hakomori and Kannagi 1983). The structural complexity and the developmentally regulated expression of these carbohydrate sequences suggest that they may carry structural information required for cell–cell or cell–substratum interactions during embryogenesis (Bird and Kimber 1984; Fenderson et al. 1984). Consequently, oligosaccharides that are normally involved in cell adhesion, motility and invasion during embryogenesis may serve a similar function when reexpressed in metastatic tumors. In this communication, we discuss the evidence that specific Asn-linked oligosaccharide structures expressed by malignant tumor cells are required for efficient tumor cell metastasis and serve to reduce cell adhesion to extracellular matrix proteins.

Sialylation and -GlcNAcβ1-6Manα1-6Manβ- Branching in Malignant Tumor Cells

Neoplastic transformation often leads to the synthesis of larger *N*-linked oligosaccharides (Smets and van Beek 1984). The increase in size of these structures has been attributed in part to greater sialylation of Asn-linked oligosaccharides. Sialylation of available galactose and *N*-acetylgalactosamine at the cell surface has been shown to correlate with organ colonization potential in a series of murine tumor cell lines (Yogeswarren and Salk 1981). Similarly, sialylation of SBA and PNA lectin-binding sites (i.e., GalNAc and Galβ1-3GalNAc) on lymphoid tumor cells have been shown to correlate with metastatic potential and invasiveness in vitro (Altevogt et al. 1983; Collard et al. 1986). More recently, the ability of B16 melanoma cells to spontaneously metastasize from a subcutaneous site was found to correlate with the levels of specific cell-surface saccharide structures that are penultimate to sialic acid (Passaniti and Hart 1988). These results and others (Schirrmacher et al. 1982) suggest that

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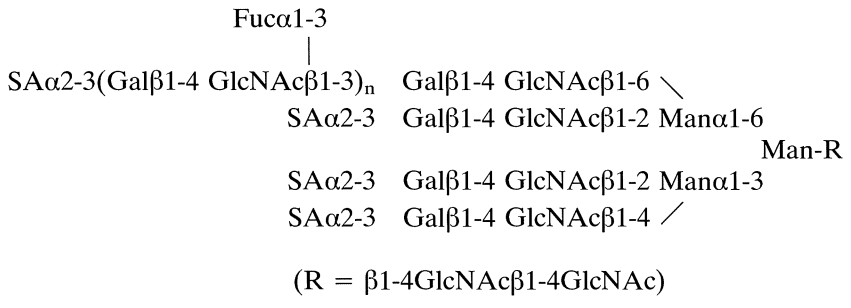
terminal sialylation of specific oligosaccharide sequences may be more important to the metastatic phenotype than amount or degree of sialylation. As outlined below, complex-type oligosaccharides with β1-6-linked antenna are a specific class of sialylated structures which have been directly associated with metastatic potential in several experimental models.

The larger Asn-linked oligosaccharides isolated from transformed fibroblasts often show increased branching at the trimannosyl core, in particular increased -GlcNAcβ1-6Manα1-6Manβ- (Yamashita et al. 1984; Pierce and Arango 1986). Consistent with this observation, UDP-GlcNAc:Manα1-6Man-R β1-6GlcNAc-transferase, (EC2.4.1.155, abbreviated GlcNAc-transferase V), the enzyme that initiates the β1-6-linked antenna was shown to be elevated in the transformed cells (Yamashita et al. 1985). At a minimum, initiation of the β1-6-linked antenna increases the overall size of the oligosaccharides by an additional lactosamine antenna. However, the presence of the β1-6-linked antenna can also engender further increases in the size and structural diversity of the oligosaccharides. In this regard, polylactosamine or repeating Galβ1-4GlcNAcβ1-3 sequences appear to be added preferentially to the β1-6-linked antenna (van den Eijnden et al. 1988). These repeating sequences are themselves a backbone for further monosaccharide substitutions which can create many of the embryonic antigens detected in tumors. An example is the Galβ1-4(Fucα1-3)GlcNAc sequence or SSEA-1 antigen depicted in the structure below.



The larger *N*-linked oligosaccharides do not appear to be required for expression of the transformed phenotype in vitro or for tumor formation in rodents. For example, chemically transformed hamster embryo cells have been shown to contain glycopeptides similar in size to those of their normal cell counterparts (Santer and Glick 1979). However, serial passage of the transformed cells in animals is accompanied by a shift to larger *N*-linked structures, suggesting that enhanced expression of β1-6-branched oligosaccharides may be a characteristic of more malignant tumor cell subpopulations that are inadvertently selected during prolonged tumor cell growth in vivo (Nowell 1976). Similarly, observations were made with the tumoroigenic, but nonmetastatic mouse mammary tumor line called SP1. Sublines of SP1, which have acquired metastatic potential, show a coincident increase in β1-6 branching of Asn-linked oligosaccharides (Dennis et al. 1987).

Glycosylation Mutants Selected from Metastatic Tumor Cells

Studies on glycosylation mutants selected from several malignant tumor cell lines also indicate that sialylated oligosaccharides either facilitate or are required for metastatic dissemination of tumor cells (Reading et al. 1980; Finne et al. 1982; Dennis et al. 1981). For example, class 1 mutants of the MDAY-D2 murine lymphoma remain highly tumorigenic, but are no longer able to metastasize following inoculation at a subcutaneous site (Dennis 1986a). These mutants synthesize prematurely truncated Asn-linked oligosaccharides that lack sialic acid and galactose due to an apparent deficiency in transport of UDP-Gal from the cytosol into the Golgi (Dennis et al. 1986). Revertants of this mutation selected in tissue culture expressed the wild-type sialylated oligosaccharides and also reexpressed the metastatic phenotype, indicating that the mutation and the nonmetastatic phenotypes were directly associated (Dennis and Laferté 1986). A mutant with the same structural deficiency selected from the human melanoma line MeWo has also been found to be poorly metastatic in athymic nude mice (Ishikawa et al. 1988).

Glycolipids are synthesized in the Golgi complex, and as expected, class 1 cells are deficient in gangliosides (i.e., GM_1 and GD_{1a}) and show a corresponding increase in neutral glycolipids (i.e., glucosyl- and lactosyl-ceramide; Laferté et al. 1987). The class 3 mutants, on the other hand, show a normal ganglioside profile, but are deficient in the branching enzyme GlcNAc-transferase V (Dennis et al. 1987). For the latter mutants, tumorigenicity is unchanged, but the cells are poorly metastatic, similar in phenotype to the class 1 mutants. The class 3 mutation is particularly interesting since it appears to specifically inhibit the synthesis of β1-6-branched, Asn-linked structures and suggested that this class of oligosaccharide was required for tumor cell metastasis. The β1-6-branched oligosaccharides purified from metastatic MDAY-D2 cells also contain polylactosamine sequences and are highly sialylated (Dennis et al. 1986). In support of this conclusion, swainsonine and castinospermine, which specifically inhibit *N*-linked oligosaccharide processing prior to the initiation of the β1-6 antenna, also inhibit experimental and spontaneous metastasis (Humphries et al. 1986; Dennis 1986b).

β1-6 Branching in Oncogene-Transformed Fibroblasts and Human Breast Carcinomas

The plant lectin leucoagglutinin (L-PHA) has recently been used to compare the levels of branched oligosaccharide structures in oncogene-transformed tumor cell lines and in a series of human breast carcinomas. L-PHA requires galactose as well as the β1-6-linked antenna for high-affinity binding as shown in the underlined portion of the structure shown above (Cumming and Kornfeld 1982). Further substitutions of α2-3-linked sialic acid or polylactosamine onto the antenna do not inhibit L-PHA binding to tumor cell glycoprotein immobilized on nitrocellulose (Dennis 1986a). However, the affinity of β1-6-branched oligosaccharides for L-PHA affinity columns has recently been shown

to be reduced by substitution of the antenna with α2-6-linked sialic acid or α1-3-linked fucose (Bierhuizen et al. 1988). Therefore, prior removal of sialic acid and fucose may also enhance the specificity of the lectin as a probe for the detection of β1-6-branched oligosaccharides in crude glycoprotein extracts from cells or tissues.

We have used L-PHA as a probe to detect increased β1-6 branching of oligosaccharides in rat2 fibroblasts transfected with activated forms of c-*myc,* c-H-*ras* or v-*fps,* which correspond respectively, to representative members of the nuclear, GTPase, and cytoplasmic tyrosine kinase groups of oncogenes (Dennis et al., in press, 1989). Cells expressing activated c-*myc* were nontumorigenic in nude mice and did not show elevated levels of β1-6-branched oligosaccharides, whereas transfectants carrying H-*ras* or v-*fps* were tumorigenic and generally exhibited metastatic potential which was associated with increased β1-6 branching. Elevated levels of β1-6-branched oligosaccharides in malignant rat2 cells were associated with increased β1-6 GlcNAc-transferase V activity as has previously been reported for polyoma virus-transformed baby kidney hamster cells (Yamashita et al. 1985).

Normal human breast tissue and benign lesions showed low expression, whereas 50% of the primary malignancies examined expressed elevated levels of L-PHA-reactive oligosaccharides (Dennis and Laferté 1988). GlcNAc-transferase V activities in the human breast carcinoma samples also correlated with the levels of L-PHA-reactive oligosaccharide in the tissues. Amplification and overexpression of the tyrosine-kinase oncogene *erbB-2/Her-2/neu* in human breast carcinomas has recently been shown to correlate with early relapse in node-positive patients (Slamon et al. 1987). Since activated tyrosine-kinases such as *src* and *fps* enhance β1-6 branching in rodent cell lines, overexpression or mutation of *neu* and other dominantly acting oncogenes might be expected to correlate with increased β1-6 branching of oligosaccharides and decreased patient survival time. Probes for an oncogene-induced phenotype, such as increased β1-6 branching, which appears to be functionally associated with the malignant phenotype may have considerable prognostic value. In this regard, increased branching of Asn-linked oligosaccharides has been shown to correlate with invasiveness in a series of human uroepithelial cell lines (Debray et al. 1986).

β1-6-Branched Oligosaccharides and Tumor Cell Adhesion

The β1-6-linked antenna appears to be the preferred site for the addition of polylactosamine. These repeating sequences have been found in the Asn-linked oligosaccharides of tumor cells, embryonic cells and anchorage-independent cells, including hematopoietic cells, and may contribute directly to reducing cellular adhesion to extracellular matrix (Zhu and Laine 1985). This view is supported by studies on several adhesion-mediating glycoproteins which show developmentally regulated expression of these embryonic carbohydrate structures. For example, loss of embryonic polylactosamine sequences from placental fibronectin enhances fibronectin-collagen binding (Zhu and Laine 1985). Simi-

larly, loss of sialic acid and polylactosamine in Asn-linked oligosaccharides of lysosomal membrane-associated glycoprotein (LAMP-1) enhances binding of the glycoprotein to several extracellular matrix proteins (Laferté and Dennis 1988). And finally, the class 1 mutants of MDAY-D2 which were deficient in sialic acid and polylactosamine sequences showed increased cell adhesion to the extracellular matrix proteins fibronectin, laminin and collagen type IV (Dennis et al. 1982).

β1-6-branched oligosaccharides containing polylactosamine and terminal sialic acid may modulate the interaction between adhesion receptors and their ligands by altering the conformation of the glycoproteins or by steric interference at binding sites of the glycoproteins. The modulating effects may be restricted to glycoproteins such as fibronectin and LAMP-1 which possess the oligosaccharides. Alternatively, the antiadhesive effect may be longer range, interfering with ligand binding by neighboring receptors which lack these carbohydrate sequences. The latter mechanism is favored in a recent report on the antiadhesion effects of polysialic acid sequences in Asn-linked oligosaccharides of embryonic neural cell adhesion molecule (Rutishauser et al. 1988).

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Cell Shape and the Metastatic Phenotype

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The acquisition of the ability to metastasize is the ultimate and most deadly level of the tumorigenic progression. Metastatic cells are able to dissociate from the primary tumor, invade through the surrounding tissue and endothelial layer into the bloodstream, migrate through the bloodstream to the site of metastasis and then reinvade and proliferate to generate a new tumor lesion (Poste and Fidler 1980). Throughout this process the metastatic cell assumes a number of different morphological configurations varying from densely packed within the tumor mass, deformed and elongated in invasion, and free-floating in the bloodstream. During each of these stages it must acquire distinct functional capabilities requiring the expression of distinct molecular entities.

Cell Shape and the Transformed Phenotype

Normal or untransformed cells are unable to proliferate under nonadherent conditions, a phenomenom termed anchorage dependence (Stoker et al. 1968). When normal cells are grown in a spherical configuration by either suspension in a viscous medium containing agar or methyl cellulose or on tissue culture plates coated with a nonadhesive substrate such as poly(hydroxymethacrylate), poly(HEMA), the macromolecular metabolism of the cells is shut off and the cells cease to proliferate (Folkman and Moscona 1978). Normal cells in suspension culture are able to proliferate in the presence of small glass fibrils longer than 20μ in length which allow the cells to assume a spread configuration, indicating that it is not anchorage or attachment, but cell shape which regulates growth (Maroudas 1973). Decreases in macromolecular metabolism accompanying the growth of 3T3 cells in suspension are reversed by replating the cells on plastic dishes, leading to a recovery of protein, DNA, and RNA synthesis. It was shown that the signal for recovery of protein synthesis is cell surface contact with the solid substrate while recovery of nuclear events, DNA and RNA synthesis, is induced by changes in cell shape (Ben-Ze'ev et al. 1980).

Transformed cells are characterized by their ability to proliferate in a spherical configuration (Stoker et al. 1968). It has been proposed that the loss of sensitivity to cell-shape changes is a central feature of cell malignancy (Folkman and Greenspan 1975). The influence of cell shape on the transformed phenotype can be seen in studies using a stable density-inhibited line of C3H

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mouse embryo fibroblasts that can be transformed by x-irradiation. The transformed phenotype is maintained by growth at high cell density, while serial passage at low density causes the reversion of the transformed cells to the nontransformed phenotype. Growth of the transformed alls at low density on poly(HEMA)-coated plates such that the cell shape is equivalent to that of cells grown at high density prevents the reversion to the nontransformed phenotype (Brouty-Boye et al. 1980). Furthermore, investigation of the growth reponse to cell shape modulation of a series of mouse fibroblasts of increasingly transformed phenotype revealed that an increased level of transformation was progressively correlated with a loss of growth regulation in suspension culture, suggesting that the loss of cell shape-related growth restrictions may be involved in tumor progression (Wittelsberger et al. 1981).

Cell Shape and Metastatic Dissemination

The dissociation of cell shape from growth regulation caused by transformation and the marked alterations in cell morphology throughout the metastatic process suggest that cell shape modulation in vitro could influence the metastatic capability of cells in vivo. No correlation between sensitivity of growth control to cell configuration and metastatic potential was found in a study of metastatic variants of high and low colonizing ability (Raz and Ben-Ze'ev 1982). However, when grown in suspension culture on poly(HEMA)-coated tissue culture plates B16-F1 melanoma cells form large multicellular aggregates and, following disruption of the aggregates and i.v. injection of single cell suspensions into syngeneic C57Bl/6 mice, form a significantly higher number of lung colonies than do cells grown in a monolayer (Raz and Ben-Ze'ev 1983). When cells grown in a spherical configuration are replated on regular plastic plates, they reassume the flat morphology of cells grown in a monolayer and within 24–48 h lose the enhanced metastatic ability of cells grown in suspension culture and regain the metastatic phenotype of monolayer cultured cells. This effect is not limited to B16-F1 cells, but is also seen with metastatic variants of the UV-2237 fibrosarcoma and K-1735 melanoma (Raz and Ben-Ze'ev 1983). As such, cell shape modulation exerts a reversible transient effect on the lung colonizing ability of the cells.

Other investigators have also described a relationship between cell configuration and metastatic ability. Following extended propagation in culture, a cloned B16 cell line (B16-LM3/G3.26) lost its lung colonizing ability; after growth for 3 days as large multicellular aggregates in suspension culture the cell line regained its metastatic capability and formed lung colonies following i.v. injection (Stackpole et al. 1985). In vitro treatment of metastatic P-23 Lewis lung carcinoma cells with dimethylsulfoxide (DMSO) or of T-47D human breast carcinoma cells with prolactin induces both cell rounding and enhanced metastatic potential of the cells in vivo (Takenaga 1984; Shiu and Paterson 1984). A similar correlation between cell shape and malignancy is seen with established variants of differing metastatic capability. Metastatic cell variants isolated by in vivo passaging from Lewis lung carcinoma and BSp73 adenocar-

cinoma of the rat pancreas exhibit a spherical morphology in vitro (Young et al. 1985; Matzku et al. 1983). Alternatively, selected adherent cell variants from S49 mouse lymphoma grown in suspension or highly malignant T-cell lymphoma show a loss in tumorigenicity and metastatic capability (Hochman et al. 1981; Fogel et al. 1983).

Cell Shape and the Surface Properties of Metastatic Cells

As described, the effect of cell shape on internal cellular events is great, but it is the surface properties of the cell which determine and influence its metastatic capability (Nicolson 1982; Schirrmacher et al. 1982). Initial studies of the expression of surface proteins of B16-F1 cells grown under the various culture conditions had shown a significant decrease in the extent of lactoperoxidase-catalyzed iodination of a majority of cell surface proteins (Raz and Ben-Ze'ev 1983). These results indicated that growth in suspension culture decreased either the expression of exposed cell surface proteins or the accessibility of such proteins to lactoperoxidase. Glycoproteins, through their oligosaccharide moieties, are thought to play a major role in the interaction of the cell with its environment. For example, the increased size of complex *N*-linked glycopeptides resulting from increased sialylation has been shown to accompany transformation and increasing malignancy in numerous tumor systems (Warren et al. 1978; Smets and van Beek 1984). Such an alteration of surface glycoproteins does not explain the enhanced metastatic ability of spherical B16-F1 melanoma cells as chromatography on G-50 Sephadex of both fucopeptides and galactopeptides from B16-F1 melanoma cells grown as a monolayer in suspension on poly(HEMA)-coated plates or replated after growth in suspension revealed no size differences in the pronase digested glycopeptides (Fig. 1).

However, studies directed at discovering an alteration in cell surface proteins accompanying the growth of B16-F1 cells in suspension culture did reveal the increased binding of peanut agglutinin (PNA), a lectin specific for *O*-linked Gal-GalNAc oligosaccharides, to desialylated glycoproteins of M_r 78 000, 68 000, 48 000 and 38 000 (Nabi and Raz 1987). The similarity in size of proteins labeled by lactoperoxidase-catalyzed iodination with those labeled by PNA in blot analysis suggested that spherical growth did not alter the degree of expression of these glycoproteins, but rather induced increased glycosylation of these proteins, resulting in increased recognition by PNA and decreased accessibility of lactoperoxidase to the protein core. This idea was supported by the fact that polyclonal antibodies generated against the major PNA binding glycoprotein of M_r 78 000 (gp78) did not bind more strongly to lysates of spherical cells in immunoblot analysis, confirming that cell-shape modulation is associated with the increased glycosylation of gp78 and not with its increased expression. Immunoprecipitation of gp78 from lactoperoxidase-iodinated samples did reveal an increased expression of gp78 on the surface of spherical B16-F1 cells suggesting that increased *O*-linked glycosylation may influence transport to the cell membrane; however, such results are not conclusive due to the substantial influence of cell shape on the extent of lactoperoxidase iodination (Raz and Ben-Ze'ev 1983; Nabi and Raz 1987).

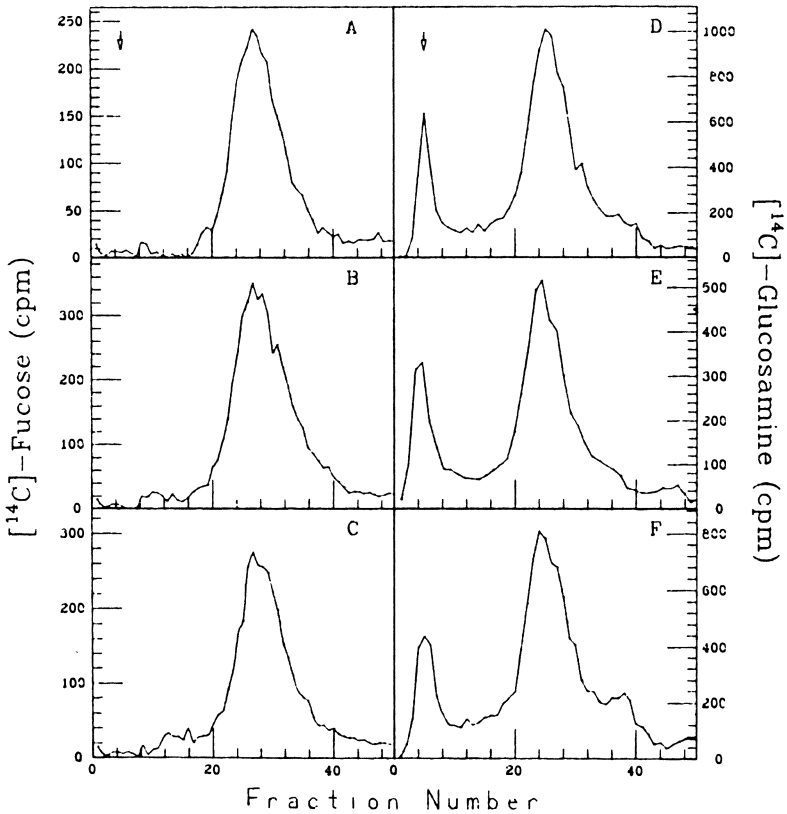

Fig. 1A–F. Chromatography of radiolabeled glycopeptides from B16-F1 cells grown under various culture conditions. Glycopeptides isolated from B16-F1 melanoma cells grown as a monolayer (**A, D**), in suspension on poly(HEMA)-coated plates (**B, E**), or replated after growth in suspension (**C, F**), which had been metabolically labeled with either [^{14}C]-fucose (**A–C**) or [^{14}C]-glucosamine (**D–F**), were chromatographed on a Sephadex G-50 column (1 × 90 cm). The *arrows* indicate the elution of dextran blue

Role of gp78 and O-Linked Glycosylation in Metastasis

A role for gp78 in metastasis is shown by the enhancing effect of F_{ab} fragments of anti-gp78 antibodies on the lung colonizing ability of B16-F1 cells (Nabi and Raz 1987). B16-F1 cells grown in either monolayer or suspension culture show a twofold increase in the number of lung colonies formed following incubation of the cells with anti-gp78 F_{ab} fragments. The use of F_{ab} fragments discounted the possibility that the increased number of lung colonies was due to antibody-mediated formation of cell aggregates and suggests that binding of antibody fragments to gp78 has a specific effect on its cellular function. That antibody binding to gp78 does not inhibit, but rather enhances lung colonization indicates that the function of gp78 and its *O*-linked glycosylation is more complex than a simple recognition process.

Confirmation of the role of sialylated *O*-linked oligosaccharides in the metastatic dissemination of spherical B16-F1 cells was obtained through studies of an adhesive variant selected from suspension-cultured cells: the B16-A10 line (Nabi and Raz 1988). B16-A10 cells adhere more strongly to poly(HEMA)-coated plates than B16-F1 cells and exhibit an organized cytoskeleton characteristic of more adherent cells. The B16-A10 cells do not acquire an increased metastatic capability in suspension culture nor do they exhibit the decreased lactoperoxidase iodination and increased PNA binding to desialylated glycoproteins characteristic of B16-F1 cells grown in suspension culture. These results therefore serve as a negative control confirming the direct correlation between the expression of sialylated PNA binding *O*-linked oligosaccharides on spherical B16-F1 cells and their enhanced metastatic potential.

The studies with the B16-A10 cell line raised the question as to whether the altered glycosylation of suspension cultured B16-F1 cells is due to their spherical shape or rather due to their decreased surface adhesivity. There have been previous reports describing a correlation between cell-substrate adhesion and the degree of sialylation of surface glycoproteins. Selection of a plastic adhesive variant from the highly metastatic ESb lymphoma resulted in the isolation of a low metastatic variant with decreased expression of terminal sialic acid residues (Fogel et al. 1983). Lectin-binding studies of the cells suggested that there is a specific increase in sialylation of *O*-linked oligosaccharides accompanying the change in adhesiveness (Altevogt et al. 1983). Recently the idea of transfer of sialic acid residues between *N*- and *O*-linked oligosaccharides was proposed based on studies of two glycoproteins from MDAY-D2 lymphoma cells in which the expression of sialylated *O*-linked oligosaccharides was correlated with binding to collagen Type I (Laferte and Dennis 1988). The authors suggested that such transfer could be regulated by differential expression of sialyltransferases specific for *N*- or *O*-linked gylcosyl linkages.

The increased expression of PNA binding desialylated glycoproteins of B16-F1 cells grown in suspension culture does not result from increased sialylation of existing core oligosaccharides since no unsialylated PNA-binding oligosaccharides are present on B16-F1 cells grown in either configuration and PNA binding is seen only after neuraminidase treatment. Furthermore, no exposed galactose residues were detected using the galactose oxidase-NaB^3H_4 technique for surface labeling of glycoproteins in the absence of neuraminidase (Nabi and Raz 1987). The B16-F1 melanoma is therefore heavily sialylated and likely has excess expression of sialyltransferases. Thus, the adaptive mechanism to nonadhesive growth conditions which induces increased surface sialylation may require the increased expression of Gal-NAc- and galactosyl transferases resulting in novel glycosylation of serine and threonine residues.

The B16-F1 cell line is heterogeneous and not all cells, such as those selected to form the B16-A10 cell line, respond similarly to cell-shape changes. Increased expression of *O*-linked oligosaccharides on surface glycoproteins upon growth in a spherical configuration in culture is directly related to the enhanced ability of the cell to colonize the lung. Such an ability to modulate the cellular phenotype to altered growth conditions in vivo must be considered a critically important property of the metastatic cell.

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Cell Surface Plasminogen Activation

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The assumption that the plasminogen activation system, through a breakdown of extracellular matrix proteins, plays a role in invasiveness and destruction of normal tissue during growth of malignant tumors is supported by a variety of findings. These include a close correlation between transformation of cells with oncogenic viruses and synthesis of urokinase-type plasminogen activator (u-PA), the finding that u-PA is involved in tissue destruction in many nonmalignant conditions, and the immunohistochemical localization of u-PA in invading areas of tumors (for reviews, see Danø et al. 1985; Saksela 1985). Further support for this hypothesis has come from studies with anticatalytic antibodies to u-PA in model systems for invasion and metastasis. Such antibodies were found to decrease metastasis to the lung from a human u-PA-producing tumor, HEp-3, transplanted onto the chorioallantoic membrane of chicken embryos (Ossowski and Reich 1983; Ossowski 1988), penetration of amniotic membranes by B16 melanoma cells (Mignatti et al. 1986), basement membrane invasion by several human and murine cell lines of neoplastic origin (Reich et al. 1988), and formation of lung metastasis after intravenous injection of B16 melanoma cells in mice (Hearing et al. 1988). In some of these studies (Mignatti et al. 1986; Reich et al. 1988), a plasmin-catalyzed activation of procollagenases (see Tryggvason et al. 1987) appeared to be a crucial part of the effect of plasminogen activation.

A requirement for the regulation of a proteolytic cascade system in extracellular processes is the precise localization of its initiation and progression. For example, in the complement and coagulation systems, cellular receptors for various components are known and serve to localize reactions that either promote or terminate the reaction sequence (Müller-Eberhard 1988; Mann et al. 1988). In the plasminogen activation system, the role of fibrin in localization of plasminogen activation catalyzed by the tissue-type plasminogen activator (t-PA) is well known (Thorsen et al. 1972; Hoylaerts et al. 1982).

Immunocytochemical studies have suggested that u-PA in the invasive areas of tumors is located at the membrane of the tumor cells (Skriver et al. 1984), and recent findings indicate that u-PA at cell surfaces generally is bound to a specific receptor and that this localization may be crucial for regulation of u-PA catalyzed plasminogen activation in time and space (see Blasi et al. 1987). Preliminary reports suggest that also t-PA may bind to cell surface receptors, retaining enzymatic activity (Beebe 1987; Barnathan et al. 1988; Hajjar and

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Nachmann 1988; Kuiper et al. 1988). This phenomenon, however, awaits further clarification concerning the nature of the binding sites, and the following review will be limited to plasminogen activation catalyzed by u-PA.

Surface Receptor for u-PA

The cellular receptor for u-PA (u-PAR) was originally identified in blood monocytes and in the monocyte like U937 cell line (Vassalli et al. 1985). Its presence has been demonstrated on a variety of cultured cells, including several types of malignant cells (Stoppelli et al. 1985, 1986; Vassalli et al. 1985; Plow et al. 1986; Boyd et al. 1988a; Nielsen et al. 1988), human fibroblasts (Bajpai and Baker 1985), and also in human breast carcinoma tissue (Needham et al. 1987). The receptor binds active 54-kDa u-PA, its one-polypeptide chain proenzyme, pro-u-PA (see below), as well as 54-kDa u-PA inhibited by the active site reagent DFP, but shows no binding of the low-molecular-weight (33 kDa) form of active u-PA (Vassalli et al. 1985). Thus, binding to the receptor does not require the catalytic site of u-PA, and in agreement with these findings, the binding determinant of u-PA has been identified in the amino terminal part of the enzyme, in a region which in the primary structure is remote from the catalytic site (Appella et al. 1987). This region shows homologies to that part of epidermal growth factor (EGF) which is responsible for binding to the EGF receptor (Appella et al. 1987).

Binding of u-PA to u-PAR is specific in the sense that as yet no other protein has been found to compete for binding to the receptor, though several proteins structurally related to u-PA, including t-PA and plasminogen, have been tested (Stoppelli et al. 1985; Vassalli et al. 1985; Nielsen et al. 1988). The number of receptors reported varies strongly among the cell types studied, from a few thousand molecules per cell on normal monocytes (Miles and Plow 1987) up to $3 \cdot 10^5$ on some colon carcinoma cell lines (Boyd et al. 1988a). Some variation apparently also occurs in the binding affinity, which is in the 0.1–10 n*M* range (for a review, see Blasi 1988). Further, on certain cell lines the number of receptors can be regulated by the addition of various agents, like phorbol myristate acetate in U937 cells (Stoppelli et al. 1985, Nielsen et al. 1988), EGF in A431 cells (Blasi et al. 1986) and dimethylformamide in colon carcinoma cells (Boyd et al. 1988b). In the first-mentioned case, a large decrease in affinity for the ligand occurs concomitantly with an increase in the number of receptors (Nielsen et al. 1988; Picone et al. 1989).

Preliminary molecular studies on the u-PA receptor have been carried out. A u-PA receptor assay has been developed and an approximately 2200-fold purification has been accomplished, using metabolically labeled material and affinity chromatography with immobilized pro-u-PA (Nielsen et al. 1988). Characterization of the purified protein has shown that the receptor is a 55–60-kDa glycoprotein, the molecular weight of which is unchanged after cleavage of disulfide bonds, suggesting that it consists of a single polypeptide chain. Nothing is known as yet about the structural properties of the receptor responsible for binding to the ligand.

Proenzyme to u-PA

Several studies have indicated that u-PA is released from many types of cultured cells as a single-chain proenzyme with little or no plasminogen activating capacity (Nielsen et al. 1982; Skriver et al. 1982; Eaton et al. 1984; Kasai et al. 1985; Pannell and Gurewich 1987). By limited proteolysis with catalytic amounts of plasmin, this proenzyme can be converted to its active two-chain counterpart. The proenzyme nature of single-chain u-PA is also reflected in the finding that it has essentially no amidolytic activity with synthetic substrates (Wun et al. 1982; Eaton et al. 1984; Lijnen et al. 1986; Stump et al. 1986a, b; Nelles et al. 1987; Pannell and Gurewich 1987), and that it has little or no reactivity with macromolecular (Eaton et al. 1984; Vassalli et al. 1984; Andreasen et al. 1986; Stephens et al. 1987) and synthetic (Nielsen et al. 1982; Skriver et al. 1982; Wun et al. 1982; Gurewich et al. 1984; Kasai et al. 1985) inhibitors.

This picture of single-chain u-PA as an essentially inactive proenzyme is in contrast to the interpretation reached by some other investigators (Collen et al. 1986; Lijnen et al. 1986; Stump et al. 1986a, b). They concluded that single-chain u-PA from several sources had considerable plasminogen-activating capability and that recombinant single-chain u-PA had an activity that was even higher than that of two-chain u-PA. For these studies, a coupled plasminogen activation assay was used in which the activity of generated plasmin was measured with a chromogenic substrate. Such assays for pro-u-PA are self-activating and are strongly influenced by small amounts of contaminating or generated two-chain u-PA or plasmin. As discussed in detail elsewhere (Petersen et al. 1988), it is therefore possible that the high activity of one-chain u-PA found in these studies was apparent and not due to intrinsic activity of single-chain u-PA. Consistent with this interpretation is a report on a variant of recombinant single-chain u-PA which by site-directed mutagenesis was made partly resistant to plasmin cleavage. This variant of single-chain u-PA had an activity that in coupled assays was ≈200-fold lower than that of two-chain u-PA (Nelles et al. 1987).

Recent kinetic studies, which included measures to prevent self-activation in the assays for pro-u-PA, has confirmed the low intrinsic activity of pro-u-PA (Ellis et al. 1987; Petersen et al. 1988; Urano et al. 1988). In one study with a highly purified preparation of pro-u-PA from HT 1080 fibrosarcoma cells, it was shown that the pro-u-PA had a capacity for plasminogen activation that was lower than that of a 250-fold lower concentration of two-chain u-PA. It was not possible to decide whether this low activity was intrinsic or due to contamination (Petersen et al. 1988).

In the intact organism, pro-u-PA is the predominant form of u-PA in intracellular stores, and it also constitutes a sizable fraction of the u-PA in extracellular fluids (Skriver et al. 1984; Kielberg et al. 1985). Extracellular activation of pro-u-PA may therefore be a crucial step in the physiological regulation of the u-PA pathway of plasminogen activation. The plasmin-catalyzed activation of pro-u-PA provides a positive feedback mechanism that accelerates and amplifies the effect of activation of a small amount of pro-u-PA. The initiation of the u-PA pathway of plasminogen activation under physiological condi-

tions, however, may involve as yet unknown triggering factors that activate pro-u-PA.

u-PA at Focal Contact Sites

At the surface of HT-1080 fibrosarcoma cells and human fibroblasts, u-PA has been found to be unevenly distributed, distinctly located at cell-cell contact sites and at focal contacts that are the sites of closest apposition between the cells and the substratum (Pöllänen et al. 1987, 1988; Hébert and Baker 1988; Figs. 1, 2). u-PA was not detected in the two other types of cell-substratum contact, i.e., close contacts and fibronexuses, making it an intrinsic component at focal contact sites (Pöllänen et al. 1988). u-PA at the focal contact sites is receptor bound (Hébert and Baker 1988) and virtually all surface-bound u-PA is in the pro-u-PA form (H. Tapiovaara and R. Stephens, unpublished results).

The focal contact sites are located at the termini of actin-containing microfilament bundles, the so-called stress fibers or actin cables (Burridge 1986). These sites contain several structural components (actin, talin) and regulatory factors (the tyrosine kinase protooncogene products $P60^{src}$, $P120^{gag\text{-}abl}$, $P90^{gag\text{-}yes}$, $P80^{gag\text{-}yes}$) that all are located on the cytoplasmic side (see Burridge 1986).

The location of pro-u-PA at the focal contact sites suggests that u-PA-catalyzed plasminogen activation is involved in breakdown of the contacts, e.g., during cell movement. A selective activation of pro-u-PA at some contact sites would provide a means of obtaining a directional proteolysis, and it is tempting to speculate that such a pro-u-PA activation might be intracellularly initiated and mediated by a transmembrane signal through the u-PA receptor.

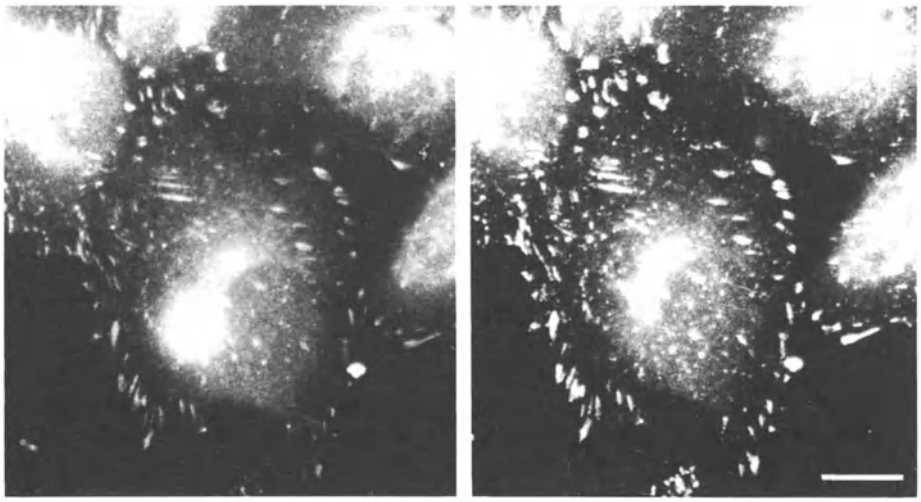

Fig. 1a, b. Colocalization of vinculin and u-PA in HT 1080 fibrosarcoma cells. Double immunofluorescence labeling with monoclonal antibodies against vinculin **a** and polyclonal antibodies against u-PA **b**. *Bar* 15 μm. (From Pöllänen et al. 1988)

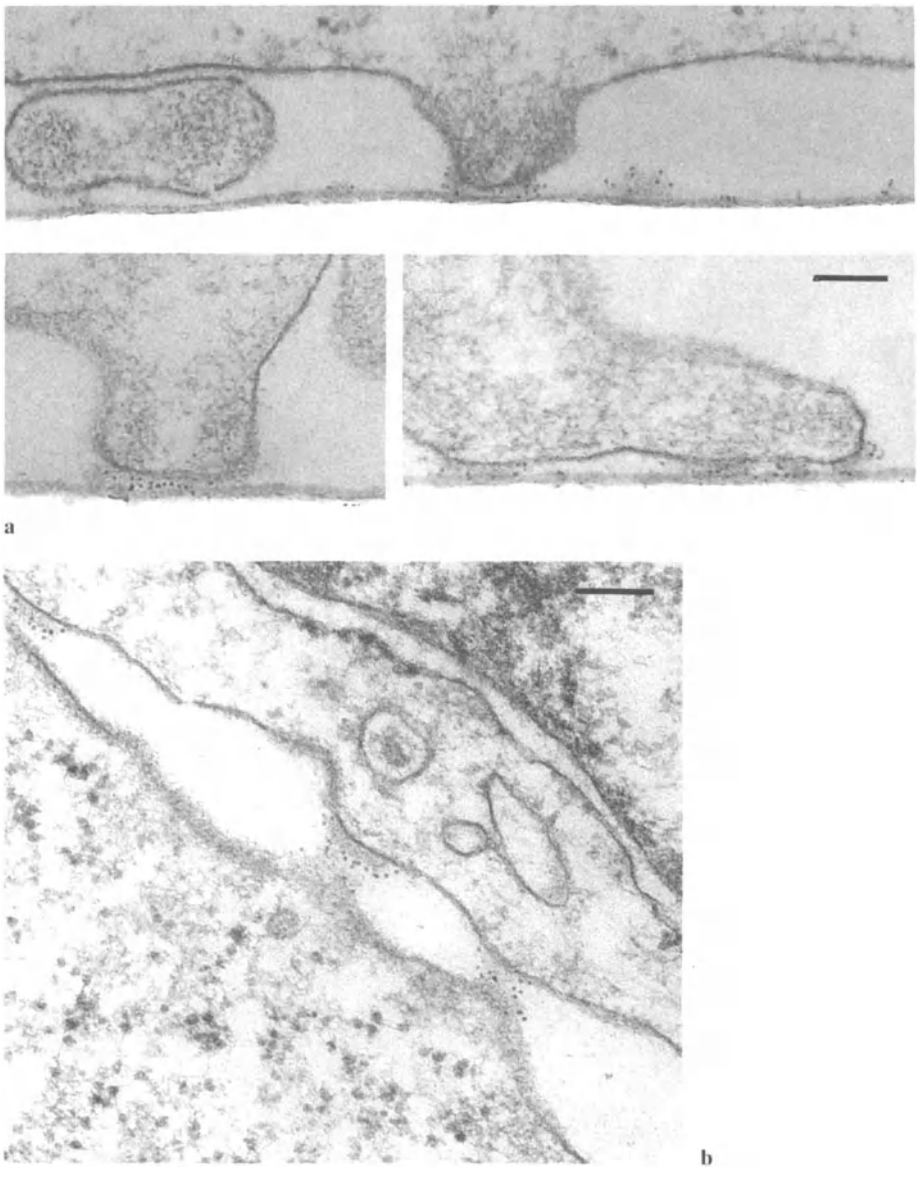

Fig. 2a, b. Electronmicroscopic localization of u-PA at focal cell-substratum **a** and cell-cell **b** contact sites in HT 1080 fibrosarcoma cells. The cells were stained live with the immunoferritin bridge procedure and then fixed with glutaraldehyde. *Bar* 100 m*M*. (From Pöllänen et al. 1988)

Plasminogen Binding Sites on Cell Surfaces

Plasminogen, as well as plasmin, binds to many types of cultured cells, including thrombocytes, endothelial cells, and several cell types of neoplastic origin

(Miles and Plow 1985; Hajjar et al. 1986; Plow et al. 1986; Miles and Plow 1987; Burtin and Fondaneche 1988). The binding is saturable with a rather low affinity for plasminogen ($k_D \approx 1$ μM). At least in some cell types, binding of plasmin appears to utilize the same site as plasminogen, but the binding parameters for plasmin indicates that more than one type of binding site for plasminogen and plasmin may exist. Thus, on some cell types, plasmin and plasminogen bind with almost equal affinity (Plow et al. 1986), while on others plasmin apparently binds with a higher affinity ($k_D \approx 50$ nM) than plasminogen (Burtin and Fondaneche 1988). The binding is inhibited by low amounts of lysine and lysine analogues and appears to involve the kringle structures of the heavy chains of plasminogen and plasmin (Miles and Plow 1986; Miles et al. 1988).

The binding capacity varies between cell types and in many cell types is quite high (10^5–10^7 binding sites per cell). The chemical nature of the binding sites are not known. A membrane protein GPIIb/IIIa seems to be involved in binding of plasminogen to thrombocytes (Miles et al. 1986) and, particularly on thrombin-stimulated thrombocytes, also fibrin may be involved in plasminogen binding (Miles et al. 1986). In purified form, the thrombocyte protein thrombospondin forms complexes ($k_D \approx 35$ nM) with plasminogen (Silverstein et al. 1984). Also immobilized laminin (Salonen et al. 1984) and fibronectin (Salonen et al. 1985) binds plasminogen ($k_D \approx 3$ nM and ≈ 90 nM, respectively).

Surface Plasminogen Activation

Some cell types bind both u-PA and plasminogen (Plow et al. 1986; Miles and Plow 1987; Burtin and Fondaneche 1988; Ellis et al. 1988). Receptor-bound pro-u-PA can be activated by plasmin (Cubellis et al. 1986) and receptor-bound two-chain u-PA at least in part retains its ability to activate plasminogen (Vassalli et al. 1985).

Addition of u-PA and plasminogen to cells holding binding sites for both molecules leads to the occurrence of cell-bound plasmin (Plow et al. 1986; Burtin and Fondenache 1988). These studies did not allow rigorous discrimination between an activation process occurring in solution or between surface-bound reactants. A recent preliminary report by Ellis et al. (1988), however, does strongly suggest that plasminogen activation occurs when both reactants are cell bound. Furthermore, this report suggests that in the system studied, the rate of plasmin generation is greatly enhanced when both pro-u-PA and plasminogen are cell bound and that this increase is mainly due to an increased rate of pro-u-PA activation. In addition, other recent studies of formation of plasmin on HT 1080 cells in serum-containing culture medium showed that a large part, if not all, of the cell surface plasminogen activation was catalyzed by surface-bound u-PA, and that binding of plasmin to the surface was necessary for the activation of pro-u-PA (R. Stephens, unpublished results).

