CANCER MANAGEMENT IN MAN

Detection, Diagnosis, Surgery, Radiology, Chronobiology, Endocrine Therapy

Cancer Growth and Progression

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Cancer Management in Man

Detection, Diagnosis, Surgery, Radiology, Chronobiology, Endocrine Therapy

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CONTENTS

INTRODUCTION

Previous volumes in this series have discussed the current state of our knowledge concerning the pathophysiology of cancer growth and progression. The complexity of the interaction of malignant neoplasms and the host, the heterogeneity of malignant cell subpopulations, and the existence of metastatic tumor cells resistant to drug therapies remain as significant clinical challenges to clinical oncologists. Indeed, conventional treatment regimens of chemotherapy, surgery and radiology are often ineffective for the therapy of a large variety of established metastatic cancer in patients. When one considers the insidiousness of progressive neoplastic growth and the emergence of continuously more aggressive and malignant cellular subpopulations one is overwhelmed with the challenges inherent in attempting to control malignant neoplasms. Nevertheless, as summarized in the earlier volumes of this series, substantial knowledge and insights have recently been obtained pertaining to the molecular and biochemical mechanisms involved in tumor progression and growth as well as in host antimalignant responses. While there is clearly an urgent need for improvements in cancer management in man, major advances in our understanding of cancer growth and progression have identified a variety of targets and strategies to allow these goals to be realized. This volume critically reviews approaches towards cancer management in man at the levels of: detection, diagnosis, surgery, radiology, chronobiology and endocrine treatment.

Several chapters review selected methods of cancer diagnosis. In addition, a variety of on-going and novel approaches for cancer treatment are also presented in this volume. Progress in the early detection of malignant neoplasms, coupled with novel approaches for the therapy of such neoplasms, may ultimately yield safe and well-tolerated agents for the selective therapy of solid malignancies. New therapeutic approaches, directed towards the biochemical and molecular targets identified in the earlier volumes of this series, may ultimately lead to the generation of new modalities which will yield a high margin of safety and be significant advances over currently available therapeutic measures.

Series Editor
Hans E. Kaiser

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1

SELECTED DIAGNOSTIC METHODS

HANS E. KAISER with a contribution by ERIC T. FOSSELL

INTRODUCTION by H.E. Kaiser

The neoplasm itself is not the whole disease, known under the collective term "cancer," neither the beginning nor the end. The host body reflects the development of the different types of cancer and related diseases in the form of changes occurring on the basis of tumor-host relationship. The diagnostic usefulness of these facts will be briefly discussed in this chapter with respect to the applicability of such methods as computed tomography (CAT scans = CT); magnetic resonance imaging; and nuclear magnetic resonance for early cancer detection. Other studies discussed in this chapter are those dealing with antigens and antibodies. The first two methods may be called morphological whereas the latter may be referred to as immunological. Of special interest is the usefulness of these methods in the diagnosis of progressive neoplastic stages prior to their actual development. The intraspecific effectiveness of these diagnostic tools differs with the various types of neoplasms and their various stages. The applicability of these methods and their evaluation are discussed in the several chapters of this book with reference to the particular tissues from which the tumors arise. In this chapter some recent developments are briefly summarized. Computed tomography, also known as computerized axial tomography, CT is the gathering of anatomical information from a cross-sectional plane of the body, presented as an image generated by computer synthesis of x-ray transmission data obtained in many different directions through the given plane (201).

Magnetic resonance imaging or nuclear magnetic resonance (NMR) is a method for defining the character of covalent bonds by measuring the magnetic moment of the atomic nuclei involved (201). Antigens (Ag) are substances foreign to the native state of the body, usually proteins, carbohydrates, or fat-carbohydrate complexes that, as a result of coming in contact with appropriate tissues of an animal body, induce a state of sensitivity and/or resistance to infection or toxic substances after a latent period (8 to 14 days), and which react in a demonstrable way with tissues. A well-known example from oncology is the carcinoembryonic antigen (CEA), oncofetal a.: a glycoprotein constituent of the glycocalyx of embryonic entodermal epithelium, generally absent from adult cells with the exception of some carcinomas, in which case it may also be detected in a patient's serum (201). Antibodies or sensitizers are immune or protective proteins evoked in man or other animals by an antigen and characterized by reacting specifically with the antigen in some demonstrable way, but it is now assumed that antibodies may also have natural existence without being present as the result of a stimulus provided by the introduction of an antigen (152, 201).

COMPUTED TOMOGRAPHY by H.E. Kaiser

Recent advances in imaging, such as CT and magnetic resonance imaging, together with new computer developments will be beneficial to cancer therapy (84). This is also the case with ultrasound (US) and positron emission tomography (PET). The augmentation of precision should ameliorate the effect of radiation therapy (83). MR is able to give information concerning location, size and margins of neoplasms not available through other imaging modalities (187). A particular region to study with CT and MR is the portacaval space with its multiple anatomic structures (232). Soft tissues are well characterized by differences of intensity between tissues with variable T1 and T2 relaxation time. Distinction of vessels and soft tissue masses can be accomplished by NMR-CT. Contrast material need not be employed. The combination of both methods offers positive prospects (94). Vital and necrotic zones of a neoplasm are also distinguishable by NMR (90). NMR may be used in the detection of neurotoxicity, a potential complication arising from the treatment of the whole brain with a chemotherapy-radiotherapy combination in long-term carcinoma patients (77). MRI showed no improvement in the accuracy of diagnosis in cases of malignant epithelial and lymphoid tumors of the orbit (42). The method is also not very useful in body regions with extensive respiratory or vascular motion in those structures which require exquisite spatial resolution and those where angulation of the viewing plane is necessitated, or in cases where fresh blood and calcification are present inside a lesion (14). It was found in recent studies that most antibodies able to recognize neoplasm-associated antigens are altered carbohydrates. For the past 25 years, the definition of surface alterations connected with neoplasia has been investigated. The comparative studies dealt with normal animal tissues and human tumor cells, cultured cells before and after transformation by oncogenic agents, neoplastic and nonneoplastic transformed cells, metastatic and nonmetastatic cells, variants with high and low metastatic potential, and neoplastic cells before and after induction of differentiation to a less malignant phenotype. Nucleotide sugar biosynthesis and glycosyltransferase alterations have been observed (173). For discussion of selected and specific

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applications of computed tomography, see (22, 28, 75, 76, 80, 107, 190, 208, 209).