An interaction between binding sites for u-PA and plasminogen is suggested by the finding that u-PA binding in two cell lines led to an increased binding capacity for plasminogen. Binding of plasminogen in these studies had no effec

on the binding capacity for u-PA (Plow et al. 1986). An enhancement of plasminogen binding caused by u-PA was also found by Burtin and Fondaneche (1988) in a cell line of neoplastic origin, even though the plasminogen binding sites demonstrated in the two studies were apparently not identical (see above).

Plasmin bound to the fibroblast cell line GM 1380 appears to be protected from binding to the inhibitor α_2-antiplasmin (Plow et al. 1986).

Conclusions

Binding of u-PA to its receptor appears to localize u-PA not only to the cell surface, but to focalize it to distinct parts of the surface, that at least in some cell types are the cell-cell and cell-substrate contact sites. Selective activation of pro-u-PA at these sites may provide a means of obtaining directional pericellular proteolysis.

Plasminogen also binds to cell surfaces, and recent findings suggest that both u-PA catalyzed plasminogen activation and plasmin-catalyzed pro-u-PA activation may occur at cell surfaces and furthermore that the binding of the reactants enhances the overall rate of plasmin generation, particularly by the enhancement of pro-u-PA activation. In addition, surface-bound plasmin appears to be inaccessible to at least one macromolecular inhibitor.

Little is as yet known of the structural properties of the cell surface binding sites, and particularly of those responsible for plasminogen binding. The available data do, however, indicate that cell surfaces may be physiological sites for u-PA-catalyzed plasminogen activation, analogous to the role of fibrin deposits as the physiological sites for t-PA catalyzed plasminogen activation. The findings also point to inhibition of receptor binding of u-PA as a means of inhibition of some of its physiological functions, e.g., in relationship to therapeutic attempts to prevent invasion and metastasis of cancer cells.

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*Adhesion Mechanisms in Lymphoma Invasion and Metastasis**

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Invasion and Metastasis of T cell Hybridomas

Activated T cells are invasive in vitro (Roos and van de Pavert 1983; Savion et al. 1984; Naparstek et al. 1984). T cell hybrids generated by fusion of such T cells with noninvasive and nonmetastatic BW5147 T lymphoma cells are invasive and metastasize widely and extensively, in particular to the liver but also to kidneys, ovaries, and lymph nodes (de Baetselier et al. 1984; Roos et al. 1985). Part of the hybrids segregate most of the acquired extra chromosomes shortly after fusion. These cells are either noninvasive or exhibit low levels of invasiveness as compared with the vast majority of highly invasive hybrids. Noninvasive cells do not metastasize, whereas low invasive cells do form metastases, but only in a limited number of animals, usually restricted to lymph nodes, and only occasionally in liver (La Rivière et al. 1988). This observation suggests that high levels of invasiveness are a prerequisite for extensive metastasis formation by T lymphoma cells, especially to the liver. Furthermore, the partial or complete loss of invasiveness after extensive chromosome segregation indicates that multiple genes are required for the maintenance of such high levels of invasiveness. As discussed by John Collard (this volume), analysis of human x mouse hybrids has shown that an essential gene is located on human chromosome 7 (Collard et al. 1987b). The notion of a close relation between invasiveness and metastasis formation is supported by the effects of pertussis toxin (Roos and van de Pavert 1987) and of a transfected *ras* oncogene (Collard et al. 1987a).

Invasion into Fibroblast Cultures

T cell hybrids are highly invasive, not only in hepatocyte cultures, but also in monolayers of fibroblasts (Verschueren et al. 1987; Collard et al. 1987b). Strikingly, MB6A lymphosarcoma cells that are highly invasive in hepatocyte cultures (Roos et al. 1981) as well as in the liver in vivo (Dingemans 1973), do not invade fibroblast cultures (La Rivière et al. 1988) and do not form extrahe-

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patic metastases (Dingemans 1973). Apparently, MB6A cells lack properties, in addition to those necessary for invasion of hepatocyte cultures, required for invasion of fibroblast monolayers. Lack of these properties might also be responsible for the limited tissue distribution of MB6A metastases in vivo.

Adhesion to Hepatocytes: Involvement of Leukocyte Function-Associated Antigen-1

Invasion into hepatocyte monolayers proceeds in two steps: adhesion to the exposed surface, and subsequently penetration between hepatocytes. We have shown that the first step is mediated in part by the leukocyte adhesion molecule leukocyte function-associated antigen-1 (LFA-1; for reviews see Springer et al. 1987; Martz 1987). Anti-LFA-1 antibodies reduced the number of lymphoma cells interacting with hepatocytes to approx. 30% of controls. The fraction of interacting cells that infiltrated within 4 h was not diminished, and thus the total number of infiltrated cells was reduced to 30% as well (Roos and Roossien 1987). We have concluded from these results that LFA-1 is involved in adhesion to the exposed surface, but not in adhesion to the lateral hepatocyte surface during the penetration step.

LFA-1-Deficient Mutants

To investigate the relative importance of LFA-1 for the establishment of metastases, we have recently generated a series of LFA-1-deficient mutants of the TAM2D2 T cell hybridoma. (Roossien et al., 1989) TAM2D2 cells were exposed to ethyl methane sulphonate, and mutants selected by panning on a substrate coated with monoclonal anti-LFA-1 antibody. Nonadherent cells were cultured, selection was repeated 6–10 times, and finally the resulting cells were cloned. LFA-1-negative clones were identified by fluorescence-activated cell sorter (FACS) analysis.

Pulse-chase experiments with ^{35}S-methionine revealed that the mutants were of two types. In some clones the synthesis of the α-chain of the LFA-1 heterodimer was impaired, in others the β-chain was not produced. Among the α-chain mutants part had also lost expression of Thy.1. Thus, at least three independently arisen mutant clones could be distinguished. The invasive potential of all mutants was low and comparable to that of parental TAM2D2 cells in the presence of anti-LFA-1 antibodies. For two of the three mutants this was evidently due to reduced adhesion since the infiltrated fraction of interacting cells was comparable to that of TAM2D2 cells.

Metastatic Potential of the Mutants

Within approx. 21 days after tail-vein injection of 10^6 TAM2D2 cells in syngeneic AKR mice, all the animals were moribund due to extensive and wide-

spread metastasis. After injection of mutant cells, however, metastases were seen in only 10% of the animals. The rest of the mice were alive and healthy after 100 days. The mutants were tumorigenic upon s.c. injection, albeit after a longer latency period (18 instead of 12 days). These results strongly suggest that LFA-1 is required for sufficiently strong adhesion to tissues in vivo, not only in the liver but also, e.g., in kidneys, ovaries, and lymph nodes. However, definite proof that the reduced metastatic potential of the mutants was solely due to lack of LFA-1 and not to additional mutations, requires reinsertion of the defective LFA-1 subunit. Attempts to transfect the LFA-1 β-chain into the appropriate mutant are in progress.

LFA-1 on Lymphoma Cells

We have found that LFA-1 is also expressed on highly metastatic lymphoma cells such as MB6A (Dingemans 1973, ESb (Schirrmacher et al. 1979), and MDAY-D2 (Kerbel et al. 1978). Invasion of these cells into hepatocyte monolayers is inhibited by anti-LFA-1 antibody (Roos and Roossien 1987). Thus, LFA-1 is also likely to be involved in metastasis formation by these cells. However, expression of LFA-1 is not sufficient for metastasis to occur. For instance, several nonmetastatic T cell hybrids as well as the nonmetastatic BW5147 cells used to generate the T cell hybrids do express LFA-1. Such cells adhere to hepatocytes to different extents, but do not proceed to penetrate between hepatocytes. A possible explanation is that they lack the ability to extend pseudopods. This notion is supported by the inhibition of invasion by pertussis toxin (Roos and van de Pavert 1987) since pertussis toxin is known to interfere with pseudopod formation and motility (Liotta et al. 1986).

Adhesion to Fibroblasts

As compared with hepatocytes, the interaction of T cell hybridoma cells with fibroblasts is slightly different. Adhesion to the monolayers is relatively weak, so that adherent cells can be easily removed by agitation. Nevertheless, also here adhesion is likely to be a prerequisite for invasion. Indeed, we have recently found that invasion into fibroblast cultures is strongly inhibited by anti-LFA-1 antibody and also that the LFA-1-deficient TAM2D2 mutants are much less invasive than the parental TAM2D2 cells (see Table 1).

Previously we observed that pertussis toxin inhibits invasion in both in vitro assays (La Rivière et al. 1988). Thus, all reagents that we have found so far to influence invasion in hepatocyte cultures also affect invasion in fibroblast monolayers. We have not observed a difference that might explain the inability of MB6A lymphoma cells to invade between fibroblasts. Our observation that LFA-1 mediates adhesion of lymphoma cells to multiple cell types is in keeping with results obtained with nontransformed leukocytes (Springer et al. 1987) and explains the low probability of metastasis formation by LFA-1-deficient mutants in all of the normal target tissues of T cell hybrid metastasis.

Table 1. Infiltration of parental and LFA-1 deficient TAM2D2 T cell hybridomas in rat embryo fibroblast (REF) monolayers

	Synthesis of LFA-1 precursors		Percentage of infiltrated cells	
Cell line	α-chain	β-chain	Anti-LFA-1 MoAB	
			–	+
TAM2D2	+	–	28 ± 7	9 ± 6
TAM2D2-A1A4.1	+	–	2 ± 2	2 ± 1
TAM2D2-B10	+	–	1 ± 1	1 ± 1
TAM2D2-1C9	–	+	3 ± 1	2 ± 1
TAM2D2-2B10	–	+	4 ± 2	2 ± 1

Tumor cells were incubated for 3 h on REF monolayers. Noninfiltrated cells were removed by repeated washings of the monolayers. Infiltrated cells were counted by light microscopy. Results are expressed as the percentage of added cells that have infiltrated. Where indicated, infiltration was measured in the presence of anti-LFA-1 monoclonal antibody

Conclusion

Interaction of blood-borne tumor cells with the cells in target tissues is only one step in the complex metastatic process. Here we have shown that this step is quite complicated in itself. Adhesion to the cultured cells' upper surface, and subsequently to the lateral contiguous surface during invasion, is mediated by different and multiple adhesion molecules. Furthermore, efficient invasion depends on additional events like the generation of a pertussis toxin-sensitive signal, possibly required for induction of motility.

For lymphoma cells we have identified LFA-1 as one of the molecules involved in the adhesion, but not the invasion step. We have also shown that LFA-1 is indispensable for efficient metastasis formation in vivo. It is clear, however, that other cell surface proteins are also involved in initial adhesion. Furthermore, our results suggest that yet other adhesion molecules are responsible for interaction with contiguous surfaces during invasion. It is still possible that the expression of one of these molecules is the crucial feature of the metastatic phenotype in lymphoma cells. It seems more likely, however, that high metastatic capacity depends upon the simultaneous expression of a set of features such as the right number and type of adhesion molecules, including in some cases LFA-1.

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The Role of Macrophages in the Regulation of Tumor Growth and Metastasis

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Introduction

Macrophages are a major component of the lymphoreticular infiltrate of rodent and human tumors (Key 1983; Mantovani et al. 1987). Since these cells are situated at the very interface between tumor and host, they may represent a strategically located target for therapeutic intervention. Interest in these cells is stimulated by the knowledge that macrophages have the potential to kill neoplastic cells, including drug-resistant variants surviving conventional chemotherapy (Giavazzi et al. 1984; Allavena et al. 1987).

Tumor-associated macrophages (TAM) derive from circulating monocytic precursors and tumor-derived chemoattractive factors (TDCF) have been identified (Bottazzi et al. 1983). Studies conducted in rodent and human tumors indicate that there is a correlation between release of TDCF and macrophage content of neoplastic tissues, thus suggesting an in vivo role for TDCF in the regulation of TAM accumulation. In addition to releasing a 10-kDa chemoattractant, various human tumor lines release an inhibitor of chemotaxis antigenically related to the transmembrane protein P15E of murine retroviruses (Wang et al. 1986). Hence, the regulation of monocyte infiltration in tumors is complex and may involve a balance of factors with opposing influences on leukocyte migration.

The functional properties of macrophages infiltrating murine and human metastatic tumors have been characterized in an effort to obtain indications as to the role played by these cells in the immunobiology of neoplastic tissues (Mantovani et al. 1987). This analysis has indicated how TAM can contribute to important aspects of tumor tissue biology, such as fibrin deposition and angiogenesis. Moreover, TAM in certain tumors are a source of growth factors which actually provide optimal conditions for tumor growth. More in general, this type of analysis has shown how TAM, within the mononuclear phagocyte system, represent a cell population with peculiar phenotypic and functional properties. Here, we will summarize our recent work on protooncogene expression in TAM from murine metastatic sarcomas which was conducted in an effort to obtain clues as to the molecular basis of the reprogramming of mononuclear phagocytes which enter neoplastic tissue. This led to the identification of a paracrine circuit involved in the regulation of the proliferation of TAM. Macrophages have the potential of dual (stimulatory and inhibitory)

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influence on tumor growth and metastasis and we will briefly mention recent work on the influence of a macrophage mediator, interleukin 1 (IL-1), on tumor cell adhesion and invasion.

Protooncogene Expression in TAM

Myelomonocytic differentiation is associated with the orderly expression of a set of protooncogenes (Gonda and Metcalf 1984; Mitchell et al. 1985; Muller et al. 1985). Moreover, functional activation of mononuclear phagocytes (Introna et al. 1986; Radzioch et al. 1987; Collart et al. 1987), as well as that of cells showing properties of macrophages, endothelial cells, and NK cells (Colotta et al. 1988 a, b), is signaled by early transient expression of certain protooncogenes (e.g., c-fos).

In an effort to elucidate the molecular basis of the peculiar functional properties of TAM we focused our attention on the expression of c-fos protooncogene. TAM were purified from a series of murine tumors which have been extensively characterized in this laboratory (Mantovani 1978, 1981). They expressed appreciable levels of c-fos transcripts. c-fos expression in TAM was considerably higher than in control peritoneal exudate macrophages (PEM). However, in contrast to peritoneal macrophages, exposure of TAM to lipopolisaccharides (LPS) failed to augment c-fos expression. Similarly, treatment with phorbol myristate acetate (PMA) results in an increase in c-fos expression in PEM, but has no effect on TAM. After culture for 18–20 h in medium, TAM completely recover their responsiveness to LPS, at least as far as the expression of c-fos is concerned.

Since it has been shown that signals which augment c-fos expression in peritoneal macrophages are followed by a period of unresponsiveness to stimulation (Introna et al. 1987), it seems likely that macrophages entering tumors are triggered to express c-fos by as yet unidentified signals (e.g., chemoattractants; Ho et al. 1987) and that heightened expression and unresponsiveness to stimulation are a reflection of previous in vivo stimulation.

Paracrine Regulation of TAM Proliferation

The mechanisms involved in the maintainance of constant levels of macrophages in growing tumors are complex and involve various factors. It has been reported that TAM have increased proliferative capacity (Stewart and Beetham 1978; Stewart 1983; Evans and Cullen 1984; Mahoney and Heppner 1987) and in situ proliferation may contribute to the macrophage content of tumor tissues.

The proliferative capacity of TAM from two murine sarcomas was investigated by cytofluorography: the frequency of cells in the S phase of the cell cycle was 7%–11% for TAM and 1%–2% for resident or elicited macrophages. The proliferation and differentiation of mononuclear phagocytes is regulated by the growth factor M-CSF, which is also active on differentiated macrophages. The

c-fms protooncogene encodes for a transmembrane glycoprotein which is probably identical to the M-CSF receptor (Sherr et al. 1985). We therefore examined c-fms expression in TAM. TAM showed levels of c-fms mRNA higher than PEM. Having established that TAM express c-fms at levels higher than PEM, it was of interest to investigate M-CSF expression in TAM and sarcoma cells. TAM did not express appreciable levels of M-CSF mRNA, while tumor cells from both sarcomas used in this study showed high levels of M-CSF transcripts. Moreover, supernatants from the two fibrosarcomas have M-CSF activity, as detected by the growth of macrophage colonies in murine bone marrow.

These observations outline the existence of a paracrine circuit in the regulation of TAM proliferation, involving M-CSF secreted by sarcoma cells and acting on c-fms expressing TAM.

TAM and Metastasis

While appropriately activated mononuclear cells have the potential to inhibit tumor growth, under in vivo conditions TAM may play an ambiguous role in the regulation of primary tumor and metastasis (for review see Mantovani et al. 1987). It has been formally demonstrated that under certain circumstances macrophages provide optimal conditions for the growth of primary tumors and for implantation of secondary foci. This may in part depend upon the capacity of TAM to produce in situ growth factors. More recently monokines, IL-1 in particular, have been shown to change the adhesive properties of vascular endothelium by augmenting its capacity to act as a substratum for tumor cell adhesion (Dejana et al. 1988). Table 1 shows the increase in adhesion of human colorectal carcinoma and melanoma cells to cultured human umbilical vein endothelial cells pretreated with IL-1 compared with untreated cells. In contrast, normal human fibroblasts did not change their adhesion patterns. The induction of the adhesion process reached a peak at 4 h and was reversed within 24 h (data not shown). Moreover, this effect appeared specific for

Table 1. Cell adhesion to IL-1-treated endothelial cells

Cell line	% Attachment to	
	e.c.-(IL-1)	e.c.+(IL-1)
HT-29 colorectal carcinoma	34.0 ± 6.5	51.3 ± 2.1
HCC-P2998 colorectal carcinoma	7.6 ± 1.0	23.1 ± 2.1
HCC-M1410 colorectal carcinoma	22.9 ± 0.2	43.5 ± 1.4
A 375 melanoma	14.4 ± 4.4	25.9 ± 4.3
MR-5 fibroblasts	16.0 ± 1.1	15.5 ± 0.4

Human natural purified IL-1 10 U/ml was added on endothelial cell monolayer for 4 h at 37 °C and the percentage of ^{111}Indium-oxine-labeled tumor cells was determined after 30 min.
e.c., endothelial cell monolayer

endothelial cells and was not inhibited by antibodies to extracellular matrix components.

During metastatic spread the arrest of tumor cells to vascular endothelium precedes their extravasation. With this in mind, the modification of endothelial cells' reaction to tumor cell attachment may play an important role in tumor cell invasion and ultimately in metastasis formation. Preliminary results have shown that IL-1 can indeed augment metastasis formation in vivo (Giavazzi et al., in preparation). These findings further highlight the complexity and ambivalence of macrophages in the regulation of primary tumor growth and metastasis.

Concluding Remarks

Phagocytes infiltrating neoplastic tissues have peculiar membrane phenotype and functional properties. As briefly summarized here, analysis of protooncogene and growth factor gene expression in TAM is beginning to unravel the molecular basis of the peculiar reprogramming of TAM. TAM play a complex, ambiguous role in the regulation of primary tumor growth and metastasis (for review see Mantovani et al. 1987). Yet these cells are strategically located at the very interface between tumor and host and represent a potential target for immunomodulation. A better understanding of the regulation and function of TAM may provide a better theoretical, less empirical basis for a rational design of therapeutic approaches.

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Symposium IV: Tumor Immunology

The Influence of Murine and Human Major Histocompatibility Complex Class I Expression on Tumor Growth and Metastasis

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Introduction

The major histocompatibility complex (MHC) encodes a group of highly polymorphic cell surface antigens that serve as important recognition structures in the immune system. According to the peptide model of MHC class I-restricted antigen recognition cytotoxic T lymphocytes (CTL) can recognize antigens only in the form of peptidic fragments bound to the peptide-binding groove of MHC class I molecules (reviewed by Bjorkman et al. 1988). Therefore it is conceivable that tumor cells with low or absent expression of MHC antigens cannot be recognized by the T lymphocytes of the immune system.

In the first part of this brief review we will summarize some of our H-2 gene transfection studies with murine tumor models. These studies demonstrate that indeed only MHC class I positive tumor cells are immunogenic, resulting in the rejection of primary tumors or metastasis in vivo. In the second part we describe transfection studies, providing evidence that expression of MHC class I antigens can also have the opposite effect because enhanced MHC class I expression reduces the sensitivity for NK cell-mediated lysis. In the third part findings are mentioned, suggesting that in certain cases expression of H-2D antigens can result in an increased metastatic potential. In the final part the importance of HLA on human tumors is discussed.

Results

MHC Antigens and Recognition of Tumor Cells by T Lymphocytes

As a murine model system in previous studies the Meth-A-induced T10 sarcoma from heterozygous (C3H × C57BL/6)F_1 mice was chosen. Previously two T10 subclones were isolated with differential H-2 class I expression and with distinct metastatic properties (De Baetselier et al. 1980). Clone IC9 is not metastatic. Of the four different H-2 antigens which should be present on an F_1 cell the IC9 tumor expresses both D^b molecules, but not K^b, K^k, or D^k. Clone

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Table 1. Growth and metastasis of H-2 gene transfected IC9 and IE7 tumor in syngeneic F_1 mice

Cell line	Transfected gene	H-2 phenotype	Tumor growth	Metastasis nodules/lung	CTL induction
IC9	–	–, D^b	Fast	0	No
IC9.K^k-1	K^k	K^k, D^b	Fast	0	Yes
IC9.K^b-1	K^b	K^b, D^b	None/slow	0	Yes
IE7	–	–, D^b, D^k	Fast	>100	No
IE7.K^k-1	K^k	K^k, D^b, D^k	Fast	0	Yes
IE7.K^b-1	K^b	K^b, D^b, D^k	Medium	0	Yes

IC9 is a (C3H × C57BL/6)F_1-derived sarcoma which expresses only the D^b molecule. Restoration of H-2 K^k, D^k or K^b expression was achieved by transfection with H-2 genes (Wallich et al. 1985)

IE7 is metastatic and expresses both D^b and D^k, but lacks completely K^b and K^k. Restoration of K^b and K^k expression was achieved by transfection with cloned syngeneic H-2 genes (Wallich et al. 1985). Inoculation of the transfectants into syngeneic (C3H × C57BL/6)F_1 mice demonstrated that the de novo expression of the K^b antigen abrogated the tumorigenicity of the IC9 cells. In contrast, transfection of either the K^k gene, the D^k gene, or the neo^r gene alone did not significantly alter the tumorigenicity of the IC9 cells (Table 1). In the case of the metastatic IE7 variant the de novo expression of K^b and K^k antigens led to a complete abrogation of metastasis (Table 1). In both cases it could be demonstrated that the H-2K-transfected tumor cells were highly immunogeneic and led to the induction of tumor-specific CTL which were restricted by the H-2K molecules. These CTL did not lyse the parental H-2K negative IC9 or IE7 tumor cells (Wallich et al. 1985).

Similar results have been obtained in other tumor systems, such as the K^b-deficient lung carcinoma 3LL from C57BL/6 mice (Plaksin et al. 1988; Hämmerling et al. 1987), the K^k-negative AKR thymoma K36 (Hui et al. 1984), an H-2-deficient adenovirus 12 transformed line (Tanaka et al. 1985), the $H\text{-}2^b$ low melanoma B16 (Tanaka et al. 1988), and an HLA transfected tumor (see below). It can be concluded from all these studies that H-2 negative tumors cannot be attacked by the T lymphocytes of the immune system and that there exists a hierarchy among H-2 class I antigens to serve as an H-2 restriction molecule for the presentation of a peptide of the putative tumor antigens.

MHC and NK Lysis

There are several reports that tumor lines with low MHC class I expression are more sensitive to NK lysis than cells with high class I expression (e.g., Kawano et al. 1986; Harel-Bellan et al. 1986; Kärre et al. 1986). The latter authors have described that class I-negative variants of the YAC and RBL-5 lymphoma cell

Table 2. MHC class I expression decreases NK lysis

Cell line	Transfection	Surface MHC			NK lysis (%)	Tumor growth in B6 mice
		D^b	K^b	IA^b		
EL4	–	D^b	K^b	–	20	Yes
EL4.0	–	–	–	–	40	No
EL4.0β_2	β_2m, neo	D^b	K^b	–	19	NT
EL4.0.IA	IA^b, neo	–	–	IA^b	44	NT

From the EL4 thymoma an H-2 negative EL4.0 line was selected which had a β_2m defect. EL4.0 was transfected with β_2m neo or with IA^b α and β genes. NK cells were obtained from poly I:C (polyinosinic : polycytidylic acid) treated B6 mice (effector : target ratio = 200:1) (K. Sturmhöfel and G. J. Hämmerling, unpublished).
NT, not tested

show a strongly reduced growth capability in syngeneic mice and that this phenomenon is due to an increased sensitivity to NK-mediated lysis. In order to address the question if the MHC molecules are directly responsible for the increased resistance to NK-mediated lysis we have chosen the H-2 positive EL4 thymoma line derived from C57BL/6 mice. A class I-negative variant was selected by treatment with anti-H-2^b antibodies and complement followed by multiple sortings with an Ortho-50 H cell sorter (K. Sturmhöfel and G. J. Hämmerling, in preparation). Table 2 shows that the resulting H-2 surface negative EL4 variant was highly sensitive to NK-mediated lysis and that it failed to grow in C57BL/6 mice, which is in contrast to the H-2 positive parental EL4 line. When C57BL/6 mice were used in which the NK cells were depleted by means of injection of the monoclonal anti-NK1.1 antibody (Koo and Peppard 1984) the H-2-negative EL4 variant was able to grow again, suggesting that the lack of growth in normal mice was caused by NK cells.

The molecular analysis of the EL4.null variant demonstrated that this cell was defective for β_2-microglobulin, whereas K^b and D^b proteins were present in the cytoplasm. Transfection of this EL4.null variant with a β_2m gene restored cell-surface expression of K^b and D^b antigens. This was accompanied by a decrease in NK-mediated lysis (Table 2). Transfection with I-A^b α and β genes did not influence the NK lysis.

These data are the formal proof that

a) MHC class I molecules are directly responsible for resistance to NK-mediated lysis,
b) cytoplasmic expression of the class I antigens is not sufficient, and
c) expression of class II antigens has no effect.

The relevance of MHC antigens and NK lysis for metastasis is exemplified in a study where treatment of the H-2-deficient spontaneous lung carcinoma CMT with IFN-γ strongly increased the expression of the H-2b class I antigens. This

was accompanied by a decreased sensitivity to NK lysis and a higher metastatic potential in vivo (Hämmerling et al. 1987). Similar studies were also performed by several other investigators.

The mechanism by which MHC class I molecules render cells more resistant to NK lysis remains speculative because neither the receptors of NK cells nor the respective target structures are known. A possible explanation is that there exist several different NK target structures. On MHC-positive cells some, but not all of these target structures could interact with the MHC molecules, resulting in a conformational change of the target structures in such a way that they cannot be recognized anymore by NK cells. Since not all target structures are affected by such an interaction, one would not expect a complete effect. This would explain why the effect of MHC antigens on NK lysis is always only partial (e.g., reduction from 40% to 20% lysis, see Table 2) and why in several systems no influence of MHC on NK-mediated lysis was observed (e.g., Dalianis et al. 1981).

H-2D-Encoded Antigens and Metastasis

There is evidence that certain H-2D molecules contribute to the metastatic potential of tumor cells. For example, Eisenbach et al. (1983) have reported that the ratio of D^b to K^b expression in the murine 3LL tumor correlates with the metastatic potential of various subclones. Another notable example is the T10 fibrosarcoma described above. De Baetselier et al. (1980) have observed that all metastatic subclones isolated from T10 express not only the D^b, but also the D^k molecule, whereas all nonmetastatic subclones express only the D^b, but not the D^k molecule. Two experiments were performed in order to address the question whether the D^k molecule was responsible for the metastatic potential. First, from D^b- and D^k-positive clone IE7 variants were selected with anti-D^k antibody and complement which no longer expressed the D^k molecule. These variants showed a strongly reduced metastatic potential (see Table 3). Second, the D^k-negative IC9 cell was transfected with the D^k gene. Some, but not all of the D^k transfectants showed increased metastatic potential in syngeneic (C3H $\times$ C57BL/6)F_1 mice, whereas control transfectants which had received a neo^r gene alone never displayed increased metastasis (Table 3; Y. Gopas, S. Segal, and G. J. Hämmerling, unpublished). However, it should be emphasized that not all of the D^k transfectants were metastatic. Therefore the question cannot yet be answered whether the D^k molecule is directly responsible and sufficient for metastasis in the T10 tumor system. But it is obvious from the results that the D^k molecule plays a very important role, and it will be of interest to unravel the mechanism and to determine whether D end molecules in general contribute to metastasis. So far no clear correlation between NK sensitivity and expression of the D^k molecule in the transfectants could be observed (B. Rager-Zisman, unpublished).

Table 3. Influence of D^k on the metastatic phenotype

		H-2 phenotype				
Cell line	Selection	D^k	D^b	K^k	K^b	Metastasis
IE7	–	D^k	D^b	–	–	High
IE7.D^kminus.1	Anti-D^k	–	D^b	–	–	No
IE7.D^kminus.2	Anti-D^k	–	D^b	–	–	No
IE7.D^kminus.3	Anti-D^k	–	D^b	–	–	Medium
IC9	–	–	D^b	–	–	No
IC9-D^k.1	Transfected D^k	D^k	D^b	–	–	High
IC9-D^k.2	Transfected D^k	D^k	D^b	–	–	High
IC9-D^k.2	Transfected D^k	D^k	D^b	–	–	No

D^kminus variants were obtained from IE7 by treatment with anti-D^k antibody and complement followed by fluorescence-activated cell sorting. IC9 was transfected with a cloned D^k gene (Stephan et al. 1986). Metastasis was determined after intrafoot pad injection into syngeneic (C3H × C57BL/6)F_1 mice.
High = >100 nodules/lung; no metastasis = <10 nodules/lung

MHC Antigens on Human Tumors

In view of the unique importance of MHC expression for tumor growth and metastasis of murine tumors it is of importance to determine whether HLA antigens have a similar role for human tumors. The dilemma with the human studies is that they all have to rely on a statistical correlation between MHC expression and malignant properties because in humans no direct experimental evidence can be provided. Therefore it was important to establish model systems which allowed us to determine whether HLA molecules can mediate tumor rejection. For this purpose transgenic C57BL/6 mice expressing the HLA-Cw3 gene were chosen. In previous studies it was shown that in these mice the HLA-Cw3 molecules served as efficient restriction molecules for the recognition of viral antigens by CTL (Dill et al. 1988). The C57BL/6 derived 3LL tumor grows rapidly in these mice and forms metastasis. However, when the 3LL tumor was transfected with the HLA-Cw3 gene the resulting transfectants did not form metastasis anymore (G. Strauss and G. J. Hämmerling, unpublished; see Table 4). These data are similar to the 3LL tumor transfected with a K^b gene mentioned above (Plaksin et al. 1988; Hämmerling et al. 1987) and they establish that HLA molecules can also be involved in tumor rejection.

There are numerous reports that on various human tumors a downregulation of HLA-A,B,C class I antigens has been observed. However, in some cases also an induction of MHC expression was reported. Attempts to correlate the loss of HLA class I antigens with malignant properties of these tumors showed that a correlation indeed exists in several tumor systems, such as porocarcinoma of the skin, malignant melanoma, colorectal carcinoma, and B lympho-

Table 4. Transfection with the HLA-Cw3 gene prevents metastatic growth of the 3LL tumor in HLA-Cw3 transgenic mice

Tumor	Transfected gene	Lung metastasis
3LL	–	Yes
3LL.neo	neo^r	Yes
3LL.Cw3-1	Cw3 + neo^r	No
3LL.Cw3-2	Cw3 + neo^r	No

The C56BL/6-derived metastatic tumor 3LL was transfected with the human HLA-Cw3 gene and metastasis was determined in syngeneic transgenic C57BL/6.Cw3 mice which express the Cw3 gene (G. Strauss and G. J. Hämmerling, unpublished)

Table 5. Correlation of decreased HLA-A, B, C expression with malignant properties of human tumors

Human neoplasm	Level of MHC class I antigens	Reference
Epidermal tumors[a]	Decreased with increase of malignancy	Turbitt and Mackie 1981 Holden et al. 1984
Malignant melanoma	Decreased in highly invasive primary tumors and metastases	Ruiter et al. 1983 van Duinen et al. 1988
Mammary carcinoma[b]	Decreased in poorly differentiated tumors	Sawtell et al. 1984
Colon carcinoma[b]	Decreased in poorly differentiated tumors	Momburg et al. 1986
B cell lymphoma[b]	Decreased in tumors of high-grade malignancy	Möller et al. 1987

HLA-A, B, C and/or β_2m expression was determined by immunochemical staining. Correlation with malignant phenotype was done by

[a] comparison of malignant with benign tumors

[b] statistical evaluation of a large number of cases in relationship to histopathological criteria of malignancy

mas. Some examples are presented in Table 5. However, in a follow-up study of patients after surgical removal of the colorectal carcinoma no correlation between decreased HLA-A,B,C expression on the tumor and survival or relapse-free time was evident (Stein et al. 1988; F. Momburg et al., unpublished).

All of these studies have been performed with antibodies directed against HLA-A,B,C framework determinants. Therefore it was necessary to extend these studies with allele-specific antibodies. In the case of colorectal carcinoma we observed that about 19% of all tumor samples (80 samples tested) had lost

expression of one or more A or B alleles (Momburg et al. 1989). In about 20% of colon carcinomas, tumor cells occur with loss or downregulation of all A,B,C antigens due to loss of β_2m expression (Momburg and Koch 1989). So far no correlation between loss of individual HLA alleles and malignant properties could be made because the numbers were still too small.

Another major problem concerning the attempts to correlate HLA expression with malignancy are the opposing effects of MHC class I molecules on CTL, NK cells, and metastatic properties as described above for murine tumor systems. Similar effects can also be expected for human tumors regardless of the type of tumor. Therefore careful studies will be required to separate these different effects for each tumor before the knowledge about the HLA status can be exploited for potential diagnostic or therapeutic approaches.

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Identification of Point Mutations in Genes Coding for tum-Antigens. A Step Towards the Understanding of Mouse and Human Tumor-Specific Transplantation Antigens?

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Introduction

Most experimental tumors induced with chemical carcinogens or UV radiation express individually specific transplantation antigens that elicit a T cell-mediated immune rejection in the syngeneic animals (Prehn and Main 1957; Klein et al. 1960; Kripke 1981; Uyttenhove et al. 1983). The characterization of these transplantation antigens has proved very difficult and several different approaches have been followed. One involves the search for specific antibodies to isolate the antigen by immunoprecipitation. Unfortunately, tumors seldom elicit antibodies directed against their specific transplantation antigens. One notable exception is UV-induced tumor 1591, and the molecules that were isolated with these antibodies proved to be modified H-2 class I molecules (Phillips et al. 1985; Linsk et al. 1986). Another approach involves the biochemical fractionation of membrane constituents and the assay of their ability to induce in vivo a specific immune protection against the tumor. It was applied to methylcholanthrene-induced sarcomas and a family of 96-kDa surface glycoproteins was recently reported to induce a specific immunity (Srivastava et al. 1987). We have used yet another approach aimed at the direct isolation of genes coding for "tum$^-$" transplantation antigens, which are observed on mouse tumor cells treated with mutagens. We begin therefore with a brief description of this system.

In vitro mutagen treatment of mouse tumor cell lines generates at high frequency stable immunogenic mutants that are rejected by syngeneic mice (Boon and Kellerman 1977; Boon et al. 1980; Contessa et al. 1981; Frost et al. 1983). Since they fail to form tumors, these mutants have been named "tum$^-$" as opposed to the original "tum$^+$" cell, which forms progressive tumors. Most tum$^-$ mutants express transplantation antigens not found on the tum$^+$ cell. The existence of these "tum$^-$ antigens" could be demonstrated in vitro with cytolytic T lymphocytes (CTL) on a series of tum$^-$ mutants derived from mouse mastocytoma P815 (Boon et al. 1980). For most P815 tum$^-$ mutants, stable

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CTL clones have been obtained that show a strict specificity for the immunizing mutant (Maryanski et al. 1982). Using these CTL clones, we demonstrated that the diversity of P815 tum$^-$ antigens is considerable and that many P815 tum$^-$ variants express at least two tum$^-$ antigens (Boon 1983).

The three major features of the tum− variants are their high stability, their high frequency, and their high diversity. It appeared possible that the tum$^-$ antigens were coded by a family of genes that were particularly sensitive to the mutagen treatment, resulting in rearrangements of the kind observed in immunoglobulin genes. An alternative hypothesis was that mutations in a very large number of unrelated genes created tum$^-$ antigens. The solution to this issue required the isolation of the antigens or the cloning of the relevant genes. The complete lack of specific antibodies directed against tum$^-$ antigens prevented the first approach. We therefore followed the genetic approach.

Cloning of tum$^-$ Gene P91A

P815 tum$^-$ variant P91 expresses two distinct tum$^-$ antigens : P91A and B. We undertook to clone the gene that determines the expression of antigen P91A, using the DNA transfection approach. P1.HTR, a highly transfectable cell, was selected by submitting P1, the P815 tum$^+$ cell line, to multiple cycles of transfection (Van Pel et al. 1985). To detect the transfectants expressing the tum$^-$ antigen we developed a test system wherein transfectants were detected by their ability to stimulate the specific CTL clone. This enabled us to detect one antigen-expressing transfectant in a pool of 30 independent transfectants (Wölfel et al. 1987).

By transfecting P1.HTR with DNA of tum$^-$ variant P91 and pSVtk-neoβ we obtained antigen-expressing transfectants at a frequency of 1 per 13.000 geneticin-resistant transfectants (Wölfel et al. 1987). A library of cosmids was prepared with cosmid c2RB and DNA of a transfectant-expressing antigen P91A. Nineteen groups of 35.000 cosmids were transfected. Two produced transfectants expressing P91A. The DNA of the cosmid transfectants was packaged directly in lambda phage heads (Lau and Kan 1983) and by this method, one cosmid was obtained which was capable of transferring the expression of the antigen (De Plaen et al. 1988).

A restriction map of this cosmid was obtained and various restriction fragments were transfected. A 800-bp fragment capable of transferring the expression of P91A was identified. By hybridization with this probe, we obtained cosmids that contain the entire P91A gene for both the tum$^+$ cell and the tum$^-$ variant. cDNA clones of the same gene were also isolated. Using these various sources, we were able to obtain the entire sequence of gene P91A.

Structure of Gene P91A and Presence of a Point Mutation

Gene P91A (Fig. 1) is 14 kb long and contains 12 exons. It contains a long, open-reading frame coding for a putative protein of 60 kDa. This protein does

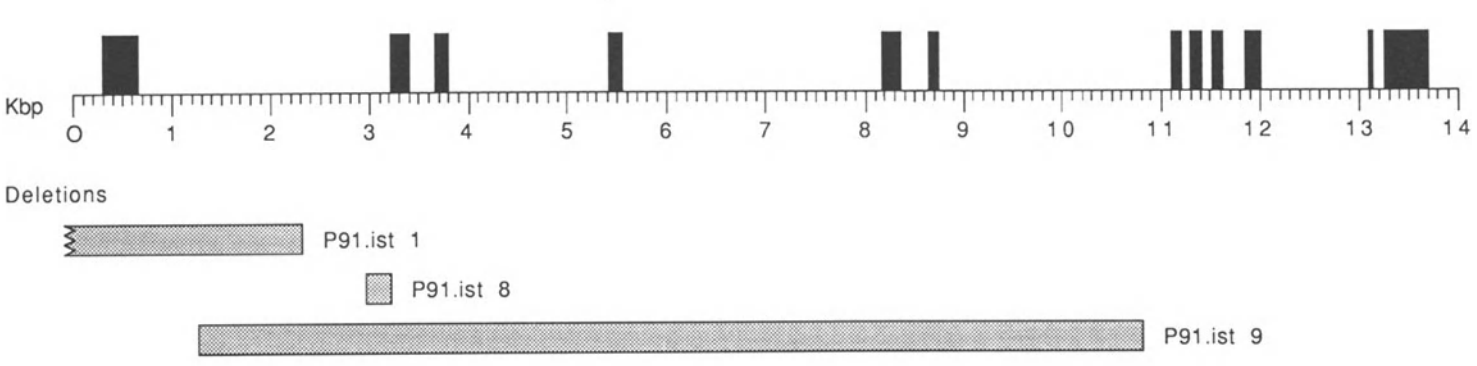

Fig. 1. Structure of gene P91A. The *asterisk* indicates the exon where a mutation was identified. The *lower part* of the figure indicates the limits of the deletions observed in three antigen-loses variants

Fig. 2. The sequence of exon 4 of gene P91A

not contain at its N-terminus a typical signal sequence. It is therefore unlikely that it is a membrane protein, even though this is not rigorously excluded.

Fragments of the tum^- gene containing only exon 4 and parts of the surrounding introns are capable of transferring the expression of the antigen. The sequence of exon 4 differs from its tum^- counterpart by a point mutation (Fig. 2). This G to A transition codes for a change from arginine to histidine. It was shown by site-directed mutagenesis to be responsible for the expression of antigen P91A.

On the basis of the work of A. Townsend (Townsend et al. 1986a) and his associates, who demonstrated that CTL directed against an influenza nucleoprotein antigen can lyse cells incubated with a small peptide corresponding to a short segment of the nucleoprotein, we examined whether peptides derived from the region surrounding the tum^- mutation could have similar effects. The peptide shown in Fig. 2 causes the lysis of P1 cells by anti-P91A CTL, thereby demonstrating that gene P91A codes directly for the antigen.

Gene P91A shows no homology with any gene presently recorded in DNA sequence data bases. We have recently cloned two additional tum^- genes. They do not show any homology either between themselves or with P91A.

Antigen-Loss Variants

Occasionally tum^- variants form tumors, which develop very slowly (Maryanski et al. 1982). When the cells of these tumors are readapted to culture and tested with anti-tum^- CTL, they are usually found to have lost the expression of one or several tum^+ antigens. Such antigen-loss variants were obtained with P91. When the P91A genes of these variants were examined, they were found to have undergone deletions (Fig. 1). This is important because it indicates that the loss of the expression of a transplantation antigen usually requires a deletion in the relevant gene.

Conclusions

Our results indicate that the acquisition of new transplantation antigens by tum^- variants is the result of point mutations. This explains the stability of the tum^- variants. The complete lack of homology between the first three tum^- genes that were cloned suggests that the high frequency and diversity of tum^- variants may be due to the fact that a very large number of genes, coding or not for membrane proteins, can be mutated to produce strong transplantation antigens. This interpretation is in line with the remarkable work of Townsend, who demonstrated that CTL can recognize class I-associated peptides derived from influenza proteins that are not present on the surface of the target cells (Townsend et al. 1985, 1986b).

It would be unreasonable to draw general conclusions regarding the nature of tumor-specific transplantation antigens on the basis of our results regarding tum^- antigens. We will therefore try to extend the methods which ensured the

cloning of tum^- antigens to the cloning of mouse tumor-associated antigens. However, at this point it is not unreasonable to consider seriously the possibility that many tumor-specific transplantation antigens are caused by mutations that occur throughout the whole cellular genome. What could be the origin of these mutations? First, there are those that cause the tumoral transformation. We consider this to be unlikely because it would limit the diversity of tumor-specific transplantation antigens, whereas this diversity appears to be very considerable. Moreover, the loss of tumor-specific antigens does not seem to reduce the transformed character of mouse tumor cells (Uyttenhove et al. 1983). A second interpretation is based on the high frequency of tum^- mutants. Conceivably, at the time cells become tumoral, they are submitted to highly mutagenic influences producing several mutations. One of these would cause the tumoral transformation event, while others, functionally unrelated, would produce the tumor-specific antigens. In our opinion, this hypothesis is still very plausible, but we believe now that a third source of mutation should also be considered. It seems probable that upon ageing, all the normal cells of the organism accumulate mutations. The resulting antigens are probably not detected by immunosurveillance as long as the progeny of each mutated cell remains small. However, if it becomes cancerous and undergoes considerable clonal expansion, a sufficient amount of the same antigen may be available to generate an immune response.

Future Perspectives

Human Tumor-Associated Transplantation Antigens

Several studies of melanoma patients have shown that by cultivating autologous PBL with the melanoma lines, it was possible to obtain cytolytic T cells that lyse the autologous tumor and do not lyse autologous EBV-transformed lymphoblastoid cells, autologous fibroblasts, or NK target K562. These CTL show therefore some specificity for the tumor cell line, even though it is difficult to decide how strict this specificity is (Hérin et al. 1987; Mukherji and MacAlister 1983).

To decide whether or not these autologous CTL recognize bona fide human tumor-specific transplantation antigens, we must demonstrate that these antigens are present on the tumor cells in the patient, as opposed to being a culture artefact. Then we must demonstrate that these antigens are completely or almost completely absent from other cells of the organism, so that a rejection of the tumor cells due to these antigens would not degenerate into an autoimmune disease.

The first point is almost impossible to demonstrate by lysis because fresh tumor samples usually contain too much necrotic material and foreign tissue to serve as target for ^{51}Cr release test. However, it was recently demonstrated for one melanoma that tumor cells from a surgical sample were capable of stimulating the proliferation of a CTL clone showing specificity for the tumor

(Degiovanni et al., in preparation). If generalized, this result would establish the relevance of the cultured cells.

The specificity issue is a very difficult one since it is clearly impossible to test the autologous anti tumoral CTL on every cell of the organism. But it could be solved completely by genetic analysis: if genes coding for the CTL-recognized antigens could be cloned and were found to carry mutations that are absent on a number of normal cells of the patient, then it would become almost certain that the mutation is absolutely specific for the tumor. However, this is presently far from being achieved. A first step in this direction is the analysis of the CTL-recognized antigens by immunoselection. This was performed on melanoma MZ-2. Six different antigens were observed and several CTL clones were found that were directed against each antigen (Hérin et al. 1987; Van den Eynde et al., in preparation). We intend to use the immunoselected cells as transfection recipients for the purpose of cloning the genes that code for these human tumor-associated antigens.

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Recognition of the HLA Class II-Peptide Complex by T cell Receptor: Reversal of MHC Restriction of a T Cell Clone by a Point Mutation in the Peptide Determinant

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Introduction

The recent demonstration that peptide fragments of immunogenic proteins are bound by MHC class II molecules has provided an important explanation for the genetic basis of immune responsiveness (Babbitt et al. 1985; Guillet et al. 1987; Buus et al. 1986). Although not the sole factor, the formation of a peptide-MHC complex appears to be a necessary requirement for both the generation of a cellular immune response and the T cell-dependent humoral response (reviewed by Moller 1987). Even though these experiments have generated a unified model of T cell recognition, not all issues have been resolved. One confusing is if binding to one of an individual's MHC proteins is critical to the generation of an immune response and subsequent protection from infection, how can a very limited number of binding sites interact with a sufficiently large percentage of peptides from the multitude of proteins from pathogens to protect the individual? The ability of an MHC protein to interact with manifold peptides is quite different from the specificity characteristic of other known membrane receptors. In contrast with receptors of the endocrine system, which have evolved to optimize their specific interaction with ligand, the MHC proteins appear to have developed to bind a diverse range of ligands.

A possible explanation is that peptides that bind the MHC proteins and are recognized by T cell receptors are limited in their diversity. Such a proposal has been supported by the structural similarities found in the defined helper and cytotoxic T cell determinants (Rothbard and Taylor 1988). Potential allele specific motifs also can be seen as the number of determinants has increased. At the present time at least two general patterns can be identified. The most common are homologous residues found in relative positions that would constitute a face of the helix if the peptide adopts a helical conformation (examples are shown in Fig. 1 a, b). The similarities can be optimized if selected sequences are reversed, implying that some determinants could bind in the opposite direction of others. The second pattern is less common and appears to be less

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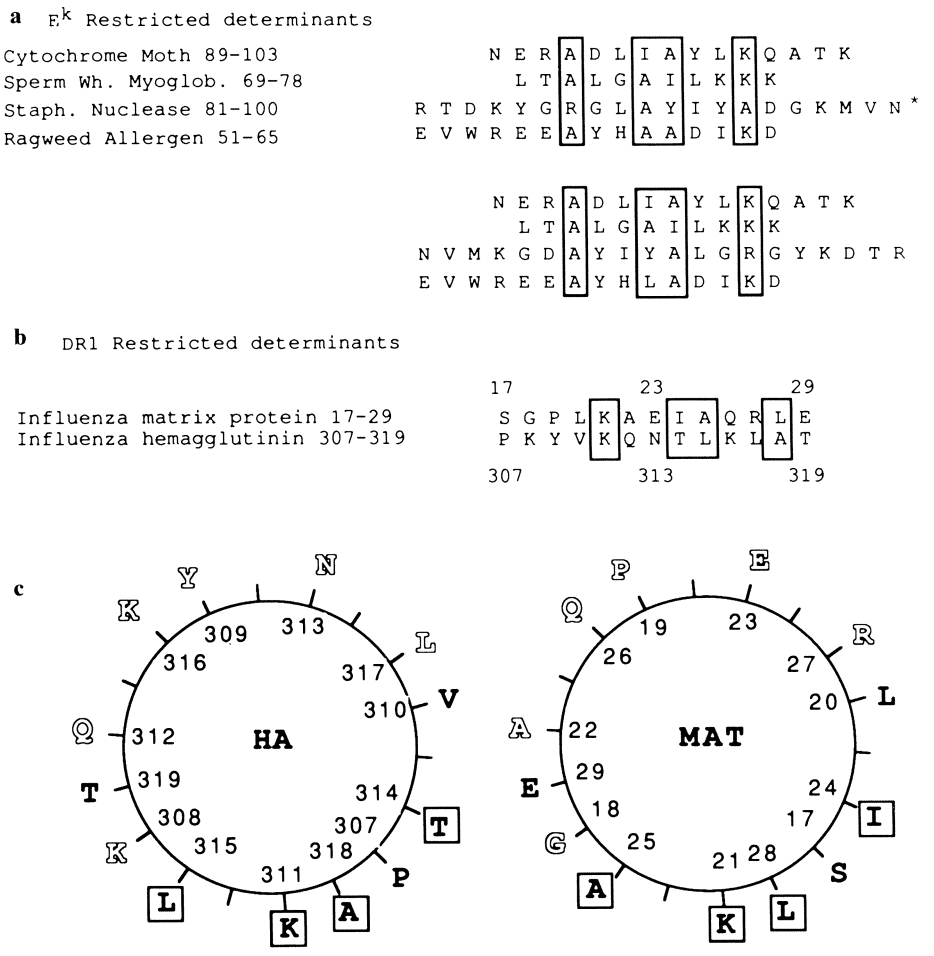


Fig. 1a–d. Structural similarities found in selected T cell determinants. **a** The sequences of four E^k-restricted T cell determinants (Heber-Katz et al. 1983; Livingstone and Fathman 1987; Finnegan et al. 1986; Kuisaki et al. 1986) aligned to emphasize structural similarities at relative positions 1,4,5 and 8 (enclosed in *boxes*). If selected sequences (*) are reversed, similarities can be enhanced. **b** Two DR1-restricted determinants aligned to reveal structural similarity. **c** Two DR1-restricted determinants displayed on *helical wheels* demonstrating the residues enclosed in boxes in **a** and **b** constitute the face of a helix. The outlined amino acids could be exchanged between determinants with maintanence of T cell recognition (Rothbard et al. 1988). **d** Selected T cell determinants oriented to demonstrate sequence similarity around a proline. The hemagglutinin determinant is E^d restricted (Hackett et al. 1982), the murine cytomegalo virus determinant is L^d restricted (Del Val et al. 1988), while the acetyl choline receptor peptide is DR3 restricted (Brocke et al. 1988)

compatible with a helical peptide. It is composed of five amino acids; the first is a charged amino acid which is followed by two hydrophobic amino acids, then a proline, and concludes with a polar amino acid (Fig. 1 d).

To determine whether these structural similarities have any merit, several different strategies have been used to examine the significance of a putative motif for DR1-restricted determinants. The initial experiments examined the shared pattern between the defined I-E^k, E^d and DR1 determinants (Rothbard and Taylor 1988). Successful presentation of peptide antigens to DR1-restricted T cell clones by L cells expressing either the murine I-E alpha chain and the DR1 beta chain or the intact murine I-E^k molecule demonstrated significant structural similarity in the antigen-combining sites of these two class II molecules (Lechler et al. 1988).

The simple motif of a positively charged amino acid and three hydrophobic residues (Fig. 1) also has been used to define previously unidentified DR1-restricted determinants in the ragwed E protein and the 19-kDa protein of *M. tuberculosis* (Rothbard et al. 1988; Lamb et al. 1988). In the latter case, recognition of the peptide in association with DR1 and 4 was confirmed using T cell clones and transfected murine L cell lines expressing DR molecules. As impressive as these predictions were, not all peptides containing this sequence motif will be guaranteed to be restricted by DR1 and 4, most likely because these four amino acids do not represent all the contact points between the peptide and the MHC protein. Nevertheless, the experiments provided further support for the importance of these residues in DR1- and DR4-restricted recognition and represent the potential of using detailed structural requirements to identify determinants a priori.

The third approach for studying the molecular requirements for recognition by DR1 was based on the assumption that if the structural similarity found in DR1-restricted determinants is involved in binding to the MHC protein, then the two influenza peptides, shown in Fig. 1 b, might interact with similar DR1 residues and also adopt an identical conformation and be localized in the same position in the binding site. Consequently, residues interacting with DR1 should be able to be exchanged between the determinants without loss of recognition. The experiment presumes that such an exchange will not prevent the hybrid peptides from adopting the correct conformation by removing important intrapeptide interactions.

For each T cell clone, a hybrid peptide created from such exchanges based on the alignment shown in Fig. 1 was stimulatory (Rothbard et al. 1988). Substituting six residues from the hemagglutinin peptide (printed as hollow characters in Fig. 1 c) for amino acids in the corresponding positions in the matrix sequence resulted in a peptide that was recognized by the hemagglutinin-specific clone. However, an equivalent proliferative response required approximately 100 times as much peptide as the natural sequence. The reciprocal exchange of six residues did not result in a stimulatory peptide for the matrix-specific clone. A possible explanation was the presence of two prolines in the hybrid peptide which was hypothesized to interfere with its ability to adopt a helical conformation. Replacement of one (corresponding to 307 in the hemagglutinin sequence), but not the other (proline-19 in the matrix pep-

tide) with alanine resulted in a peptide that could stimulate the matrix-specific clone.

As can be seen in the simple helical wheel representation, the substitutions necessary to generate stimulatory peptides constituted the complete upper facade of the peptide. Our interpretation was that only if an intact constellation of residues interacting with both the MHC protein and the antigen receptor of the T cell were present will the clone be stimulated. Point mutations at critical positions in either set of amino acids can prevent the peptide from being recognized.

This report further examines both the proposed conformation and location of the hemagglutinin peptide in the DR1 binding site.

Methods

Isolation of Antigen-Reactive T Cell Clones

The isolation and characterization of human T lymphocyte clones HA 1.7 have been described previously (Lamb et al. 1982a, b).

Proliferation Assays

Cloned T cells (5×10^4/ml) were cultured with soluble antigen in the presence of irradiated histocompatible peripheral blood mononuclear cells PBMC (1.25×10^5/ml), autologous Epstein-Barr virus (EBV)-transformed B cells (10^5/ml), or mitomycin C-treated, transfected murine L cells (10^5/ml) in a total volume of 200 μl of complete medium in 96-well round bottom plates. After 72 h of incubation, tritiated methyl thymidine (1 μCi, [^{3}H]TdR; Amersham International, Amersham, U.K.) was added to the cultures for 8–16 h and then harvested onto glass fiber filters. Proliferation as correlated with [^{3}H]TdR incorporation was measured by liquid scintillation spectroscopy. The results are expressed as mean counts per minute (cpm) plus or minus percentage error of the mean for triplicate cultures.

Peptide Synthesis, Analysis, and Purification

Peptides were synthesized using solid phase techniques (Barany and Merrifield 1979) on an Applied Biosystems 430A peptide synthesizer utilizing commercially available reagents as described previously (Rothbard et al. 1988). The purity of the peptides were assayed using amino-acid analysis and high-pressure liquid chromatography on an Aquapore RP-300 reverse-phase column (Brownlee Labs). Each peptide was purified on a RP-300 column using a water-acetonitrile-trifluoroacetic acid gradient prior to circular dichroism measurements. The structures of the analogues were confirmed using fast atom bombardment mass spectroscopy.

Circular Dichroism Measurements

Circular dichroism measurements were performed at room temperature of peptides (0.15 mg/ml) dissolved in differing mixtures of water and trifluoroethanol on a Jasco 41C spectropolarimeter equipped with a model J-D DPY data processor. The values shown are a result of an average of at least three scans.

Results and Discussion

Modification of the Peptide to Improve Potency

If the hemagglutinin peptide binds the restriction element in a helical conformation, then stabilization of this conformation might improve the potency of the peptide. Recent experiments have demonstrated that stabilization of the macrodipole of an alpha helix can result in a significant increase in the helical content of relatively short peptides (Shoemaker et al. 1987). One way to stabilize the macrodipole is to remove the charges at the amino and carboxyl termini of the peptide by acetylation and amidation.

The natural hemagglutinin peptide and several analogues were examined for secondary structure content by circular dichroism in a range of water-trifluoroethanol (TFE) mixtures. The unacetylated and unamidated hemagglutinin peptide exhibited only low helicity even at high TFE concentration (Fig. 2a) as judged by absorption of circularly polarized light at 222 nm. Amidation and, to a greater extent, combined acetylation and amidation substantially increased its helicity of the hemagglutinin peptide. The peptides underwent simple helix-coil transitions, with helicity increasing with greater TFE concentration. The transition was not concentration dependent (data not shown) suggesting that aggregation is not important in the induction of the alpha helix.

Analogues containing alternative amino acids for proline-307 also were examined for greater helical propensity. Replacement of the proline at 307 with arginine does not affect the ability of the peptide to form a helix. However, amidation and acetylation does. Consistent with the hypothesis that the improved helical content is due to stabilization of the macrodipole, acetylation and amidation of the arginine containing peptide results in greater helical content, but not as much as when the positively charged arginine is not at the amino terminus.

When tested in proliferation assays with the T cell clone, HA 1.7, the acetylated and amidated peptide can stimulate the clone at approximately two orders of magnitude lower concentrations than the natural sequence (Fig. 2b). Replacement of the amino terminal proline by either lysine or arginine also results in more potent peptides (Fig. 2b), but the acetylated, amidated peptide containing arginine at 307 is only recognized as well as the natural sequence acetylated and amidated (data not shown). That the effects of exchanging proline for a positive-charged residue and blocking the charged end groups are not additive indicates that the two factors are acting by separate mechanisms.

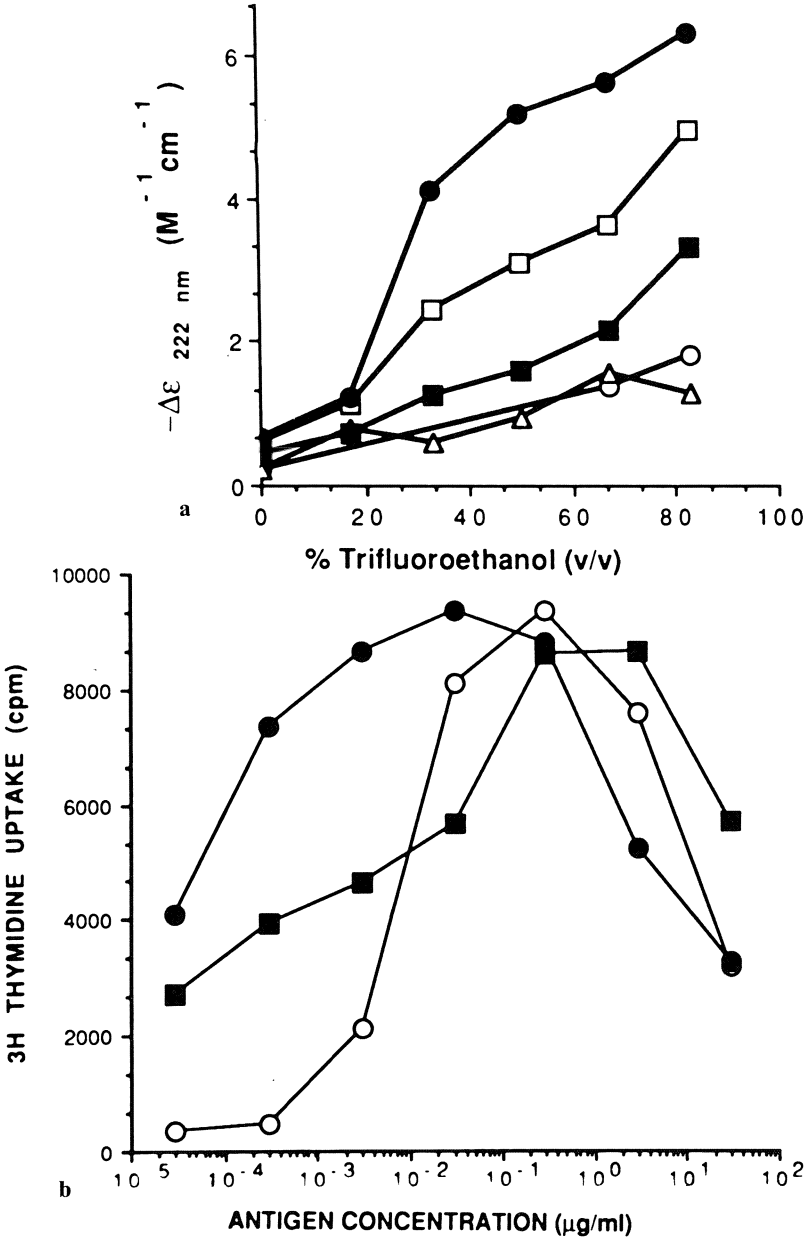


Fig. 2a, b. Increased potency of analogues of HA 307–319 correlates with greater propensity of the peptide to form a helix. **a** Differences in absorption between lift and right circularly polarized light at 222 nm for HA 307–319 *(open circles)* HA 307–319 acetylated and amidated (HA Ac/NH_2, *solid circles*), HA 307–319 with arginine at 307 (HA R-307, *triangles*), HA 307–319 with arginine at 307 only amidated (HA NH_2 R-307, *solid squares*), and HA 307–319 with argine at 307 both acetylated and amidated (HA Ac/NH_2 R-307, *open squares*) in differing water-trifluoroethanol mixtures. On this scale, a CD value of 10 is conventionally taken to be equivalent to 100% helix. **b** Proliferation of HA 1.7 in response to HA 307–319 *(open circles),* HA 307–319 acetylated and amidated (HA Ac/NH_2, *solid circles*), and HA 307–319 with arginine at 307 (HA 307–319 R-307, *solid squares*)

The most likely explanation is that a positive charge at 307 results in a superior interaction with a residue in the binding site of DR1, but as shown, does not stabilize the macrodipole.

Even though these results can be interpreted to be due to the increased propensity of the peptide to be helical, an alternative explanation is that by removing the end charge groups a peptide is created that can bind the restriction element more effectively than the natural sequence. Because these two issues are so closely related, they cannot be easily distinguished at this time.

Identification of Specific Contact Residues in the DR1 Binding Site

If the proposed model of the orientation of peptide relative to the two macromolecules is valid, complementary residues in the binding site to those of the peptide should be able to be identified. To identify aspects of the peptide sequence that were critical for interaction with DR1 and the T cell receptor, peptide analogues of the hemagglutinin peptide containing point mutations were synthesized and assayed for their ability to stimulate the T cell clone.

To identify an approximate position of the peptide in the antigen-combining site of DR1, a model of DR1 was generated based on the published HLA A2 structure (Bjorkman et al. 1987a, b; Fig. 3a). As previously detailed (Brown et al. 1988) alignment of the MHC class I and II alleles, whose sequence has been determined, reveal that the two classes of proteins share a number of structural features. In particular, the antigen-combining sites of both are composed of identical secondary structural units, even though the class II site is composed of residues from both the α and β chains, while that of class I is generated by the folding of a single polypeptide chain. In addition, many of the residues exhibiting sequence variation between alleles can be placed in similar positions in the binding site for both class I and class II MHC proteins.

Generating a working model of the peptide ligand in the proposed binding site involved several major assumptions. We assumed that the antigen receptor of the T cell clone recognizes the peptide in a unique conformation and location in the site, making specific contacts with the residues composing the cleft. These assumptions do not preclude the possibility that the peptide might be able to bind to DR1 in several different locations and perhaps even with different conformations, but these are assumed not be recognized by the antigen receptor of this particular clone. The previous helix exchange experiments were interpreted to have established that the peptide bound in a helical conformation. In the orientation shown in Fig. 1c, the residues forming the lower face of the helix are believed to interact with DR1, while the residues on the upper facade contact the T cell receptor.

Based on analyses of helices packing onto β-pleated sheets (Cohen et al. 1982; Janin and Chothia 1980; Chou et al. 1985) a 13-amino acid peptide folded as three turns of an α-helix can bind in a limited number of ways. Our goal was to generate a working model of the peptide interacting with specific residues in the site, both to understand MHC restriction and to increase binding affinity.

Even though we will speak of potential contacts and even postulate a preferred location of the peptide in the binding site, the details of binding can only be approximated both because of the uncertainty of the class II model and the flexibility of the side chains of both the restriction element and the ligand.

The dimensions of the proposed antigen-combining site are sufficient to bind a helical peptide with its helical axis approximately parallel with those of the alpha-1 helix of class I or the helix of the alpha chain of class II, which form one side of the cleft. In such a model, the residues composing the lower facade of the helical peptide would be expected to closely pack onto the four central strands of the β-pleated sheet of the restriction element. A potential diversity of molecular contacts can result because of the length and flexibility of the amino acid side chains of both the MHC molecule and the peptide. Thus, the corresponding amino acids of two different peptides bound in the same location in the site might not necessarily interact with the identical residues of the β-strands. The amino acids of the determinant forming the sides of the helix could interact with corresponding residues of the helices of the α and β chains of DR1. Steric constraints also will be important, and size limitations were apparent in modeling the helix-helix contacts.

The degeneracy of restriction of the hemagglutinin-specific T cell clone for both DR1 and 4 (Eckels et al. 1984) allowed us to use the natural variants of DR4 to help localize the peptide in the site. EBV-transformed B cells representative of the DR4 dw subtypes were used to present HA 307-319 to the cloned T cells. Marked variations in the efficiency of antigen presentation were observed when the subtypes were compared. B cells expressing dw4 and dw15 were indistinguishable from cells expressing the parental restriction element DR1 (Table 1). However, dw13, 14, and more dramatically dw10, were less effective in presenting antigen. Cells expressing dw13 and dw14 were capable of eliciting approximately 25% of a response generated by the high-responding alleles, whereas dw10 elicited less than 10% of the maximal response at 3 μg/ml. The differences cannot be accounted for by variation in the level of cell surface DR since by flow cytometric analysis the DR density on all the EBV-B cells was not the result of differential processing.

If amino acid sequences of the β1 domain of the DR4 subtypes are compared with DR1, there are multiple differences in the amino terminal half of the domain which contribute to T cell antigen recognition (Table 1). However, the

Table 1. Partial primary sequence of the beta chains of DR1 and DR4 dw 15 (Gregerson et al. 1986; Bell et al. 1987)

		10	20	30
DR1	G D T R P R F L W Q L E F E C H F F N G T E R V R L L E R C			
DR4 dw15	– – – – – – – – E – V – H – – – – – – – – – – – – F – D – Y			
		40	50	60
DR1	I Y N N Q E E S V R F D S D V G E Y R A A T E L G K P D A E			
DR4 dw15	F H J – – – – Y – S – –			
		70	80	90
DR1	Y W N S Q K D L I E Q R R A A V D T Y C R H N Y G N G E S F			
DR4 dw15	– –			

Table 2. Differential response of cloned T cells to HA 307-319 presented by EBV-transformed B cells expressing the DRdw subtypes (Gregerson et al. 1986)

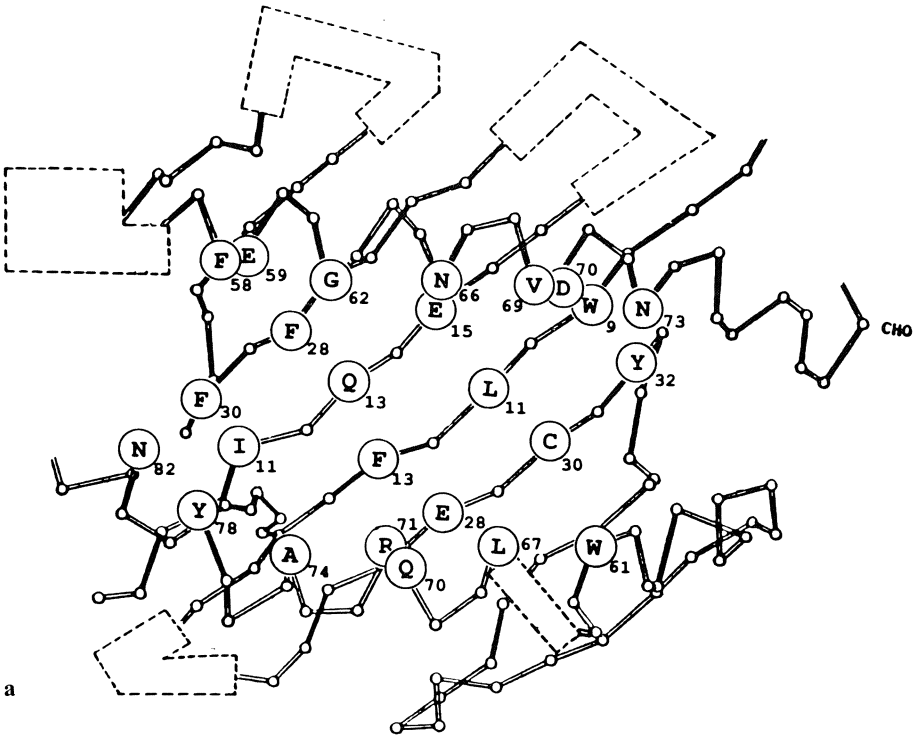
HLA-DR specificity	Amino acids at β chain positions							Proliferative response (Δ cpm) (3 μg/ml)
	57	67	69	70	71	74	86	
DR1	D	L	E	Q	R	A	G	34288
DR4 dw4	D	L	E	Q	K	A	G	22793
dw10	D	I	E	D	E	A	V	2435
dw13	D	L	E	Q	R	E	V	17554
dw14	D	L	E	Q	R	A	V	14562
dw15	S	L	E	Q	R	A	G	38416

Culture conditions and proliferation were determined as described in Methods. Results expressed as Δ cpm with the background response of T cells cultured with EBV-transformed B cells (< 3500 cpm) subtracted from proliferation observed when antigen is present

sequence identity between the DR4 subtypes in this region proves that these differences cannot account for the functional variation seen between the dw4/15- and dw10-expressing cells. Only sequence differences present in the β1 helix can account for the different abilities to present antigen. When the sequence differences between the DR4 dw subtypes are analyzed, prominent variations between the low responder allele, dw10, and the remaining subtypes occur at positions 70 and 71 (Table 2). DR4 dw10 contains an aspartic acid at position 70 and a glutamic acid at position 71. In contrast, the other alleles have glutamine at 70 and a positively charged residue at 71. In the working model of class II proteins, both positions are centrally located in the bent helix composing the side of the proposed antigen-combining site. Residue 70 has been modeled to point up towards the T cell receptor, while residue 71 points in towards the combining site.

The peptide can be placed in the binding site in either of two directions, either parallel or antiparallel with the alpha-chain helix. In the antiparallel direction, a location can be found with lysine-308 and lysine-311 next to two negatively charged amino acids in the binding site, glutamic acid-15 of the alpha chain, and glutamic acid 28 of the beta chain (Fig. 3 b). In this orienta-

Fig. 3a, b. Schematic representation of the amino terminal domains of the α and β chains of HLA DR1. **a** The proposed antigen-combining site of DR1 represented as initially done by Brown et al. 1988. The model is based on the crystal structure of HLA A2. The areas enclosed in *dashed lines* correspond to positions with either additions or deletions of residues between consensus class I or II sequences. Residues enclosed in *circles* are believed to point to the putative antigen-combining site. **b** A potential location of HA 307–319 in the combining site. The peptide is modeled as three turns of a helix and placed in the site antiparallel with the α chain helix. The orientation of the peptide relative to the MHC protein and the antigen receptor is consistent with the experiments exchanging residues between determinants (Rothbard et al. 1988). The residues of the peptide believed to be facing down in the site are enclosed in *diamonds*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E 59
G 62
N 66
E 15
V 69
D 70
W 9
N 73
CHO
F 28
F 30
Q 13
L 11
Y 32
N 82
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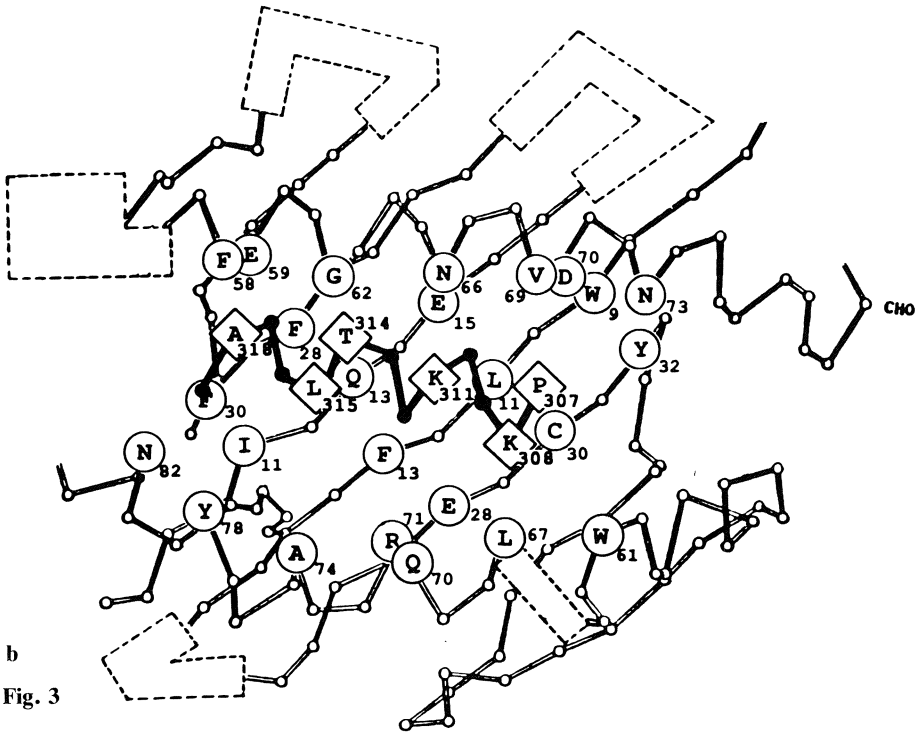
A 74
R 71
Q 70
L 67
W 61

a


F 58
E 59
G 62
N 66
E 15
V 69
D 70
W 9
N 73
CHO
A 316
F 28
T 314
L 315
Q 13
K 311
L 11
P 307
Y 32
F 30
I 11
K 308
C 30
N 82
F 13
Y 78
E 28
A 74
R 71
Q 70
L 67
W 61

b

Fig. 3

tion, residue 71 of the beta chain is directed at glutamine 312 of the peptide. If the peptide binds DR4 in the same location as proposed for DR1, and if the low-responder phenotype arose from the inability of the peptide to interact with the restriction element, responsiveness might be recovered by mutating the peptide at position 312.

When glutamine-312 was converted to a negatively charged amino acid, the peptide analogues were recognized by the T cells as well as the native peptide when presented by DR4 dw15 (Fig. 4a). However, these analogues failed to stimulate the cloned T cells when dw10 cells were used as presenting cells (Fig. 4b). In contrast, a peptide containing an arginine at 312 stimulated the clone when presented by dw10, but not DR4 dw15 APC. Interestingly, peptides containing lysine at 312 were able to be presented by both dw10 and dw15 EBV-B cells.

The reversal of restriction of the T cell implies that residue 312 of the peptide interacts with residue 71 of the β chain of DR4. However, before the interaction is proven, mutations at other positions in the peptide need to be analyzed and additional binding and/or competition experiments need to be performed. The results also demonstrate the subtle nature of the peptide-MHC interactions. There is significant difference between recognition of a peptide containing arginine and lysine at the same position. The differences can be rationalized by differences in the length, chemical character, and flexibility of the amino acid side chains, but at the present time the exact reason or reasons for the variations remains to be determined.

The functional importance of residues 70 and 71 in the β1 helix in T cell recognition has been demonstrated previously in murine T cell responses restricted by I-A^b and the I-A^{bm12} mutants (Kanamori et al. 1984; Ronchese et al. 1987). Also, position 71 has been identified to be critical in the DR4-restricted T cell responses found in rheumatoid arthritis patients (Todd et al. 1988).

The strategy of using mutant HLA molecules to present antigen and to complement the mutations with changes in the peptide appears to be an extremely useful method to analyze both the location of the peptide in the binding site and also to examine the structural requirements for binding and recognition by T cell receptors.

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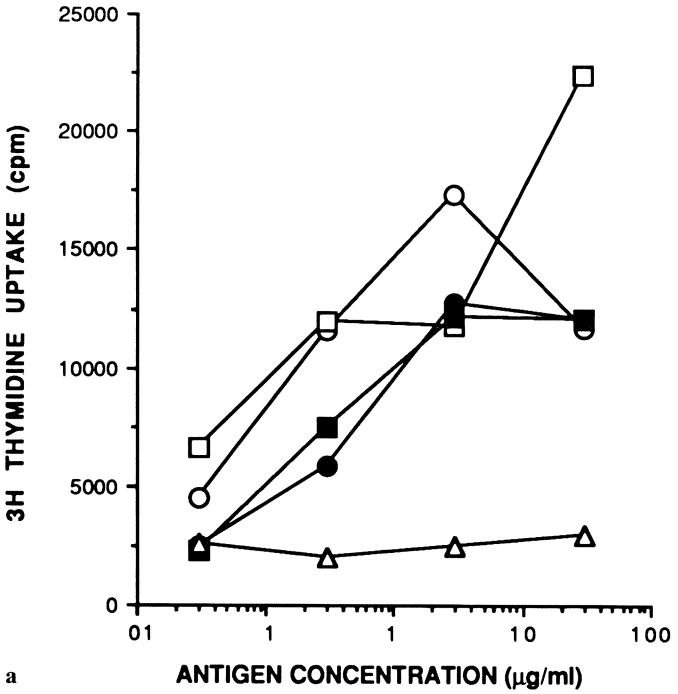
Fig. 4 a, b. Recognition of analogues of HA 307–319 with mutations at residue 312 by HA 1.7 when presented by either by **a** DR4 dw15 or **b** DR4 dw10. EBV-transformed B cells. Proliferation was measured as described in the Methods. 307–319 Q-312, *open circles;* 307–319 D-312, *solid circles;* 307–319 E-312, *open squares;* 307–319 K-312, *solid squares;* 307–319 R-312, *open triangles*


25000
20000
15000
10000
5000
0
3H THYMIDINE UPTAKE (cpm)
01
1
1
10
100
ANTIGEN CONCENTRATION (μg/ml)

a

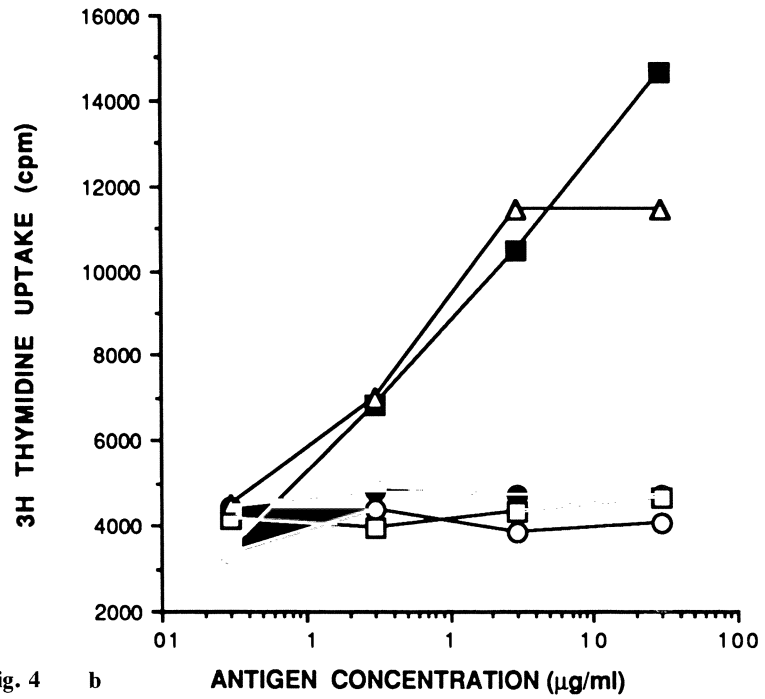

16000
14000
12000
10000
8000
6000
4000
2000
3H THYMIDINE UPTAKE (cpm)
01
1
1
10
100
ANTIGEN CONCENTRATION (μg/ml)

Fig. 4 b

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*Regulatory Aspects of the Antitumor Immune Response**

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Many transplantable animal tumors, by virtue of their possession of tumor-specific transplantation antigens, are capable of evoking an antitumor immune response in their syngeneic hosts. The existence of tumor-specific antigens has been revealed by demonstrating that the tumors that possess them are capable of specifically immunizing their hosts against the growth of a subsequent implant of tumor cells. Thus, the method for determining whether or not a murine tumor is immunogenic has been a transplantation rejection assay that cannot be used in humans. Therefore, in the absence of evidence to the contrary, there is no justification for the statement that human tumors are nonimmunogenic. In fact, on the basis of studies prompted by results first obtained with murine tumors (Rosenberg et al. 1986), it has recently been demonstrated (reviewed by Itoh et al. 1988) that certain human tumors contain specifically sensitized, tumor-infiltrating T lymphocytes (TIL) that can be expanded in number in vitro under the influence of interleukin 2 and used for therapy. This procedure for expanding tumor-sensitized T cells in vitro is likely to supply a great deal of additional evidence to support the view that many human tumors are capable of inducing a T cell-mediated immune response in their autochthonous hosts.

Concomitant Immunity During Progressive Growth of Murine Tumors

It has been demonstrated repeatedly over many years (reviewed by Gorelik 1983) that mice bearing progressive immunogenic tumors can acquire a paradoxical state of concomitant immunity that is capable of preventing the growth of an implant of cells of the same tumors given at a distant site. It was established in the 1970s that concomitant immunity is essentially specific and that it can be passively transferred locally with T cells, according to the Winn assay. More recently, it was demonstrated (North and Bursuker 1984; North and Dye 1985) that concomitant immunity can be passively transferred systemically and can cause regression of established tumors in sublethally γ-irradiated recipients. The donor T cells responsible for tumor regression in these studies were shown

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to be predominantly of the $CD8^+$ phenotype and to be generated progressively between days 6 and 9 of tumor growth. It was also revealed that after day 9 of tumor growth, tumor-sensitized T cells capable of passively transferring immunity are progressively lost, and this is in keeping with the well-documented finding (North 1985) that specific concomitant immunity to a tumor implant decays after a primary tumor grows beyond a certain critical size.

Concomitant Immunity and Temporary Retardation of Tumor Metastasis

Mice die quickly of their transplantable metastatic tumors, in spite of the fact that these tumors evoke the generation of concomitant immunity. It can be demonstrated with ease, however, that mice die much sooner if they are rendered incapable of generating concomitant immunity. For example, mice that have been made T cell deficient by thymectomy and irradiation and restored with bone marrow die much sooner than immunocompetent control mice from intradermal implantation of a standard number of tumor cells (Kearney et al. 1973; Dye 1986). Figure 1 illustrates results obtained in the case of two of the immunogenic tumors under study in this laboratory.

Convincing evidence that concomitant immunity is important in retarding tumor dissemination comes from recent studies of mice bearing the P815 mastocytoma. It was demonstrated (Dye 1986) that this tumor disseminates from a site of intradermal implantation to the draining lymph nodes and spleen during the first 6 days of tumor growth. After day 6, however, tumor dissemination ceases and metastases that are already present in lymph nodes and

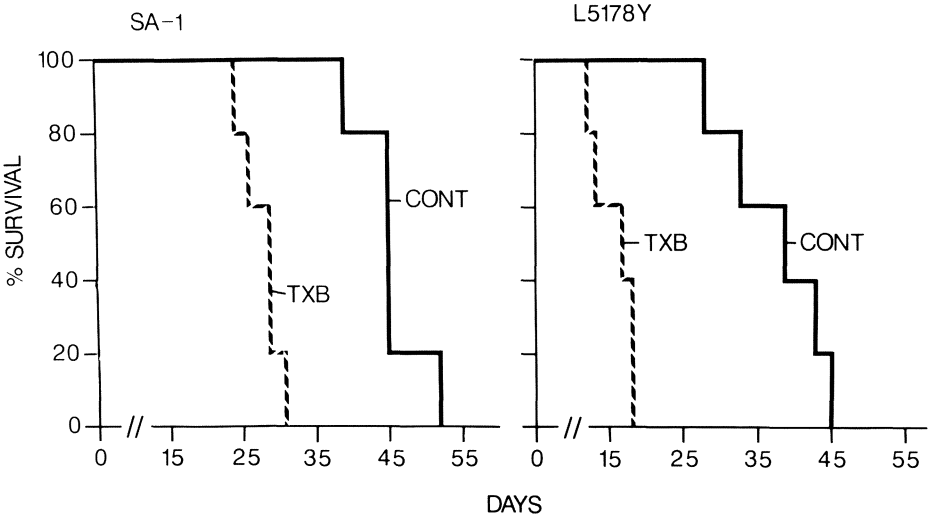

Fig. 1. Evidence that host concomitant immunity serves to retard the development of systemic disease. Immunocompetent control mice *(CONT)* all eventually died of their SA1 sarcoma *(left panel)* or L5178Y lymphoma *(right panel)*. However, T cell-deficient mice *(TXB)* that were incapable of generating an immune response died twice as fast

spleen are destroyed as concomitant immunity is progressively generated by the host. Soon after this occurs, tumor metastasis resumes and progressive systemic disease develops as host concomitant immunity undergoes progressive decay.

Suppressor T cells and Decay of Concomitant Immunity

In view of the preceding discussion, it goes without saying, that the loss of concomitant immunity represents the loss of the host's key defense against metastasis of immunogenic tumors. It is important to understand, therefore, why concomitant immunity is lost progressively after an immunogenic tumor grows beyond a certain critical size. Available evidence strongly supports the view that the loss of concomitant immunity is caused by tumor-induced suppressor T cells. Tumor-induced suppressor T cells have been the subject of several review articles (Naor 1979; Schatten et al. 1984; North 1985).

It is apparent that the most convincing evidence that suppressor T cells are produced in response to the growth of immunogenic tumors consists of the general finding that adoptive immunotherapy of solid tumors with tumor-sensitized T cells from immunized donors requires that the tumor-bearing recipients be immunodepressed by

a) exposure to sublethal ionizing radiation (Eberlein et al. 1982; North 1984),
b) by treatment with cyclophosphamide (reviewed by Awwad and North 1988), or
c) by being rendered T cell-deficient by thymectomy and irradiation (North 1985).

That the recipients need to be immunodepressed in order to remove or prevent the generation of suppressor T cells that act as a barrier to adoptive immunotherapy is evidenced by the demonstration that adoptive immunotherapy in irradiated, cyclophosphamide-treated, or T cell-deficient mice can be inhibited by an infusion of T cells from tumor-bearing, but not from normal donors (North 1984). The T cells that suppress the expression of adoptive immunity were shown to be of the $CD4^+$ phenotype and to be specific for the tumor that evokes their generation (Dye and North 1985).

The major reason for believing that these $CD4^+$ suppressor T cells are responsible for the loss of concomitant immunity is that they are generated progressively as concomitant immunity is progressively lost (North and Bursuker 1984; North and Dye 1985). In the case of two tumors studied in detail in this laboratory, suppressor T cell production was shown to commence after about 9 days of progressive tumor growth, when the tumors reach 8–10 mm in diameter.

Elimination of Suppressor T Cells and Tumor Regression

The finding that suppressor T cells, as defined by physiological adoptive immunization assays, are progressively acquired as effector T cells are progressively

lost does not represent causal evidence that suppressor T cells are responsible for the loss of effector T cells. To obtain such evidence, it would be necessary to show that selective removal of suppressor T cells results in sustained production of effector T cells and in tumor regression. Suppressor T cells have been removed from a tumor-bearing hosts in three different ways:

a) by exposure to sublethal γ-radiation,
b) by treatment with cyclophosphamide, and
c) by infusion of anti-CD4 monoclonal antibody.