CT alters clinical decisions in 14 to 30% of patients (106). CT scans are especially valuable in cases of the superior vena cava syndrome to demonstrate the tumor and, in exclusion, the recurrent tumor in previously treated patients (181). CORRELATE, a new computer software program for CT, is an accurate tool for the localization of neoplasms (188). Sources of error in CT volumetric measurement of extravisceral tumors were evaluated by Staron and Ford (200), and Gatenby and co-workers (79) described a technique allowing *in vivo* measurement of oxygen in neoplasms using CT to guide probes; see also Zink *et al.* (231). It should be possible to improve diagnosis by a combination of CT and sonography, but up to now this goal was only partially achieved (182). Percutaneous aspiration, drainage, and biopsy techniques can be performed safely and effectively in infants and children (212). Automatic techniques for comparison of irradiation and simulation setup are feasible (145). The use of CT numbers in dose calculations for radiation therapy shows an accuracy of 5%. These conversions turn out to be possible also within an accuracy of 5%. These limited accuracies cause errors in the photon beam dose calculation of less than 1% of the dose maximum, and errors in electron beam dose calculations of less than 2% of the dose maximum (105). CT-guided percutaneous biopsies are useful in the tissue diagnosis of pediatric disease, and in guiding the subsequent treatment (16). Clinical studies suggest the usefulness of radiofrequency capacitive hyperthermia together with radiotherapy to treat refractory deep-seated tumors. Intratumor low density areas appearing on posttreatment CT occur as a good parameter for the assessing of tumor response to thermoradiography (101, 103). Tumors in the head and neck, thorax, lower abdomen and pelvis can be heated better than tumors in the upper abdomen (102). The annular phased array shows no preferential heating of superficial over deep-seated neoplasms (166). Tumor and organ contours can be delineated by projection radiography, an important supplement to treatment planning by radiography (104). Low-LET charged-particle radiation has a potential in cancer research and therapy; it should be checked for its clinical advantages (85). The transcutaneous approach of CT is useful in guiding surgery of neoplasms (20). The cross-sections displayed by computed tomography are an ideal basis for radiotherapy planning, because the body contour, tumor target and adjacent normal tissues are accurately visualized (12). Although the cost of CT is high, it is very useful in radiotherapy planning treatment, and also in effective diagnosing of tumor sinuses, nasopharynx, and bladder (2).

SELECTED ASPECTS OF CT APPLICATION IN DETECTION AND TREATMENT PLANNING OF NEOPLASMS WHICH DERIVE FROM PARTICULAR TISSUES:

3/4* Simple cuboidal/simple columnar epithelium

Characterization of natural history and staging of prostate cancer with regard to survival, malignant potential, extent of neoplasm and treatment response are enhanced by CT and related methods (225). During irradiation treatment, the sparing of one-third of the kidney parenchyma prevents sequelae. Studies of one hundred CT scans were made to evaluate the possibility of using the vertebrae as landmarks in the planning of therapy (26). In intra-arterial chemotherapy, the end of the catheter with concomitant arterial opacification, as in the case of pelvic pathologies, can be checked by CT scan (29).

5* Pseudostratified epithelium

Appropriate initial interpretation of the chest roentgenogram should be followed by selective use of computed tomography and surgical mediastinal exploration in potentially resectable cases of non-small cell lung carcinoma (11). Physicians regularly engaged in punctures control by roentgenotelevision and computed tomography of patients should consider dosimetric control as obligatory for themselves (24). CT is not useful in the evaluation of patients with a peripheral tumor, such as bronchogenic carcinoma; however, it is useful in determining which patients with a central tumor do not require a surgical staging procedure prior to thoracotomy (143).

28* Desmal epithelium

CT has also been applied, together with two-dimensional echocardiography, angiography, and inferior venocavography following tumor resection of the heart in children under cardiopulmonary bypass and/or radiation and chemotherapy (40). Vascular malformation are often diagnosed by CT in other regions including tumors, resulting often in inflammation where otherwise angiography is more selective and accurate (217).

32* Reticular connective tissue

CT is used for diagnosing splenic involvement, providing these pathological-anatomical findings with a valuable non-invasive method with a specificity of 86%, sensitivity of 77%, and accuracy of 83% (98). Seventy-four long-term survivors of childhood cancer were examined. The results were related to (1) the effects of CNS irradiation on cognitive development; (2) the specificity of these effects; and (3) the relationship of age at diagnosis to treatment effects. In any case, CNS irradiation reduces performance especially in cases of children below the age of five (49). Juvenile patients who had received central nervous system prophylactic treatments at an earlier age exhibited poorer performance on verbal IQ scores. Comprehension and arithmetic subscores were most affected. Patients who had received cranial radiotherapy plus intrathecal methotrexate exhibited a decrease in 6 out of 7 categories of instruction. Combined groups of

*After Kaiser HE (ed): *Neoplasms – Comparative Pathology of Growth in Animals, Plants, and Man.* Baltimore: Williams & Wilkins, p. 653, 1981.

patients with leukemia had a lower gradepoint average and poorer school attendance (130). Neurologic disabilities are often a sequela of cancer, such as metastases to brain, spinal cord, leptomeninges, peripheral nerves, or nonmetastatic lesions due to cancer, such as infections, vascular problems, metabolic abnormalities, side effects of therapy or paraneoplastic syndromes, in which the definitive diagnosis is improved by CT (164).

45* Transverse striated musculature

An eight-year old boy suffering from untreatable epilepsy and slowly progressing hemiparesis together with personality changes exhibited months later a rhabdomyosarcoma of diffuse type involving the leptomeninx. The late development of a tumor mass explains the delay in diagnosis (127).

47* Neurons of central nervous system

Sixty patients with known metastatic cancer or high risk primary cancer exhibited tumor progression detected by Tc99m bone scans. These were compared with plain radiographs, spinal computer tomography, and computed tomographic myelography. The plain radiographs identified three groups of which the first was a normal radiograph; in the second, a compression fracture was seen; and in the third evidence of metastasis was indicated. In the first group with computed tomography, 335 of the patients had benign disease and 67% metastasis; epidural compression was seen in 25% of the patients with metastasis as indicated by computed tomographic metrizamide myelography. In the second group 38% presented with benign compression fracture and 62% with metastasis, and of the latter, 63% of the patients with metastases had epidural compression. In the third group, spinal computed tomography showed that 15 patients had metastases and one, benign disease. Epidural cord compression was found in 47% with metastatic disease. This approach permits the diagnosis of spinal metastasis, epidural tumors, and benign diseases (160). Forty-eight cases (96%) of 55 patients with malignant disease investigated on an emergency base due to clinical signs of spinal cord or nerve root compression could be accurately diagnosed by computed tomography alone (221). Dynamic computed tomography is important for the early demonstration of infarcts discerned as regions of hypoperfusion not easily detectable with conventional computed tomography. A quantitative assessment of vasogenic edema and hypoperfusion helped in establishing the diagnosis of infarction and neoplasia; orbital and parasellar neoplasms can be separated accurately from vascular lesions, as well as jugular neoplasms from vascular malformations (222).

50* Meninges

The fourth case described in the literature of diffuse primary leptomeningeal gliomatosis diagnosed by CT (hydrocephalus with enhancement of the cerebral cisterns) and the atypical cells in cerebrospinal fluid showed some improvement following radiotherapy and chemotherapy (121). CT and angiographic features of 15 histologically proven primary extradural juxtasellar tumors showed in the case of 5 chordomas prominent bone erosion with significant posterior fossa component; four trigeminal nerve neuromas with bone erosion around Meckel's cave, with contrast enhancement; two meningiomas of the cavernous sinus with moderate contrast enhancement, expansion of the sinus and angiographic stain; two cavernous hemangiomas of the same sinus were clearly discerned and presented angiographic stain. The sphenoid sinus showed opacification and prominent bone destruction (150).

In cases of malignant lymphoma, Hodgkin's and non-Hodgkin's alike, 6.6% showed adenopathy of cardiophrenic angle lymph nodes by CT. Chest radiography alone revealed only three of these as positive (44). Percutaneous computed tomography biopsy procedures are more and more popular (134). Sixty-eight punctures guided by CT resulted in 63% interpretable cytologic data. The method is useful for detection of post-therapy residual masses and is most effective (78%) when used for thoracic masses and visceral locations (133).

53* Peripheral glia

A 68-year-old woman had rapid progressive visual loss in 7 weeks due to a malignant glioma, the diagnosis of which was masked by usual methods (192). Thirteen patients with echographic and CT evidence of optic nerve or sheath enlargement in which the clinical and radiographic findings pointed to nerve sheath meningioma, showed histologically that in four patients no meningioma could be demonstrated, because two had inflammatory infiltration of the dural sheath and two had only endomatous or dense fibrous tissue. Idiopathic inflammatory perioptic neuritis producing optic nerve sheath enlargement may simulate sheath meningioma (64).

The first case of cystic teratoma of the diaphragm in the English literature showed a similar CT appearance as in teratomas in the ovaries. This teratoma contained soft tissue, fat, spots of calcification, and a tooth (151).

MAGNETIC RESONANCE IMAGING** (NUCLEAR MAGNETIC RESONANCE***) by H.E. Kaiser

Magnetic resonance imaging (nuclear magnetic resonance). See especially reports (45, 66, 74, 81, 93, 117, 132, 156, 183, 195).

Damadian was the first to explore the use of NMR techniques in the diagnosis of cancer. He differentiated spin-lattice and spin-spin relaxation times (T1 and T2) as determined *in vitro* with NMR spectrometers. The problem at this time was that biopsies had to be performed to obtain the material for investigation. It was Lauterbur who showed that NMR signals could be spatially encoded to produce images of the object under examination. It was possible to measure T1 and T2 without biopsy. Initial efforts are still disappointing, but NMR imaging will be very important in the evaluation of patients with malignant disease due to the unique anatomic information gained without the use of ionizing radiation (213). The main purpose of nuclear medi-

cine in clinical oncology is tumor imaging, to evaluate selected organs or the whole body for the presence of a neoplasm. In the case of intraarterial chemotherapy, the catheter tip can be monitored as well as tumor vascularity, arteriovenous shunts in the tumor bed, if bilateral arterial catheters are employed. Angiocardiography is useful in the assessment of doxorubicin toxicity; the effect of chemotherapy on such organs as lung and kidney can also be monitored. Radionuclide venography is important for the detection of thrombi; serial bone scans can identify the response of bone metastases during systemic therapy. The same holds true for hepatocellular dysfunction (119). The longitudinal relaxation time (T1), the transverse relaxation time (T2), and the water content of 16 tissues from normal adult rats were measured at 10.7 MHz and 29 degrees C and the differences in T1 and T2, and water content values between normal and malignant liver tissue, were studied. The differences in T1 and T2 values between neoplastic and normal tissues correlated with differences in the volume fraction (amounts) of extracellular fluid volumes and in the amounts of membrane and fibrillar surface area in the cells (33). It is possible to obtain nuclear magnetic resonance spectra from spatially localized regions of live animals and of patients, enabling the investigator to measure biochemical processes *in vivo* with the use of surface coils. High energy phosphates and related studies have been largely preclinical but diagnostic possibilities have been emerging (30). Moving images of the beating heart can be produced by electrocardiogram gating; blood flow can be imaged. NMR is potentially important to the study of therapeutic response; mental states and dementia; tissue generation; and discrimination of body fat and body fluids. Images of T1 values are diagnostically very useful, but the numerical volumes are less important for pathologic evaluation. Fifteen companies manufacture NMR imagers and over 200 are used in hospitals. The technique is rapidly becoming established in diagnostic clinical practice (138). Paramagnetic pharmaceuticals are significant in the assessment of organ perfusion and in some specific organ functions. Dilute iron solutions for the contrast-enhancement of the gastrointestinal tract are used; ferrioxamine B seems to permit the diagnosis of focal blood-brain-barrier defects and renal excretory function; and gadolinium-DTPA is in use for contrast enhancement in several lesions (224). Floating fluids are characterized by a strong signal during slow flow by unsaturated protons which enter the imaging volume (220). The effects of radiations, hyperthermia and chemotherapeutica can be precociously detected because spectral changes occur before the mass of the neoplasm decreases (136). NMR relaxation times are of great significance in the characterization of tissues and the localization of neoplasms (122). T1 values yielded improved discrimination of normal and malignant tissue compared to previous results at higher frequencies (142). The relationship between NMR spin-lattice relaxation times for human tumor tissue at 24 and 6.25 MHz was investigated by Ekstrand *et al.* (68). The measurement of control parameters is experimentally difficult, does not promise success in all cases but is the only noninvasive measurement possibility (125). Cells in tissues that are deficient in oxygen are relatively resistant to radiation inactivation and may not be accessible to some systemic chemotherapy. The premise that hypoxic tumor cells do, indeed, control the radiocurability of some cancers is supported by some clinical evidence. Novel procedures (some of which are noninvasive) for detecting hypoxic regions within solid tumors have been proposed and are based upon two recent developments: (1) the discovery that some radiosensitizing drugs become selectively bound by metabolism to the molecules of viable hypoxic cells, and (2) the growing availability of new imaging procedures based upon positron-emission tomography, single-photon emission tomography, and nuclear magnetic resonance spectroscopy (41). Iron and gadolinium chelates can readily change NMR image contrast (36); see (38 regarding stizolicin). The use of liposomes permits a choice of several routes to administer imaging agents. Especially important is the subcutaneous administration for lymph node visualization (35). The elevation of T1 and T2 values in uninvolved tissues and in the blood of tumor-bearing animals is known as the systemic effect. T1 values showed significant elevation in colorectal and stomach cancers. No effect was seen in acute myeloid leukemia, chronic lymphatic leukemia, chronic myeloid leukemia, plasma cell myeloma, or in pancreatic and lung cancers. Noncancerous states of cirrhosis, chronic hepatitis and monoclonal gamopathies showed no T1 elevation (17). The present ability of nuclear magnetic resonance to monitor the metabolic channeling of fluoropyrimidines in intact tumor cells suggests that future spectroscopic imaging of patients treated with fluorinated antimetabolites may provide clinically important information about tumor biochemistry and drug sensitivity (116). Magnetic resonance images of transplanted human colon carcinoma in athymic mice are enhanced by metalloporphyrin contrast agents for NMRI (165). Many trace metals are connected with enzymes involved in vital physiological roles. Data concerning transition elements of Fe, Zn, Mn and Cu in human cancers in unaffected regions of the body show that they have a value as markers of malignancy (171).

Magnetic resonance imaging of periventricular hyperintensity was investigated by Sarpel and co-workers (179). Triventricular hypersensitivity was concluded to be high and increasing with age, or in the presence of cardiovascular disease or extracranial malignancy. Magnetic resonance imaging can be used repeatedly due to its low x-ray exposure based on the low field strength. This is advantageous in the treatment of children (100). The hope that MRI relaxation time signatures would identify tissues, specifically, malignancies, has not been realized (50). Specific antimitochondrial agents, such as gossypol, rhodamine-123, and lonidamine, might be selectively administered on the basis of tumor LDH isozyme content, and noninvasively monitored for antiproliferative activity by 31P spectroscopy (19). Water-suppressed proton nuclear magnetic resonance spectroscopy is a potentially valuable approach to the detection of cancer and the monitoring of therapy (73).