In the case of γ-irradiation-induced tumor regression, it was shown (North 1986) that exposure of mice bearing any one of three different tumors to 500 rad of γ-radiation resulted, after several days delay, in complete tumor regression and in long-term host survival. The additional key findings from this study were

a) that γ-irradiation failed to cause regression of tumors growing in T cell-deficient mice and
b) that regression could by inhibited by infusing the irradiated mice with $CD4^+$ suppressor T cells from tumor-bearing donors.

The findings confirm and add to those originally made by others (Hellström et al. 1978).

The ability of cyclophosphamide (Cy) treatment to cause immunologically mediated regression of an immunogenic tumor was demonstrated with the L5178Y lyphoma, a tumor that is resistant to the direct cytotoxic action of Cy (Awwad and North 1988a). It was shown (Awwad and North 1989) that injection of 150 mg/kg of Cy at any time between 4 days prior to or 2 days after implanting 10^6 L5178Y tumor cells intradermally resulted, after 10 days of progressive tumor growth, in complete tumor regression and in long term host survival. The most relevant additional finding from this study was that the therapeutic action of Cy could be inhibited by infusing the Cy-treated host with Cy-sensitive $CD4^+$ T cells from tumor-bearing donor mice.

As for the removal of $CD4^+$ suppressor T cells by treating mice with anti-CD4 monoclonal antibody, this was suggested as a possibility by the result of preliminary experiments showing that concomitant immunity to the L5178Y lymphoma is exclusively mediated by $CD8^+$ T cells and is suppressed by $CD4^+$ T cells. It was demonstrated (Awwad and North 1988b) that infusion of anti-CD4 monoclonal antibody into mice bearing a 9-day (1 cm in diameter) L5178Y tumor resulted after several days delay in complete tumor regression and in long-term host survival. In contrast, treatment of tumor bearers with anti-CD4 plus anti-CD8 monoclonal antibody or with anti-CD8 monoclonal antibody alone resulted in an increased rate of tumor growth and in greatly reduced time of host survival. These and additional results are totally consistent with the interpretation that removal of $CD4^+$ suppressor T cells enabled $CD8^+$ effector T cells to be produced in large enough numbers to cause regression of the L5178Y lymphoma. These results were not obtained with a different immunogenic tumor that evokes the generation of $CD4^+$ T cell-mediated immunity.

Conclusion

It seems likely that all immunogenic tumors evoke, to a greater or lesser degree, a concomitant immune response in their hosts. It goes without saying, however, that concomitant immunity is of insufficient magnitude to cause tumor regression because tumor bearing mice eventually die from systemic disease. Concomitant immunity serves, nevertheless, to temporarily retard the establishment and growth of metastases. The purpose of this communication is to show that in the case of some immunogenic murine tumors, failure of host concomitant immunity to cause tumor regression is the result of the negative regulatory influence of tumor-induced suppressor T cells. In some cases, selective removal of suppressor T cells can lead to tumor regression. In others, however, it cannot. Regardless of the outcome of removing suppressor T cells, it is important that their influence be taken into account when attempting to cause tumor regression with immunomodulators and chemotherapeutic agents.

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Symposium V:
Biological Tumor Therapy

Active Specific Immunotherapy with Autologous Tumor Cell Vaccines Modified by Newcastle Disease Virus: Experimental and Clinical Studies

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Active Specific Immunotherapy

Active specific immunotherapy (ASI) procedures usually involve active vaccination with intact, but inactivated tumor cells or with tumor-derived subcellular membrane fractions. Such material is modified in various ways to make it more immunogenic. Modified tumor cell vaccines which have been applied in cancer patients were prepared for instance by polymerization of autologous tumor tissue (Tallberg 1974; Tykkä et al. 1974; Tallberg et al. 1986; Tallberg and Tykkä 1986; Tallberg et al. 1987; Kurth et al. 1987; Fowler 1986), by neuraminidase treatment (Wunderlich et al. 1985), by chemical modification (Skornick et al. 1984), by the admixture of viruses (Cassel et al. 1977, 1983, 1986; Murray et al. 1977; Hersey et al. 1986, 1987; Wallack et al. 1986; Freedman et al. 1983; Sinkovics 1977), bacteria, in particular *C. parvum* (McCune et al. 1979, 1984), and bacillus Calmette-Guerin (BCG; Mathe et al. 1986; Hoover et al. 1984, 1985), fungi (Schärfe et al. 1986; Fowler 1986), Freund's adjuvant (Hollinshead 1987), carrier proteins such as Keyhole Limpet hemocyanine (KLH; Klippel et al. 1981) or of biological response modifiers (Cortesina et al. 1988; Forni et al. 1984) as adjuvants. Some concepts of ASI procedures have been developed over years in well-defined animal models. Major contributions to the understanding of the principles of active-specific immunotherapy were made in the line 10 hepatocarcinoma guinea-pig tumor model (Hanna et al. 1979, 1980; Hanna and Key 1982), and in the well-characterized, highly metastatic ESb mouse lymphoma model (Schirrmacher et al. 1979, 1982, 1986; Bosslet et al. 1979; Heicappell et al. 1986). In the guinea-pig model a series of studies demonstrated that BCG, admixed with tumor cells, could induce a degree of systemic tumor immunity that would eliminate a small, disseminated tumor burden when the vaccine was carefully controlled for such variables as the number of viable, but nontumorigenic tumor cells (10^7 optimal), the ratio of viable BCG organisms to tumor cells (1:1) and the vaccination regimen (3 vaccines, 1 week apart; Hanna et al. 1980). We reported previously on the

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prevention of metastatic spread by postoperative immunotherapy with virally modified autologous tumor cells in the ESb tumor model (Heicappell et al. 1986; Schirrmacher et al. 1986). A nonlytic, avirulent strain of the paramyxovirus Newcastle disease virus (NDV) was used to infect the tumor cells at a low virus to tumor cell ratio. Such NDV-modified tumor cells were found to have increased tumor immunogenicity and to be effective as tumor vaccines for antimetastatic therapy in combination with surgical removal of the primary tumor. Postoperative immunization with ESb-NDV led to the establishment of long-lasting systemic antitumor immunity (Schirrmacher and Heicappell 1987). Important parameters for optimal therapeutic effects were the time of operation of the primary tumor, the remaining residual disseminated tumor burden, and the dose of virus admixed with a standard dose of 10^7 irradiated tumor cells.

Why NDV?

While there have been occasional reports in the clinical literature (Salmon and Baix 1922; Bluming and Ziegler 1971) that certain viruses can cause tumor destruction in human cancer patients, a first wave of interest in treating cancer patients with viruses developed between the years 1950–1971 (Moore 1955; Southam 1960; Lindenmann 1974). A major drawback to this type of therapy was, however, the observed neurotropism of the most effective viruses which caused encephalitis in some patients. In an attempt to eliminate this side effect, Cassel and Garett (1965) utilized the oncolytic strain 73-T of NDV, which was isolated after passage 73 times in vitro and 13 times in vivo in Ehrlich mouse ascites tumor cells. This paramyxovirus caused no side effects when high doses were injected to humans (Cassel et al. 1977, 1983; Murray et al. 1977). In 1983 Cassel and Murray reported on a phase II study of the postsurgical management of stage II malignant melanoma with an NDV oncolysate (Cassel et al. 1983). Of 32 patients which had been treated over a long time interval with an NDV oncolysate (consisting of a concentrate of live virus and tumor cell membranes from allogeneic or autologous tumor material) 90% remained disease free after 3 years, in contrast to less than 10% of control patients. Meanwhile Cassel has repeated this study (Cassel and Murray 1988), this time involving 51 stage II melanoma patients with three or more (average 3.5) involved lymph nodes. The results of this new study (Group 2), which have not yet been published, are illustrated together with those of the first study (Group 1) in Fig. 1. About 75% of the patients in Group 1 and 63% in Group 2 remained free of disseminated disease over a 5-year period. In Fig. 1 the results of the two studies are compared with each other and with 10 historical control groups involving no less than 624 patients, in which only 17% remained free of disease and survived within the same period. Because of the low pathogenicity of NDV for humans (Waterson et al. 1967; Rolle and Mayr 1984) and these promising clinical results, we became interested in using NDV for active-specific immunotherapy purposes.

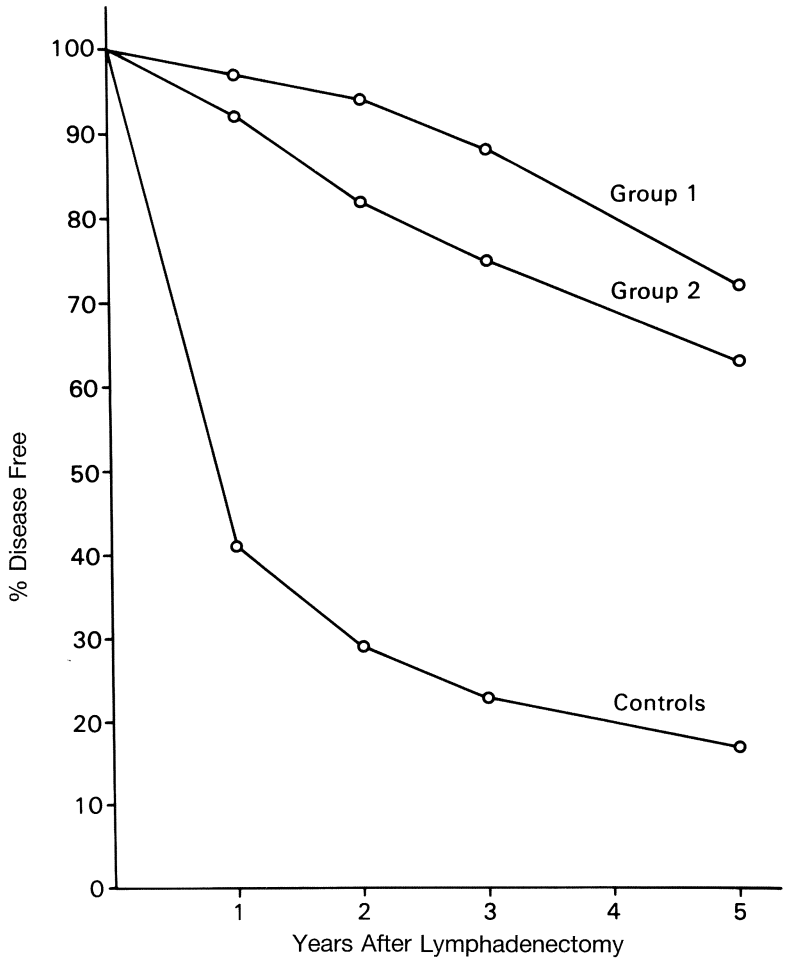

Fig. 1. Results in terms of disease-free survival from two independent phase II studies on the postsurgical management of stage II malignant melanoma with a Newcastle disease virus (NDV) oncolysate. Study 1 *(upper curve)* involved 32 patients with an average number of surgically removed, histologically proven positive lymph nodes of 2.1, study 2 *(middle curve)* performed thereafter involved 51 patients with an average number of surgically removed, positive nodes >3.5. The survival curves of the two groups of treated patients are compared with each other and with 10 historical control groups *(lower curve)* involving 624 patients, where the line is plotted through the mean value at each yearly interval. The survivors in group 1 have remained well for at least 9 years and as long as 13 years, half of the survivors in group 2 for 7–8 years. (Data from W. A. Cassel, personal communication)

New Gene Products Induced by NDV

Why then should NDV be such an effective adjuvant agent for ASI procedures? Through evolution, viruses have as infectious agents stimulated immune responses in their hosts. This has led to the selection of various protection

Table 1. NDV: induction of new mRNA species

New Species induced	Study
Interferon-α,β	Brown et al. 1980; Marcus et al. 1983
Tumor Neurosis Factor-α	Lorence et al. 1988
Adrenocorticotropic hormone and β-endorphin	Westley et al. 1986
Stress (heat shock) proteins	Collins and Hightower 1982
EPA/TIMP	Gewert et al. 1987

mechanisms. Such protective host antivirus immune responses involve the activation of lymphokines, of natural defense reactions, and of specific T and B cell-mediated immune responses. It is thus likely that there is not one single property of a virus like NDV that could explain its efficacy, but several. A study of the literature revealed that the infection of cells by NDV can lead to the novel induction of several distinct genes. These are listed in Table 1. NDV is a potent inducer of interferon (IFN)-α,β (Brown et al. 1980; Marcus et al. 1983), and from the study of DeMayer-Guignard (1984) and others (Merigan et al. 1970) it is clear that IFN-α,β affects cell-mediated immunity as manifested by delayed hypersensitivity and allograft rejection responses. Recently it was found that NDV can also induce tumor necrosis factor (TNF)-α. Infection of tumor cells by NDV can lead to the augmentation of their sensitivity to TNF-α cytotoxicity (Lorence et al. 1988). Another recent finding was that NDV-infected splenocytes expressed the proopiomelanocortin gene (Westly et al. 1984). This is the first evidence that the precursor protein for adrenocorticotropin (ACTH) and β-endorphin can be synthesized by lymphoid cells. The report states: "The physiological significance of hormone secretion by lymphoid cells may be to serve as an afferent signal to the central nervous system or a target organ during viral or bacterial invasion. Synthesis and secretion of biologically active ACTH from lymphocytes would signal the adrenal gland to synthesize and secrete glucocorticoids, and glucocorticoids are well known to play an important role in lymphocyte regulation. ACTH and its derivative β endorphin can also function as a paracrine immunomodulator, since it was found to increase proliferative response of lymphocytes to T cell mitogens, to enhance natural cytotoxicity and interferon production by natural killer cells and to stimulate chemotaxis of human peripheral blood mononuclear cells" (Westly et al. 1984). In another study it was reported that NDV stimulates the cellular accumulation of stress (heat shock) mRNAs and proteins (Collins and Hightower 1982), and recently yet another gene homologous to human erythroid potentiating activity (EPA) identical to tissue inhibitor of metalloproteinases (TIMP) was found to be induced by the virus in mouse cells (Gewert et al. 1987). The 5′ flanking sequences of this gene revealed a set of repeated elements with structural similarity to those previously described as inducer-responsive elements in the human IFN-β1 gene. This list illustrates a multitude of activities of the virus and for the scientists, identifying all the possible interactions is like working a puzzle. Nevertheless, it appears likely that several

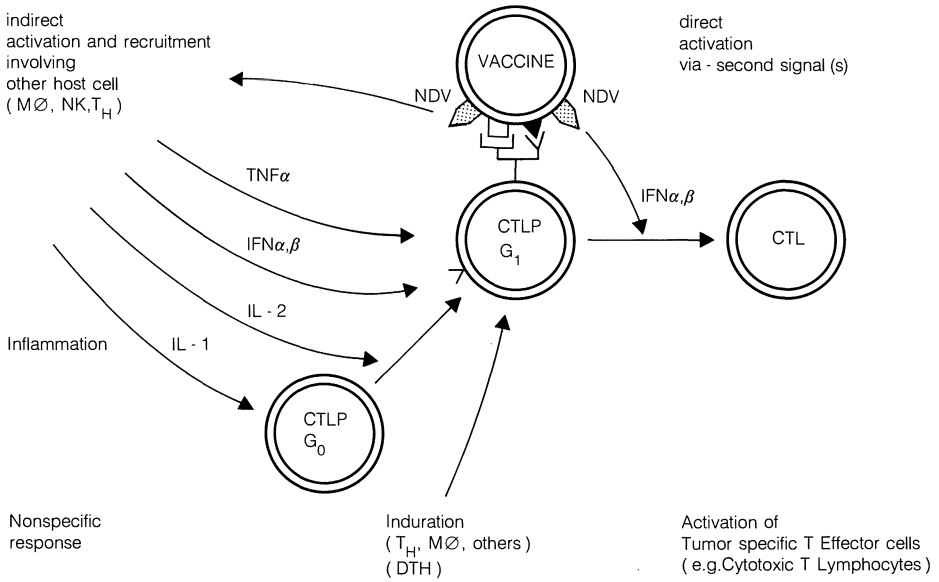

Fig. 2. Model of action of autologous tumor cell vaccine (ATV) modified by NDV. Tumor antigen (▶) associated with a restricting class I major histocompatibility complex (MHC) molecule (□); the MHC-bound tumor antigen is recognized by an antigen-specific T cell receptor ⊥Y expressed on a few T cells within the repertoir of the T cell pool. At the site of application of ATV-NDV, the cell surface-bound virus leads to a nonspecific inflammatory response which is associated with the release of various cytokines (e.g. interleukins , *IL-1, IL-2,* interferons, *IFN-*αβ, tumor necrosis factor, *TNFα).* This may facilitate the local recruitment of T cells with specific T cell receptors (TCR) for the tumor antigen. Antigen–TCR interaction leads to further lymphokine release and to a delayed-type hypersensitivity (DTH) skin response. In the course of such a T cell-mediated immune response the presence of the virus may facilitate the activation of tumor-specific precursor cells *(CTLP)* to cytotoxic T lymphocytes *(CTL)* via second signals. Activated tumor-specific CTL or other effector cells might then recirculate via draining lymph nodes and blood and be able to extravasate, detect, and destroy tumor-derived micrometastases in internal organs if they express the same tumor-associated antigen. Macrophages (MØ), natural killer cells *(NK),* T helper cells *(T_H),* G_0 and G_1 (stages of the cell cycle)

of the virus-induced new gene products play an important role in inflammatory responses such as delayed-type hypersensitivity (DTH) skin reactions. This is schematically illustrated in Fig. 2.

Effects of NDV on Natural Immunity and on Tumor-Specific Immune Responses

In Table 2 we have summarized experimental and clinical findings relating to the effects of NDV on the immune system. Components of the natural immune system, such as natural killer (NK) cells and macrophages, can be activated by

Table 2. NDV: effects on the immune system (experimental and clinical findings)

Findings	Study
Enhancement of NK cell activity	Rees et al. 1987
Stimulation of macrophage phagocytosis	Hamburg et al. 1980
Increase in frequency of tumor-specific CTLP	von Hoegen et al. 1988b
Augmentation of specific killer T cell activity	von Hoegen et al. 1988a
Augmentation of specific helper T cell activity	Schild et al. 1988
Enhancement of local DTH responses of patients to an autologous tumor cell vaccine	Lehner et al. 1988; Table 4, this chapter
Induction of inflammatory mononuclear cell infiltrates in cerebral metastases during concurrent therapy of melanoma patients with NDV oncolysates	Cassel et al. 1986

NDV to increase their respective functions, namely, cytotoxicity (Rees et al. 1987) and phacocytosis (Hamburg et al. 1980). Viruses like NDV attached to the tumor cell surface seem to activate natural immune reactions even in T cell-deficient nude mice (Schirrmacher et al. 1986).

More importantly, NDV can also selectively affect T cell-mediated, tumor-specific immune responses (von Hoegen et al. 1988a, b) which are of primary importance for tumor rejection (Evans and Duffy 1985, Zangemeister et al. 1989). This we have analyzed and elaborated in detail in the murine ESb tumor model. First, we confirmed findings already reported previously for other viruses that viral modification of tumor cells can increase their immunogenicity (Kobayashi 1979; Schirrmacher et al. 1986; Hirsch et al. 1988). Animals inoculated with 5×10^4 live ESb cells into the external ear (pinna) – the only site at which DBA/2 mice can spontaneously reject the syngeneic malignant tumor ESb – developed systemic immunity and rejected in a TD50 assay 1.8×10^4 live tumor cells upon challenge in the back. For comparison, animals immunized similarly with NDV-modified ESb cells, rejected as many as 5×10^6 live ESb tumor cells. The immunogenicity was thus increased by a factor greater than 250 by adding 160 hemagglutinating units of NDV Ulster per 2.5×10^7 ESb tumor cells (Schirrmacher et al. 1986). When we analyzed in detail the immune response of animals inoculated in the pinna with either live ESb or live ESb-NDV cells, we found three levels where NDV exerted an amplifying effect on the immune response:

1. An increased $CD4^+$ T cell-mediated helper response (Schild et al. 1989)
2. An increased frequency of tumor-specific cytotoxic T lymphocyte precursors (CTLP) at the peak of the response (9 days after immunization; von Hoegen et al. 1988)
3. An increased stimulatory capacity of NDV-modified ESb cells for activating tumor-specific CTLP to mature cytotoxic CTL in vitro (von Hoegen et al. 1988b).

Table 2 also contains conclusions from clinical data. As will be shown below in more detail, it appears that NDV enhances the local DTH responses of

patients to an autologous tumor cell vaccine. This conclusion is derived from studies which were initiated about 1 year ago in three clinics in the Federal Republic of Germany (FRG) (Homburg/Saar, Stuttgart, and Heidelberg). Furthermore, in melanoma patients, vaccination with NDV oncolysates was reported to lead to the induction of inflammatory mononuclear cell infiltrates at the sites of metastases (Cassel et al. 1986). In this study, the metastases were even located in the brain, so that the mononuclear cells that infiltrated the metastases had to cross the blood-brain barrier. From recent studies by H. Wekerle (personal communication) there is indeed evidence that activated T lymphocytes can cross the blood-brain barrier.

Antitumor Effects Following Modification of Tumor Cells by NDV

In Table 3 we have summarized the antitumor effects so far observed following modification of tumor cells by NDV. Firstly, when NDV-infected human melanoma cells were mixed with an excess of nonmodified melanoma cells and implanted subcutanously into nude mice, a striking retardation of melanoma tumor growth was observed in comparison with another group of animals inoculated with uninfected melanoma cells alone (Schirrmacher et al. 1986). Secondly, we observed in a variety of metastatic tumor systems that the intravenous inoculation of NDV-modified tumor cells in comparison with nonmodified tumor cells led to a dramatic reduction in their organ-colonizing potential. It was furthermore reported that neoplastic cells infected by NDV become more sensitive to the cytolytic effects of TNF-α (Lorence et al. 1988). As already mentioned the application of NDV-modified ESb tumor vaccines in ESb tumor-bearing animals in which a locally growing tumor had been surgically removed led to long-term survival (in different experiments between 30% and 75% of tumor-bearing animals, Schirrmacher, 1986), whereas control animals which had only been operated on all died from the outgrowth of ESb

Table 3. NDV: antitumor effects

Effects	Study
Retardation of the local growth of human tumor cells in nude mice	Schirrmacher et al. 1986
Reduction of organ colonization potential of tumor cells	Schirrmacher et al., unpublished data
Antimetastatic effects in the ESb animal tumor model by postoperative ASI vaccination with ESb-NDV	Heicappell et al. 1986; Schirrmacher et al. 1986; Schirrmacher and Heicappell 1987; Schirrmacher 1986; von Hoegen et al. 1988b
Enhancement of the sensitivity of neoplastic cells to the cytolytic effects of TNF-α	Lorence et al. 1988
Significant increase in disease-free survival of 83 melanoma patients after therapeutic lymphadenectomy and immunotherapy with NDV oncolysates	Cassel et al. 1983; Cassel and Murray, 1988

metastases. Similarly, the clinical data from Cassel et al. (1983) showed a highly significant increase in disease-free survival after 5 years of altogether 83 melanoma patients in two separate studies after therapeutic lymphadenectomy followed by immunotherapy with NDV oncolysates.

Clinical Studies With NDV-Modified Autologous Tumor Cell Vaccines

Based on the ASI concept developed from our animal tumor model we have tried to establish a similar vaccination protocol for cancer patients. The vaccine consists of freshly isolated autologous intact tumor cells inactivated by irradiation and modified by coincubation with a small amount of NDV. In a clinical phase I study, ASI with ATV-NDV was given to cancer patients postoperatively to activate antitumor immune responses with the aim of prolonging disease-free survival and to reduce the number of recurrences. Following potentially curative surgical resection, the tumor was dissociated mechanically and enzymatically and cryopreserved. Prior to inoculation into patients, the frozen cells were thawed carefully and inactivated by x-irradiation with 200 Gy using a ^{137}Cs source (Gammacell 1000, Atomic Energy of Canada Ltd., Canada). The cells were than incubated at 37° C for 1 h with the nonvirulent Ulster strain of NDV. The patients were immunized intradermally into the thigh according to a special protocol at least three times at weekly intervals. The study had been approved before hand by all three ethic commissions of the three clinics involved. The effect of the vaccine was documented by DTH skin reactivity. Figure 3 illustrates the vaccine application and the typical skin reactions observed.

Results

Here we want to summarize our experience with the preparation of the vaccines, their characteristics, as well as the cell-mediated immune responses observed in the treated cancer patients. No statements will be made yet about therapeutic effects since it is far too early and the results have to be evaluated in a randomized prospective trial. The autologous tumor cell vaccine (ATV) was characterized by cytological as well as immunocytochemical methods. About 5×10^7 cells were obtained from colorectal cancer tissues per gram. The average viability of the cells was 75%. There were no differences before and after thawing. As demonstrated by electronmicroscopy, the vaccine was a mixture of different cell types. The average tumor cell content was 60%, the rest were erythrocytes, leucocytes and other cell types, as well as cell debris. The vaccines contained intact autologous tumor cells and a few virus particles absorbed to the cell surface (Liebrich et al. 1988).

Starting about 3 weeks after operation patients were vaccinated in a chessboard test using different amounts of tumor cells and virus, either separately or in combination. We also occasionally used normal colon mucosa cells for specificity control. The number of tumor cells ranged from 1×10^6 to 2×10^7

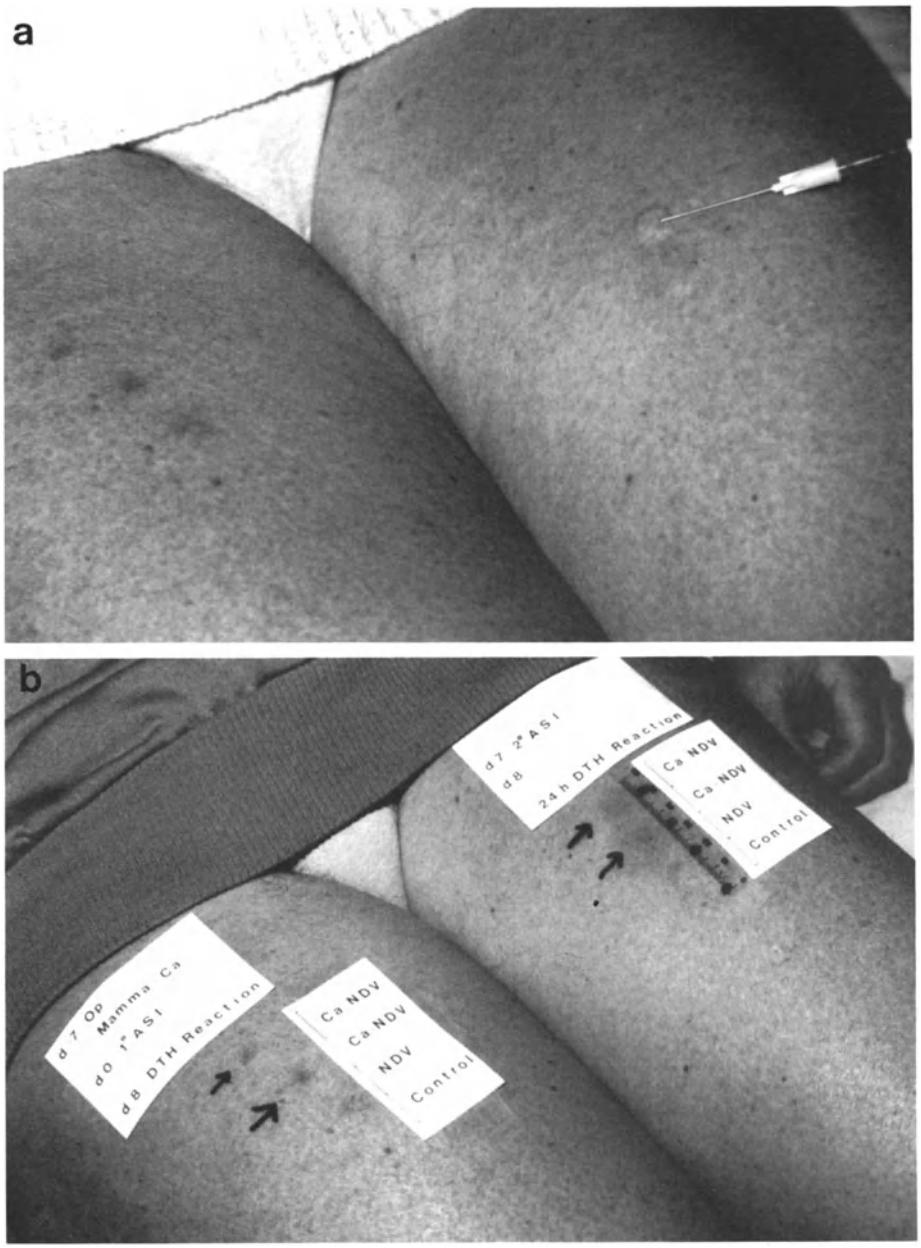

Fig. 3a, b. Local DTH skin responses of a mammary carcinoma patient. A mammary carcinoma (T 2 NO MO) was operated (day-7) and 40×10^6 cells isolated by a special procedure. Most cells stained with a monoclonal antibody specific for mammary and ovarial carcinoma cells. We started the first ASI vaccination 1 week after operation (day 0), 2 weeks later the second, and 3 weeks later the third one. **a** *Right,* second inoculation of autologous tumor vaccine 7 days after the first vaccination in the other thigh. *Left,* skin reaction 7 days after first vaccination. **b** Skin reactions 24 h later. Positive skin reactions with central induration indicated by *arrows.* Control inoculations with medium alone showed no reaction, injections of NDV virus alone resulted in slight inflammation without induration

Table 4. Enhancement via NDV of local DTH responses of cancer patients to an autologous tumor vaccine

	DTH response[a] against			
	ATV		ATV-NDV	
Patients[b]	Response 24–48 h after first vaccination	Strongest response altogether	Response 24–48 h after first vaccination	Strongest response altogether
Mamma CA				
S.J., f, 73	n.d.	+	+	+++
M.B., f, 40	–	+	+	+
B.K., f, 52	–	–	++	++
Ovarian Ca				
D.J., f, 41	n.d.	n.d.	+	++
M.H., f, 61	+	+++	++	+++
B.E., f, 24	n.d.	n.d.	++	+++
Stomach Ca				
K.S., m, 65	–	+	+	++
O.K., m, 48	–	+	–	+
F.F., m, 79	+	+	+	++
Colon Ca				
M.J., f, 41	+	+	++	++
K.T., f, 59	+	++	+++	+++
M.B., f, 65	–	–	++	+
E.R., m, 61	++	+++	++	+++
I.W., f, 55	–	–	–	–
D.R., m, 53	+	n.d.	+	+++
R.O., m, 61	–	+	–	+
Sigmoid/rectum Ca				
K.E., m, 55	–	–	–	+++
R.W., f, 56	+++	+++	+++	+++
S.S., m, 53	++	+++	++	+++
Kidney Ca				
E.G., f, 65	n.d.	+	++	+++
Malignant melanoma				
R.K., m, 62	–	+	+	+
D.M., f, 29	–	+	++	++
I.S., f, 52	+	++	+	+++
P.H., m, 29	–	–	–	+
A.F., f, 55	–	–	+	+++
MH.S., m, 33	–	–	–	+++
AML				
M.B., f, 25	–	+	+	+

DTH, delayed-type hypersensitivity; ATV, autologous tumor vaccine; ATV-NDV, virus-modified ATV; Ca, carcinoma; AML, acute myelogenous leukemia; n.d., not done

[a] Intensity of induration in mm in diameter, whereby –, < 2 mm; +, 3–5 mm; ++, 5–10 mm; +++ > 10 mm.

[b] Patients are categorized according to type of tumor and their initials, sex, and age indicated.

and NDV concentrations from 2 to 64 hemagglutinating units (HA). The first clinical phase I study involved 20 patients with colorectal cancer following potentially curative surgical resection of their tumors (Lehner et al. 1988). Of these, 17 patients responded with a DTH reaction to the vaccine. Best DTH reactions were obtained by using a vaccine consisting of 1×10^7 tumor cells and 32 HA of NDV. This produced a mean induration of 7 mm, ranging from 3 mm to 18 mm. Responses to NDV alone were seen in three patients only, with a mean induration of 2 mm; 13 patients responded to tumor cells (1×10^7) alone. Mean induration was 4 mm. In 8 patients DTH responses to normal colon mucosa was also tested. Four of the patients responded weakly with a mean induration of 3 mm. DTH responses to the vaccine increased throughout the repeated vaccination and median induration at the end of the vaccinations was 8.5 mm with the vaccine of 1×10^7 tumor cells and 32 HA NDV. There were no severe side effects besides mild fever and headache in 25% of the patients.

In Table 4 we have summarized our latest data on DTH skin responses of 27 patients with different types of cancer (carcinomas of different origin, melanoma, and leukaemia), all of which had been subjected to the above ASI procedure with either ATV or ATV-NDV. Table 4 shows the skin responses after the first inoculation of the vaccine, as well as the strongest skin responses seen during the whole course of treatment. Of the patients inoculated with ATV 39% showed DTH reactivity after the first inoculation, while with ATV-NDV this percentage was raised to 78%. Also the intensity of the skin response as measured by the diameter of induration was increased by the presence of the virus, so that on the average the diameter was twice that seen with ATV alone. The strongest skin reactions were seen with ATV-NDV after several vaccinations and involved 96% of the patients.

Although it seems too early to report on clinical outcome, our trial shows the induction of immune responses to ATV modified with NDV without concomitant side effects. We believe that these positive findings warrant further investigations of this new type of vaccination procedure. Many questions need to be resolved, such as the specificity and the type of cells involved in the response, the optimal time intervals for long-term vaccination protocols, and finally an evaluation of the antimetastatic effects.

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The Effect of Interferon on Experimental Tumor Metastases

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Introduction

The finding that potent preparations of mouse interferon α/β inhibited the development of several viral-induced leukemias of mice, inhibited the growth of transplantable ascitic tumors, and markedly increased survival time (reviewed in Gresser 1977) prompted us to determine the effect of similar interferon preparations on the development of tumor metastases.

Studies and Results

Pulmonary Tumor Metastases

Interferon inhibits the development of pulmonary tumor metastases. Daily administration of interferon markedly inhibited the growth of the Lewis lung (3LL) tumor implanted subcutaneously (s.c.) and the number of pulmonary metastases. Interferon was effective in inhibiting the development of pulmonary metastases even when treatment was initiated 6 days after inoculation of 3LL cells, a time when palpable s.c. tumor nodules were already present (Gresser and Bourali-Maury 1972). Since this time, there have been a few reports showing an inhibitory effect of mouse interferon (Glasgow and Kern 1981; Sakurai et al. 1986; Ueda et al. 1986) or human recombinant hybrid interferon αA/D (Brunda et al. 1984; Nishimura et al. 1985; Yokoyama et al. 1986; Ramani et al. 1986) on experimental pulmonary metastases in mice. In most of these studies, interferon treatment was most effective or only effective when administrated prior to injection of tumor cells (Brunda et al. 1984; Nishimura et al. 1985; Yokoyama et al. 1986; Ueda et al. 1986), and in only 2 studies (Gresser and Bourali-Maury 1972; Ramani et al. 1986) was a clear-cut therapeutic effect observed when interferon treatment was initiated several days after tumor inoculation.

More recently we have also shown that daily interferon α/β treatment (about 1.6×10^6 units/day per mouse) initiated 24 h after the i.v. inoculation of B16 melanoma cells inhibited the development of pulmonary metastases and increased mouse survival time (Fig. 1). These results are in contrast to those of Brunda et al. (1984) and Ueda et al. (1986) who found that interferon treat-

V. Schirrmacher, R. Schwartz-Albiez (Eds.)
Cancer Metastasis

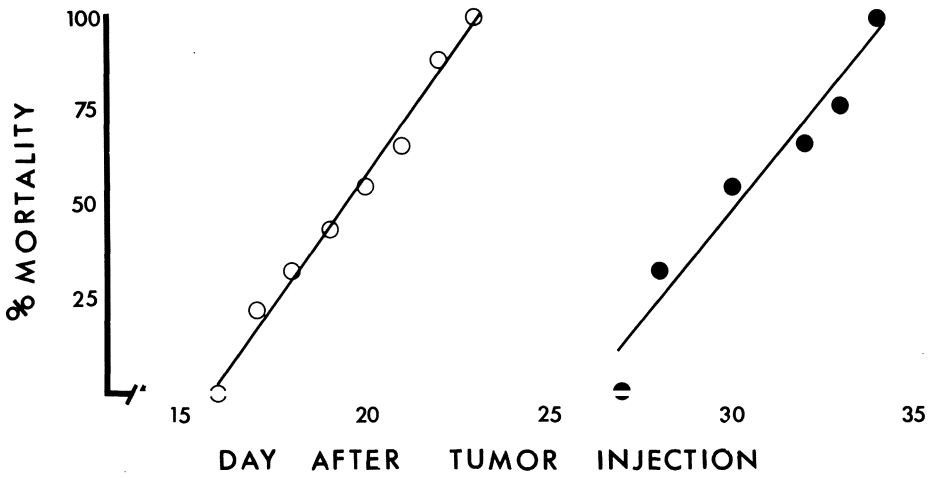

Fig. 1. Increased survival in C57Bl/6 mice injected with B16 melanoma cells. Male 7-week-old C57Bl/6 mice were injected intravenously with 10^6 B16 melanoma cells passaged in cell culture (approximately 300 LD_{50}). Nine mice were injected i.p. with bovine serum albumin (BSA; *open circles*) or 1.6×10^6 units of natural mouse interferon α/β (specific activity approximately 2×10^7 reference units/mg protein; closed circles) 24 h later. There were nine mice in each group. All dead mice had massive melanoma nodules in both lungs and often in the kidney, abdominal lymph nodes, peritoneum, and especially in the adrenal glands. The mean day of death for BSA-treated mice was 19.9 ± 0.8 days and for interferon-treated mice 30.8 ± 0.8 days ($P < 0.001$)

ment was ineffective when initiated after i.v. inoculation of B16 melanoma cells. One of the reasons for this difference may be that these investigators used interferon preparations that were probably about 40- to 100-fold less potent than those used in our studies.

Liver and Splenic Metastases

Interferon inhibits the development of liver and splenic metastases. I should like to summarize our recent studies on the effect of interferon on the development of liver metastases induced by Friend erythroleukemia cells. DBA/2 mice injected subcutaneously (s.c.) with in vivo-passaged interferon-sensitive or -resistant Friend erythroleukemia cells (FLC) usually died 3 weeks later with extensive liver and spleen metastases (Belardelli et al. 1983, 1984; Amici et al. 1984). Injection of established s.c. interferon-resistant 3Cl8 FLC tumor nodules with mouse interferon α/β resulted in an ischemic tumor necrosis, regression of the primary tumor and inhibition of liver and spleen metastases in some mice, and an increased survival time (Belardelli et al. 1983). In contrast, injection of FLC nodules with tumor necrosis factor (Gresser et al. 1986a), histamine, or serotonin also induced necrosis of the primary tumor, but did not inhibit the

development of metastases (I. Gresser, unpublished observations). Although these findings suggested that interferon treatment might be exerting an effect not only on the primary tumor, but also on the development of metastases, we could not rule out the possibility that intratumoral injection of interferon might have impeded the egress of tumor cells from the tumor nodule. Accordingly, we undertook experiments in which we excised the established s.c. primary FLC tumor at a time when tumor cells had already metastasized to the liver and then treated the mice daily with interferon. All control-treated mice died in the ensuing 2 weeks with extensive tumor metastases in the liver and spleen. Interferon treatment resulted in an inhibition of the development of liver and spleen metastases and a markedly increased survival time (Gresser et al. 1987). These results showed that interferon α/β was effective as adjuvant therapy after surgery for metastatic disease in mice.

To study further the effect of interferon on the development of FLC tumor metastases in the liver and spleen, we injected mice intravenously (i.v.) with very large numbers of FLC which resulted in rapid and massive tumor growth in the liver and spleen, and death of all mice in the ensuing days (illustrated in Fig. 2). Daily treatment of mice with potent preparations of mouse interferon α/β was initiated 3–72 h after i.v. inoculation of tumor cells. It is important to emphasize that at these times, FLC had already implanted in the liver and spleen (Gresser et al. 1988a). One other point should be emphasized: in these studies we used the interferon α/β resistant 3Cl8 clone of FLC (Affabris et al. 1982) shown to be resistant to several effects of interferon α/β not only in vitro, but also within the peritoneum (Gresser et al. 1986b; Locardi et al. 1987). Despite the resistance of these FLC to a direct effect of interferon, interferon treatment of mice resulted in a 100- to 1000-fold inhibition of FLC multiplication in the liver and spleen and a marked increase in the mean survival time. Figure 3 summarizes the results of four experiments. The mean day of death for interferon-treated mice was 82.7 ± 10.8 days compared with 9.2 ± 0.1 days for control mice. It was of interest that several interferon-treated mice in these experiments died with macroscopically normal livers and spleens, but showed FLC tumors elsewhere. (Thus, several mice had renal FLC tumors, or intra- or retroperitoneal tumors involving mesenteric, mediastinal, and retroaortic lymph nodes, massive soft-tissue tumors, or tumor invasion of the vertebral column, ovary, or meninges). Nevertheless, despite this overwhelming tumor

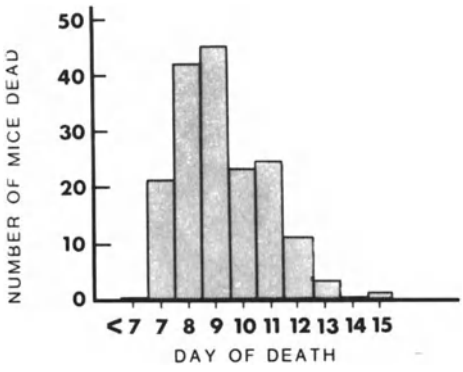

Fig. 2. Summary of results of experiments showing the rapid onset of death in DBA/2 mice injected with 2×10^6 Friend erythroleukemia cells (FLC; equivalent of approximately 5×10^5 LD50). There were 18 experiments conducted over a time period of 18 months involving a total of 170 mice. The mean day of death was 9.2 ± 0.12

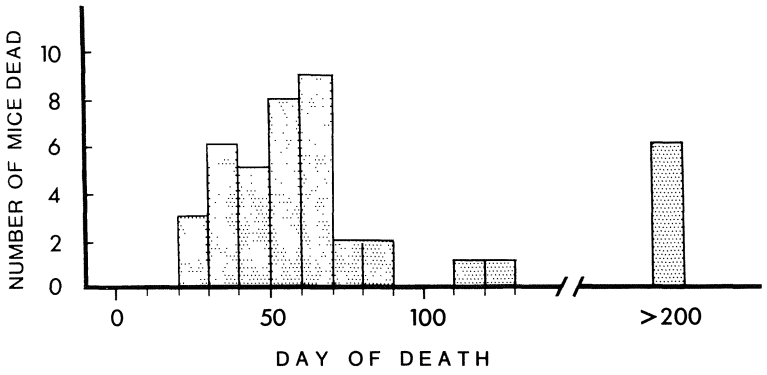

Fig. 3. Summary of results of experiments showing the effect of daily interferon α/β treatment (1.6×10^6 units/day per mouse for at least 1 month) on the survival time and overall survival of DBA/2 mice injected i.v. with 2×10^6 FLC. There were 4 experiments conducted over a time period of 2 years involving a total of 43 mice. The mean day of death was 82.7 ± 10.8

challenge (2×10^6 FLC i.v.), 6 of 43 (14%) interferon-treated mice were considered cured, as they were apparently tumor free for more than 200 days after tumor inoculation (Fig. 3; Gresser et al. 1988a).

Interferon is far more effective than single-agent chemotherapy in increasing survival time of mice after intravenous inoculation of Friend erythroleukemia cells

In view of the very marked inhibition by interferon of the development of visceral metastases, it was of interest to compare its efficacy with that of various cytostatic drugs in increasing mouse survival time after i.v. inoculation of FLC. In contrast to the marked therapeutic effects of interferon α/β, treatment of FLC injected (i.v.) mice with cyclophosphamide, 5-fluorouracil, and methotrexate increased survival time by only a few days; and treatment of mice with cisplatin, vincristine, doxorubicin, bleomycin, or etoposide was ineffective. However, when FLC were injected i.p., both cytostatic drugs and interferon exerted an antitumor effect (Gresser et al. 1988b).