DETECTION OF MALIGNANCY BY WATER-SUPPRESSED PROTON NMR SPECTROSCOPY OF PLASMA by ERIC T. FOSSEL

I. In 1986 we reported a sensitive and specific blood test for cancer based on water-suppressed NMR spectroscopy (73). We now expand on our initial report as follows:

The water-suppressed proton nuclear magnetic resonance (NMR) spectrum of plasma is dominated by the resonances of plasma lipoprotein lipids. We measured the mean linewidths of the methyl and methylene resonances, which were found to be correlated with the presence or absence of malignant tumors. Values for the average linewidth were lower in patients with cancer. We analyzed plasma from 2,127 people (normal controls, patients with malignant and benign tumors, patients without tumors, and pregnant patients); NMR analysis and measurement of linewidths were blinded to diagnosis or patient group. The mean linewidth for 747 normal controls ($\pm$ SD) was 40.1 $\pm$ 3.2 Hz. For 330 patients with untreated cancer, demonstrated by biopsy, the linewidth was 30.5 $\pm$ 2.7 Hz. Patients with malignant tumors were reliably distinguished from normal controls by this method ($p < 0.0001$), and differed from 461 patients with diseases that did not involve tumors (linewidth, 37.4 $\pm$ 3.6 Hz; $p < 0.0001$). Patients with benign tumors (e.g. those of the breast, ovary, uterus, and colon) had linewidths of 36.8 $\pm$ 4.1 Hz and were different from those with malignant tumors ($p < 0.0001$). However, pregnant patients and those with benign prostatic hyperplasia had linewidths consistent with the presence of malignant tumors. The mechanisms of this effect are presently uncertain. However, certain data suggest that the narrowing of lipoprotein-lipid resonances with cancer is consistent with the response of the host to tumor growth. In our cohort described above, our overall sensitivity was 97% and our specificity was 96%.

II. We have recently expanded this study in a group of patients with lung and breast disease. Data for lung disease patients are shown in Figure 1. There was no difference in the average linewidths for normal controls, inflammatory lung disease, and benign lung tumors; but the average linewidth for malignant lung tumors was different from the benign lesions ($p < 0.0001$). NMR data correctly differentiated between malignant and nonmalignant lung diseases in 195/204 patients. Sensitivity was 97% and specificity was 92%.

Agreement was also observed in patients with breast disease (Figure 2). These patients were categorized according to the type of benign (Figure 2A) or malignant disease and patients whose blood was obtained after treatment began (Figure 2B). Patients with benign disease were well differentiated from those with malignant infiltrating breast tumors ($p < 0.0001$) and from breast carcinoma *in situ* ($p = 0.001$). The NMR linewidth data correctly differentiated 177/187 patients with breast disease whose blood was obtained prior to therapy. Sensitivity was 93% and specificity was 97%. Linewidth measurements of single samples from 164 patients posttreatment were uninformative. Such patients should be followed serially, including pretreatment values, in order to define the usefulness of this procedure in the setting of therapy.

III. We have also studied two models in which hepatocellular tumors (line 1 and line 10) are injected into syngenic guinea pigs. Growth characteristics of these tumor models are known (65, 78). The primary purpose was to define the relationship between linewidth and the number of viable tumor cells present in the experimental animals. NMR spec-

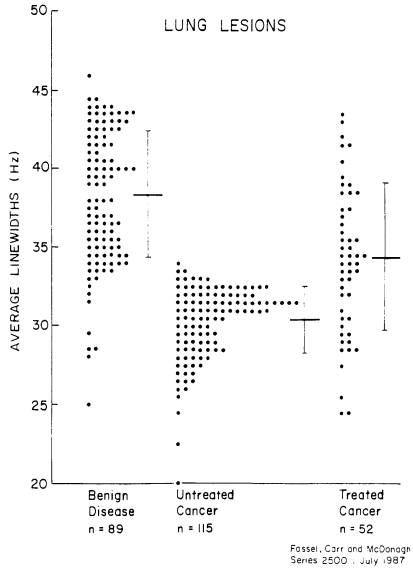

Figure 1. NMR data from patients with benign or malignant lung disease.

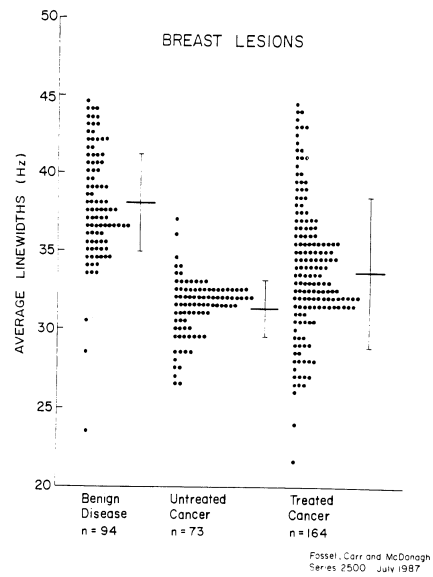

Figure 2. NMR data from patients with benign and malignant breast disease.

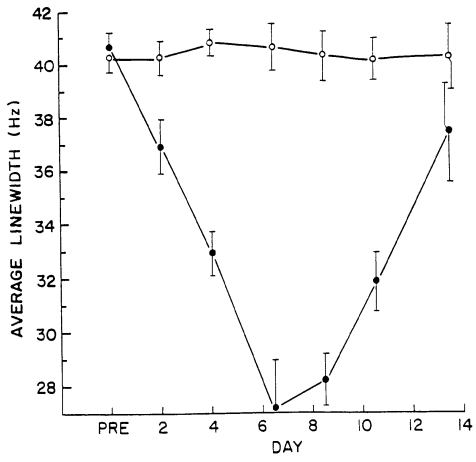

Figure 3. Water-suppressed proton NMR data for methyl and methylene resonances from plasma in which animals were injected with viable line 1 tumor cells (●), or heat-killed tumor cells (○).

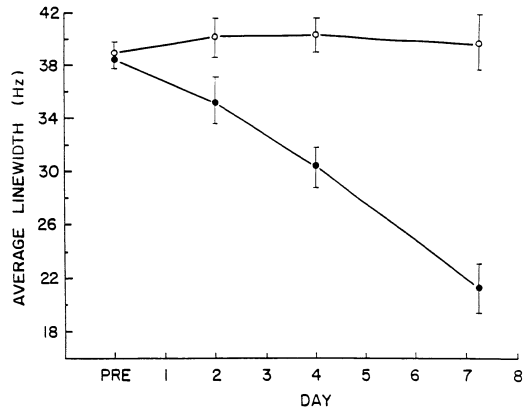

Figure 4. Average methyl and methylene linewidths as a function of tumor number. Animals were injected with line 10 viable tumor cells (●) or heat-killed tumor cells (○).

tra were obtained on citrated plasma at 360 MHz, as reported (73).

Figure 3 shows the time course of the average plasma methyl and methylene linewidths for guinea pigs injected subcutaneously with viable line 1 tumor cells or with heat-killed cells (3×10^6 cells). From day 0 to day 7–8, as the number of tumor cells increased, the average proton NMR linewidth of the methyl and methylene groups decreased progressively. On days 2 and 4, the tumor mass was palpable and by day 7, it had reached its greatest mass (12×15 mm). On day 7–8, there was evidence of tumor necrosis and regression. By day 14, the tumor was no longer palpable. The linewidths of the methyl and methylene groups in these animals began to broaden on day 8, and with tumor regression, became normal. Thus, measurements of proton NMR spectra of plasma in these animals exactly parallels the histology and natural history of this experimental tumor.

Similar data are shown in Figure 4 for line 10 tumor cells injected intraperitoneally (3×10^6 cells). On day 2, the linewidths became smaller and continued to decrease as the tumor grew. On day 2, there were 1.3×10^7 tumor cells in the peritoneum. On day 4, there were 2.4×10^7 cells, and on day 7 there were 1.2×10^8 tumor cells. Line 10 tumor cells continue unconstrained growth.

Inbred guinea pigs were used. All were identically treated except for the tumor cells they received. The average methyl and methylene proton NMR resonance linewidths accurately reflect the natural history of these tumors, and under these carefully controlled conditions, there is a reciprocal relationship between the linewidths and the number of viable cells.

ANTIBODIES by H.E. Kaiser

See reports (31, 69, 144, 204).

Natural antibodies are considered as mediators of T-independent or B-independent natural resistance to neoplasms and microbes (87). The generation of increasing numbers of well-characterized polyclonal antisera and monoclonal antibodies directed to a variety of antigenic determinants has the phenotyping of neoplasms a reality (55). The application of antibody-drug conjugate binding by flow cytometry in cases of several drug and toxin conjugates which defines a human adenocarcinoma-associated antigen demonstrates that these assays are able to monitor the effects of covalent modification of monoclonal antibody as antigen-binding reactivity (140). Corresponding tumor antibodies of different tumors (colorectal, melanoma, testicle, ovary, bladder, carcinoid, lungs) should be selected on the basis of immunohistochemical studies of the primary tumor prior to performing radioimmunoscintigraphy to screen for recurrences of metastases (23).

6-Phosphofructokinase is important for regulating glycolysis in normal and neoplastic cells, mediating glycolysis and respiration. Aerobic glycolysis enhanced in malignant cells is known as the Warburg effect. 6-Phosphofructokinase of humans and the rat occurs in multiple tetrameric isozymic forms showing 3 unique subunits under separate genetic controls, M, L, and P types. Isozymic alterations closely parallel the quantitative increases in total 6-phosphofructokinase activity, which in turn is closely related to the rate of replication of cancer cells and hence an increase in metabolism. Human 6-phosphofructokinase is both a transformation- and a progression-linked discriminant of malignancy (219). Typing by antibodies to intermediate filament proteins is valuable to diagnosis in clinical cytology because these antibodies are able to distinguish between the major groups of neoplasms in man, according to studies with sectioned human material (6). Antibodies to epiglycanin and radioimmunoassay are able to detect epiglycanin-related glycoproteins in the body fluids of cancer patients (46). The subtraction method for radioimmunodetection of neoplasms is important as diagnostic tool (86). Activation of T-cell immunity *in vivo* may result in humoral immunosuppression (158). Anti-idiotypic antibodies may induce the formation of antigen-specific anti-idiotypic antibody, prob-

ably because it appears as the internal image of the tumor-associated antigen. These anti-idiotypic antibodies may therefore have potential for modulating the immune response of cancer patients to their tumors (97). Antibodies observed in human sera were indistinguishable from anti-O-phosphotyrosine antibodies raised experimentally in rabbits or mice (157). The elevated levels of circulating tissue polypeptide antigen antigenicity present in the sera of patients with carcinoma, which are often used to monitor tumor progression, correspond to soluble proteolytic fragments originating from this particular keratin subgroup (223).

Protein A of *Staphylococcus aureus* was used in cancer treatment for 3 years due to its potential for removing serum blocking factors in patients with malignant disease. Solal-Celigny and co-workers (197) reviewed the problems of this type of immunotherapy: the basis of Protein A treatments according to the known effects of Protein A on the immune system; different techniques of plasma adsorption and especially the various Protein A carriers that have been used; the toxic effects which complicated the treatment course in several studies and their mechanisms; treatment results in animal models; and the results of phase I and II trials in patients. *Cis*-diamminedichloroplatinum (II) (*cis*-DDP), the antitumor drug, is cytotoxic *in vitro* primarily by binding to DNA and disrupting its normal functions (169). The regulation of lipid metabolism by a lipid-carrying protein was investigated by Dempsey (56). Diseases manifested by abnormal immune regulation represent perturbations of a system of heterologous genetic recombination (218); regarding the *myc* gene product see (18). Studies with interferon were done (1, 96, 198).

Concerning specific tissues, some additional data may be given in other appropriate chapters.

2* Stratified squamous epithelium

No correlation between the presence or absence of anti-BMZ antibody in the serum and the development of malignant disease was observed (3). Two polyclonal rabbit antibodies to epithelial membrane antigen (EMA), two mouse monoclonal antibodies (E29 and HMFG-21), and a "cocktail" of these two monoclonals have been compared, using an indirect immunoperoxidase technique. The polyclonal antibodies produced stronger staining in colorectal carcinomas and lactating breast, whereas staining with the monoclonal antibodies was stronger in nonneoplastic pleural mesothelium and in pulmonary alveolar cells (99). In 308 consecutive cases, the reliability of ultrasound-guided needle aspiration biopsy for detection of intraperitoneal and retroperitoneal malignancies was evaluated. The method was clearly improved with regard to the accuracy of diagnosis (62). Monoclonal antibodies detected a 16.5 K mol. wt. polypeptide in cells derived from a human cervical carcinoma (15). Mouse monoclonal antibody 17–1A may be a good candidate for use in clinical trials because it retains the tumor antigen specificity and human effector cell recognition of the native 17–1A, would presumably have a five- to ten-fold increase in the circulating half-life in man, and should be considerably less immunogenic as compared with native murine immunoglobins (185).

5* Pseudostratified columnar epithelium

A radioimmunometric assay from P3 lung carcinoma target cells to detect antilung cancer antibodies showed the following: of 100 sera from lung cancer patients tested, 80 (80%) were positive. However, only 6/30 (20%) sera from cancer patients wtih other cancers, 1/25 (4%) of sera from patients with nonmalignant lung disease, and 0/20 sera from healthy donors were positive (95).

Immunized small cell and non-small cell lung cancer cells exhibit different responses to monoclonal antibodies; IgG in regard to the first and IgM, the second type of lung cancer (59).

8* Transitional cell epithelium

In a study by Takahashi and co-workers (210) monoclonal antibody no. 10 was the most appropriate for selection of human transitional cell carcinoma of the bladder; it was identified as IgN with kappa-light chains, by enzyme immunoassay.

10* Mammary glands

Electron dense granules and other markers for neuroendocrine cells are important in diagnosis of various types of breast cancer but the difficulty of small cell and spindle cell tumors has to be considered (154).

20* Testis

The cluster distribution of human placental alkaline phosphatase is a general phenomenon and probably influenced by the physiological function of the enzyme, which has yet to be defined (112).

A monoclonal antitesticular carcinoma antibody obtained via the somatic cell fusion technique by immunization of BALB/c mice with freshly prepared single cell suspension from a patient with testicular embryonal carcinoma with choriocarcinoma components had selective reactivity with the surface of tumor cells from embryonal carcinoma (testicle) and choriocarcinoma both *in vitro* and *in vivo* (118).

28* Desmal epithelium

The Cal antibody was used in an alkaline phosphatase immunocytochemical method on cells obtained from 150 specimens of pleural and ascitic fluids (191).