ESb Tumor Metastases in the Liver

Interferon fails to inhibit the development of ESb tumor metastases in the liver.

We concluded from these experiments that interferon α/β was particularly effective in inhibiting the development of liver and spleen metastases and in increasing mouse survival time after i.v. inoculation of FLC. The use of inter-

feron-resistant tumor cells in these experiments indicated to us that the antitumor effect of interferon was most probably mediated by the host and did not stem from a direct effect of interferon on the tumor cells themselves. Was the liver unusually sensitive to this effect of interferon, or was the efficacy of interferon related to the use of FLC? V. Schirrmacher kindly sent us his line of mouse ESb cells which also metastasize to the liver (Schirrmacher et al. 1979). Like FLC, ESb cells were resistant in cell culture to the inhibitory effect of even large amounts of interferon α/β on cell multiplication (I. Gresser, unpublished observations). We injected DBA/2 mice intravenously with 2×10^5 ESb cells and found that the same interferon preparations that had so effectively protected DBA/2 mice injected with 2×10^6 FLC did not increase the survival time of ESb injected mice by even 1 day. When ESb cells were injected intraperitoneally, they rapidly implanted in the liver and killed the mice. Interferon treatment did not affect the survival time of mice. Likewise, although interferon induced necrosis of established s.c. ESb tumors, it again failed to increase the survival time as all the mice died with extensive liver metastases.

Patterns of Liver Invasion

FLC and ESb show different patterns of liver invasion.

I cannot explain why interferon proved so effective in inhibiting the development of liver metastases in mice injected with FLC and so ineffective in mice injected with ESb cells, but I would like to suggest that the differences in the pattern of invasion of the liver by these two types of tumor cells has some relevance. Sequential histologic examination of the livers of mice injected i.v. with FLC showed that at 5 days there were discrete foci of tumor cells that enlarged and increased in number in the ensuing days. Tumor foci consisted of large numbers of FLC that remained in close contact (Figs. 4, 5). This pattern of tumor invasion of the liver is characteristic of metastatic carcinomas. In contrast, the ESb tumor cells infiltrated the liver diffusely. They did not form clear-cut foci, but instead lined up as individual cells mostly within the sinusoids (or in Disse's space) (Fig. 6). This pattern of tumor invasion of the liver is often seen in leukemic infiltration of the liver.

Speculations on how interferon inhibits liver metastasis

Why does interferon inhibit the development of FLC liver metastases but not ESb liver metastases?

It may be that the different patterns of invasion of the liver by FLC and ESb cells are related to the presence of particular glycoproteins on the cell surface. For example, Benedetto and coworkers have shown that there are marked differences in surface glycoproteins between the highly tumorigenic and liver

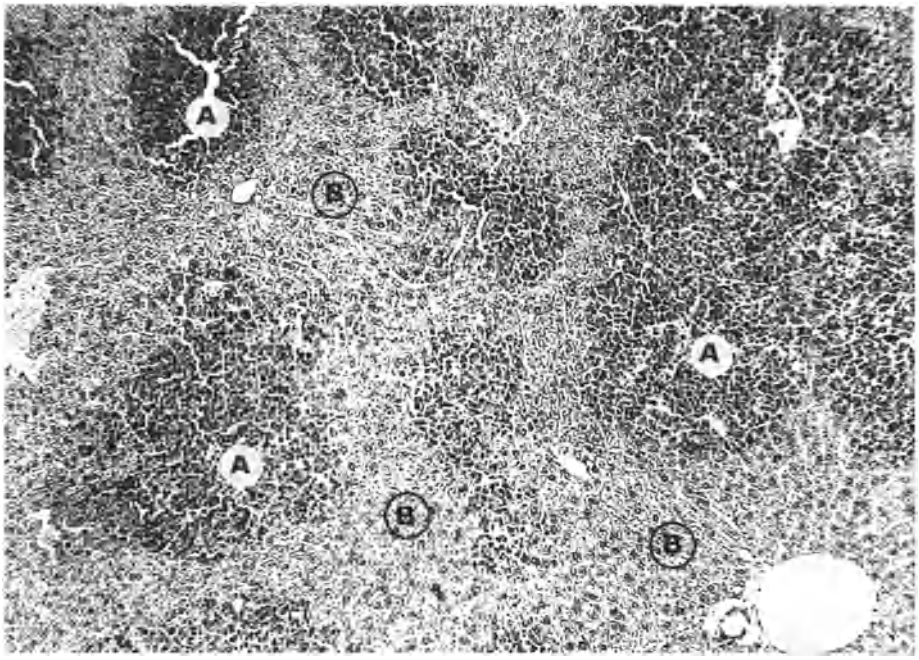

Fig. 4. Liver of a DBA/2 mouse 7 days after inoculation of 2×10^6 3Cl8 FLC. Note prominant multiple foci of FLC (*A*) and normal liver parenchyma (*B*)

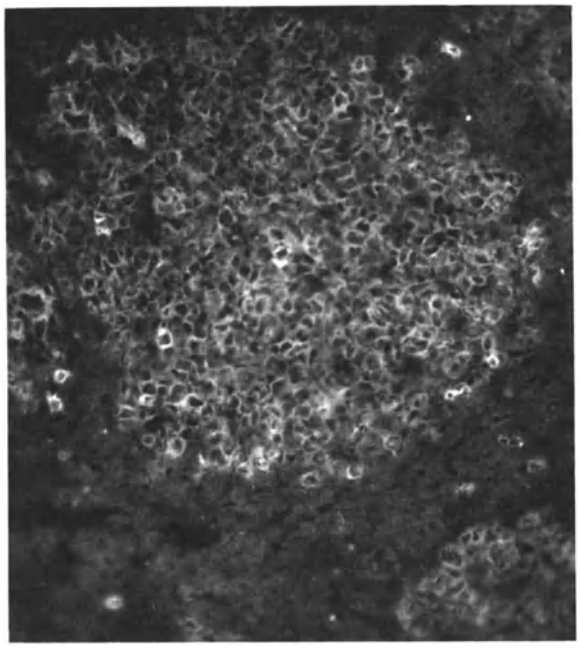

Fig. 5. Immunofluorescent staining of FLC in the liver of a DBA/2 mouse 7 days after i.v. inoculation of 2×10^6 3Cl8 FLC. Indirect immunofluorescence labeling FMuLV p30 antigen on FLC, x 125. (From Gresser et al. 1988a)

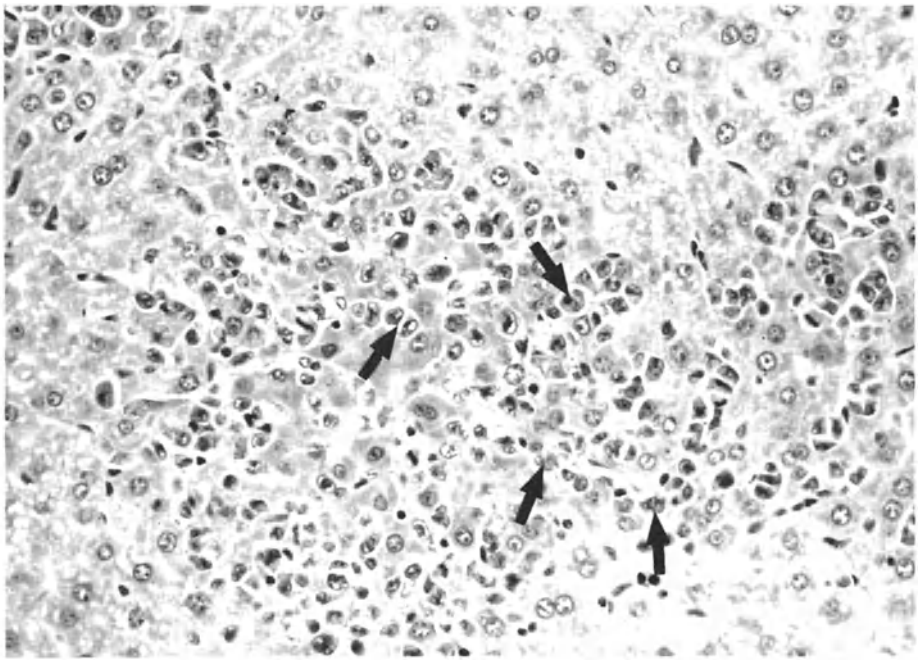

Fig. 6. Liver of a DBA/2 mouse 5 days after injection of ESb tumor cells. Note piling up of individual ESb cells within the sinusoids *(arrows)*

metastatic FLC and the poorly tumorigenic in vitro-passaged lines of FLC (Elia et al. 1988; Benedetto et al. 1988). These differences consisted in an increased amount and altered pattern of high molecular weight wheat germ agglutinin (WGA)-binding glycoproteins on the cell membrane of the tumorigenic line of FLC. The intense WGA-binding pattern appeared to be due to an increased glycosylation and sialylation of some surface proteins in the 100–200 kDa region occurring in the highly metastatic line of FLC (Elia et al. 1988). Likewise, using different techniques, Schirrmacher suggested that "not only the amount but also the specific positioning of sialic acid at the cell surface may be important". These changes could "lead to blocking or unblocking of cellular adhesion sites which might influence metastases" (Schirrmacher et al. 1982, p. 321; Altevogt et al. 1983; Schirrmacher 1985). Thus, it seems possible that differences between the surface properties of FLC and ESb cells determines how they interact with each other (i.e., piling up together or remaining as individual cells) or with host parenchymal or stromal cells. This in turn may influence the efficacy of interferon.

To speculate further: it seems possible that the blood supply of FLC and ESb liver metastases may be different. Thus, as FLC form tumor clumps there may be angiogenesis. We have shown that interferon induces an ischemic necrosis of established subcutaneous FLC nodules (Belardelli et al. 1983), possibly by

damaging the endothelial cells of newly formed tumor blood vessels (Dvorak and Gresser, 1989). In interferon-treated mice we have observed necrosis of FLC tumor foci in the liver that resembled the ischemic necrosis induced by interferon in s.c. FLC tumors. Necrosis of FLC foci was never observed in the livers of untreated mice even at a stage in which the liver parenchyma was totally replaced by tumor. In contrast, ESb cells which infiltrate the liver lie either within the established liver sinusoids or just outside the sinusoids and would presumably not depend on the formation of new blood vessels.

Mechanisms of the Interferon-Induced Inhibition of the Development of Metastases

Interferon can inhibit the multiplication of a variety of tumor and normal cells, so that it is entirely possible that in some instances interferon acts directly on the metastasizing tumor cells themselves. We have used lines of interferon-resistant tumor cells only to demonstrate that there is a host-mediated component in the inhibition of the development of tumor metastases. At present we do not know the mechanism of this host-mediated component (Gresser 1985). Some investigators have provided evidence that the immune system and especially NK cells are an important, if not essential element in the mediation of interferon's inhibitory effect on metastases (Brunda et al. 1984; Nishimura et al. 1985; Sakurai et al. 1986; Ueda et al. 1986; Yokoyama et al. 1986). Others have been less sanguine and have spoken of host-mediated effects, but have failed to find evidence that the immune system is involved (Belardelli et al. 1983; Gresser 1985; Ramani et al. 1986; Gresser et al. 1988a). In our work on the effect of interferon in inhibiting the development of liver metastases, we are beginning to suspect that there is initially an antitumor effect which is independent of the immune system, but that long-term suppression of tumor growth may require normal functioning of the immune system (I. Gresser, unpublished observations).

Conclusions

There is now evidence that interferon can exert an inhibitory effect not only on the development of a primary tumor but also on the development of pulmonary and visceral metastases even after the tumor cells have implanted and multiplied in the target organ. We do not understand how interferon exerts an antitumor effect on the primary tumor or on the development of tumor metastases.

Based on experimental results, interferon might well be considered as adjuvant therapy after surgical removal of the primary tumor or combined with chemotherapy. It would seem to us worthwhile envisaging the use of interferon in the treatment of hepatic tumor metastases in patients, possibly by increasing the concentration of interferon in the liver through in situ delivery systems.

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*Injections of Interleukins Around Tumor-Draining Lymph Nodes: A New Mode of Immunotherapy**

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Objective

There are two main kinds of immune strategy which can be used against neoplasia. The first potentiates a selected effector arm. In vitro culture of lymphocytes with exogenous interleukin-2 (IL-2) generates lymphokine-activated killer (LAK) activity or leads to the expansion of T cytotoxic lymphocytes. Their reinfusion together with high doses of IL-2 mediates the regression of a variety of murine and human tumors (Rosenberg et al. 1986, 1987). Alternatively, the host immune system can be "helped" to recognize and react against the tumor itself. Here the therapeutic maneuver is regulatory.

The purpose of our ongoing studies is to evaluate the ability of lymphokines injected around tumor draining lymph nodes – and in a few cases also around the neoplastic lesion – to promote the activation of an immune reaction powerful enough to hamper the growth of the primary tumor and its metastatic diffusion.

Tumor-Specific Helper T Lymphocytes and the Lymphokine-Activated Tumor Inhibition Phenomenon in Mice

Tumor-specific helper T lymphocytes can be generated from the spleen of mice harboring clinically evident tumors of very poor – or apparently nil – immunogenicity. In vivo, these are able to protect against a lethal tumor challenge and to confer a systemic immunity when admixed with challenging tumor cells. As a result of specific tumor antigen recognition, helper T lymphocytes secrete various lymphokines by which they recruit specific as well as nonspecific host reaction to the tumor (Forni and Giovarelli 1984). On the basis of that, in order to mimic helper T lymphocyte function, we next tried to trigger

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host reactivity by injecting the tumor growth site with small amounts of IL-2. A small, but consistent tumor inhibition was found when the tumor challenge was followed by daily local injections of 10–20 U of IL-2 during the first 10 days after challenge. However, almost complete tumor inhibition took place when these injections were performed in mice challenged with tumor cells admixed with nonreactive lymphocytes directly obtained from tumor-bearing mice.

Several features of this lymphokine-activated tumor inhibition (LATI) have been analyzed. The effectiveness of the system is not increased by using higher doses of IL-2. Crisscross-type experiments showed that LATI is not a tumor-specific phenomenon. It is not effective in sublethally irradiated recipients, showing that radiosensitive mechanisms of the host immune system play a crucial role. Moreover, it does not take place when the helper T lymphocyte and natural killer (NK) cell reactivity of the host is removed. When LATI is taking place, the tumor growth area is heavily infiltrated by mononuclear cells and granulocytes. Eosinophils are frequently found, mostly in close contact with both lymphocytes and tumor cells. Draining lymph nodes show progressive enlargement with expansion of cortical and paracortical zones. The wall of epithelioid venules is infiltrated by actively migrating lymphocytes. Lastly, a significant number of mice acquire a tumor-specific immune memory after LATI (Forni et al. 1985, 1987).

Towards a Molecularly Defined Helper System

Currently we are investigating whether the association of distinct lymphokines allows a helper system which is fully defined at the molecular level to be built up. Indeed, not adding lymphocytes admixed to the tumor would remove a critical variable since their efficiency in LATI is affected by the kind of tumor borne by the mice.

First, we considered the use of IL-1 as it plays key roles in the regulation of T and B lymphocyte responses (Dinarello 1988). In parallel, we tested the activity of a highly hydrophilic nonapeptide fragment (fragment 163–171, VQGEESNDK) synthesized by Centro Ricerche Sclavo, Siena, Italy (hereafter referred to as 163–171 peptide), which maintains most of the immunostimulatory activites of the entire IL-1β molecule and is devoid of the inflammatory activity (Boraschi et al. 1988).

Normal mice were challenged with the minimum lethal dose of various weakly immunogenic tumors. Starting 4 h later, they were injected in the peritumoral area with picograms of IL-1β or micrograms of the 163–171 peptide daily for 10 days. A marked inhibition of tumor takes was observed with IL-1β, whereas only a small, but nonetheless consistent and statistically significant reduction was found in mice receiving the 163–171 peptide.

To test whether IL-2 or IFN-γ and 163–171 peptide are synergistic, mice challenged with distinct tumors received two daily injections: 163–171 peptide first, then IL-2 or IFN-γ 4 h later. An efficient LATI was obtained when IL-2 was associated with the 163–171 peptide. This is true synergism since both

molecules are rather ineffective when injected alone. By contrast, IFN-γ and the 163–171 peptide do not synergize, but antagonize.

In the presence of the 163–171 peptide and IL-1, alone or in combination with IL-2, NK cells, TH1 and TH2 lymphocyte subsets are turned on and release various lymphokines by which a nonspecific, inflammatorylike reaction is elicited. Nonspecific immunity dominates this initial phase, which appears to provide a favorable environment for local activation of a few tumor-specific T lymphocytes. The passage from early, nonspecific to late, tumor-specific immunity is clinically significant. Following inhibition of a primary, weakly immunogenic tumor, the efficient outcome of specific immune reactivity is of importance in hampering tumor metastatic diffusion.

Perilymphatic Injections of IL-2 in Patients with Head and Neck Tumors

The LATI system is of potential clinical interest since low doses of lymphokines locally injected avoid most of the side effects associated with systemic injection of large doses. The experimental results promoted the initiation of pilot clinical trials with IL-2 in recurrent head and neck squamous cell carcinomas.

Patients with recurrent carcinomas no longer amenable to conventional treatment receive daily injections of IL-2 from the Jurkat T cell line purified by high-pressure liquid chromatography for 10 days. From a solution containing 400 units/ml of IL-2 0.5 ml were injected to a depth of 15 mm at a point 15 mm from the insertion of the sternocleidomastoid muscle on the mastoid. It has been shown that the injection of contrast solution at this point ensures maximum visualization of the neck lymphatic network. When possible, courses of treatment were repeated at 45-day intervals. A total of 20 patients have been or are currently being treated. IL-2 is ineffective in patients who have already undergone functional or radical lymphatic neck dissection. By contrast, in patients with contralateral or bilateral cervical lymph nodes, complete or partial tumor disappearance was observed in 70% of cases. However, remissions only last 4–6 months.

Even the presence of contralateral neck lymph nodes only induced tumor shrinkage. A direct toxic effect of IL-2 on tumor cells can be ruled out since no clinical effects were observed in patients who had undergone bilateral lymph node dissection and since the injections were always administered in the vicinity of the mastoid at the junction of two lymphatic channels, and not into the tumor. Previous irradiation of the tumor growth area and draining lymph nodes performed at least 5 months earlier did not counteract the therapeutic effect of local IL-2 treatment, while a good performance score appears to be important for its effectiveness (Cortesina et al. 1988).

In a companion set of experiments, we investigated both the cell reaction mechanisms and the complications elicited by low doses of IL-2 injected in patients with advanced primary squamous cell carcinoma of the head and neck which, technically speaking, were operable, but at high risk of relapse and death. For 10 days, these hospitalized patients routinely prepared for surgery

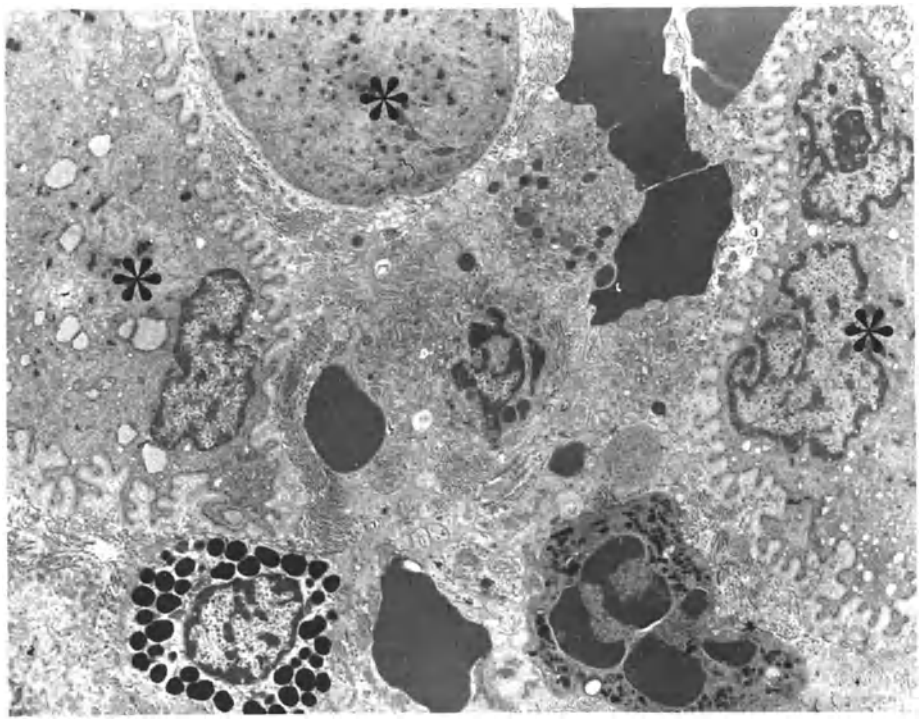

Fig. 1. Electron micrograph of a tumor-invaded lymph node removed at surgery. A patient with advanced squamous cell carcinoma of the tongue received two daily inoculations of 100 U of IL-2 between days 15 and 6 before surgery. From day 5 until surgery he was observed without IL-2 treatment. Eosinophils are closely associated with the metastatic tumor cells (*) that exhibit a large cytoplasm with numerous electron-dense granules of keratohyalin, × 2700

received two daily inoculations of 100 units of IL-2. The first injection was administered around the tumor growth area, the second around the mastoid as described above. Histologic and ultrastructural examination of tumor fragments and tumor-invaded lymph nodes recovered during surgery showed that in the great majority of the fields neoplastic cells were intermingled with numerous leukocytes (mostly lymphocytes and eosinophils). Lymph nodes displayed hyperplasia of both cortical and paracortical areas and epithelioid venules with a thick endothelium infiltrated by lymphocytes and granulocytes (Fig. 1). On a few occasions, disappearance of neoplastic lesions was also documented clinically and histologically.

Conclusions

Even on its own, exogenous IL-2 appears to elicit a local reaction by activating a few reaction pathways of patient immunoreactivity, the efficiency of which is potentially high, as shown by the tumor regressions often observed. However,

apart from this dramatic and immediate effect, an efficient and systemic antitumor reactivity is apparently not achieved since three patients showed recurrences or metastases in the early follow-up. Nevertheless, it should be remembered that these tumors were already advanced, and the efficacy of immune reactivity is marginal under these conditions. Furthermore, patients with head and neck squamous carcinomas display a marked immunosuppression that may impair the establishment of systemic reactivity (Cortesina et al. 1988).

The synergistic combination of IL-2 with 163–171 peptide currently being investigated in murine systems may constitute an important improvement of these clinical trials. The use of this short, synthetic IL-1β peptide in clinical practice is both feasible and attractive since it does not activate prostaglandin production, acute phase responses and inflammatory reactions, and is not pyrogenic (Boraschi et al. 1988).

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Autolymphocyte Therapy: An Outpatient, Low-Toxicity Approach to Adoptive Immunotherapy

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Introduction

Adoptive immunotherapy of cancer is a treatment approach in which immune cells with antitumor reactivity are administered to a tumor-bearing host and mediate regression of the tumor (Rosenberg 1984). In doing so, a presumably inadequate host immune response to the tumor may be overcome.

It is uncertain why malignant tumors do not provoke an effective immune response in the host. It may be that in some hosts, tumor antigens simply do not exist. Second, tumor antigens may exist, but effective immunization has not occurred, either because the tumor antigens are not sufficiently immunogenic, or the proper conditions to allow spontaneous immunization are not present. Lastly, it is possible that immunogenic tumor antigens do exist, and initial immunization has taken place, but a clinically effective immune response is blocked by the concurrent presence of host suppressor cells.

Consequently, we formulated an immunotherapeutic approach that had two goals: activation of autologous lymphocytes specifically directed against autologous tumor, and second, the reduction of in vivo immunosuppression. Furthermore, we intended this therapy to be safe and well tolerated, with low-cost delivery on an outpatient basis, unlike LAK/IL2 therapy with its very significant toxicity and cost (Rosenberg 1987). This also would result in an appropriate risk:-benefit ratio in patients with minimal disease as well as to those with disseminated cancer and thus allow for use in an adjuvant setting where immunotherapy may prove to have its greatest impact.

We believe that in vitro immunization (IVI) is superior to in vivo because it allows for better control of several variables including the concentration of antigen, the duration of antigen exposure, the nature of antigen presentation, the depletion or enrichment from the cells to be immunized of various lymphocyte subpopulations, and the addition to the immunizing cultures of various lymphokines, or other drugs, that cannot be used in vivo.

Previous reports (Cavagnaro and Osband 1983) of our technique for the successful IVI of human peripheral blood lymphocytes note three requirements. Autologous serum, rather than allogeneic or xenogeneic serum must be used. Specific antigen, as well as a nonspecific lymphocyte activator, such as phytohemagglutinin, pokeweed mitogen, or the supernatant from a mixed lymphocyte reaction must be present in the cell culture. Finally, in vitro inacti-

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vation of suppressor cells bearing histamine H2 receptors is also necessary. We found that the H2 receptor antagonist cimetidine accomplishes this last task and is safe and well tolerated in the clinical setting (Khan et al. 1985; Osband et al. 1981).

Phase I Study

In a phase I study 25 patients with disseminated cancer (20 with renal cell carcinomas, and one each with breast adenocarcinoma, glioblastoma, rhabdomyosarcoma, melanoma, and transitional cell carcinoma of the bladder) were treated with three successive weekly infusions of 50–100 million immunized autolymphocytes, plus daily oral cimetidine, 2400 mg, in divided doses. Toxicity was minimal, consisting solely of low-grade, self-limited fever and chills in 4 of the 75 infusions. Patient acceptance was excellent. At the 3-month time point 16 patients were evaluable. There were three partial responses, two minor responses, and four patients with stable disease, all in patients with renal cell carcinoma. Three patients with renal cell carcinoma survived longer than 3 years, representing a long-term survival tail of 15%–20%, a rate far superior to that expected from the natural history of stage IV renal cell carcinoma (Carpinito et al. 1986).

Phase II Study

General Results

In 1986, a phase II trial was begun; it was similar in study design to the initial study, but required six monthly infusions. In all, 55 patients were entered (36 with renal cell carcinoma, 4 with melanoma, and 2 with breast, 5 with colorectal, 4 with pancreas, and 4 with miscellaneous cancers). A total of 273 lymphopheresis/infusion cycles were completed. Technical feasibility was again demonstrated and toxicity was minimal.

Of the cell infusions 25% were accompanied by mild transient fever and chills. No other adverse effects were noted. Approximately 30% of patients have shown either an objective decrease or long-term stabilization of disease.

Treatment of Renal Cell Carcinoma

As part of this phase II study, 36 patients with biopsy-proven renal cell carcinoma were enrolled consecutively between May 1985 and April 1987. All had measurable disease as demonstrated either by physical examination or standard diagnostic imaging studies. In addition, all patients had been documented to have progressive disease in the 3 months preceding the initiation of autolymphocyte therapy. The age range was 22–74 years with a median age of 59; 78% were male. All patients had measurable disease: 14% of patients had residual

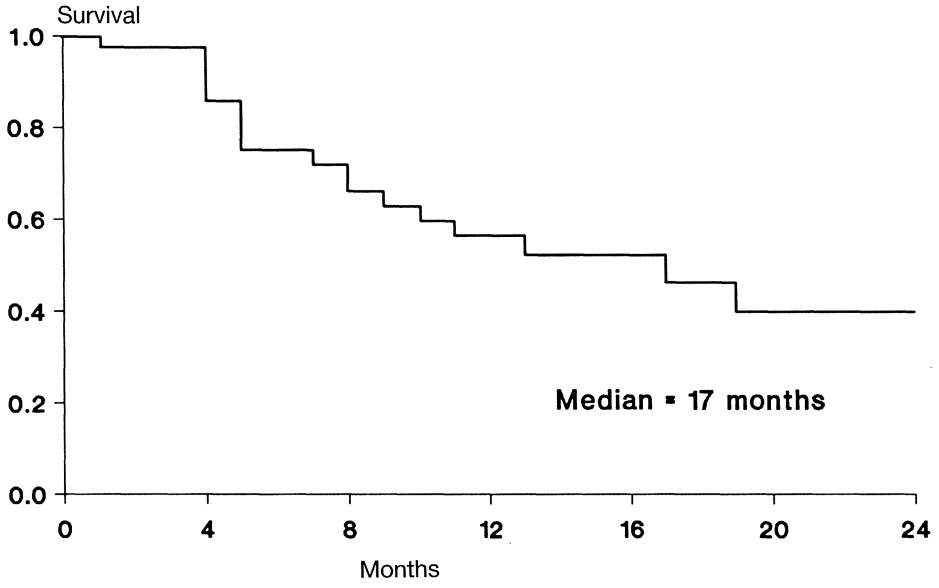

Fig. 1. Survival after autolymphocyte therapy in 36 patients with renal cell carcinoma. Median survival is 17 months

localized disease while the remaining 86% had metastatic disease in the following sites: lung (65%), bone (38%), liver (19%), brain (16%). A diagnosis of metastatic or residual disease within the 2 months prior to study entry was made in 76% of the patients.

The survival curve for the 36 renal cell carcinoma patients is shown in Fig. 1. The median survival is 17 months, and there appears to be a long-term survival tail in the range of 25%–30%.

It is impossible to develop new medical treatment without the use of correlative diagnostics that can identify patients likely to respond and monitor their course during therapy. In these patients with renal cell carcinoma two correlative diagnostic markers were noted: serum interleukin 2 receptors (IL2R) and serum immunosuppressive acid protein (IAP). Serum IL2R receptor is a 55 kDa protein shed from activated T cells, and IAP is an alpha-1 glycoprotein similar to several acute phase reactants. Patients with normal baseline serum IL2R levels (<500 U/ml) have a significant survival advantage ($P<0.005$) in comparison to those with higher levels (Fig. 2). A similar survival advantage is seen in patients with normal baseline IAP (<1000 μg/ml; Fig. 3).

Using both the IL2R and IAP baseline values, we constructed the following algorithm which correctly predicted 9-month survival outcome for 26 of the 29 patients at risk for that timepoint.

In addition, this algorithm was used to assign patients to the good (i.e., expected to be alive) or bad (expected not to survive) risk groups. These groups also correlated significantly with survival as shown in Fig. 4. As seen,

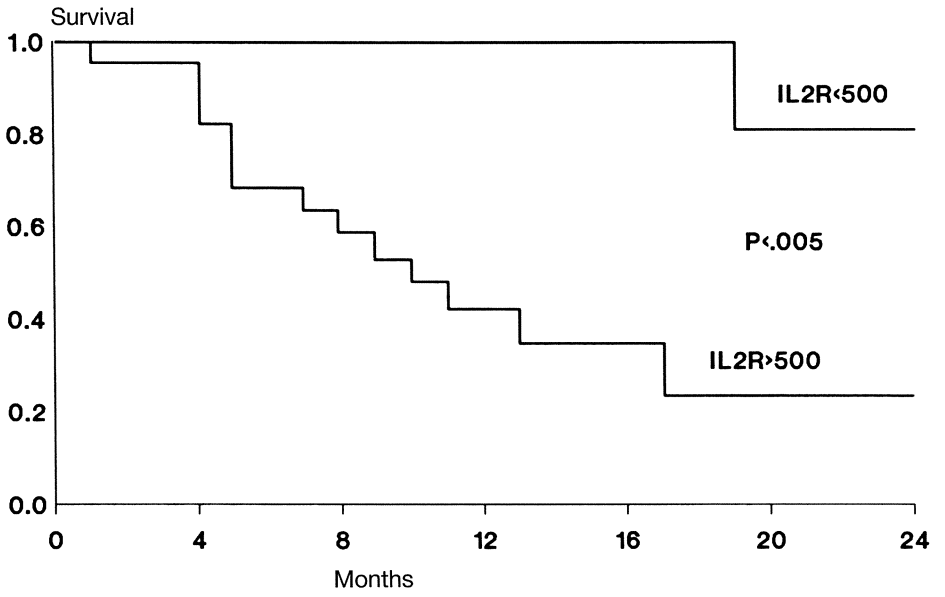

Fig. 2. Survival after autolymphocyte therapy in patients with renal cell carcinoma based on serum interleukin 2 receptors (IL2R). *Upper curve,* IL2R < 500 U/ml; *lower curve,* IL2R > 500 U/ml. $P < 0.005$

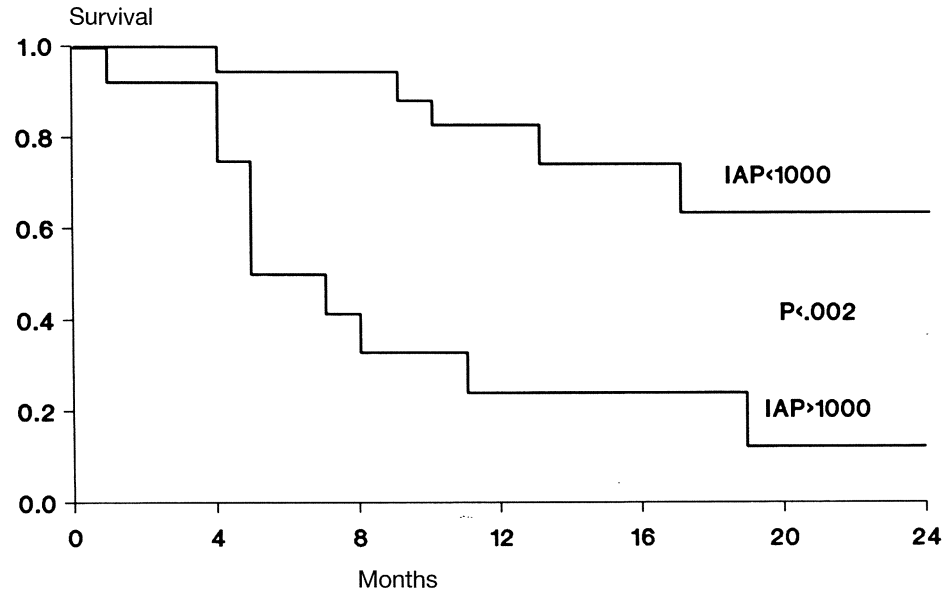

Fig. 3. Survival after autolymphocyte therapy in patients with renal cell carcinoma based on serum immunosuppressive acid protein (IAP). *Upper curve,* IAP < 1000 μg/ml; *lower curve,* IAP > 1000 μg/ml. $P < 0.002$

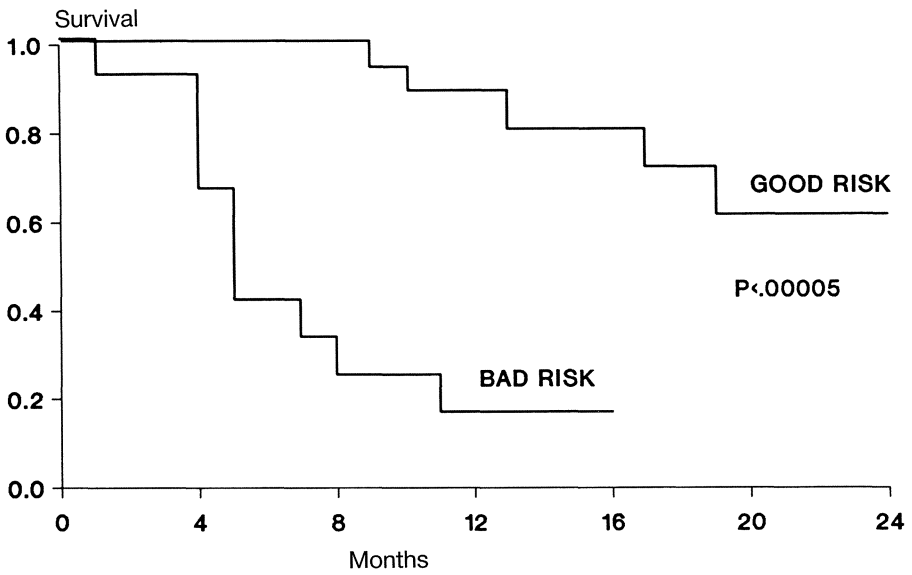

Fig. 4. Survival after autolymphocyte therapy based on interleukim 2 receptors / immunosuppressive acid protein. *Upper curve,* good risk; *lower curve,* bad risk. $P < 0.0005$

patients in the bad risk group have a median survival of 5 months while those in the good risk group have not yet reached their median survival ($P<0.00005$; Fig. 4).

The percentage of the immunized T4 cells positive for IL2 receptors was also predictive of survival. Those patients who were still surviving after 9 months had a significantly higher percentage of T4+/IL2R+ cells than patients who expired before 9 months had passed ($P<0.0001$; Table 1).

Table 1. Correlation of status at 9 months to T4+/IL2R+ lymphocytes

	Alive	Dead
Mean (%)	10.1	3.8
< 6%	2	10
> 6%	19	2

$P < .001$

Future Work

Based on our results with renal cell carcinoma patients, we are currently conducting an FDA-approved multisite study comparing autolymphocyte therapy plus cimetidine versus cimetidine alone in patients with stage IV renal cell

carcinoma. We will soon begin two additional multisite trials of autolymphocyte therapy in previously untreated stage IV nonsmall cell lung cancer (NSCLC) and also as adjuvant treatment in stage III NSCLC.

Autolymphocyte therapy may have other clinical applications in areas other than cancer (Osband and Carpinito 1988). The prevention of transplantation rejection, the prophylaxis of infectious disease, the treatment of autoimmune disease and allergy, and use as an imaging reagent may all be potential applications of this technology.

In conclusion, autolymphocyte therapy is a hybrid product that merges the concepts of adoptive immunotherapy with in vitro immunization. Results thus far indicate safety and feasibility as an outpatient treatment modality. Biological activity has been demonstrated. Serum markers predictive of clinical outcome have been identified and should prove useful in selecting patients best suited for autolymphocyte therapy. Further studies are justified and are ongoing at this time.

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Active Specific Immunotherapy of Colorectal Cancer

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Introduction

In spite of improvements in surgical approaches, colorectal cancer patients with transmural extension of tumor and/or metastases to regional lymph nodes still have 5-year survival rates of only 20%–50% when treated by operative resection alone. Adjuvant radiation therapy can lower the incidence of local recurrence in rectal cancer, but does not control systemic metastasis. Toxicity and drug resistance continue to limit the usefulness of chemotherapy, although some benefit is suggested in a recent study (Wolmark et al. 1988).

Our efforts have focused upon using a patient's own tumor, presuming the presence of tumor-associated antigens that are capable of eliciting an immune response. This approach is known as active specific immunotherapy (ASI).

The impetus for this work was the development and biological characterization of an experimental model showing the requirements for effective immunotherapy of established tumors. A series of studies in a guinea pig line-10 hepatocarcinoma model (Hanna et al. 1979; Peters and Hanna Jr 1980) have demonstrated that BCG admixed with syngeneic tumor cells can induce systemic immunity capable of eliminating a limited disseminated tumor burden when the vaccine is carefully controlled for variables such as the number of tumor cells, ratio of viable BCG organisms to tumor cells, viability of the tumor cells, and vaccination regimen.

We have translated the principles and procedures of ASI, as learned in the guinea-pig hepatocarcinoma model, into a randomized, controlled trial of ASI in patients with colorectal cancer. The objectives of this study have been to determine whether ASI could:

a) enhance the delayed cutaneous hypersensitivity (DCH) responses to autologous tumor cells, and
b) prolong the disease-free interval and survival of these patients.

We have reported (Hoover Jr et al. 1984) that immunized patients showed a significant increase in their DCH response to autologous tumor cells. In 1985, we reported the preliminary clinical results in the first 40 patients with a mean follow-up of 28 months (Hoover et al. 1985). This report updates that clinical trial with additional patients and follow-up to a median of nearly 5 years.

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Material and Methods

Vaccine Preparation

The methodology of this clinical protocol has been described in detail (Hoover Jr et al. 1984; Hoover et al. 1985). All tumor tissue not required for staging and distal normal colon mucosa was processed by the tissue dissociation and cryopreservation procedures of Peters and Brandhorst (1979).

Clinical Protocol

Patients with tumors of the appropriate pathologic stages (B2-C3) were randomized postoperatively either to receive the autologous tumor cell: BCG vaccine or to have no further therapy. Colon cancer patients were randomized separately from rectal cancer patients with substratification for each pathologic stage. The vaccines were started at 4 to 5 weeks after tumor resection following skin testing of both control and treatment patients with standard recall antigens and with autologous tumor and mucosa cells. Induration of greater than 5 mm at 48 h was considered positive. Skin testing was repeated at 6 weeks, 6 months, and 1 year following vaccination.

Treated patients received one intradermal vaccination per week for 2 weeks consisting of 10^7 viable, irradiated, autologous tumor cells and 10^7 fresh-frozen Tice BCG. In the third week, patients received one vaccination of 10^7 irradiated tumor cells alone. The first two vaccines were given in the anterior thighs with the third in the right deltoid area. Before injection, the tumor cells were irradiated with 20 000 rad. The third vaccine was similarly prepared, omitting the BCG. Control patients did not receive a placebo injection.

All patients with lesions below the peritoneal reflection (rectal) received 5040 rad of pelvic irradiation starting after immunotherapy was completed. Both control and immunized patients were monitored at 3-month intervals for the first 2 years and every 4–6 months thereafter. At relapse, patients were candidates for surgical resection, systemic chemotherapy, or infusional chemotherapy depending upon the extent and sites of recurrence.

Results

This report includes all patients ($n = 74$) participating in the trial between March 1981 and November 1987 with a minimum follow-up of 1 month and a maximum of 81 months. The median follow-up time was 56 months. No patients have been lost to follow-up. The two treatment groups were remarkably similar with respect to age, sex, race, location of primary tumor, pathologic stage, size of primary, extent of colon or rectal resection, and number of positive regional lymph nodes. As expected, all patients developed a superficial ulceration at the sites of the first and second vaccinations containing BCG. These healed within 3 months. Liver and renal function tests were not altered. Total lymphocyte and absolute lymphocyte counts were not changed.

Clinical Outcome

We considered two outcomes: time to recurrence and time to death. Among the 44 colon cancer patients, 21 received ASI (2 deaths and 3 recurrences) and 23 were in the control group (5 deaths and 8 recurrences). Among the 30 rectal cancer patients, 14 received ASI (6 deaths and 7 recurrences) and 16 were in the control group (7 deaths and 7 recurrences). One patient from each group died disease free of causes unrelated to cancer.

Comparing treatments among all patients, there is a moderately significant difference in the distributions of time to recurrence (one-sided P value by Mantel-Haenszel test is 0.037) and a comparably significant difference in the distributions of time to death (one-sided P value is 0.031); both comparisions favor the ASI group. As shown in Figs. 1 and 2, most of the difference was due to the subgroup with colon cancer.

Perhaps because of small sample sizes and insufficient statistical power, less clear conclusions emerge from the separate examination of colon and rectal patients. Colon patients obtain a moderately significant benefit ($P = 0.041$) from ASI in delaying recurrence (Fig. 1a), but not for time to death ($P = 0.085$; Fig. 1b). It should be noted, however, that three control patients with colon cancer currently remain alive with extensive unresectable metastases, whereas only one patient in the treated group has known disease. One treated patient is clinically free of disease 2 years after resection of a mesenteric recurrence. Therefore, the number of deaths in the control group is likely to increase substantially in the near future. No significant treatment differences are seen among the 30 rectal cancer patients (Figs. 2a, b) despite a 7:1 ratio of recurrence rates between 9 and 15 months in favor of ASI. But, as noted above, upon combining these patients with the colon cancer patients, stronger conclusions emerged than from either group alone.

Treated patients that relapsed demonstrated significantly less disease ($P = 0.44$) at the time of recurrence. Local recurrence was defined as patients who had surgically resectable recurrences or metastases, whereas diffuse recurrence meant unresectable recurrences or metastases. Of treated patients 60% had localized disease at the time of recurrence, of control patients only 20%.

Discussion

With such small numbers of patients, we cannot conclude that ASI is of proven therapeutic benefit. That will require completion of the multi-institutional trial currently underway through the Eastern Cooperative Oncology Group. We are encouraged by the more limited extent of recurrence in ASI-treated patients. It is assumed that some of these patients with resectable recurrences will be cured, giving eventual survival differences greater than the disease-free differences. It should be noted that our control patients had disease-free and overall survival similar to other recent reports (Wolmark et al. 1988).