32* Reticular connective tissue

Antibody heteroaggregates were used to render human peripheral blood T-cells lytic for specified targets. A strategy

is suggested in which heteroaggregate-coated T cells could be used *in vivo* to mount a lytic response against pathogenic cells, such as tumor cells or virus-infected cells (167). Large granular lymphocytes from patients with carcinomatous pleural effusions suppress the capacity of autotumor-recognizing T-lymphocytes to proliferate and develop autotumor cytotoxicity. No cytolytic activity to autologous tumor occurs (214). A remarkable diversity exists with regard to the specificities of monoclonal antibodies classified by conventional criteria as anti-Id antibody, indicating the potential value of several antibodies to analyze the clonal diversity in normal and abnormal B-cell development (123).

36* Melanogenic system

A melanoma-associated proteoglycan antigen is expressed by primary cutaneous and ocular melanomas, metastatic melanomas, nevus cells, some astrocytomas, and fetal fibroblasts, and it is shed into culture supernatant by both melanoma and nevus cells (177). Met 72, a 72,000 dalton glycoprotein, reveals a high correlation with the metastatic activity of several B16 melanoma clones. Met 72 antigens are in fact surface markers of B16 melanoma metastatic variants and may provide the means of monitoring their presence, influence, and autonomy during tumor progression (228).

A cytoplasmic glycoprotein, originally identified by monoclonal antibody 465.12S in melanomas, is increased in epithelial cells of different histotype after transformation. The cytoplasmic melanoma-associated antigen (cyt-MAA) is drastically enhanced in lymphoid cells by polyclonal and allogeneic stimulation, and transformation. Among transformed lymphoid cells, the expression of the antigen correlates not with lineage, but the stage of differentiation. The expression of the cyt-MAA is shared by cells of various embryological origin in early stages of differentiation and/or proliferation (82).

47* Neurons of central nervous system

Sera from patients with paraneoplastic cerebellar degeneration have been shown to contain high titers of antibody to human Purkinje cells. Although human anticerebellar antibodies react with cerebellar tissue from other animal species, patient-to-patient sera might be suitable for passive transfer expression of the cyt-MAA is shared by cells of various by reacting patient sera with nonhuman cerebellar tissue could be negative where these antibodies are in fact present and could be demonstrated using human material (89).

ANTIGENS by H.E. Kaiser

Interaction of genotoxic chemicals with their intracellular target, i.e., DNA, may result in the formation of covalent adducts. Adducts were measured in DNA from various organs of rats treated with the liver carcinogen 2-AAF (10). For neoplasm antigens, see also reports (5, 48, 51, 54, 178, 186).

The present status and value for immunodiagnosis of human cancer-associated antigens was evaluated by Sulitzeanu (206) and the activity of natural killers and macrophages regarding their activity in leukemias, by Fi (72).

Tumors express generally oncofetal antigens which generate several immune responses in the tumor-bearing host of animals and man which are related to neoplastic induction by protooncogenes and oncogenes (47). Cellular and humoral tumor-growth-enhancing factors are present, causing immunosuppression and neoplastic growth. These factors are elicited by neoplastic cells or induced by immunocytes of the host. Circulating immune complexes appear of predominant importance. Plasma adsorption of CIC and IgG protein A of *Staphylococcus aureus* was reported to cause neoplastic regression. Plasma adsorption with protein A-collodion charcoal, protein A-silica, or protein A-sepharose also induced tumorilytic reactions. Even direct infusion of protein A induced tumor regressions in rat mammary tumors. A number of staphylococcal agents are leached by *S. aureus* plasma adsorption. Staphylococcal agents, protein A, enterotoxin and others seem to induce reactions which lead to neoplastic destruction (172). The development of monoclonal antibodies that recognize tumor-associated antigens has led to significantly greater practical possibilities for producing highly specific radiolabeled antibodies for diagnosis and therapy of human tumors (131). Aspects of tumor specificity have also been amenable to an increased level of objectivity, as based upon the probe-like characteristic of monoclonal antibodies (163). Incubation of viable tumor cells in single-phase aqueous solutions of 1-butanol releases a subset of peripherally-associated membrane proteins. The extracted components contain tumor-specific and neoplasm-specific antigens of several human and experimental neoplasms. Viability of extracted cells is not destroyed. The denuded cells permit studies dealing with cellular communications and hematogenous metastasis (135). The production of human anticlonal bodies and their application to kidney transplantation and genito-urinary oncology are evaluated by Guiter (92). Prostatic proteins of seminal plasma in dog and man are related enzymes of the serine-protease class. Their enzymatic activity seems similar. Toward protein substrates the enzymatic activity appears similar, whereas the enzyme of the dog is trypsin-like and the one of man, chymotrypsin-like in its activity toward synthetic substrates. Arginine esterase and prostate specific antigen are closely related proteins (63). Despite their embryonic fibroblastic origin and their infinite life span in culture, C3H 10T1/2 C18 (10T1/2) cells are capable of a wide range of responses to carcinogens and modulators of carcinogenesis that correspond closely to those observed *in vivo*, for the most part, in epithelial tissues (21). Sewell and co-workers (184) made cautionary points and broad recommendations with regard to the use of anti Lew M1 antibody in normal and neoplastic epithelia.

Two chemically induced BALB/c sarcomas shared a tumor-specific antigen on gp96, a M_r 96,000 glycoprotein isolated from Meth A cytosol. It is functional in tumor rejection assays (161).

2/4* Stratified squamous/simple columnar epithelium

Immunohistochemical demonstration of keratins of different molecular weight offers a method of assessing squamous differentiation at the cervical transformation zone and in cervical cancer and precancer (203).

3/4* Simple cuboidal/simple columnar epithelium

Lectins may be useful for estimating the characteristics of renal cell carcinoma, including its malignant potentials; antibodies to renal tubular antigens and intermediate filaments seem to be available for the diagnosis of the tumor in metastatic lesions (108). A good correlation was found between histologic grade in prostatic cancer and presence or absence of R1881-binding protein in the tissue (110). In nonmetastatic disease of carcinoma of the prostate, serum prostatic specific antigen greater than 10 ng/ml at presentation, with or without a coincidentally raised prostatic acid phosphatase, carried an increased risk of progression within two years (189).

4* Simple columnar epithelium

It was concluded that differences in the expression of membrane antigens, differences in the glycosylation of membrane components, and the selective phosphorylation and/or dephosphorylation of cellular proteins exist in subpopulations of intratumoral colonic carcinoma cells with different biological properties. These biochemical alterations of cellular proteins may play an important role in the generation of phenotypic diversity and heterogeneity of malignant cells (39). A new carbohydrate antigen CA 19-9 is a marker of epithelial cancers, does not vary with the smoking status, and is superior to CEA in detecting gastrointestinal malignancies, especially those arising from the pancreatic gland (7). For tissue antigens in large-bowel carcinoma, see Arends and co-workers (8). An intestinal antigen was revealed in 94.3% of primary and metastatic colonic neoplasms, 50% of gastric tumors, and in all samples of the enterolyzed gastric mucosa but not in colonic carcinoid (170).

8* Transitional epithelium

Three cases with papillary adenocarcinoma of the prostatic urethra were prostate-specific antigen positive. Two of these patients were treated hormonally and one radiotherapeutically (227).