That colon cancer patients would benefit more than rectal cancer patients is not surprising and would have been predicted from our animal model in which

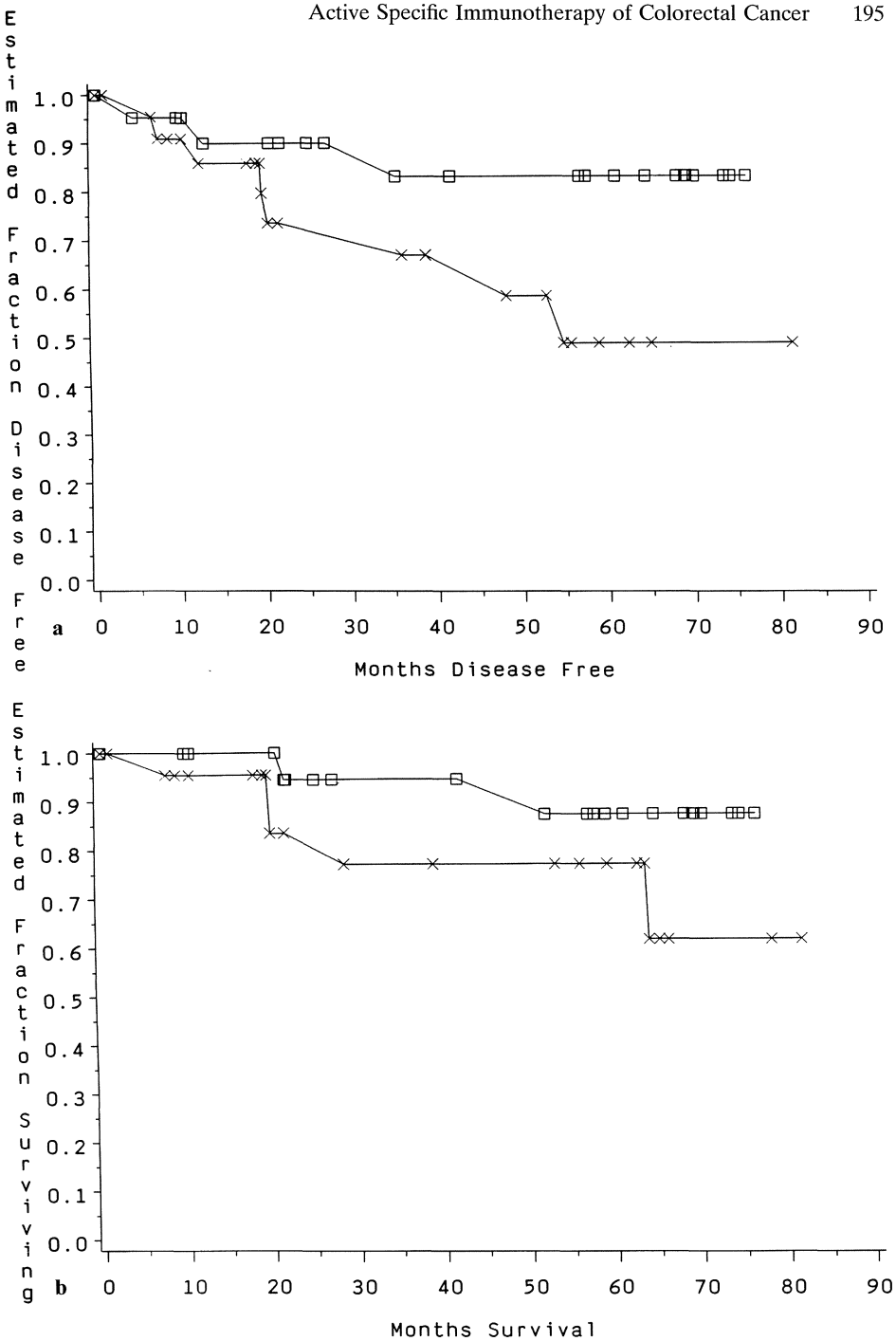

Fig. 1a, b. Among 44 colon cancer patients, actuarial estimate of **a** time-to-recurrence (one-sided *P* value 0.041, Mantel-Haenszel test) and **b** time-to-death distributions (one-sided *P* value 0.085, Mantel-Haenszel test) by treatment. Control patients at stages B and C, *crossed line;* treated patients at stages B and C, *boxed line*

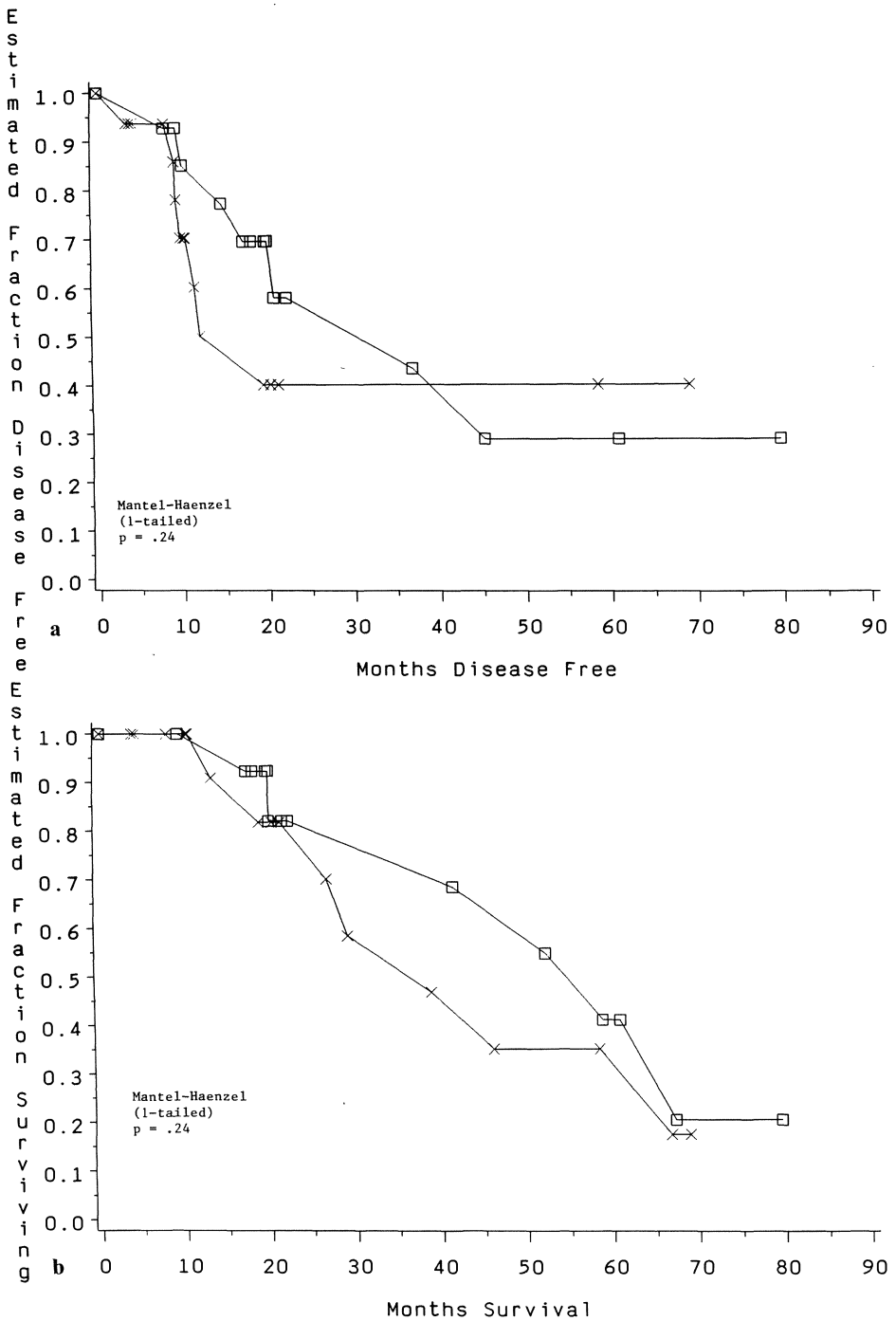

Fig. 2a, b. Among 30 rectal cancer patients, actuarial estimate of **a** time-to recurrence distributions or **b** death by treatment. No significant differences are seen ($P = 0.24$, Mantel-Haenszel test). Control patients at stages B and C, *crossed line;* treated patients at stages B and C, *boxed line*

the draining lymph nodes are imperative to the efficacy of the vaccine for 21 days following immunization. Rectal cancer patients were started on pelvic irradiation treatment, including the draining lymph nodes, within a few days to 1 week after completing immunization. Theoretically, the lymph nodes that had been stimulated by immunotherapy were destroyed by the radiation before they could impact fully upon the immunologic response. Our plan is to obviate this problem in future protocols by immunizing via lymph node basins that are not within the radiation field.

We now have three lines of evidence that ASI has immunologic impact in patients with colorectal cancer:

1. a significant boost in reactivity to autologous tumor cells in vaccinated patients as already reported (Hoover Jr et al. 1984);
2. the use of peripheral blood lymphocytes from these immunized patients as sources for the development of stable clones of human B lymphocytes that produce colon tumor-specific monoclonal antibodies as shown by Haspell et al. (1984);
3. the sustained differences in clinical outcome between immunized and nonimmunized groups of patients which are very encouraging in this study.

How can we explain our modest success in the face of so many past failures of attempts to control malignant disease by immune manipulations? Most human trials with tumor vaccines have had at least one major variation from the requirements found in the guinea-pig model for successful immune stimulation with ASI. Most have used tumor cells of low viability, an absolute predictor of failure in our model. Such cells disintegrate rapidly and do not provide the necessary sustained antigenic stimulus to the regional lymph nodes. Others have used allogeneic cell lines. Unless cross-reacting tumor antigens are shared by the donor and recipient, such vaccines are destined to fail.

It is presumed that the optimal vaccination and treatment protocols have not yet been achieved. To date, efforts to develop therapeutic vaccines have concentrated primarily on vaccine preparation and administration. Another equally important aspect of immunization is the capacity of the host to respond adequately to potentially immunogenic vaccines. Cancer patients frequently display varying degrees of generalized immunosuppression. Even in immunocompetent patients, there may exist specific tolerance toward their tumor. Obviously, patients develop their malignancy in spite of an intact immune system as we routinely measure it. Although it has not been demonstrated unequivocally in humans, it is now recognized that in certain murine tumor models there exists a state of specific tolerance to the tumor induced by suppressor cells (Dye and North 1981). Pretreatment of animals with cyclophosphamide (CY) has been shown to augment specific immune reactions to a variety of antigens, including tumor antigens, presumably by depleting suppressor cells (North 1982). Thus, pretreatment of tumor-bearing hosts prior to vaccination may enhance subsequent immune reactivity to tumor cell vaccines. In the guinea-pig model, animals treated with CY prior to immunization showed significantly increased immune reactivity. Other investigators (Berd et al. 1986) have demonstrated increased immunization with a human melanoma

vaccine by pretreatment with CY. In the past year, a third arm has been added to our colorectal trial with one group receiving a single dose of 300 mg/m^2 CY 4 days prior to their first vaccine to potentially abrogate suppressor activity and increase the capability of immunization against their tumor.

Addendum: A recent review of this study has revealed an inappropriate patient being carried as a failure and death in the control arm of the rectal group. Since no benefits are being reported in rectal cancer patients, the data are not affected by this error so the statistics and curves have not been recalculated.

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Review of Experience in Clinical Trials of Specific Active Tumor-Associated Antigen Immunotherapy of Lung Cancer

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Materials and Methods

Tumor-Associated Antigens

The preparation of tumor-associated antigens (TAA) has been described in detail (Hollinshead 1978, 1980; Hollinshead et al. 1981, 1979, 1987, 1988, 1980; Stewart et al. 1976, 1986; Takita et al. 1985). Two categories of TAA are prepared for usage in immunotherapy (ITx) and clinical and laboratory evaluations: pure TAA, in which membranes are separated from carefully characterized tumor cells, the membranes are subjected to gentle, low frequency sonication procedures, and the solubilized proteins are separated into active components, according to size, charge, affinity. After identification, characterization, and preclinical testing, selected components are used for therapy; and ultrapure TAA, in which the above preparations are subjected to further purification, especially by isotachophoresis, and alternately by monoclonal antibody-affinity chromatography. These preparations are used in particular for characterization, initial clinical studies, for hybridomas, and epitope preparations. Both forms of TAA are biologically active and immunogenic. Some of the methods used throughout for identification and characterization are:

1. Stepwise, gradient polyacrylamide gel electrophoresis (PAGE) and SDS-PAGE, using well-defined control preparations of known MW determination; migration and densitometry profiles and other chemical studies are performed for usage in comparison with libraries of previously studied specimens. These standard studies are optionally followed by studies with 2D slab gel or blotting techniques.
2. Another standard study is the use of immunodiffusion/immunoelectrophoresis testing of component vs. battery of sera, either monoclonal antibodies, or immune or hyperimmune or control sera from our serum bank.
3. Titration assays to ascertain the ability of the TAA candidate to induce cell-mediated immunity (CMI) include specifically controlled studies of the induction of measured levels of delayed hypersensitivity (DHR) by skin tests, and, when possible, backup studies using specific lymphocyte stimulation assays.

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4. Two types of enzyme immunoassays are now employed:
   a) Initial studies in which the candidate TAA is screened against a battery of cancer and control sera, and preparations of TAA for clinical usage are titrated.
   b) As recently established, a reverse enzyme immunoassay using selected TAA monoclonal antibody-derived epitopes is employed in monitoring immunotherapy.
5. Stability of each TAA is studied at various temperatures and times.
6. Isotope tagging studies are selectively performed to study localization on tumor cells.
7. Immunofluorescence or immunocytochemistry is used to evaluate certain TAA in tissues, cells, and sometimes in cell subsets.

Pretesting of TAA

We have presented elsewhere the steps taken in the final assessment of those TAAs which alone or in combination produce the strongest CMI. These studies enabled the efficient production of the major synergistic antigens of good quality and quantity for usage in clinical trials (Hollinshead 1978/1980; Hollinshead et al. 1981, 1979, 1987, 1988, 1980; Stewart et al. 1976, 1986; Takita et al. 1985). In brief, cross-testing of ultrapure TAAs in patients without lung cancer as well as 952 tests in 56 patients with lung cancer permitted the selection of those TAAs obtained from primary lung tumors which shared activities with 75% or more of primary tumors of the related histological type. Synergistic testing (ST) was performed in order to compare single and combination TAAs in 45 lung cancer patients in order to detect the strongest CMI per given protein concentration.

Clinical Preparation of TAAs

For efficiency and economy, after selectivity of desired ultrapure TAAs, the MW range selection of these components in a pure TAA preparation permitted a threefold better recovery, with only 14%–16% impurity. Based upon the studies of ultrapure TAA, we can calculate that each cell contains, on average, approximately 0.3 pg or approximately 2% soluble membrane protein. Thus, 1×10^9 cells yields approximately 300 μg TAA. The clinical preparations are essentially free of nucleic acids, major tissue antigens, pyrogens, bacteria, and viruses. In both qualitative and quantitative analyses, the TAAs essential for good CMI test with good reproducibility between batches.

Clinical Trials

Each of the three clinical trials is described in detail elsewhere (Hollinshead 1978, 1980; Hollinshead et al. 1981, 1979, 1987, 1988, 1980; Stewart et al. 1976, 1986; Takita et al. 1985). In Table 2 the pertinent common features of all three trials, as previously agreed to by the principle investigators, are summarized.

Table 1. Synergistic TAAs present in each vaccine

Type of lung cancer vaccine	TAA mw	Description	Chemical composition
Squamous cell (epidermoid)	37 kDa	Unique[a]	Protein
	49 kDa	Fetal	Protein
	100 kDa	Fetal	Lipoprotein
Adenocarcinoma	51 kDa	Unique	Protein
	77 kDa	Unique	Protein
Oat cell	51 kDa	Unique	Protein
	69 kDa	Unique	Lipoprotein
Bronchioaveolar	77 kDa	Unique	Protein
	100 kDa	Fetal	Lipoprotein
Large cell	37 kDa	Unique	Protein
	51 kDa	Unique	Protein

Note that some antigens are shared between strictly evaluated histologic types
[a] not found in this form in normal lung

Table 2. Common features of three clinical trials

Selection criteria
No previous treatment prior to curative resection
No history of other malignancy in the past 5 years
Contraindications: pregnancy; stages 3 and 4 disease
Stage 1 and 2 disease fully resected; all excised lymph nodes diagrammed and numbered
Staging
Surgical and pathological reports. AJC TNM.
Overview of pathology: review records and slides and final staging determination
Randomization
Eligible patients divided into two groups: control and immunotherapy[a]
Follow-up
After 5 years

AJC TNM, American Joint Committee staging.
[a] Therapy-immunotherapy was administered monthly × 3. 500 μg TAA well emulsified with FCA per dose. TAA were matched histologically

The principle of specific active immunotherapy is based upon the well-described, classic dual effect of appropriate immunization (Hollinshead 1978). Proper emulsification of antigen and adjuvant is crucial. The effects are based upon a two-phase antibody formation, with immediate release of the aqueous portion of emulsion followed by boosting from the emulsified portion; this is coupled with systemic effects, which are reflected in delayed hypersensitivity reactions. Our routine, small biopsy studies of immunized sites show very striking 90%–100% mononuclear cell infiltration. Our DHR-ST measurements

Table 3. The 5-year survival distribution according to stage of disease of a total of 234 patients with stage 1 and 2 disease, including any violations. Data is from centers in northern New York, north New Jersey, western Pennsylvania and east Canada

	Controls	Immunotherapy
T1N0	47	37
T2N0	43	44
T1N1	9	13
T2N1	17	22
T2N2	0	2
Total:	116	118

reported in detail show the patient mounting specific immune responses which last even as long as 12–14 years. Classic studies show that antigen incorporated in adjuvant remains at the site of injection for several months, is adjuvant-transported to subpleural parts of lungs and regional lymph nodes, and that antibody is detected not only in regional, but also in distant lymph nodes. So far, other adjuvants have not yet been shown in the clinical setting to induce long-lasting CMI. Toxicity includes a possible overnight fever, although emulsification reduces the tendency toward febrile reactions; skin ulcers similar to that seen for smallpox vaccination occur, and these generally heal within 4 to 6 months; lately we have been immunizing in three spots to reduce this effect. The distribution according to stage (Hollinshead et al. 1987), including two patients with T2N2 initially staged incorrectly, is shown in Table 3.

Results and Discussion

As reported (Hollinshead et al. 1987), the first two successful trials and a third unsuccessful trial were individually reported in their entirety. Two centers in the third trial had acceptable levels of protocol violation and could be analyzed in a pragmatic overview (Hollinshead et al. 1987). An update of the successful second trial in January 1988 has been reported recently (Hollinshead et al. 1988). It is now possible to study both control and immunotherapy groups in all trials of postsurgical patients with stages 1 and 2 lung cancer. A comparison of these (Table 4) indicates a favorable effect of immunotherapy, and we have reported (Hollinshead et al. 1987, 1988) P values all below 0.00 according to Wilcoxon and Savage tests; Kaplan-Meier density analyses were highly significant. Crucial to effective therapy is slow intradermal injection of well-mixed antigen and adjuvant. A strong CMI response is associated with effective immunization to TAA (good prognosis).

Table 4. Specific active immunotherapy trials (January 1988) in patients with lung cancer

	5-year survival (%)	
	Immunotherapy	Control
Phase II Ottawa – stage 1 disease	78	46
Phase III USA – stages 1 and 2 disease	75	33
Phase III Ottawa Pgh. – stages 1 and 2 disease	72	53
Summary	75	49

Optional studies in the individual trials, and the results of these according to the literature are summarized in Table 5.

Table 5. Optional studies in trials

Trial	Type of study	Results
1	MTX added to both groups	Drug alone: no statistical difference or effect seen Combination of MTX and TAA: effect seen, but group too small for statistical evaluation
2	Separate site treatment	After 5 years survival rate was 53%. The group using adjuvant plus multiple skin tests was included in overall analysis of immunotherapy in March 1986, which brought the survival rate down from 75% to 69%
3	Analysis of group using adjuvant alone	No statistical difference from control group was seen after 5 years

One of the important needs in solid tumor immunotherapy trials is precise monitoring. We have reported recently (Hollinshead et al. 1988) a correlation between monitoring for long-lasting CMI and early humoral responses to immunotherapy. A reverse enzyme immunoassay utilizing selected TAA epitopes appears to discriminate between patients who respond to therapy and those who fail therapy 5–6 months after commencement of immunotherapy. In our studies with this assay, we identified a cohort of patients with adenocarcinomas who appeared to respond to combination chemoimmunotherapy with stronger or earlier signs of specific humoral immunity. Coupled with our studies of chemoimmunotherapy in the first clinical trial, these studies indicate the importance of a larger trial to test this possibility. In the laboratory we are evaluating TAAs in metastatic tissues with the aim of more comprehensive polyvalency in our preparations. Further studies of genes which express selected TAA epitopes may permit future therapeutic strategies. For those less familiar with immunotherapy, we have included a simple illustration (Fig. 1). Now that

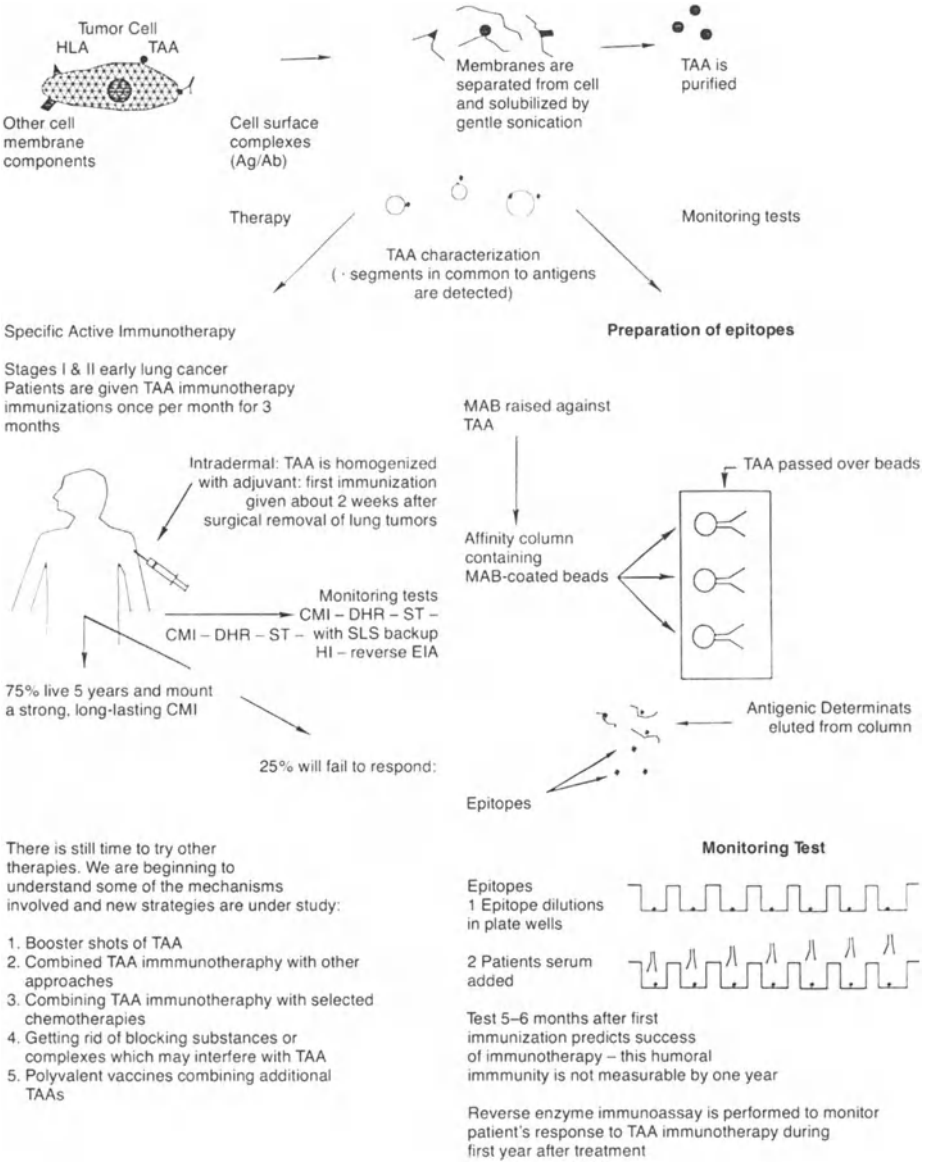

Fig. 1. Lung Cancer TAA specific active immunotherapy TAA, tumor-associated antigens; CMI, cell-mediated immunity; DHR, delayed-hypersensitivity reaction; HLA, histocompatibility antigens; MAB, monoclonal antibody; ST, skin testing; SLS, specific lymphocyte stimulation; HI, humoral immunity; EIA, enzyme immunoassay.

immunotherapy has been tested alone, its use may be considered, along with other important approaches, in well-thought-out combination strategies for the treatment of lung cancer.

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Summary of Poster-Sessions and Mini-Symposia

Summary of Poster Session and Mini-Symposium A

Metastasis: Growth and Pathogenesis, Models, and Mechanisms

L. Milas

The session consisted of 19 posters, of which 6 were also orally presented. The topic of the posters varied widely as reflected by the mere title of this session. Most studies were performed with human tumors, in which patients' material was analyzed or human tumors were studied as xenografts in nude mice or rats.

The largest number of posters considered the tissue of metastatic cell variants and organ or tissue selectivity for metastasis formation. It is well known that breast carcinoma frequently spreads to bones. The findings were presented, showing that this may be due to the existence of a tumor cell clone with a high metastatic propensity to the bone (3). The conclusion was based on the observation that 28% metastatic lesions contained membrane-bound, electron-dense cytoplasmic granules, which is a feature of cells with endocrine or paracrine activity, whereas only 3% primary tumors contained such granules. A rat prostatic carcinoma, designated R3327-MatLyLu, gives rise to metastases selectively to the bronchus-associated lymphoid tissue in the lung (7). Already within minutes after intravenous (i.v.) injection, R3327-MatLyLu cells accumulate in this tissue. Upon injection into the internal carotid artery, cells from a number of murine and human tumors develop tumor lesions at distinct sites of the brain, which in the case of human tumors apparently correlates with their clinical metastatic behavior (17). Cells from murine tumors were injected into syngeneic mice, whereas those from human tumors were injected into nude mice. The human melanoma cell line FEMX produced extrapulmonary metastases only (lymph nodes, brown fat, medulla of the adrenal gland) when tumor cells were injected into nude mice i.v., intraperitoneally (i.p.), into the spleen or footpad (6). This is in contrast to the other human melanoma cell line, LOX, which prefers to give metastases to the lung. An attempt was made to select a cell subpopulation from the FEMX metastases with increased metastatic potential. This was done by repetitive i.v. injections of tumor cells for several transplant generations. The result was, however, the opposite of the expected one: with each subsequent transplantation, the metastatic ability of the injected cells decreased, which was attributed to the increase in cell differentiation (5). No organ selectivity in metastatic spread was observed with a human ovarian carcinoma, designated IGROV1, when transplanted into nude mice (16). Growth of tumor nodules was limited to the lung when tumor cells were injected i.v., was limited to the liver when tumor cells were injected into the spleen, and was limited to the peritoneal cavity linings when tumor cells

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were injected i.p. Using intrasplenic injection, it was reported that both colonization and the growth of metastatic nodules of the B16 melanoma and of Lewis lung carcinoma cells in the liver can be modified by pretreatment of mice with bleomycin, cyclosporin A, immunostimulating agents, etc. (13). As one would expect, different treatments affected differently and unequally cell seeding as well as microenvironmental growth support in the liver. It is clear from these posters that the metastatic behavior of either rodent or human tumors is dependent on the intrinsic, inherent properties of individual tumor types. Cell variants with either increased or decreased metastatic proclivity do exist, and some tumors seek specific organ or tissue sites for their metastases, and some tumors spread according to the stochastic principle.

Several studies dealt with the expression of antigenic and cell differentiation markers in metastasis. Metastatic spread of gastric cancer to the local lymph nodes with respect to the type of tumor histology and antigen expression was analyzed in humans (1). Irrespective of the histological type, the metastases expressed the same antigens (and similar quantity of antigens) as the primary tumor. Expression of HLA Class II antigens, cell differentiation, and cell progression markers of human melanoma lines with different metastatic potential was analyzed when these lines were grown in vitro or when they were transplanted into nude mice subcutaneously (s.c.), i.v., or intrasplenically (9). The expression of phenotypic markers was determined at different stages of metastatic growth. Some markers which were detected in vitro were not expressed in vivo. Also, there was a change in markers' expression during the metastatic process. There was some indication that highly metastatic lines are associated with a specific marker. In another study (8) human rabdomyosarcoma cell lines were injected i.v. into nude mice to generate tumor deposits in the lung. Since these tumor cells undergo cell differentiation along the myogenic pathway, which can be readily assessed by cell differentiation markers, the model can be useful in research on the role of cell differentiation in metastasis development.

Two studies evaluated the growth rate of metastases. The tumor doubling time of lung metastases in patients with testicular cancer ranged from 7.7 to 94 days (median 29.9 days) and that of lung metastases in patients with malignant melanoma ranged from 7.6 to 359 days (median 35 days, 12). There was a wide range in tumor growth rate not only among different patients, but also among metastases within individual patients. Frequently, metastases which responded poorly to therapy or metastases in the preterminal phase showed an increased growth rate. Growth rate of a murine adenocarcinoma, designated E0771, and its paraaortic lymph node metastases was assessed (14). Both the primary tumors and metastases exhibited the Gompertzian mode of growth. However, the growth rate of metastases was more rapid than that of the simultaneously growing primary tumors.

Studies on the metastatic behavior of human tumors often require transplantation of such tumors in immunodeficient rodents, primarily nude mice. There were two studies advocating the use of rats for such studies. In one study (15), the use of nude rats is seen as being more suitable than the use of mice because of their larger size, more robust constitution, and longer life span. However, a

greater number of tumor cells is needed to produce tumors (or experimental metastases) in nude rats than in nude mice, which might imply that such xenotransplants in rats are less representative of their original tumors than xenotransplants in nude mice. In laboratories where nude rats or mice are not available, one can still successfully transplant human tumors in neonatal outbred rats immunosuppressed with antithymocyte serum (4). In rats treated in this way transplanted tumors grow progressively and produce spontaneous metastases.

A study (18) was presented in which the influence of cell volume on spontaneous metastatic potential of 10 cell lines from a murine fibrosarcoma was investigated. Opposite to the author's expectation, an inverse correlation was found to exist between cell volume and metastatic propensity. One poster dealt with the influence of angiogenic factors on tumor growth (19). Multiple injections of an angiogenic factor (isolated from lectin-activated porcine leukocytes) into human tumor xenografts growing in nude mice enhanced tumor growth, which was attributed to stimulated neovascularization. Finally, the modes of metastatic spread to the brain of malignant tumors in children were presented (2). These include spreading by continuous growth through bone channels and foramina, by direct extension of bone metastases, or by hematogenous route. Which of these will dominate depends mainly on the anatomic localization of the primary tumor.

References

Citations refer to the individual abstract numbers in the collection of abstracts of the Metastasis Congress published in Clin Exp Metastasis 6 [Suppl 1], 1988

Summary of Poster Session and Mini-Symposium B

Metastasis: Diagnosis and Targeting, Genetic Changes

S. A. Eccles

This session comprised 16 posters, 7 of which were selected for oral presentation in the symposium.

The posters could be considered to fall roughly into two main groups: those which utilized monoclonal antibodies (MAB) to detect metastatic deposits in vivo and those investigating gene expression/karyotype/DNA changes in malignant cells. There was a healthy balance between clinical and experimental presentations.

Two papers from the same group (22, 30), had used a MAB (CK 2) recognising human cytokeratin 18 to detect tumor cells in the bone marrow of patients with breast, colorectal and gastric cancer at the time of surgery. Positive results were obtained in 9.8–30.4% of patients without distant metastases, and correlated with other known risk factors, suggesting that the presence of such bone marrow 'micrometastases' was a predictor of subsequent relapse.

In the plenary session, Sugarbaker had challenged investigators to prove whether clusters of cells detected, e.g. in marrow or lymph nodes, were necessarily precursors of overt metastases since a proportion of patients who gave 'positive' results survived disease free for extended periods. The second poster (22) addressed this critically important question, and results showed that the carcinoma cells in the marrow expressed proliferation-associated antigens and loss of HLA-class I determinants and were capable of growth in vitro and tumorigenicity in nu/nu mice.

Two complementary papers had utilized animal model systems in attempts to quantitate localisation of MABs in disseminated tumour deposits and to determine some of the factors influencing their biodistribution. Schmidt et al. (32) had developed a novel technique based on quantitative autoradiography in order to determine MAB uptake in very small (< 500 μm diameter) lesions. The model employed was the ESb-MP lymphoma and its liver metastases, and although the system has some drawbacks, (e.g. the target antigen is also expressed in normal lymphoid organs), the method clearly was potentially of more general applicability. The authors were able to show that up to 34% of the injected dose of MAB per gram was localised in tumour deposits of 0.6–4 mm diameter, whereas in larger lesions the figure was approximately 7% overall, although areas of higher uptake – "hot spots" – could be discerned. Surprisingly, perhaps, very small lesions (< 0.3 mm diameter) seemed to have a reduced uptake compared with the intermediate sized metastases.

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The following paper by Eccles et al. (33) utilized a rat sarcoma model from which cells with enhanced capacity for hepatic, pulmonary and lymphatic colonisation/metastasis had been developed. A panel of syngeneic, tumour-specific MABs was employed to clarify some critical parameters in MAB biodistribution. Tumour site was found to be extremely important; MAB localisation in hepatic tumours was up to 20-fold higher than that in s.c. deposits, and, in spite of higher blood flow (as estimated by ^{86}Rb uptake), pulmonary tumour colonies did not accumulate specific MABs to a significant degree. Similar observations had been made by Schmidt et al. (32) in their model. Also in accordance with the previous poster, Eccles et al., showed that with increasing lesion size and total tumour burden, the percentage MAB/gram tumour dropped sharply, i.e. tumours of less than 0.1 g weight accumulated up to 22%/g, those of 0.1–0.3 g only 11%/g. The data in these two papers have implications for the results that may be expected in clinical immunoscintigraphy/immunotherapy, where 'micrometastatic' (perhaps better defined as 'subclinical') disease may include lesions of several grams in weight.

A major preoccupation of many investigators is the phenotypic and ultimately genotypic parameters which determine the metastatic capacity of tumour cells. Several approaches have been taken in attempts to determine which gene(s) may be active in facilitating this complex process: fusion of metastatic and non-metastatic cells and genetic analysis of the resulting hybrids; transfection of genomic DNA/selected genes; correlation of karyotypic characteristics with malignancy; analysis of expression of oncogenes/regulatory genes in metastatic versus non-metastatic cells. A critical factor in many of these experimental studies is an *accurate* determination of 'metastatic capacity' since the expression of this phenotype may be influenced by many factors such as the host animal, implantation site, growth period etc.

Two papers had examined DNA/karyotypic characteristics of human tumour material. Siracky et al. (25), using flow cytometry, had measured ploidy and S-phase fractions in cervical and vulvar carcinomas and their lymph node metastases. Variations between primary and secondary tumours were noted, but there appeared to be no clear-cut association between aneuploidy/high S phase fraction and increased risk of tumour progression as had been illustrated by Sugarbaker in a variety of tumour types. Bertrand et al. (37) implanted a human melanoma metastasis into a nude mouse and obtained a 'primary' xenograft tumour and pulmonary and lymphatic metastases. In these and subsequent metastases numerous copies of chromosome 7 or 7q, and an increase in translocations involving chromosome 7 were found. These observations are of interest in connection with data presented by Collard (this volume), showing that in mouse-human T cell heterohybrids, only those retaining human chromosome 7 were capable of invasion and/or organ colonisation.

Pfluger and Schirrmacher (26), also using a T lymphoma system, Eb, showed that fusion of 'low' and 'high' metastatic variants in the majority of cases led to hybrids expressing the characteristics of the more malignant parent, suggesting a dominance due to the selective segregation of the chromosomes of the less malignant partner. Similarly, Miller and McEachern (34) in a mouse mammary carcinoma model, demonstrated showed that hybrids formed by the fusion of

parental cells resistant to either 5FU or ouabain and 6-thioguanine showed resistance to all three compounds and were more resistant than either parent to methotrexate. One of the fusion partners was highly tumorigenic, yet rarely metastasized. The other was poorly tumorigenic, but capable of dissemination to lungs and liver; the hybrid progeny were both highly tumorigenic and metastatic.

These papers both suggest that the malignant phenotype, and the propensity for drug resistance, may be dominantly expressed, and that cell fusion, which may occur spontaneously, could lead to the evolution of tumour cells which are at once both more aggressive, and more refractory to treatment than their antecedants.

Two posters examined the properties of cells subsequent to H-RAS transfection. Van Roy et al. (29), used two epithelial cell lines, canine kidney MDCK and mouse mammary gland NMuMG. The former cells, when transfected, demonstrated either fibroblastic or epithelial morphology. These lines varied in their expression of v-RAS, uvomorulin and cytokeratin and in their invasive capacity (assayed in vitro) and metastatic capacity in vivo, indicating that the effects of the H-RAS gene are complex, and that, as previously suggested in the literature, the type of recipient cell may influence its response to such transfections. The data also caution against the assumption that an in vitro assay for one component of the metastatic cascade (e.g. invasion) will predict the successful development of spontaneous metastases in vivo.

Gingras et al. (36) transfected mouse fibroblasts with H-RAS and injected these cells i.v. into syngeneic recipients. The cells in the lungs were subsequently recovered at various times from 30 min to 9 days later. The cells which survived the 'early' phase had an enhanced propensity to lodge in the lung, but this was not extended to 'metastatic' capacity. Cells recovered later, however, produced around 26 times more colonies than parental cells. Patterns of gene expression also differed significantly between the two cell populations, suggesting that early selection pressures following i.v. inoculation favour cells with low protease expression and enhanced secretion of extracellular matrix components, whereas ultimate survival and growth is favoured by other, as yet unidentified, attributes. Again, these data emphasize that gene expression may be *transiently* altered by genetic manipulation, but that such changes may have a significant effect on ultimate survival.

Two further papers examined endogenous gene expression in cells of differing metastatic potential. Ponton et al. (35) studied the expression of the TIMP (tissue inhibitor of metalloproteinases) gene and found that mRNA levels were lower in metastatic cell lines in serum-free medium and more responsive to induction by addition of serum. The authors speculate that the action of this gene product in regulating proteinases such as collagenase may influence invasion, but its role in vivo remains to be established. Boyano et al. (27) investigated c-onc expression in cell clones derived from a Ni-induced rhabdomyosarcoma and the tumours and metastases producted in vivo. All c-oncs assayed were over-expressed, and the level of expression (particularly of *myc* and *ras*) was correlated with the metastatic potential. Differences in expression in cells growing in vivo and in vitro on different substrates were noted, and in a drug-

resistant subline with enhanced pulmonary metastases, the proto-oncogenes were activated.

References

Citations refer to the individual abstract numbers in the collection of abstracts of the Metastasis Congress published in Clin Exp Metastasis 6 [Suppl 1], 1988

Summary of Poster Session and Mini-Symposium C

Metastasis: Adhesion, Lectin-Carbohydrate Interaction, Organotropism, Growth Factors

J. P. Thiery

There is increasing evidence for the mechanical as well as for the seed and soil hypothesis to account for the pattern of tumor metastasis.

After injection in the mesenteric vein B16 F10 melanoma cells almost exclusively localized in the periportal region of the liver lobule (50), therefore emphasizing a specific role for the microcirculatory system. Another study using B16 derived melanoma showed that a direct contact with hepatocytes was necessary for the proliferation of the metastasic cells (51). So far, however, it has not been possible to identify the mitogenic factors. Convincing experiments by Breilloux et al. (59) have clearly established that epidermal growth factor (EGF) contribute to the development of already formed micrometastasis of rhabdomyosarcoma cells; the action of EGF could however be more complex, implying functions not directly related to growth.

Organotropism involves many different parameters, including chemotaxis, selective adhesion, and growth. These different mechanisms have been discussed extensively by Fidler and Nicolson (this volume). There is indeed an increasing body of data showing that not all types of tumors metastatasize at random. In this respect selective adhesion must be an important element in the arrest and retention in defined organs. Over the last decade much effort has been made to understand the molecular nature of cell adhesion. Several reports showed the importance of lectins and their interacting surface glycoconjugates. Gabius et al. (54) evidenced qualitative as well as quantitative differences in the profile of sugar receptors in several different tumors with varying metastatic ability. They also showed that targeting to the lung correlated with surface expression of mannan- and galactoside-binding protein, whereas β-galactoside-binding proteins were expressed on tumor cells metastasizing to the liver.

Our knowledge on the structure and functions of lectins have made considerable progress recently. The galactoside-binding lectins form a family of well-conserved proteins found in normal and tumor cells. Lotan et al. (55) were able to demonstrate a correlation between the prevalence of two well-defined lectins (14.4 and 34 kDa) and malignancy and propensity to metastasize. In fact the 34-kDa lectin is downregulated when metastatic melanoma cells are induced to differentiate. The recent cloning of the 34 kDa lectin by Raz (this volume) will permit defining the functional domains of this molecule which could play a key role in various aspects of metastasis, including formation of microemboli.

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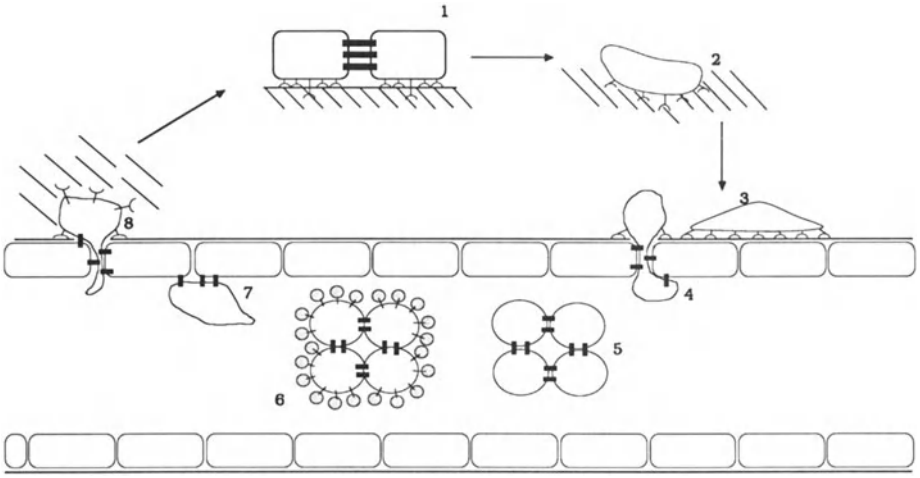

Fig. 1. Adhesion mechanisms in cancer invasion and metastasis. *1,* epithelial-mesenchymal transition; *2,* clusters or solitary cells invading the adjacent tissue; *3, 4, 7,* selective adhesion between tumor and endothelial cells and their subendothelial matrix; *5, 6,* adhesion mechanisms; *8,* extravasation from vessels

The homing mechanism was analyzed by Hamann et al. (58) in the case of lymphoma. They stressed that different types of interactions could occur in in vitro and in vivo experimentations. In vitro, a family of homing receptors described by Butcher et al. contribute to specific interactions with "addressin" molecules expressed by high endothelial veinules of lymph nodes. However, in vivo these interactions may involve other adhesive systems including LFA-1 to allow cells to extravasate and reside in lymph nodes.

On the basis of their primary function, adhesion molecules have been classified either as CAMs for cell–cell adhesion or as SAMs for cell–substrate interactions. As shown in Fig. 1, several distinct adhesion mechanisms must operate a different steps of tumor invasion and metastasis.

For instance, in carcinoma CAMs must be downregulated during the epithelial-mesenchymal transition which leads to clusters or solitary cells invading the adjacent tissue. In this new environment as well after extravasation from vessels migratory cells must interact specifically with the extracellular matrix via specific surface receptors. As already mentioned selective adhesion is also occuring between tumor cells and endothelial cells and their subendothelial matrix, and in the blood aggregation of cells and interaction with platelets must involve several adhesion mechanisms.

These different modes of adhesion have been discussed in this mini symposium as well as by Van Roy and Birchmeier (this volume; see also 104).

Altevogt et al. (52) have been able to identify the presence of L1 in a low metastatic lymphoma ESb variant (a CAM of the Ig superfamily normally found in the nervous system). Interestingly, L1 as well as LFA-1 and LFA-2 were de novo expressed in this clone which forms mostly large primary tumors.

A strong emphasis was put upon an immunologically related (IR) IIb/IIIa receptor expressed by tumor cells. IIb/IIIa expressed by platelets is a SAM belonging the integrin family. Honn et al. and Chopra et al. (53, 57) provided good evidence to for a role of this IR IIb/IIIa in tumor cell–platelet interactions; these receptors undergo a profound surface modulation by patching and capping, while cytoskeletal elements, particularly vimentin filaments, are reorganized. They also provided evidence that different lipoxygenase products have opposite effects on the dynamics of these receptors, possibly interfering with their capability to arrest and metastasize. Finally, some studies were carried out on laminin, a predominant SAM in basement membranes which has been repeatedly considered to be important during early stages of tumor invasion. Skubitz et al. (56) have found a new adhesive site on the B1 chain; this site preferentially interacts with heparin. Since several other binding sites have already been described on this particularly large SAM, it is tempting to consider that a subset of these sites will be recognized by different cell types. Lissistsky et al. (40) have indeed found that this is the case.

In conclusion this mini-symposium has brought a series of new results, strengthening the primary function of adhesion in cancer invasion and metastasis. Future studies will certainly permit us to decipher the cascade of events leading to the control of the expression of the different adhesion modes and possibly offer new strategies in cancer research.

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Citations refer to the individual abstract numbers in the collection of abstracts of the Metastasis Congress published in Clin Exp Metastasis 6 [Suppl 1], 1988

Summary of Poster Session and Mini-Symposium D

Invasion, Matrix Degradation, Angiogenesis, Motility

M. M. Mareel

Definition of the invasive and metastatic phenotypes is based on a number of in vivo and in vitro techniques which is close to the number of papers presented (61–81). This variety of techniques, admittedly choosen in accordance with the question raised, comprises: s.c. injection of cells into nude mice; i.v. injection into nude mice or into the chorioallantoic vein of chick embryos; seeding on human amnion basement membrane, on artificial substrata coated with extracellular matrix molecules, or on fibrin gels; confrontation of cells with embryonic tissue fragments in organ culture or with monolayers of fibroblast-like cells on tissue culture substrate. Strategies to elucidate cellular and molecular functions implicated in invasion and metastasis fall into three categories:

a) Tumors of known phenotype or cell lines derived from these are analyzed without counterpart of the opposite phenotype;
b) Couples of cell populations with the same origin, but with qualitatively or quantitatively different expression of phenotypes are compared with each other;
c) cost attractively, individual cell populations are manipulated either genetically or epigenetically to change their invasive or metastatic phenotype.

The results presented are summarized in Fig. 1. A glance at the figure produces the following impressions:

a) Current interest of researchers is in molecules rather than in cellular activities.
b) The number of molecules associated with the expression of the invasive or metastatic phenotype by far exceeds the number of molecules associated with maintenance of the noninvasive or nonmetastatic phenotype. Similarly, the number of inhibitors of invasion or of metastasis and their associated activities exceeds the number of inducers. Interestingly, we now seem to dispose of at least some tools to manipulate the phenotypes of interest in both directions.
c) The components associated with invasion and metastasis are not new. However, much emphasis is now put on the regulation of the activities of these components either by other components or by shifts in their disposition.

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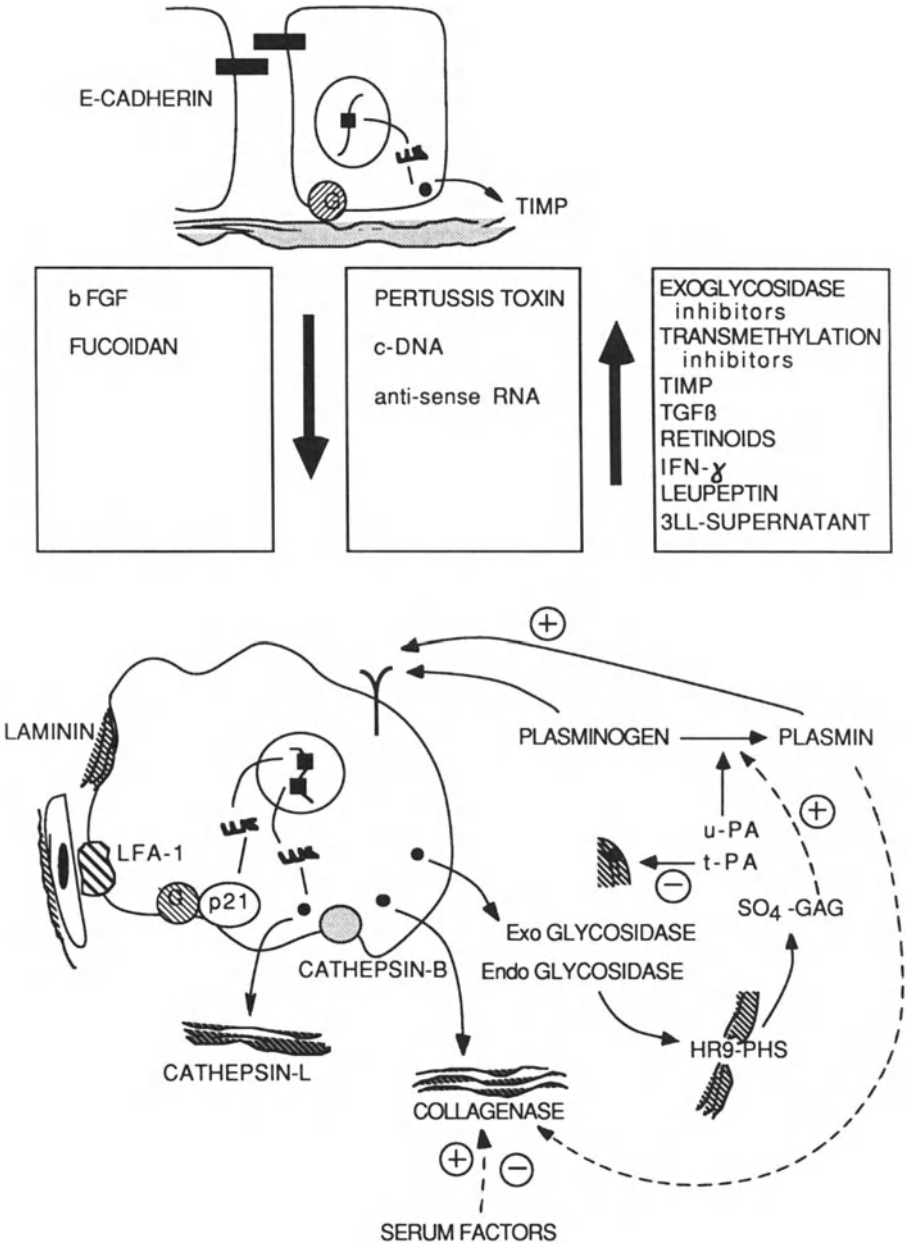

Fig. 1. Schematic representation of components associated with expression of the invasive or metastatic phenotype *(bottom)* or with maintenance of the noninvasive or nonmetastatic phenotype *(top)*. *Boxed in* are components that affect expression in one direction (*left* and *right)* or in both directions *(middle)*. *Solid lines* indicate displacement of components; *dashed lines* molecular interactions, resulting in activation (⊕) or inhibition (⊖)

It is fortunate that invasion and metastasis has been taken to the molecular level. It is hoped that definition of the phenotypes will remain as strict as possible.

Acknowledgement. I thank G. DeBruyne for the computer-generated scheme.

References

Citations (61–81) refer to the individual abstract numbers in the collection of abstracts of the Metastasis Congress published in Clin Exp Metastasis 6 [Suppl 1], 1988.

Summary of Poster Session and Mini-Symposium E

Metastatic Phenotype and Cell Differentiation

T. Boon

Many interesting results were presented. A very interesting observation was reported by Eisenbach et al. (96), who found that metastatic mouse tumor cells express little or no major histocompatibility complex (MHC) class I H-2K molecules. This may allow these cells to avoid the expression of a transplantation antigen that could induce the rejection of metastatic cells. In the course of studying this phenomenon, they made the remarkable observation that the *fos* oncogene promotes the expression of H-2K.

A number of reports compared the glycoproteins of high and low metastatic cells (82–86, 92, 95). Differences in sialic acid content (98) and oligosaccharide composition (87, 88) and in glycosaminoglycans conjugated to proteins (89–91, 100, 101) were discussed.

Some studies of membrane proteins of metastatic cells were particularly worthy of attention because they gave us hints of function or because they achieved a complete characterization of the molecules. Vollmers et al. (103) obtained autologous antibodies against a surface protein that may be involved in the motility of the metastatic cells. The antibodies inhibit the motility of several stomach carcinoma cell lines. Lehmann et al. (99) were able to use antibodies directed against a melanoma surface protein to screen a cDNA expression library. By doing so, they determined the complete sequence of the relevant genes coding for a 113-kDa glycosylated protein which seems to belong to the immunoglobulin superfamily.

Finally, Bover et al. (104) offered a very different and interesting approach. A culture line derived from a bladder carcinoma behaves in vitro as an epithelium. However, when it is grown on a surface where collagen I is present, these cells adopt the morphology of mesenchymal cells and loose the characteristic proteins of epithelia. This transfiguration is reversible. This system appears, therefore, very attractive to study the changes occurring when carcinoma cells engage in the metastatic process.

References

Citations refer to the individual abstract numbers in the collection of abstacts of the Metastatis Congress published in Clin Exp Metastasis 6 [Suppl 1], 1988

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Summary of Poster Session and Mini-Symposium F

Microenvironmental Factors, Hormones, and Signal Transduction

M. M. Burger

The results reported in this session do not fit under a common title, but can be grouped into
a) physical effects of the environment,
b) hormonal effects,
c) the effects of external factors, and
d) the effects of the environment and of general microenvironmental agents upon metastasis or tumorigenicity.

By far most of the effects cannot as yet be defined in molecular terms and are descriptive. They are nonetheless interesting since they challenge us to unify the vast number of speculations about mechanisms of metastasis. The danger that the questions raised will remain unanswered and that descriptive phenomena will be accumulated is a real one and should be kept in mind when abandoning a project in the descriptive stage to start a new one, rather than digging deeper into possible molecular mechanisms.

Physical Effects

Among the physical effects temperature was described as promoting growth of tumor cells in mice tails (107). Hypoxia was considered the main reason for poorer growth of tumor cells more remote from vessels (122), although a poorer availability of nutrients will have to be considered as well.

Hormonal Effects

Two presentations were concerned with estrogen effects. Estrogen-responsive rat mammary tumor showed fewer spontaneous metastases than did nonresponsive cells, and when the animal was supplemented with estrogen, metastases from responsive cells were increased, while no metastases developed under tamoxifen (120). Some of these effects may be explained by alterations in adhesion properties: human breast cancer cells displayed an increased adhesion to endothelial cells, extracellular matrix and its components (collagen I and IV, laminin, fibronectin) in vitro when estrogen-dependent cells were subjected to

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estrogen. Again, tamoxifen abolished the effect and estrogen was able to overcome the effect of tamoxifen. Since it does not seem that a secreted component is responsible, extracellular matrix receptor changes will now have to be considered – or still more interesting – cytoskeleton alterations as systems which are subject to the estrogen (115).

B16 melanoma cells were previously found to demonstrate a direct correlation between metastasic potential and intracellular response to melanocyte-stimulating hormone (MSH). The same has now been demonstrated in a hamster fibrosarcoma cell line (118). In addition, in melanoma cells differentiated functions like melanin production and tyrosinase activity stimulated by MSH were expressed less in more metastasizing cells, a not unexpected result (114). In one presentation α-MSH secretion in culture was observed and it was proposed that this hormone might function as an autocrine factor (121). Some of the cautiousness in evaluating all these correlations may come from well-established observations in the literature that melanogenesis is a very unstable property in melanoma cells and does not correlate with either malignancy or metastasis.

Effects on metastasis after treatment of whole animals with hormones like postaglandins cannot be interpreted as being directed towards the cell (113) since neural and other hormonal systems may well be interspersed between agent and tumor cell, as was pointed out in a paper discussing sympathoadrenal-medullary systems (117).

The Involvement of Protein Growth Factors

Epidermal growth factor (EGF) treatment of rat mammary adenocarcinoma cells does not lead to preferential phosphorylation of any protein fraction. EGF treatment and subsequent analysis of the phosphorylation of cytoskeletal elements in vitro with labeled ATP revealed a factor-dependent, 280-kDa cytoskeletal protein phosphorylation (110). It will be interesting to make a detailed analysis as to whether such changes have anything to do with metastasis. The latter question will pertain to the well-documented EGF receptor truncations in chicken fibro- and erythroblasts where ligand site deletion leads to constitutive growth activation, although less than that seen with v-erb B (111). Not only EGF but also transforming growth factor (TGF) production was found in many metastasizing human tumors like melanoma, breast, and colon adenocarcinoma (109). This again supports the concepts of autocrine growth, but first, enough receptors have to be found, and second, the relevance to metastasis still remains to be seen.

Effects of the Microenvironment and of General Environmental Agents upon Metastasis or Tumorigenicity

Murine sarcoma 180 grown in ascitic form expresses cytokeratins of MW 55 kDa, 49 kDa and 40 kDa while grown as subcutaneous tumor no such cytokera-

tins were found. This organ environmental effect turns out to be due not to transcriptional control or mRNA stability, but to differential translation (105). It remains to be seen whether this is purely an organ and not a suspension growth effect. A change in DNA index from growing mouse myofibrosarcoma cells in liver versus thigh (106) seems also to be organ specific.

Microenvironmental influences may also explain why small-cell carcinoma of the lung displays thrombin-like surface activity and consequently thrombin-specific cleavage sites, while human colon does not show any thrombin cleavage sites (108). Calcium ionophore or phorbol ester mimic cell surface modifiers acting under some circumstances from the extracellular space. They increase metastatic potential of poorly metastatic mouse mammary tumor cells. The manner in which the elevation of Ca^{++} and the translocation of kinase C bring about the activation of gene expression (stress genes, matrix degradation enzymes) is of general interest, not only to cancer researchers (119).

Proteoglycans are not only elements of the extracellular matrix and thus part of the immediate microenvironment of tissue cells, but they have specific binding sites on cells and matrix glycoproteins. Lewis lung carcinomas do bind several glycosaminoglycans, but only internalize heparin sulfate. Highly metastatic cells degrade glycosaminoglycans already on the surface and do so better than those cells which are less metastatic and which better interact and bind the glycosaminoglycan (112). The significance of such subtleties for tumorigenicity and metastasis remain to be revealed in detail.

Surgical stress has long been suspected of promoting tumor spread in humans. Experimental evidence was criticized in earlier times as being insignificant and due to massage during surgery and mechanical implosion into vessels. Since surgery in areas remote from the tumor site promotes spread from the primary tumor site and since anesthesia alone does not, the concept of surgical stress has been widely accepted and has led to innumerable speculations as to its mechanism. A new one was presented which assumed that NK cells and macrophages temporarily disappear after surgery. In the experiments presented rat adenocarcinoma cells were applied to the foot pad and laparatomy was performed 2 days prior to leg amputation, whereupon massive lung metastases developed. This effect on metastasis was ascribed to a decrease in peripheral NK cells both in blood and peritoneal cavity after 2 days and a subsequent raise or overshoot in both locations, but in the latter instance by immature, poorly functional NK cells and macrophages. These cells remain for a while until replaced by functional and mature cells. That their incompetence is at least part of the explanation was demonstrated when their weakness was overcome after stimulation with *Corynebacterium parvum* which led to reduced metastases (116).

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Summary of Poster Session and Mini-Symposium G

Tumor Immunology

G. Parmiani

The session on tumor immunology included 25 posters of high scientific level, although unavoidably heterogeneous as far as the subject was concerned. To discuss at least most of these contributions, I decided to group them according to the following topics:

1. Major histocompatibility complex (MHC) class I molecules of tumors as determinants of tumor progression and metastasis (both in mouse and man)
2. Tumor-associated or tumor-specific antigens in murine (B16, Eb), rat (colon carcinoma), and human tumors
3. Mechanism(s) of NK and macrophage reactions to metastasis; factors which may suppress lymphocyte activation in metastatic cancer patients

As for the first subject group, several, and apparently controversial, findings have been reported in the literature on which role (if any) class I antigens expressed by tumor cells may have in the control of tumor progression. At a certain point I had the impression that, according to different tumor models (3LL, T10, B16, and now the Garrido GR9 fibrosarcoma) one may reach a different conclusion as to whether expression of K or D antigens would favor or prevent metastasis.

After Michael Feldman's presentation in the general session we now certainly have a clearer picture of this phenomenon. In fact, with some tumors (e.g., T10 and some B16 sublines), downregulation of MHC gene products (particularly those of the K-end) would result in a higher number of metastases, possibly due to impairment of host cytotoxic T lymphocyte (CTL) activity, whereas with other neoplasms (e.g., 3LL and B16) the low expression or absence of class I antigens causes a reduction in metastasis.

Segal, Hämmerling and colleagues (124), who work with the T10 F1-hybrid (K^b K^k, D^b D^k) sarcoma, saw an inverse correlation between metastaticapacity and NK susceptibility of tumor cells in two clones whose difference was limited to the presence or absence of D^k antigens. These authors, however, conclude that there is no simple quantitative correlation between metastatic potential, NK resistance, and MHC expression. By working with a more recently induced BALB/c fibrosarcoma and its clones, Garrido's group (126, 137) confirms previous studies done by Kärre with the B16 melanoma, indicating an inverse correlation between class I expression and NK susceptibility, but the mechanism at the basis of such an observation remains unclear. A simple immunolo-

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gic explanation based on the negative role of H-2 expression in the recognition of tumor cells by NK cannot fit all data. In an attempt to explore such phenomenon at the molecular level several authors have looked at the possible interactions between epxression of MHC genes and of oncogenes. An interesting association was reported by Eisenbach between high expression of class I and c-fos in several clones of the 3LL tumor and metastatic activity. In our session the association between *K-ras,* class I, and metastaticapacity was explored by Har-Vardi et al. (125), who found that, although imperfect, an inverse association may exist between metastatic capacity, high expression of *K-ras,* and low expression of D^k in the T10 sarcoma clones. Such findings are at variance with published reports of *ras* transfection which appears to augment the metastatic activity in other cell lines.

In my view one should not only consider the immunological importance of MHC gene products, but also keep in mind two other potential functions of such molecules:

a) nonimmunological cellular interaction function, which might be useful to reexamine after several reports of the 1970s, and
b) that class I are transmembrane molecules with a tail which interacts with G proteins or other intracellular signal transducing targets.

The study of metastasis, therefore, is contributing to the discovery of other nonimmune functions that MHC molecules may have at the molecular level. Data on the expression and biological role of MHC molecules on human cells are even less clear. Immunohistochemical observations of transitional cell carcinomas, colon adenocarcinomas and of their normal epithelia (128, 129) are rather surprising and at variance with other reports in revealing that 80%–100% of normal tissues have undetectable class I HLA products. If confirmed, such a finding may represent a challenge to the idea that class I are necessary to cope with infectious agents attacking our body.

The observation by Turner (127) that truncated MHC molecules can be released by metastatic tumor cells indicated yet another interesting possibility of how neoplastic cells may interact with their environment, thus directly or indirectly influencing their own progression.

The second topic, tumor-specific antigens, represents an exciting, but also frustrating area of investigation for tumor immunologists since, until 2–3 years ago, no defined genes encoding tumor-specific antigens were available. But the patient work of Schreiber, Boon and Old has opened the way to the cloning of genes coding for such elusive molecules in the mouse and perhaps in the human system as well.

In our session, Frixen and Birchmeier (123) have shown that a 49–112-kDa molecule can be precipitated from B16 cells by a monoclonal antibody. These molecules have previously been defined by Birchmeier's group as adhesive structures but, remarkably enough, now these authors show that at least the 49-kDa molecule behaves like a tumor transplantation antigen since vaccinations with it significantly reduces the number of B16 lung colonies in syngeneic mice. It will be interesting to see whether an immunological mechanism is solely responsible for this effect.

In an interesting contribution, Apt and Altevogt (138) addressed the question of whether or not retroviral genes may be involved in the appearance of new antigens in MNNG-treated murine Eb lymphoma. A detailed study clearly showed that this was indeed the case since a group of gp70-related antigens appears to be expressed de novo in these cells, whereas a second group of viral genes coding for similar antigens was found to be amplified. These results are important in establishing that mutation and/or amplification of retroviral genes may give rise to cytotoxic T lymphocyte-defined, tumor-associated antigens. Since the antigen derived from gene amplification was able to induce a CTL reaction against the original Eb line, this model opens the way to the application of a similar approach in human tumors. In conjunction with this, the study presented by Traversari et al. (139) showed that a single passage of a variety of nonantigenic tumors in nude mice can induce the appearance of antigen(s) which is seen by CTL and detectable in transplantation assays and, more importantly, can be due to the infection of the transplanted cells by host retroviruses. This finding may be considered a disturbing one in view of the widespread use of nude mice as carriers of xenotransplanted human neoplasms. Further studies are necessary to evaluate the host range of these viruses and to assess whether they can also alter the antigenic profile of human cell xenografts.

Back to the xenogenization induced by mutagens, Hainault et al. (140) have shown the feasibility of such an approach in humans. By immunizing a patients with his own mutagenized rhabdomyosarcoma cells, these authors were able to obtain tumor-specific T cell clones hardly detectable in the patient's lymphocytes before vaccination. This work, together with those of Knuth et al. (142) and Wölfel et al. (143) who showed the presence of multiple, CTL-defined, specific antigens in human melanomas support the concept challenged over the past years that tumor-specific autoimmunogenic antigens are expressed in several human tumors, both primary and metastatic.

Such a conclusion was corroborated also by the findings of Notter et al. (130) who took a different approach. By isolating protein fractions from both autologous melanoma and lymphoblastoid cells they showed that some of the peptide-containing fractions of the neoplastic, but not of normal cells could specifically stimulate autologous lymphocyte clones in the presence of antigen-presenting cells.

Evidence that tumor-specific antigens exist, but that different antigens or different epitopes of the same antigens may cause either a rejection or a growth facilitation of the tumor was presented by the group of F. Martin (132). They also showed a peculiar in vivo sequence of immune reactions in the liver metastases of a rat colon carcinoma involving CTL and NK first which are subsequently replaced by macrophages (Mø) (131).

In the last group of presentations, Remels et al. (135) have shown that Mø obtained from Mø-resistant or -sensitive 3LL sublines (the former being more tumorigenic and more metastatic than the latter) differ in terms of antitumor lysis. In fact Mø derived from Mø-resistant tumors have high lytic potential and produce higher amounts of tumor necrosis factor-α. Within the framework of factors which are produced by tumor cells directly or indirectly and which may

interfere in the host reaction to neoplasia, many, sometimes ill-defined substances have been reported. It was interesting then to know from Alino et al. (134) that some, but not other laminin peptides may reduce the NK activity boosted by indomethacin treatment. Although it is unknown whether a similar mechanism may work in vivo, the reported production of laminin by tumor cells lends support to such a possibility. We know that immunogenic tumors may grow despite the presence of CTL due to their ability to activate lymphocyte suppressor. Rees et al. (133) reported a mean that has perhaps been underestimated by which T suppressors can enhance tumor growth and metastasis, i.e., as a consequence of cryosurgery of the primary tumor mass. Finally, Bartolini et al. (186), Guidi et al. (200), and Serrano et al. (197) reported on factors which may be either found in patients serum, alone or combined in immunecomplexes, or released by tumor cells and which can inhibit several immune functions through different mechanisms, most notably by impairing the IL-2 production (200). What the impact of such factors is on the progression and metastasis of tumor cells is difficult to tell at this stage of investigation. One cannot ignore, however, the complex picture which emerges from these results and which emphasizes, more than before, the active role that tumor cells may develop in contrasting the many immune or nonimmune antitumor effector systems of our body.

The complex chain of events which lead to cells leaving the primary lesion to form a metastasis are being clarified, but along with it, new mechanisms appear by which tumor cells may escape a multiple array of biological controls.

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Summary of Poster Session and Mini-Symposium H

Immunotherapy Studies

T. Giraldi

Poster session H included a total of 13 abstracts, 3 of which were not presented. As outlined in the other plenum summaries of poster and mini-symposia, the standard level of the experimental work presented and discussed was homogeneously very high and as such deserves mention.

Poster 149, 150, and 156 were in essence dealing with biological aspects of metastasis formation, rather than with actual in vivo immunotherapy. Data presented in poster 149 show that two sublines of Lewis lung carcinoma transfected with the H-2K^{bm1} genes expressed a new immunogenicity, which is accompanied by a reduction in metastatic potential and which is independent of a reduction in tumorigenicity. In poster 156, viral modification of a highly metastatic mouse lymphoma by Newcastle disease virus NDV resulted in an enhanced T cell cytotoxic response which was dependent upon the presence of helper T cells and which was accompanied by an increased production of IL-2 by these cells. These events occured earlier in the case of virally modified tumor cells as compared with uninfected cells, and these findings were discussed in relation to strategies for in vivo antimetastatic treatment also presented in the same and other sessions. In poster 150, the transfection of cells of a murine colon carcinoma line with a bovine papilloma-virus (BVP)-expression vector containing MTHA was reported to result in the isolation of a cell clone which was highly immunogenic and nontumorigenic in syngeneic animals. The immunization of mice using this clone was capable of protecting normal mice against the challenge with otherwise lethal doses of parental nontransfected cells implanted either intracecally or intravenously in the porta cava or tail vein; the therapeutic and clinical implications of these results were also assessed.

The remaining posters dealt more specifically with therapeutic aspects of experimental and clinical interventions aiming to control tumor metastasis. Poster 147 illustrated the oxygen multi-step immunostimulation, which attempts to enhance the immune functions of cancer patients by means of the administration of thymus gland preparations and oxygen inhalation; the results obtained in reported clinical cases seem positive. Poster 148 illustrates an autologous tumor vaccine individually prepared from colon cancer patients using a nonvirulent strain of NDV. Single tumor cell suspensions were obtained by mechanical and enzymatic dissociation of tumor specimens and were stored frozen, thawed, irradiated (20 000 R) and incubated with NDV. This tumor

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vaccine has been characterized by cytological and immunocytochemical methods; after intradermal injection, it elicited a delayed-type hypersensitivity skin reaction. The vaccine has been examined clinically in a Phase I trial with 20 patients with colorectal cancer (154); several variables were explored in this study, which allowed identification of the best conditions for vaccine preparation and administration in relation to delayed-type hypersensitivity skin reactions. Mild fever and headache occured in 25% of the patients, none of which presented severe side effects.

A relatively large group of five presentations regarded the effects of lymphokines and lymphokine-activated killer (LAK) cells. Poster 144 described the effects of the administration of a crude interleukin (IL)-2 preparation, accompanied by the injection of spleen cells; this treatment was found effective in reducing the formation of lung colonies after intravenous injection of cells of an anaplastic carcinoma into rats. Poster 152 examined the influence of tumor status on the in vitro and in vivo activation of LAK cells induced by recombinant IL-2. This study focused especially on the effectiveness of adjuvant postsurgical treatment and was performed using the murine colon adenocarcinoma cell line C-26. The cytotoxicity of LAK cells on a wide panel of tumors, including C-26, was reduced in the case of donors bearing large tumors. This impairment was also evident in tumor-resected mice with pulmonary metastases and lasted for at least 2 weeks after surgery, thus showing that it is attributable to the presence of a large tumor and not to lung metastases. These results were obtained after either in vitro or in vivo LAK activation and showed important differences in IL-2 response between normal animals, animals with primary tumors, and animals with metastasis which appear significant to preclinical studies of immunotherapeutic modalities. A clinical treatment modality with IL-2 in patients with bladder carcinoma, aiming to reduce cardiopulmonary toxicity and to activate preferentially IL-2-responsive immune cells at tumor site, was illustrated in poster 153. IL-2 was topically administered as a continuous bladder perfusion for 5 days (1000 U/ml, 120 ml/h) in five patients with invasive transitional cell carcinoma after incomplete transurethral resection of the tumor. None of the patients had toxic side effects, and the number of eosinophils and IL-2-receptor-positive cells at the tumor site and in the blood was distinctly increased. One of the treated patients had a histologically confirmed complete remission; other partial responses were inherently difficult to verify. This locoregional treatment seems interesting also for the chance it offers to study the cooperation of activated immune cells at the tumor site by means of uninvasive urinary cytology. The effects of postsurgical adjuvant administration of interferon (IFN)-α/β were examined in mice bearing sublines of Friend erythroleukemia metastasizing to the liver and spleen which was either sensitive or resistant to interferon (146). The treatment strongly inhibited tumor cell proliferation in liver and spleen, with a concomitant increase in host's life span. Similar results were also obtained using the interferon-resistant tumor cell line, thus showing that host-mediated resistance factors are involved in the reported effects of interferon administration. The effects of another lymphokine, recombinant human tumor necrosis factor (TNF)-α, were examined in poster 151. The in vitro treatment of B16 melanoma cells with

TNF-α caused an inhibition of tumor cell growth and an increase of lung colony formation after in vivo injection of the treated tumor cells. Similar effects were also obtained with recombinant murine IFN-γ, and the simultaneous treatment with the two lymphokines had synergistic effects. It is not clarified at present whether these effects are caused by long-lasting cellular alterations induced by the treatment or by clonal selection of tumor cells within the parental treated line. These findings appear, however, of interest for their clinical implications, considering the unfavorable effects that an effective antiproliferative treatment with TNF-α or IFN-γ may have on tumor metastasis.

The two last posters dealt with topics unrelated to those reported so far. A conjugate of a murine monoclonal antibody with yttrium-90 targeting a variety of adenocarcinomas has been prepared and tested in nude mice xenografted with a human colon carcinoma cell line (145). This conjugate, which has many desirable features for effective therapeutic activity, caused a significant tumor growth delay in the treated mice. Finally, poster 155 reported the effects of the infusion of MVE-2 activated peritoneal exudate cells (PEC) in mice with lung tumor nodules obtained with the intravenous injection of the nonimmunogenic SA-NH sarcomas. PEC cell infusion was effective in reducing pulmonary tumor deposits. When the lungs of the tumor-bearing animals were locally irradiated (2.5–8.5 Gy), a synergism was observed when irradiation was combined with PEC infusion. This result appears to be caused by a radiation-induced accumulation of PEC in the lungs, with implications of potential relevance for the therapy of tumor deposits in the lungs.

The results presented in the mini-symposium on immunotherapy indicate that several studies continue to be performed on the efficacy of therapeutic interventions with the use of cytokines and of cytokine-activated cells, in experimental and clinical conditions. The cellular mechanisms involved also appear to be thoroughly investigated, together with new clinical treatment modalities for locoregional administration of lymphokines which allow systemic toxicity to be reduced, and to investigate the effects on immune effector cells with uninvasive methods. Investigations on viral xenogenization of tumor cells with the aim to prepare effective (autologous) tumor vaccines proceeds systematically, with the perspective of an adequate evaluation of the therapeutic potential of this approach in controlled trials. Finally, results from animal experiments suggest that attention should be directed towards possible unfavorable effects that cytokines may exert on tumor cells leading to an increase in metastatic spread, whose possible occurrence in clinical situations deserves proper attention.

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Summary of Poster Session and Mini-Symposium I

Therapy: Chemo-, Hormone- and Radiotherapy, Surgery

K. Hellmann

The posters were uniformly well presented, informative, and thought provoking. Although I examined all of them, this summary will only deal with those of special interest to me, and those that are not dealt with in the summary are by no means uninteresting, they merely reflect the fact that my special interests were different.

The poster by Mentges and Bätz (157) dealt with the important problem of the resection of liver metastases from colorectal carcinoma and factors which may influence prognosis and survival. Unfortunately the number of patients was quite small, only 51, and they were collected over a period of 8 years. This in itself demonstrates that even in a large practice the numbers of patients suitable for liver metastases resection might only be relatively small. Perhaps the most worrying result of these authors was the fact that they had an operative mortality of nearly 8%, and it is doubtful whether this high death rate would help other surgeons to conclude that this treatment was helpful. The authors examined a number of indicators for their prognostic value and came to the conclusion that neither number, localization nor volume of metastases had any influence on survival. Neither for that matter was carcino embryonic antigen (CEA) level, grading, stage or localization of primary tumour of any prognostic significance.

An interesting study by Hill and Farkas-Himsley (160) showed that colicin had no effect on the division of cells in the bone marrow but could affect the division of KHT sarcoma cells, though only when the number of cells did not exceed four. The influence therefore of colicin could be essentially on micrometastases without influencing the division of any other cells. What was not clear from these authors results was whether there was also an influence of access of the colicin to the micrometastases.

Schaal et al. (161) tested some new compounds consisting of cisplatin-linked biphosphonates, and they examined these compounds on Walker carcinosarcoma 256B after intratibial implantation in the rat. Two compounds in particular, ADP and DBB, were active in producing central tumour necrosis which might be of value in metastases growing in bone.

Flavone acetic acid was shown by Giavazzi et al. (165) to inhibit liver metastases production of a human colorectal carcinoma xenograft. The tumour used was the HCC-M-1410, which is a highly malignant colorectal carcinoma line. The compound significantly inhibited formation of liver tumour colonies

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when given 24 h before the intrasplenic injection of tumour cells. Curiously the same therapeutic conditions were not active on the same tumour when it was implanted and growing subcutaneously. The activity of the compound, moreover, depended on the tumour burden, and the authors concluded that the compound was an immunomodulator, unlikely as this may seem as its main mode of antitumour action.

In view of the importance of prostacylin and the demonstration by others that prostacyclin may influence the development of metastases, a stable prostacyclin analogue known as iloprost was examined by Giraldi et al. (167). Iloprost given i.v. up to 24 h before i.v. injection of tumour cells was quite active.

In another poster, Giraldi et al. (168) examined the metastatic potential following treatment with DTIC, DM-COOK or razoxane in mice bearing Lewis lung carcinoma. They showed that the metastatic potential of the tumour cells treated in vivo for one transplant generation remains significantly reduced for three subsequent transplant generations.

Lersch et al. (170) demonstrated that low-dose cyclophosphamide may result in immunostimulation in patients, leading to a better quality of life. Comparisons were, however, not very clear since it is very difficult to measure quality of life in objective terms.

The poster by Klenner et al. (173) demonstrated that prolongation of survival is not always linked with a parallel change in tumour size and volume. The authors used two new compounds (BAD and ADP) with and without razoxane as an antimetastatic drug. Both of these compounds (BAD and ADP) have cytostatic and osteotrophic properties which were investigated in a transplantable osteosarcoma of the rat. This tumour also metastasizes regularly to the lungs, kidneys and paraaortal lymph nodes. When the osteosarcoma was transplanted intratibially, ADP, which is a cisplatin linked biphosphonate, had a marked effect on survival and metastases, increasing the former and decreasing the latter. BAD also was highly active but both compounds increased survival when combined with razoxane. The incidence of lung metastases was reduced in all groups treated with razoxane while all control animals died of their lung metastases.

The interesting poster by Pauwels-Vergely et al. (174) demonstrated the effect of a calcium channel blocker being able to prevent the nitrosourea-induced enhancement of metastases. The calcium channel blocker was Nifedipine and it demonstrated the same reversion of nitrosourea-induced enhancement of metastases with fotemustine, chlorozotocin and BCNU when all these nitrosoureas are given at a relatively high dose. These results are of potential clinical interest as well as of fundamental importance.

The poster by Gasic et al. (176) demonstrated that a novel leech extract containing ADPase activity inhibits both platelet aggregation and experimental metastases. The experiments pinpointed the fraction in the leech extract which showed this potent action and demonstrated that there are two antimetastatic activities, one of which inhibits activated factor X which the authors called antistasin, and a second fraction which inhibits platelet aggregation named thrombostatin. Thrombostatin had potent antimetastatic effects which could be

abolished by treatment of this substance with FSDA. The authors believe that their results suggest that thrombostatin may be a valuable antimetastatic agent. It will be interesting to see whether clinical trials will support their hope.

The poster by Hilgard et al. (179) clearly demonstrated that high-dose cyclophosphamide leads to an alteration of host factors which in turn are responsible for the high incidence of metastases when a number of tumours are injected intravenously. The particular host factors which are involved are the depression of NK cells. Similar results were obtained with mafosfamide.

The poster by Merriman et al. (181) provided an interesting insight into the relationship between the development of lung metastases and survival in mice with Lewis lung carcinoma. The authors used a variety of antithrombotic agents with a wide variety of chemical structures. They were chosen for evaluation because they were inhibitors of cyclo-oxygenase thromboxane synthetase or thrombin activation. While the compounds caused complete inhibition of lung secondaries, they did not increase survival of the animals, owing to the appearance of secondaries in extrapulmonary organs, e.g. liver, kidney and brain.

References

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Laudationes

The First Paget-Ewing Award Ceremony

K. Hellmann, and L. Weiss

The Paget-Ewing Award was instituted by the Metastasis Research Society in order to recognise outstanding contributions in the field of metastasis research by any living scientist. It will be awarded at the annual or biannual meetings of the society and consists of a commemorative plaque and a cheque. The first award is being made at this conference of the society.

The award this year will be shared between Drs. Rex Coman and Irving Zeidman and an appreciation of their work will be delivered by Drs. Kurt Hellmann and Josh Fidler respectively.

The award ceremony will be somewhat unusual in that, unfortunately, neither of the recipients are able to come to Heidelberg to receive their award in person. However the secretary of the Metastasis Research Society, Dr. Luka Milas, most unstintingly took on himself the task of taking the award to Drs. Coman and Zeidman and made a video recording of the occasion. This recording will be seen after I have expressed my appreciation of Dr. Coman's work and Dr. Fidler has done the same in honour of Dr. Zeidman.

The board of directors of the society initially asked Dr. Leonard Weiss to take on the task of paying tribute to Dr. Coman's work and outstanding contributions to metastasis research, but Dr. Weiss was also unfortunately unable to come to Heidelberg. However, he did very kindly send me a brief account of Dr. Coman's seminal work, and it is an abridged version of this account, with a few alterations of my own, that I now have pleasure to deliver.

Laudatio for Professor Dale Rex Coman

Dale Rex Coman's Contributions to Metastasis Research: A Synopsis and a Tribute

L. Weiss, abridged, amended and delivered by K. Hellmann

To understand the full impact of Dr. Coman's work it is necessary to see it in the context of the time in which he worked and of our present understanding. It has frequently been said that metastasis is just one part of tumour biology and does not proceed in vacuo. It is, however, the single most important part

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of tumour biology as far as the cancer patient is concerned because it is the essential clinical feature that distinguishes malignant from benign tumours and a good from what may be a bad prognosis.

It may not be necessary to recite such self-evident truths to the converted assembled here, but it is sometimes forgotten, especially by those who may believe that the cancer problem is just one of inhibition of cell division, albeit of selective inhibition. It could be that molecular biology will, in the future, unravel the biochemical characteristics which are at the core of the differences between malignant and normal cells but in spite of the explosion of molecular biology research, it is still a fact, however humiliating, that at present, the best chance of survival for a patient with cancer is if he or she can be treated by a surgeon, not a molecular biologist.

In addition to their effects on clinical oncology, many of the older clinical observations also serve to focus research on currently unsolved, but nonetheless important problems. For example, the perplexing problem of metastatic patterns is still with us. Although pattern was discussed in terms of "seed-and-soil" by Fuchs in 1882, Paget in 1889, and in the 'mechanical' theory by Ewing in 1928 and others, the relationship of these non-exclusive mechanisms awaits clarification in specific human tumours. Another problem awaiting resolution is the role of metastasis of metastases as first identified by Travers in 1829, Walshe in 1842, Eve in 1883 and Paget in 1887. If resolved with respect to synchronous and metachronous seeding, the answers to this long-recognized problem could play an important part in the design of future therapeutic strategies.

Although it is not suggested that metastasis research or interest in metastasis had stopped by the second decade of this century, nevertheless the voluminous cancer literature of that time and before clearly indicates that metastasis research had until comparatively recently been a very minor component of the cancer research effort as a whole. It is also evident that by the early 1940s, apart from clinicopathologic observations, metastasis research had ground to a conceptual halt and that the limited, available technologies had been stretched to the limit.

From earlier histologic observations, it appeared that the release of cancer cells from primary tumors which, by definition, has to be an early step in metastasis, might be related to cohesive defects between cancer cells. The arrest of circulating cancer cells in the vasculature of target organs was also appreciated to be a vital step in metastasis. However, by the early 1940s information was sparse on the relationship between cell cohesion and adhesion to metastasis, and largely anecdotal; there had been few attempts at quantitation, and little had been done to explore metastasis at either the level of the cancer cell or the molecular level. With hindsight, it appears that the majority of cancer (metastasis) researchers at that time were unaware of the more advanced, analogous work being done in the field of embryology by Holtfreter, Paul Weiss and others. Coman's work has to be viewed against this contemporary background.

Coman's paper of major importance in the present context was entitled "Decreased Mutual Adhesiveness, a Property of Cells from Squamous Cell

Carcinomas", published in *Cancer Research* in 1944. Coman extended passive observations on fixed tissues with active observations on living cancer cells. Under the microscope, he observed that pairs of cells isolated from squamous carcinomas of the lip ". . . could be separated from one another by needles with the greatest of ease." In contrast, cells in normal lip epithelium ". . . were pulled apart only after considerable resistance had been overcome." Coman went further, by obtaining numerical estimates of the actual forces required to separate one cell from another. "Two glass microneedles were attached to a Chambers micromanipulator. One stiff and blunt served to hold one of an attached pair of cells firmly against a coverslip and remained stationary. The second (the pulling needle), flexible and sharp-pointed, was inserted into the other cell of the pair and, on being moved by the micromanipulator, pulled the cells apart. As the cells resisted separation because of their mutual adhesion the pulling needle bent, and its bending reached a maximum just before the cells had been pulled apart. The maximum bend was measured, and the value thus obtained was expressed as the force necessary to separate the pair of cells" because each pulling needle was previously calibrated by weightloading. The results were tabulated as follows:

"Forces required to separate pairs of cells by micromanipulation

Derivation of cells	Mean ± SE (mgm)
Normal lip	1.42 ± 0.041
Carcinoma lip	0.47 ± 0.051
Papilloma, skin	1.25 ± 0.032
Normal cervix	1.11 ± 0.039
Carcinoma, cervix	0.18 ± 0.022

. . . it is seen that the values for carcinomatous cells are much lower than those for normal cells and for cells from papillomas."

These experiments were unique at that time in presenting numerical data on living cancer cells and their controls, within the framework of metastasis. Although this paper is seldom quoted today, it was without doubt seminal in generating interest in cell adhesion/cohesion in relation to metastasis and is the progenitor of the subsequent hundreds of papers on adhesion. Interestingly enough, Coman once told me that his paper went down like the proverbial lead balloon in the United States, but attracted considerable attention in Great Britain.

It had been repeatedly claimed that cancers contained less calcium than normal tissues since the reports of Beebe in 1904 and Clowes and Frisbie in 1904, and including the reports by Brunschwig et al., and Carruthers and Suntzeff in 1946. In a series of studies with Zeidman and DeLong, Coman successfully correlated increased ease of cell detachment with chelation of calcium. The significant element of these studies was not primarily in connection with low calcium and cancer cell release in relation to metastasis because with improved technologies, the general concept of low calcium levels in cancers has not been substantiated.

These studies were significant conceptually because an attempt was made to forge a link between the effects of calcium removal in promoting dissociation of cells from cancers with embryologic studies on cell dissociation from embryonic sea urchins as first shown by Herbst in 1900, from sponges as shown by Galtsoff in 1925 and from other living cellular aggregates. This bridge between those reading the cancer literature and those reading the embryology and cell biology literature was directly responsible for a beneficial mutual interchange of ideas. In England, for example in the 1950s this work attracted Abercrombie, Ambrose, Curtis, Easty, Weiss and others into studying the role of cell contact interactions in metastasis-related phenomena. Once again, the historian of metastasis research can trace many of our current ideas and publications to this work.