10* Mammary glands

Human breast cancer markers were reviewed by Remennik (174). The 52 K protein is an estrogen regulated marker of cell proliferation in human mammary cells (176). T and Tn antigens in breast carcinoma play a fundamental and diagnostic role from the earliest histologic stage and throughout the disease process (199). Tumor-associated breast cancer antigen can be characterized by monoclonal antibody (67, 180). A rad monoclonal antibody YPC2/38.5 may have potential for diagnostic localization and possibly thence for the selective targeting of drugs or toxins in patients with hepatocellular carcinoma arising in a liver unaffected by significant parenchymal disease (141).

15* Pineal gland

S-antigen immunocytochemistry may be applied to characterize tumors of the pineal region (126).

20* Testis

Chemotherapeutic regime alone can lower CA 125 serum levels (162).

32* Reticular connective tissue

Human carcinomas express organ-specific cancer neoantigens as determined by *in vitro* leukocyte responses to extracts of carcinomas by the tumor host. Organ-specific cancer neoantigens of carcinomas are also expressed by fetal organs, and sufficient antigen is shed by fetal organs to sensitize pregnant women. Older fetal organs (21 weeks) and adult organs do not express immunogenic or antigenic organ-specific cancer neoantigens (91). Autologous bone marrow transplantation depends on selective removal of malignant cells from human bone marrow. Marked heterogeneity was seen in the binding of different monoclonal antibodies and in resistance to C′-dependent lysis among these different clones (53). Use of an additional monoclonal antibody FMC-7 does not contribute in the establishment of diagnostic reliability (146). The detection of MCS-2 on immature ALL blast cells might indicate a coexpression of lymphoid and myeloid markers on very immature cells, or an abnormal gene expression by malignant cells, or the identification of a so far undetected subclass of acute leukemias (61). Low-density cell fraction from the blood of patients with chronic granulocytic leukemia provides distinct advantages for the study of membrane properties of hemopoietic cells and of hemopoietic differentiation in general (129). The analysis of reactivity of normal and malignant myelomonocytic cells with monoclonal antibodies has led to refined differentiation schemes of the normal hematopoiesis (60).

35* Fibrous connective tissue

Characterization of the splenic T-DTH cells in mice primed by excision of mKSA tumor, indicated a Lyt 1+2+ phenotype of cells conferring both the DTH response and the immune protection against mKSA sarcoma in a local (Winn) adoptive transfer assay, thus reinforcing the corre-

lation between the DTH response and the antitumor protection (114). New antigens appearing during culture of rat fibrosarcoma of KMT-17 may act as helper antigens for tumor associated antigens, increasing the immunogenicity and decreasing the tumor genicity of the cultured cells (229).

53* Peripheral glia

The techniques described by Krajewski *et al.* (128) are suitable for the identification of an astrocytic subpopulation within gliomas, and may improve the understanding of antigen expression in various stages of astrocytic dedifferentiation.

SUMMARY AND CONCLUSIONS

As selected examples of diagnostic methods in clinical oncology, computed tomography, nuclear resonance imaging, cancer antigen methods and methods dealing with antibodies have been described briefly. In addition to illustrating the diagnostic value of these scientific tools, it was intended to elaborate on the different changes paralleling tumor progression in the host not directly affected neoplastically. It can be seen clearly that tumor development, especially metastasis, is indicated or paralleled by metabolic, immunologic or other changes in the host due to the progression of the tumor. These changes may occur before new steps of tumor progression take place, or parallel them.

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2

BREAST CANCER DIAGNOSTIC IMAGING

RAUL H. MATALLANA

BREAST CANCER

For unknown reasons, breast cancer incidence has shown to be increasing in the United States maintaining a constant mortality rate for the past 40 years (9).

It is not a secret that one out of eleven women will have breast cancer, producing at least 100,000 new cases every year and over 37,000 deaths due to this disease. Nine percent of American women are destined to have breast cancer, that remains the leading cause of death among women 40–44 years of age (34). It is known that unfortunately primary prevention lies still ahead in the future. What may be called secondary prevention measures through screening appears to be as the most realistic way to achieve significant mortality reduction.

It is not new, as we learned in the past clinical experience, that breast cancer detected in an early stage has significant better prognosis than those in later stages (21, 22). The fact that 20 years ago mammography demonstrated that it can achieve detection of non-clinical breast cancer that resulted in 30% decrease mortality, has changed the original idea that periodic physical examinations only as practiced in the past can obtain similar results (30, 32).

The mammographic and clinical contribution to detection of breast cancer is different in relation to patient's age. In women under 50 years of age, omission of clinical examinations would have resulted in failure to detect 61% of the breast cancer during screening. Only 19% would have been missed by omission of mammography. In patients over 50 years of age, clinical examinations and mammography made similar contributions to detection of breast cancers.

EPIDEMIOLOGY AND HIGH RISK FOR BREAST CANCER

The value of a thorough History in Medical Diagnosis is obvious. In the field of Breast Cancer Detection, knowing what we are up against, we have used a questionnaire which directly addresses those risk factors, which researchers have shown to be significantly associated with increased incidence of breast cancer (13, 20).

In view of recent recommendations by The American Cancer Society and The American College of Radiology the usefulness of ancillary historical information relates to increased frequency of follow up, over and above the ACS/ACR's Guidelines (2), and to the prognostic significance of a clinical/mammographic finding. The relative significance of these risk factors will be briefly discussed (45).

Jensen and Wellings *et al.*, reported work in two separate articles (3, 43), showing statistically significant associations between hyperplastic lobular proliferation (HLP) and the presence of cancer in the contralateral breast. HLP was noted in a higher number per breast in those with disease in the same breast (29), or the contralateral breast (23), than in normal (non-cancerous) breasts obtained by autopsy (67). State of the art mammography detects lesions in the subcentimeter range, these patients are curable. Screening mammography has been born out of this. The work just cited was a landmark, shifting the emphasis from the carcinoma *in situ* metastatic-disease progression to the study of the precancerous lesion (which can be mammographically detectable) carcinoma *in situ* progression. The significance of biopsy-proven proliferative disease should be acknowledged as a risk factor (13).

According to Dupont and Page (13), a patient with a previous biopsy revealing non-cancerous disease, is in a higher epidemiologic risk group than the general population if there is any of the following, (1) atypical lobular hyperplasia, consisting of changes similar to lobular carcinoma *in situ*, but not quite, or (2) atypical ductal hyperplasia, atypia just less than intraductal carcinoma *in situ*, or (3) moderate and florid hyperplasia of the usual type, the most common type of hyperplasia one without the appearance of lobular or apocrine lesions.

Other researchers have expanded this subgroup to include those with papillomatosis with an increased incidence of intraductal carcinoma (3). The ultimate risk factor is previous breast carcinoma, in the contralateral breast, or in the same breast. If a lesion appears at least 5 cm from the original one and if it is histologically different, it only would be considered than a metachronous second primary.

Having discussed the histological risk factors, we will now address purely statistical associations. Most of these we addressed in out pre-mammogram survey. These are genetic (family history), nutritional-demographic (the unknown risk factor subgroup of Seidman (4, 35), hormonal (early menarche and later than usual menopause, nulliparity, delayed primiparity, and long term exposure to anticontraceptive medicinal manipulation), as well as, amongst others, environmental (including high dose radiation exposure-circa Hiroshima and Nagasaki studies) (26).

Citing once again Berg and Seidman, the fact does remain that over two-thirds of the difference in worldwide breast cancer demographics are not explained by already known risk factors. This signifies that even the North American female with "no" risk factors is at an extremely high risk for developing breast cancer, hence the need for low risk, reli-

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Table 1. Breast cancers detected during the five year breast cancer detection demonstration project with the four year health insurance plant of Greater New York screening program.