Later work by Coman and his colleagues in 1949, 1950 and 1951 was concerned with assessing Ewing's "mechanical" hypothesis of metastatic pattern and showing that cancer cells would grow in any organ, provided enough were delivered to it. Of course this does not account for all of metastatic pattern, which is surely some sort of sequential balance between the Ewing and Paget hypotheses.

At a time when we stand ready to exploit the incredible technologies of molecular biology in furthering our understanding of metastasis, it is fitting that we should take a moment to look back and honour Professor Coman for his conceptual and experimental contributions which played a seminal role in energizing subseqeunt research in metastasis.

"If we see further, it is because we stand on the shoulders of giants!"

Laudatio for Professor Irwing Zeidman

I. J. Fidler

Dear Colleagues:

It is my distinct pleasure to be here today to accept the first Paget-Ewing Award on behalf of a great scientist and a personal friend – my teacher, Professor – Irwing Zeidman. I have known Professor Zeidman for the last 20 years and state without any hesitation that he (my scientific father) and Professor Dale Rex Coman (my scientific grandfather) are the most suitable recipients of this award. It is a rare privilege for a student to accept an award on behalf of his teacher, and I do so with great pride and gratitude.

Some months ago, when the Metastasis Research Society determined to award this honor to Professors Coman and Zeidman, I was asked to say a few words about my distinguished teachers. They are the true pioneers in the field of metastasis. Both were highly trained medical pathologists who were not satisfied in merely identifying a disease state. Rather, they dared to ask questions dealing with the etiology, progression, and mechanisms of the process of metastasis. They recognized that only a better understanding of the pathogenesis of metastasis can lead to improvements in therapy of this fatal aspect of cancer. It would be impossible for me to enumerate the seminal contributions of Drs. Coman and Zeidman to our understanding of metastasis. Rather, I have asked Professor Zeidman to reflect on those years – the beginning of the renaissance of metastasis research. His letter moved me deeply, and I wish to read it to you in its entirety.

Dear Josh,

Here, at last, is your requested letter concerning my "reflections on research in metastasis". This letter also includes Professor Hellman's requested comments on "the background to my own contributions to metastasis". First, I must express my deepest appreciation to you and all members of the Metastasis Research Society for being chosen a co-recipient, with Professor Dale Coman, of the first Paget-Ewing Lecture Award. I deeply regret my inability to be present at this outstanding meeting of minds with such an important common interest.

Now, the environment which permitted my experiments in metastasis. This environment was so conducive to research that I shall be justifiably labeled a

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spoiled child. I finished my medical education at the beginning of World War II and served 4 years in a mobile pathology laboratory. At war's end, I felt that my career should be spent not in medical practice but, rather, in research and teaching. The pathology of disease was my chosen research subject as I had already done some minor work therein as a medical student. My start, with Dr. Coman soon to be my department head and advisor through much of my career. The place, the Pathology Department of the Medical School, University of Pennsylvania. This department was one of the few in the country which permitted pathologists to do full-time teaching and research. Service functions were purely voluntary. The service division of pathology was a well-staffed unit in a separate building and, as you might expect, paid much higher salaries. So, my remuneration was partially intellectual. More important, I could conduct research leisurely.

Let's continue the list of conducive environmental factors experienced during the first decade or so of my career. During World War II, the massive production of penicillin led to enormous saving of lives. This resulted in the post-war erroneous belief in the Congress that money was the only obstacle to the elimination of any problem afflicting mankind – particularly cancer. Enormous governmental research funds became available to universities and their investigators, more than could be used reasonably. Even I got a grant early in my career.

Our research day often began with coffee in the laboratory. Around the table sat Dr. Coman, the late Dr. Charles Breedis, the late Dr. Morton McCutcheon – famed for his work in chemotaxis – and Josh Fidler, who joined us during his years of Ph. D. training; associated technicians joined us at their leisure. The talk was about everything. Yes, there were remarks about our current research, past and present related research (both locally and elsewhere), remarks about the relevance or irrelevance of the research (with accent on the latter) etc. But just as often, our conversation concerned unrelated items – mostly about Coman's prodigious activities and hobbies: art, coin collecting, university problems, politics, almost all aspects of nature, particularly ornithology. Many of us, including myself, were interested in birds. After morning coffee, some research work, and lunch, we reassembled in the small botanical garden outside the medical school for a short birding expedition. We usually spotted about 100 species of birds during each year in that small patch of green. Indeed, I often heard the joking remark that an interest in birds was one prerequisite for membership in our pathology department.

There was little pressure on us to maintain standing or get promoted. The "suggested" number of publications was one a year, perhaps two. We looked askance at scientists with a large number of publications annually – considering many such papers not contributory, inadequately supervised, rehashes, etc. I personally shall never forget my visit to a dean of a reputable medical school in France. On leaving, he presented me with a small book which simply listed his publications, well over 500. I subsequently discovered that the Dean must be listed as a coauthor on any publication coming from his medical school.

At the time I started research, virtually the entire field of metastasis was open to experimentation. Our knowledge of metastasis was largely that of the

pathologists – based on autopsy findings and surgical specimes. Willis had it all summarized in his classic text. Theories on metastasis were well standardized as they were compatible with clinical and autopsy findings. The soil theory occupied its little niche in every text of pathology because certain autopsy observations could not be explained by the more popular concept that tumor emboli were blocked by the first narrow capillary bed encountered, there growing into metastases. The inquisitive mind was now ready to express its dissatisfaction with the soil theory, insisting that "soil" had no meaning unless classified on a molecular basis. The time was ripe, the research funds were available, and investigators from all walks of life suddenly became cancer researchers.

By the time I started, Coman had already made his classical contribution, demonstrating decreased adhesiveness of cancer cells, an attribute which permits invasiveness. This showed the world that we had better explore the cancer cell surface if we are to understand the mechanism of the most dangerous aspect of cancer – invasiveness and subsequent metastasis. I started with Coman's cell-pulling techniques, demonstrating that calcium was important in decreased adhesiveness as normal human cells revealed decreased adhesiveness in calcium-free solution. Methylcholanthrene, a carcinogen which decreases cell calcium, also decreased cell adhesiveness. Then I branched off on my own with assorted interesting experimental findings as the immediate and unarrested passage of some tumor cells emboli through the lung circulation, the extreme ease of transcapillary passage of leukemia and lymphoma cells through any organ, and cinematic studies of transcapillary passage. One day, I went to Coman and asked him what is known about the spread of cancer in the lymphatic system. His answer was "nothing." That started the first injection of cancer cells into lymphatics and the unfolding of the complete mechanism of the metastatic spread of cancer in the lymphatic system.

All of these contributions must seem so trivial presently. Remember, though, that in our day, ice had to be broken. Watson was wearing diapers, the gene was an unknown quantity, and even known serum proteins were few and far between. Of course, during my work, I had ample help from medical students, some of whom went on to professorships in different medical schools. Independent of my willingness to help anybody interested in doing cancer research, I also had an ulterior motive. The academic pathologists of the country were sorely in need of investigators, and I wanted to get some of these students to choose careers in academic pathology. It wasn't long before I realized that my students were graduating and were then going into ophthalmology. The situation became most noticeable when the son of the head of the Department of Dermatology worked with me on metastasis and subsequently specialized in Ophthalmology. I was jocularly given an honorary position in our eminent Ophthalmology Department as I was called on so often for letters of recommendation. I assure you that at no time when the students and I worked on metastasis did I even mention the eye. But later, I got a different sort of satisfaction.

One day, Dr. Robert Marshak, the Dean of our Veterinary School, phoned me. He said he had a young, aggresive staff member who didn't care about the limited cancer research at his school and who wanted to get a Ph.D. at our

place. Would I consider him? "Send him over and we'll talk," I said. The next day, Josh Fidler walked in and, after a brief talk, was accepted. In 3 years, Josh got his PH.D. resulting in an outstanding publication which placed him solidly in the ranks of most promising young investigators in cancer research. Then, he left me to take an academic position in teaching and research at our Dental School. From there, as everybody knows, his progress was meteoric, and I sit back in reflected glory. Hey, I taught him the ABCs of cancer research.

This plush environment surrounding our research did have a few rough edges. These were caused largely by critics outside our locus of activity who did not understand the meaning and importance of the experimental method in science. Our experiments involved the intravenous injection of tumor cell emboli and, after a few weeks, the counting of metastases produced. The primary cancer does this by invading veins locally and then releasing the emboli. We shortened the time period enormously, but ended up with the identical product – metastases. Our critics maintained that metastases slowly increase in number in cancer of man. We were injecting too many cells – and in one bolus only. This type of criticism led to the appearance in the literature of such useless substitutes for the word "metastasis" as pulmonary colonies, experimental metastases, etc. The critics, of course, did not realize they had already accepted the word "carcinogenesis" as applying to the famous experiment in which rabbit ears were tarred for a year, and they had accepted the numerous experiments in which methylcholanthrene or tar derivatives were applied to the skin of mice. Normally, these rodents never see tar or methylcholanthrene in a lifetime. In these cases, the investigators were not forced to hide the fact that a cancer was a cancer by the use of semiobscuring words. Finally, as subsequently determined, it frequently takes many tumor cell emboli to produce one metastasis. Our critics, I'm sure, are still not satisfied. So we brush them aside and regret that they never had an understanding of the word "experiment." One final note in this regard is what I consider the scientific joke of the century. About 20 years ago, a prominent investigator, with a background of one or two inconsequential Sunday experiments in metastasis, but with subsequent questionable fame in another field, remarked that there is nothing more to be discovered in the field of metastasis by using the syringe and needle.

Finally, there was a far more important rough edge in my plush research environment. In the latter part of my career, I witnessed the steady deterioration of many principles I had heretofore considered inviolate if cancer research were to continue most effectively. And the cause of all this may be found in the Congress and the U.S.P.H.S. for failure to keep up with the rising cost of biomedical research. Medical schools and other research schools in the university were left like sitting ducks. A potential young teacher-investigator was faced with a career block instead of a welcome card. First, after a relatively short period of induction, he had to get grants. These grants had to pay that part of the salary equivalent to the time used in research. This meant that grant support must be forever – as even temporary interruption could lead to a partial loss of salary. No allowances were made for the many excellent investigators who have short periods of nonproductivity – or ups and downs. Furthermore, the young investigator had two more strikes against him. First, the

difficulty for the novice to get a grant had increased enormously. Cancer research funds were inadequate, and only the highest priority grants were funded. The second strike concerned tenure. To obtain tenure, the investigator practically had to have the stature of the top young investigator in his field in the nation. So even the future financial stability of a beginner was threatened at the start. Now, what was the loss to cancer research when doctors of medicine or of other professions with similar scientific backgrounds normally guaranteed a generous remuneration for a lifetime of straight practice reached the critical time and had to decide between practice and research? I sometimes wonder whether I would have chosen an academic career under the aforementioned circumstances or, indeed, if I had chosen academia, how long I'd have lasted. The United States, the research center of the world, spending billions of dollars each year on items of questionable value, has neglected its obligation to humanity and permitted a slowing of progress in cancer research. Universities did what they could to fill the gap, but the rape of the research effort has nevertheless occurred.

Finally, in this melange of good and not so good in my reminiscences, I conclude with my best, and congratulate the "Three Musketeers" of research in metastasis and their associates for their untiring and often sensational productivity. My prolonged contact and friendship with them is my fondest memory. Josh Fidler has attained the "ne plus ultra" of cancer research, and his macrophage work will actually lead to the saving or prolonging of lives. George Poste's general cancer biology work and his contributions to the development of better therapies for cancer metastasis are impressive. I had the unforgettable privelege of collaborating with George in the last 5 years of my research. And Garth Nicolson continues his penetrating studies of the biochemical basis of metastasis with emphasis on the cell's glycoprotein coats and proteoglycans. Garth's dogged determination deserves the highest praise as he is dealing with macromolecules of seemingly unlimited diversity. But the charting of these molecules may yield the most important information needed to explain much of the behavior of the cancer cell.

Having congratulated the Three Musketeers, I now beg forgiveness for the sin of omission – the young investigator. One of the most satisfying events in my series of recollections is the continuous appearance of new, young investigators associated with distinct contributions in the field of metastasis. This guarantees that the future of our field will not be compromised and the wonderful progress to date is likely to continue unabated.

Best wishes to everyone.

Irw Zeidman

Thank you, my colleagues, for honoring two outstanding scientists

Satellite Workshop

Clinical Perspectives for the Treatment of Metastases

This satellite workshop was convened as an adjunct to the Second International Metastasis Research Society Conference in Heidelberg. Its participants comprised invited speakers and guests, members of the advisory board and local organising committee of the conference and the scientific council of SEK.

The aim was to discuss critically current and potential strategies for the early detection, control or eradication of disseminated malignant disease. Speakers were encouraged to present succinct state-of-the-art surveys of their area of expertise, highlight problems requiring further research, and to speculate and invite discussion on future developments. The measure of success of this approach will be evident in the following summaries of the presentation given and in the edited highlights of the discussions which ensued.

Session I

Surgery, Radiotherapy, Chemotherapy, Hormonal Therapy

Chairpersons: L. Milas, and G. Parmiani

P. Schlag (Heidelberg), opened the workshop by defining three clinical situations where the differing patterns of metastatic spread influenced surgical strategies: where lymph node or isolated organ metastases were present, surgery would have a role to play, whereas when intracavitary seeding was evident, surgical intervention would be inappropriate.

The surgical approach to lymph node metastases could have a therapeutic or diagnostic intent; lymph node dissection could be considered potentially curative in gastro-intestinal or lung carcinoma, but is only of diagnostic benefit in other cancers such as breast carcinoma or malignant melanoma, where such regional spread is invariably associated with the threat of disseminated disease. In gastric cancer it has been shown that surgical treatment of N1-N2 cases can yield some survivors, whereas this is not the case for N3 patients.

The case for surgical intervention where organ metastases are present required in Dr. Schlag's view one of three prerequisites: that the metastases were solitary (or very few in number), that their presence was life threatening, or that other compensative therapy was not available. Palliative indicators were listed as bone fracture, neurological emergency or obstruction, and strategies that could be considered included resection, bypass, or catheter placement for regional delivery of chemotherapeutic agents. Recently regional administration of 5-fluorouracil (e.g. via hepatic artery for liver metastases) had been shown to offer some survival advantage over systemic treatment.

Curative treatment of solitary organ metastases had been demonstrable in sarcoma, renal carcinoma and seminoma metastases to *Lung,* colorectal and endocrine tumour metastases to *Liver,* and occasionally melanoma *Skin* lesions. In addition, the recent introduction of intraoperative ultrasound had improved the detection and resection rate of abdominal metastases.

The survival rates reported for either surgical resection or catheter placement for regional chemoperfusion were encouraging; 25% of patients treated for hepatic metastases were disease free at 5 years and for melanoma, amputation or isolated limb perfusion yielded 50% survival at 40 months.

The pitfalls which Dr. Schlag perceived included metastases which were undetectable pre-operatively, residual foci which may remain following resection and the presence of micrometastases. Strategies to overcome these problems would include improved detection/diagnosis and the use of adjuvant post-operative chemo- or immunotherapy in "high-risk" groups.

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B. Kimmig and **M. Wannenmacher** from Heidelberg presented the current status and future prospects for radiotherapy. Radiotherapy, similarly to surgery, is a curative treatment modality primarily aimed at controlling primary tumors. In addition to this, however, radiotherapy has a well-established role in the prevention and management of metastatic disease. Like surgery, radiotherapeutic management of metastasis in regional lymph nodes is more frequent and more successful than that of distant metastases. Elective radiotherapy of microscopic regional lymph node metastasis given concomitantly with the radiotherapeutic treatment of primary tumours has become a routine procedure for many human malignancies, such as head and neck cancers and breast cancer. Here, modest doses of irradiation (50 Gy in 25 fractions over 5 weeks) will sterilize over 90% of occult nodal metastases in the surgically undisturbed regional lymph nodes. Selective radiotherapy can also be used for eradicating subclinical metastatic disease spread via blood, e.g. brain metastases from lung cancer, via peritoneal seeding, or peritoneal carcinomatosis from ovarian cancer, via subarachnoid fluid, or subarachnoid metastases from medulloblastoma. Radiotherapy can be curative in clinically evident metastatic disease when combined with surgery or chemotherapy. In combination with the latter, radiotherapy is used to treat sanctuary sites or used as "consolidation" treatment of areas at the highest risk of relapse.

Radiotherapy is a frequent palliative treatment for different metastatic localizations. In this role, it is highly effective in relieving patients from pain, bleeding, and in the prevention of bone fractures. Radiotherapy is also being used for treatment of overt metastatic disease in different organs, for example brain and bone metastases. Depending primarily on the tumor size and radiosensitivity of a given tumour, radiotherapy can significantly prolong the life and improve the quality of life of such patients.

In the treatment of bone metastases, pain relief can be achieved in 70%–90% of cases, and significant recalcification occurs in 40%–50% of cases, depending on the tumour histology. Radiotherapy is treatment of choice for multiple brain metastases, whereas surgery should be considered first for solitary metastases. If a patient is surgically incurable, radiation therapy is also indicated for solitary metastases. At the institution where Kimmig and Wannenmacher are working 131 patients with brain metastases were treated from 1979–1985, resulting in significant improvement of neurological symptoms for about 50% of the patients. Progression of disease was seen in 16% of the patients despite irradiation. The mean survival time was about 7 months and the 2-year survival rate 10% – patients of this group have a chance of long-term survival.

A special concept of radiotherapy, termed "radiosurgery" consists of stereotactically guided, single high-dose irradiation with a steep radiation dose gradient outside the target volume. This technique has already been used successfully in the treatment of inoperable solitary brain metastases. In Heidelberg a method for radiosurgery was developed using a moving-field technique with a modified linear accelerator facility. Some 37 patients with inoperable solitary brain metastases were treated with single high doses of 15–40 Gy. A significant improvement of neurological function resulted in 70% (26/37) patients. Com-

puter tomography (CT)-proven remission or stable disease was also found in 26 patients. The mean survival time was comparable with the results of other treatment modalities.

An interesting possibility of radiotherapy for metastatic cancer is the use of highly selective radiopharmaceuticals. Established radionuclides are ^{89}Sr for bone metastases and ^{131}I for differentiated thyroid carcinomas. Therapy with ^{131}I for thyroid cancer results in complete remission of more than 30% of the patients with metastases and more than 60% of patients with lung metastases only. A promising new pharmaceutical is MIBG (meta-iodobenzylguanidine), a compound similar to norepinephrine, with high affinity to pheochromocytomas, neuroblastomas and carcinoids. ^{131}I-labelled MIBG can be used for selective treatment of these tumours.

Prospects for the improvement of radiotherapy are seen in several areas, as for instance in the targeted radionuclide therapy in which radioisotopes (such as ^{131}I and ^{90}Y) are bound to monoclonal antibodies that specifically react with tumor cells. This approach should result in selective concentration of the radiation dose in metastatic lesions. A number of clinical trials are underway, testing the therapeutic efficacy of targeted radionuclide therapy. Other prospects discussed include total body irradiation and bone marrow transplantation, radioprotectors and radiosensitizers (such as IUdR for treatment of brain metastases) and the combination with other treatment modalities. Finally, research on tumour radioresponse prognostic factors (cell kinetics, ploidy state, intrinsic cellular radiosensitivity, etc.) was emphasized as important to improve radiotherapeutic management of cancer metastases.

The third presentation on chemotherapy was given by **P. Drings** (Heidelberg). The first point he emphasised was that in chemotherapy administered with *curative* intent, overall "complete response" (CR) rates were 60%–90%, whereas 5-year survival was 10%–80%. These results indicate that high response rates do not necessarily result in high cure rates, e.g. for testicular cancer an 80% CR is reflected in 70% "cures", yet in contrast, for patients with small cell lung cancer, (SCLC) 60% CR results in only 10% "cures".

For *palliative* chemotherapy, the mean survival time (MST) of treated patients can be 1–5 years; 3–5 for chronic lymphocytic leukaemia (CLL) and chronic myelocytic leukaemia (CML); 1–2 for other cancers. Again he cautioned that remission is *not* reflected in increased survival. Well-known chemoresistant tumours include hypernephroma, pancreatic, hepatocellular, biliary and thyroid carcinomas, malignant melanoma and other skin tumours.

P. Drings stressed the importance of well-controlled clinical trials to determine the benefits of new analogues or new combinations of existing drugs. Recently it has been recognized that survival may be improved by decreasing toxicity of treatments (e.g. by eliminating the use of procarbazine for pediatric tumours), as well as by seeking more effective anti-tumour agents. Another important factor in evaluating response was adequate staging, stratification and identification of prognostic factors in individual patients. Response in SCLC is critically dependent upon stage and markers such as carcinoembryonic antigen (CEA), neurone-specific enolase (NSE) and creatine-kinase-brain isoenzyme

(CK-BB). These have also been shown to influence response rates. In stage "extensive 2b", in which bone and liver metastases are present, if NSE and/or CK-BB are high, there will be no response to chemotherapy; if low, some responders may be anticipated. Also, tumour types shown to be chemo-resistant in vitro generally give poor in vivo responses.

The future strategy, therefore, should take account of tumour-related factors, and patients pre-identified as potential non-responders should not be treated. In addition it is likely that sequential monotherapy may show advantages over polychemotherapy, and that the use of adjuvant chemotherapy (currently under investigation in gastro-intestinal, bladder, squamous, head and neck and brain neoplasms) will become established. The existence or evolution of drug resistance and its underlying mechanisms are important areas of research, but clinically, improvements in response will be expected to come from increased dosage (possibly), better detoxification, the use of regional chemo- or combined therapy and increasing sophistication in the use of supportive treatment (bone-marrow transplantation, antimicrobial therapy, haemopoietic growth factors, control of emesis and pain). Finally Dr. Drings called for better evaluation of responsiveness, with careful documentation of remission rates and duration, time to progression, mean survival time, number of long-term survivors, and last but not least, quality of life of the treated patients.

Hormonal therapy was covered by **G. Bastert** (Homburg/Saar). He began by discussing breast cancer and reminded us that in 1889–1896, tumour remission following ovariectomy was noted. In 1902, the remission rate following ablative therapy was approximately 30%; a figure sadly still unchanged today in unselected groups. Oestrogen receptors (ER) and progesterone receptors (PGR) are thermolabile, sensitive to proteases and subject to post-translational modification. Overall, 40% of breast cancers are ER+ PGR+; 15% ER+ PGR-; 5% ER- PGR+ and 30% ER- PGR-, although the ER+ cases are 10% higher in post-menopausal patients. Receptor expression has been found to correlate with the degree of lymph node involvement, S phase index, oncogene expression, ploidy, nuclear grade and a variety of other "prognostic" factors. The expression has also been shown to be inversely proportional to that of the erb B2 gene. Based on these findings, patients could be categorized as "high risk" (ER- and PGR-, tumour > 2.5 cm; grade III, hyper-diploid; high S phase, oncogene amplification and positive lymph nodes) or low risk (ER+ PGR+; tumour < 2.5 cm, grade I, diploid, low S phase, no oncogene amplification, nodes negative).

Adjuvant therapy is tailored to the patient category: node positive premenopausal women and ER- post-menopausal women receiving tamoxifen. For node-negative patients the choices are less clear-cut, with chemotherapy being considered for "high risk" categories, or anti-endocrine agents/GnRH agonists/tamoxifen in the premenopausal group.

Tamoxifen may be administered for 2–5 years, but after this time there is a convergence of the numbers of disease-free survivors regardless of treatment;

only the ER/PGR-rich tumours seem to retain a better prognosis. The response rates are: ER+ PGR+ 71% ; ER+ PGR- 32%; ER- PGR+ 53%; ER- PGR- 9%; clearly these figures are somewhat anomalous and further work on the mode of action of tamoxifen is required.

The expression of ER and PGR can change with time; work by Nomura showed little change in ER/PGR expression between the primary and first relapse, but rapid falls in expression of both receptors during second and subsequent remissions and the preterminal phase. It is also clear that anti-oestrogen treatment can induce decreased expression – 47/71 ER+ patients reverted to ER- on treatment with anti-oestrogen drugs and 61/64 following oophorectomy. Standard therapy for pre-menopausal women is currently

a) oophorectomy or GnRH agonists;
b) tamoxifen
c) aromatase inhibitors
d) high-dose progestin
e) chemotherapy – (only if visceral metastases are present).

New trends include the development of GnRH agonists, novel anti-oestrogens/aromatase inhibitors, new anti-progesterones and the use of hormones as carriers of cytotoxic or cytostatic agents. New data is emerging on the associations between anti-oestrogenic compounds (e.g. tamoxifen) and the induction of growth regulators such as tumour growth factor (TGF). New insights into the molecular mechanisms of hormonal/growth factor induced cellular proliferation and the means by which oncogenes may "short-circuit" dependence on such factors should lead us to a better understanding of growth control in complex tissues such as breast and hopefully yield possible new approaches to intervention.

Discussion

The first point of discussion concerned the question whether the extirpation of involved lymph nodes in advanced breast cancer and melanoma is only of diagnostic or also of value for therapy decision (chemotherapy) as claimed by several American groups. At present it is felt that more refined prognostic criteria such as ploidy analysis of lymph-node metastatic cells are necessary. This is especially relevant for those cases where the disease is restricted to the primary tumour and local lymph nodes and where surgery alone would have a curative effect. Similarly there are cases of systemic disease, in which after removal of liver or lung metastasis 20%–40% survival rates have been reported. It has also been noted that successful surgical treatment was not only restricted to a single metastasis, but was also achieved in cases with up to 5 or 6 metastases. According to these reports a limited number of metastases is no apriori contra-indication for surgical intervention per se.

It was stressed by tumour biologists that one has to be cautious about the use of general categories, such as "melanomas" or "colon carcinomas", because according to more refined criteria and, for instance, the use of marker proteins

or genetic probes, each tumour may present a unique entity. This insight necessitates a more sophisticated subclassification of current tumour groups, which hopefully will also have therapeutic consequences. Furthermore, it is known from experimental tumour biology that when the tumor reaches a certain mass and is removed afterwards, metastatic growth can become accelerated. This would suggest an anti-growth effect of the primary tumour on metastasis. Such a situation may also occur in human tumours. The documented beneficial effect of chemotherapy immediately after surgical removal of a primary tumour may be a manifestation of such a phenomenon in the human system. Challenges for the future include the following problems: There is a significant group of patients with so-called "unknown primaries" (no dectable primary tumours) with extended metastases, primarily to lymph nodes. For these patients the mean survival time is only 26 weeks regardless of any therapy. Also the early diagnosis of yet undetectable micro-metastases needs further improvement. Progress is also urgently needed to distinguish subgroups of favourable from unfavourable cases, both in advanced cancer and also in local disease.

Radiotherapy as an adjuvant to other cancer treatments is currently most successful in the treatment of solitary metastases, preferentially of lymph nodes rather than of wide spread metastases. In future therapeutic schemes radiotherapy may prove useful in an alternating schedule with chemotherapy, whereby radiotherapy may eliminate those tumour cells which are resistant to chemotherapy. A new future prospective would be the use of drugs which become bifunctional alkylating agents under hypoxia, but are monofunctional under non-hypoxemic conditions, thus having a selective cytotoxicity within the tumour environment which is hypoxemic.

A better biological sub-classification of tumours would improve the specific efficacy of chemotherapeutic drugs and biological response modifiers. This was illustrated by the successful application of interferon in hairy cell leukaemias, whereas in most of the other groups of leukaemias and lymphomas the positive effect of interferon is minimal.

Session II

Bone Marrow Transplantation, Monoclonal Antibodies, Growth Factor Antagonists, Colony-Stimulating Factors

Chairpersons: E. Sugarbaker, and S. A. Eccles

M. Körbling (Heidelberg) opened session II by considering factors determining the applicability, problems and prospects of autologous and allogeneic bone marrow transplantation (BMT). With a trend towards dose excalation in chemotherapy into the supralethal range, BMT may be considered as a 'rescue' strategy if the primary toxicity is myeloid. Residual leukaemia in the patient may present problems; residual leukaemia in the marrow may be aided by a 'graft versus leukaemia' reaction of allogeneic marrow. Autologous BMT has a lower failure rate than allogeneic BMT, but there is no helpful graft versus leukaemia (GVL) reaction, and residual leukaemic cells must be purged prior to reinfusion. This is currently achieved by chemo- or immunoseparation, although long-term bone marrow culture utilizing recombinant growth factors is an attractive possibility. Different 'conditioning' regimes prior to BMT were also shown to variously influence leukaemic cell survival. Autologous BMT given in first remission for acute myeoblastic leukaemia (AML) showed 60% survival, with no further relapses after 1 year. Second remission transplants seemed to plateau at about 30%, but late remissions occur.

The possibility of obtaining healthy clonogenic stem cells from blood is being explored. Although their frequency is much less than in the marrow, they can be mobilized into the peripheral blood by transient myelosuppression or granulocyte-macrophage colony stimulating factor (GM-CSF). Leukocyte reconstitution can be achieved in two weeks following blood stem cell infusion. AML, acute lymphoblastic leukaemia (ALL) (not chronic myelocytic leukaemia, CML) and possibly solid tumour metastasis may now be considered suitable cases for BMT.

G. Moldenhauer (Heidelberg) next discussed the strategies that may be pursued for monoclonal antibody (MAB)-guided therapy. Antibodies may exert anti-tumour effects in association with complement (CDC) and cells (ADCC), when acting as carriers of toxins or short-range isotopes or as direct inhibitors by binding to growth factors or their receptors. In addition, they may exert indirect effects by activating cytotoxic T cells, and their use ex vivo in bone marrow purging is well established.

While many 'anti-tumour' antibodies actually recognize differentiation-type antigens rather than uniquely tumour-specific epitopes, recent efforts have

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focussed on a 'second-generation' approach to generate antibodies with a defined *function* rather than merely a selectivity. For example, certain antibodies which compete with ligand binding to growth factor receptors may have a direct anti-proliferative effect and bispecific MAB can be used to direct cytotoxic T cells to tumour targets. The idiotype of surface immunoglobulin on B cell neoplasia may act as a truly 'tumour-specific' target, and anti-idiotypic antibodies, armed in various ways, are being used to attack such cells. Following on from this approach is the possibility that immunization with anti-idiotypic antibodies bearing internal images of tumour antigens may evoke effective host immune responses to malignant cells. Such 'vaccines' are currently being tested in experimental models.

In most cases the major problem is that the target antigen is *not* tumour specific, but is an inappropriately expressed or over-expressed normal antigen; hence normal tissue cross-reactivity must always be assessed in vivo where possible.

The use of MAB alone as serotherapy was explored in six studies in the USA between 1981–1984. Intravenous infusion of MABs was employed in attempts to treat leukaemias and lymphomas. A partial response was seen in a few cases of cutaneous leukaemia, but effects against circulating cells were transient.

Many problems remain to be solved before MABs can be used safely and effectively in vivo for diagnosis and treatment of metastasis. These include the development of host-anti-rodent antibodies (which precludes repeated treatment), the presence of circulating antigen or modulation of its expression on target cells, heterogeneity of antigen expression, insufficient penetration of MABs to solid tumours, inadequate activation of human immune effector mechanisms and cross-reactivity with normal cells.

Access of large (150 kDa) intact immunoglobulins to solid tumours can be extremely limited. The degree of penetration can be influenced by the extent of tumour vascularization, type of endothelial cells encountered and the extracellular space. The further development of the 'customized' second-generation MABs, with bispecificity: reduced molecular size to aid extravasation, yet with modifications to reduce rapid clearance which is a problem with conventional fragments; the use of genetically engineered MABs comprising variable regions of rodent origin with the required specificity coupled to human constant regions to reduce immunogenicity; and the use of MABs to carry immuno-activating agents or drug-activating enzymes to disseminated tumour targets. Clearly, there is a long way to go, but the promise of selectivity and lack of toxicity of molecules such as MABs encourages further effort.

Discussion

One of the problems of anti-idiotypic therapy of B-cell leukaemias and lymphomas is that after initial remission, new main idiotypes arise. It was speculated that the occurrence of new idiotypes is due to the therapy against the original idiotype and its subsequent suppression. It was also critically pointed out that for the development of antibodies against specific tumour antigens the use of anti-idiotypic antibodies ('internal imaging') has never proven to be superior to

an immunization with the antigen itself. The potential problems associated with the use of mouse MABs in human therapy were shortly discussed. Few preliminary studies using human antibodies are hampered by the low affinity of human antibodies, cross-reactivity against normal tissue, the development of anti-human allotype antibodies in the patient and the difficulties of large scale production of these antibodies. A future strategy could be to "humanize" mouse MABs by molecular biology techniques, e.g. to construct hybrid molecules containing the mouse variable and the human constant part of the immunoglobulin.

Autologous bone marrow transplantation after sublethal irradiation has been successfully applied for treating lymphoproliferative diseases. There is an interesting difference between the transplantation of bone marrow-derived and of peripheral blood-derived blood stem cells. In the first case relapses could never be successfully treated by chemotherapy, in contrast to the latter case where at the moment three patients have been brought to complete remission. Autologous bone marrow transplantation could at present not be successfully applied to solid tumours.

A. Harris (Oxford) described his work in measuring growth factor receptor expression in human breast and bladder cancers, in particular epidermal growth factor (EGF)r and the related neu/erb B2 oncogene product p185. EGFr were measured in 221 primary breast cancers by ligand binding with ^{125}I EGF and high affinity sites quantitated. A highly significant inverse relationship with oestrogen receptor (ER) to EGFr was found (15 EGFr+ ER+ and 92 EGFr-ER+; 54 EGFr-ER- and 60 EGFr+ER-). The relapse-free and overall survival were significantly shorter for EGFr+ versus EGFr- tumours. When ER- tumours were substratified by EGFr status, the EGFr-ER- had a prognosis nearly as good as the ER+ tumours. EGFr positivity was correlated with poor differentiation, and usually primary and secondary tumours gave similar results. In eight patients, an EGFr- primary tumour gave rise to EGFr+ metastases, which may be indicative of 'progression'. EGFr gene amplification was rare, occurring only in 3/10 patients with the highest EGFr levels. This suggests that in the majority of patients over-expression is due to transcriptional regulation, perhaps controlled by other oncogenes.

In 31/184 cases, high expression of erb B2 correlating with amplification was found. Expression of erb B2 conferred similar poor prognosis to EGFr expression in all prognostic subgroups. Co-expression of erb B2 and EGFr had an additive adverse effect.

In bladder cancer, a positive correlation between EGFr positivity and the tumour stage/invasiveness was obtained: 13/52 pTa pT1 tumours were EGFr+. The recurrence in EGFr+ tumours was 11/100 patients months versus 1.5 for EGFr-. Stage progression occurred in 9/13 EGFr+ tumours, but only in 2/39 EGFr- tumours. Relative risk for stage progression was therefore 22-fold in EGFr+ tumours, and the risk of death 3–4-fold. In this series, multivariate analysis indicated that EGFr status had a higher prognostic value than disease stage.

The way forward may include the use of MABs for targeted therapy or EGF itself which is of much smaller size (6 kDa); antagonists to interfere with EGF-induced tumour proliferation is an attractive but unproven possibility. The presence of EGFr on normal tissues remains a problem.

E. Hersh (Tucson) gave an update on the characterization and potential applications of colony stimulating factors (CSF), a family of glycoprotein hormones necessary for in vitro proliferation and differentiation of haemopoietic cells; they include multi-CSF (IL3), granulocyte-macrophage (GM)-CSF (CSF-2), G-CSF (CSF-β) and neutrophil (N)-CSF (CSF.1).

rGM-CSF stimulates haemopoietic progenitor cells and myeloid differentiation, inhibits replication of HIV in monocytoid cell lines (it is claimed), potentiates tumoricidal capacity of monocytes and augments neutrophil oxidative phagocytosis. In patients, infusion of GM-CSF induces a rise in leukocyte count which falls rapidly on cessation of treatment. It has been used in myelodysplasia with some success (in the short term), and G-CSF has been used to prevent or reverse the myelosuppressive effects of chemotherapy. Patients suffered less neutropenia, mucositis and diarrhoea. GM-CSF is being employed in bone marrow transplantation and has been shown to induce more rapid recovery of haemopoiesis, but again, cell counts dropped to control values on cessation of treatment.

In Arizona, a comparison of i.v. bolus injection versus continuous infusion of G-CSF has been made to determine toxicity, biological effects and maximum tolerated dose. Bolus treatment was found to be ineffective, but continuous infusion effective in inducing leukocytosis.

Potential uses of these potent biological mediators, most of which are now available in recombinant form, are in primary and secondary bone marrow failure, protection against therapy related myelosuppression, and in augmentation of therapy against infection.

Discussion

One problem of the treatment with GM-CSF was the rapid decline of monocytes and neutrophils after cessation of therapy. This is in apparent contrast to the supposed mean lifetime of macrophages and monocytes in peripheral blood which is known to be longer than 5 days. A potential hazard of this kind of therapy is that certain epithelial tumours also have receptors for CSF and thus application of CSF may stimulate rather than suppress the tumour growth. Therapy with GM-CSF may further be useful in combination with autologous bone marrow transplantation by accelerating the haemopoetic differentiation. The therapeutic value of enhancing the reconstitution of the bone marrow with CSF treatment is still questionable because the critical phase immediately after irradiation is not significantly affected as could be shown by a limited number of studies.

Session III:

Adoptive Immunotherapy, Active Specific Immunotherapy, Biological Response Modifiers

Chairpersons: E. Hersh, and T. Giraldi

G. Parmiani (Milano) outlined developments in the use of adoptive immunotherapy in cancer in the last 5 years. It has been established that a type of lymphocyte known as the LAK cell (lymphocyte-activated killer) can be used as an effector to overcome the problem of the weak or non-existent immunogenicity of most malignant rodent and human tumours, and that objective responses (both 'partial' and 'complete') can be seen in a small percentage of patients treated with LAK cells plus interleukin (IL)-2. The first phase of experimental and clinical investigation is now concluding, and the limited success of such therapy has been shown to depend upon the toxicity of the high doses of IL-2 required, in the logistical complexity and high cost of the treatment, and above all, in the small number of responsive patients. The most susceptible solid tumour types appear to be malignant melanoma and renal carcinomas.

Biological responses (e.g. increased plasma and tissue levels of IL-2 and induction of other cytokines, interferon (IFN)γ, IL-2, tumour necrosis factor, TNF) do not appear to correlate with clinical responses. The latter appear to be influenced significantly by *host* factors (performance status, development of anti-LAK or anti-IL-2 antibodies, low lymphocyte rebound or circulating IL-2 receptors), *tumour* factors (total burden, altered vascularity, release of LAK-inhibitory factors), and *LAK* factors (altered circulation and homing; ratio to tumour cells; IL-2-induced suppressor lymphocytes). The factors reported as most predictive of clinical response are: performance status, tumour burden and location, lymphocyte induction by IL-2, *ex vivo* LAK activity and tumour histology.

The means by which to improve the therapeutic responses obtained include:

- Alleviation of IL-2 toxicity (?continuous infusion, lower dose)
- Identification of subsets of more cytotoxic LAK cells (?adherent LAKs, IL2+IL4 stimulated LAKs)
- Improvement in circulation and targeting of LAK cells by genetic manipulation
- Identification and removal of LAK suppressor factors
- Identification, expansion and administration of tumour-specific (?tumour-infiltrating, 'TIL') lymphocytes, although early results suggest that such cells are not more effective than peripheral blood lymphocytes

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H. Hoover (Boston) focussed on the use of active specific immunotherapy (ASI) in the prevention of metastases from colorectal cancer.

Experimental studies in a guinea-pig model showed that Bacillus Calmette-Guérin (BCG) plus tumour cells was effective in inducing a degree of systemic immunity capable of eliminating a limited disseminated tumour burden when the vaccine preparation and administration was precisely controlled. Over the past 8 years, the animal model has been translated into a prospectively randomized controlled clinical trial in patients with colorectal cancer. Past attempts at controlling human cancer by immunological means are deemed to have failed either because non-specific immunomodulators were used, or the tumour vaccines did not fulfil the requirements for a successful vaccine predicted in the animal model – e.g. cells of allogeneic origin or low viability are ineffective.

The objectives of the clinical trial were to

a) determine whether ASI in colorectal cancer patients could enhance the delayed cutaneous hypersensitivity (DCH) responses to autologous tumour cells and
b) whether treatment could prolong disease-free interval and survival.

Dr. Hoover then reported on the first 74 patients in the trial. Following standard surgical resection their autologous tumour cells were used to prepare a vaccine. No serious side effects were demonstrated in any of the immunized patients; a significant increase in DCH response to tumour cells was seen, but no responses to normal mucosa, or responses in unimmunized patients were seen.

In a median post-operative follow-up period of 56 months, distribution of the time to recommence or death for all patients using the Cox model and actuarial estimates including stage, site and treatment as predictors shows a significant improvement in both time to recurrence ($P < 0.037$) and survival ($P < 0.31$) in the immunized patients. Treated patients who failed have demonstrated less extensive disease than failed 'control' patients. However, with such small numbers of patients it is impossible to conclude that ASI ist of proven therapeutic benefit. A multi-institutional trial is currently underway which should answer this question.

Finally, **I. J. Fidler** (Houston) chose to concentrate on the use of systemically activated macrophages to treat melanoma metastases as an example of the potential of biological response modification. He emphasized that tumours are heterogeneous for a variety of phenotypes, and that it is therefore unrealistic to expect 'homogeneous' therapy to be universally effective; rather it should be accepted that multiple modalities, tailored to individual patient needs should be the norm. The use of immunotherapy – which itself has many aspects – could be used as an adjunct to many other forms of therapy.

Multiple metastases exhibit heterogeneous sensitivities to therapeutic modalities, hence successful therapy of disseminated disease must circumvent the problems of neoplastic heterogeneity and the development of resistance. Appropriately activated macrophages can fulfil these demanding criteria, but it

must be accepted that the resolution of tumours which have taken many years to develop may be a protracted process, unlike infectious diseases which generally have a more acute history.

Macrophages can be activated to become tumoricidal by interaction with phospholipid vesicles (liposomes) containing immunomodulators such as lymphokines, bacterial products and synthetic molecules. Such cells can recognize and destroy neoplastic cells (even those which have developed 'resistance' to other forms of therapy) while leaving normal cells unharmed. Such intravenously administered liposomes are cleared and endocytosed by phagocytic cells which then acquire cytotoxic properties in situ, independently of T lymphocyte involvement. Multiple administration of immunomodulator containing liposomes have been shown to be capable of eradicating metastases in several experimental models.

Macrophage destruction of metastases is limited by the ratio of effector: target cells, and hence is limited to very small lesions. For this reason, a realistic approach would be to reduce the tumour burden by conventional therapeutic regimes prior to macrophage activation. The efficiency of activation can be enhanced by the combination of two different immunomodulators within the same liposome preparation. Another problem to be overcome is to find means by which to increase the proportion of macrophages at tumour sites. Low-dose irradiation of tumour-infiltrated lungs was shown to induce inflammation which induced monocyte extravasation and increased destruction of the pulmonary tumour deposits.

Dr. Fidler concluded by reiterating an opinion voiced by several speakers, that the tumour, host and treatment *all* influence the outcome of therapy, and must all be considered in attempts to improve success. His personal view was that activated macrophages have the potential to overcome several common problems in cancer treatment (e.g. heterogeneity, resistance) and reminded us of George Bernard Shaw's words in *A Doctor's Dilemma* – "stimulate the phagocytes . . . drugs are a delusion".

Discussion

It has been discussed that chemotherapy does not always have an immunosuppressive effect, but rather can modulate the immune response. For instance, it has been shown that adriamycin can stimulate macrophages and that cyclophosphamide can modulate suppressor cell activity. It was generally felt that immunotherapy cannot be applied alone, but in most cases in combination with other treatment modalitites, for instance chemotherapy. This is particularly obvious in cases where chemotherapy is already given at ultimate doses with limited success. Here it was supposed that alternating chemotherapy with immunotherapy, e.g. ASI or macrophage activation by liposomes, is more successful than chemotherapy alone.

Subject Index

Pictures from Participants of the Metastasis Congress in Heidelberg, Sept. 1988

Photographic impressions from communications between specialists from 22 countries (mostly Europeans) during lectures, discussions and social events. The latter included a Welcome reception with folklore music, a Speaker's Dinner, the Paget-Ewing award ceremony in the "Alte Aula" of the University, a social evening with banquet and dance at Heidelberg Castle and chamber music from Mozart, Debussy, von Weber and Schumann.


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