	*BCDDP***				*HIP****			
*Suspicious modality**	*Ages 40–49 at surgery number*	*%*	*Ages 50–59 at surgery number*	*%*	*Ages 40–49 at surgery number*	*%*	*Ages 50–59 at surgery number*	*%*
Mammography only	270	35.4	540	42.1	6	19.4	27	41.5
Mammography & physical examination	381	50.0	638	49.7	6	19.4	12	18.5
Physical examination only	100	13.1	86	6.7	19	61.3	26	40.0
Unknown	11	1.4	19	1.5	0	0.0	0	0.0
Total	762+	100.0	1283++	100.0	31	100.0	65	100.0

* Includes modalities that have finding with one or more features interpreted as suspicious of malignant or benign breast disease.
** BCDDP cancers shown in this table include only those cancers detected following a surgical recommendation made at an annual or early recall screening.
*** From: Shapiro S: Evidence on screening for breast cancer from a randomized trial. Cancer 39 (suppl): 2772–2782, 1977.
+ Includes 30 breast cancer cases in which a mammogram was not performed for any reason, such as exam refused, exam not recommended for a woman under 50 years of age, or exam technically not satisfactory. Exclusion of these cases changes the distribution of suspicious modalities to: Mammography Only, 36.9 percent; and Unknown, 1.5 percent.
++ Includes 17 breast cancer cases in which a mammogram was not performed for any reason, such as exam refused or exam technically not satisfactory. Exclusion of these cases changes the distribution of suspicious modalities to: Mammography Only, 42.7 percent; Mammography and Physical Exam, 50.4 percent; Physical Exam Only, 5.5 percent; and Unknown, 1.5 percent.

able and economic screening. This will be directly addressed in the sections on Mammography and Complementary Techniques.

Risk of developing breast cancer: Many risk factors have been reported. Epidemiologic confirmation has been hard to come by in some (such as the association with a diet low in fibers and high in polyunsaturated fats). The abundant literature, the many possible permutations, and the difficulty in weighing the relative importance of all risk factors, have rendered clinically useful information hard to discern.

To this end, the large cohort study by Dupont and Page identified a subgroup with an 11-fold increased atypical hyperplasia and family history (13).

The implication, compatible with the findings of Moolgavkar and Knudson, (31) in that familiar aggregation results from high risk individuals being born with basal cells

Table 2. BCDDP program breast cancers stratified by lesion size and modality findings.

*Suspicious modality**	*Non-infiltrating breast cancers number*	*%*	*Infiltrating breast cancers < 1 cm number*	*%*	*Infiltrating cancers cancers = > 1 cm number*	*%*	*Breast cancer size not specified** number*	*%*	*Total number of breast cancers*** number*	*%*
Mammography only	461	59.0	195	52.6	631	33.7	194	36.4	1481	41.6
Mammography & physical examination	258	33.0	135	36.4	1034	55.5	252	47.3	1683	47.3
Physical examination only	43	5.5	31	8.4	161	8.6	73	13.7	308	8.7
Unknown	20	2.6	10	2.7	41	2.2	14	2.6	85	2.4
Total	782	100.0	371	100.0	1871	100.0	533	100.0	3557	100.0

* Includes modalities that have findings with one or more features interpreted as suspicious of malignant or benign breast disease.
** Breast cancer size not specified includes cancers for which the Hospitality Pathology Report did not give the specific lesion size and/or for which a project pathologist did not carry out a slide review.
*** Includes cancers detected following a surgical recommendation at an annual or early recall screening, or when a woman saw a surgeon prior to a scheduled early recall screening.

of the mammary epithelium already lined up in the direction of atypical proliferation with the total penetrance in those who develop cancer.

We hope to identify a subgroup of patients with breast cancer incidence high enough to warrant additional modifications to the currently accepted screening guidelines. On the other hand, work in progress by Matallana (17) will show that, in a group of 1,500 breast cancer screened patients, only 30% had any risk factors. This reemphasizes that all women should be screened as per ACR/ACS Guidelines, as a "minimum screening requirement", whether risk factors are present.

I.

Halsted theory of orderly progression of breast cancer is at least partially justified if we review the natural history of human breast carcinoma. This is supported by a variety of investigations, aside of clinical experience. At least there is a fairly consistent sequence of events in relatively early stages of this neoplastic condition.

It is very difficult to pinpoint when the epithelial genetic notation takes place, since the ductal epithelium has been fluctuating from by hyperplasia to repression since menarche, with every menstrual cycle, pregnancy and puerperium. Due to studies of growth rates of human mammary carcinoma and epidemiologic data showing an increase in breast cancer incidence in women under 50, it is possible to accept that the development of mammary carcinoma began premenopausally (20) therefore, the observation (5, 43) associating specific pattern of atypical proliferative hyperplasia as non-obligate form of neoplasia, since it is a common finding in patients who developed breast cancer, has generated acceptance.

II.

There is clear evidence of how important is the detection of "early" or small carcinoma while limited to the breast as compared with patients treated with larger forms, particularly with regional axillary node metastasis (16). There is also a hypothesis that all breast cancers are systemic from the beginning and, therefore, any variation in local treatment is unlikely to affect prognosis. Both hypotheses deserve attention, since breast carcinoma is a variable disease in which both hypotheses can be applied at times.

The assertion that all large infiltrating carcinomas were at some point small masses, does not absolutely imply that all small carcinomas will necessarily progress to a large one because some of this will have different biological behavior. Some critics of the screening value hypothesized that perhaps the periodic examinations are detecting only slow growing lesions ("length bias") (32) or that survival from time of detection is improved because apparently we are "pushing back" the time of death (lead time bias), therefore, erasing the significance of early detection.

It is not possible at this time to draw a line as to how much of these hypotheses are wrong or right or if they are all incorrect. Are we adding quality years of life instead of curing, in cases of infiltrating carcinoma? Are we able to cure only minimal breast cancer?

Discovery and adequate treatment of "early breast cancer" while still localized in the breast or with minimal axillary model involvement has produced statistical information of long survival free of disease Wanebo-Urban. The survival rate of patients with minimal breast cancer is 98% at five years, 95% at ten years (41) with projected 20 years survival rate of 93% (18). In addition the only randomized study or clinical trial H.I.P. program of New York (early 60's) produced results of increased survival of patients over and under 50 years of age after 14 years in comparison with the central group (20–24% survival rate).

III.

A recent randomized study in Sweden showed 31% reduction in mortality from breast cancer and 25% less proportions of stage II or larger tumors in those patients under screening mammography every 2 to 3 years, when compared with a group not offered screening (37).

Reduction of breast cancer mortality through mass screening with modern mammography (6, 40). Evaluation of screening for breast cancer in a non-randomized study (The DOM Project) by means of case control study (6). These two articles suggested, 50–70% reduction of mortality in screened women; improving the already known H.I.P. reduction in mortality.

I believed that it is quite certain that most of the "early" cancers detected have the biological potential of progressing into invasive lethal disease if not treated when they are localized.

METHODS OF DIAGNOSIS

Despite the fact that mammography is the only diagnostic system capable of detecting consistently small and/or "minimal" breast cancer, there is a percentage of the same type of tumors that can be detected by an excellent physical exam in absence of and/or associated with mammographic findings. From the BCDDP program we learned that PE positive in 8.7% of patients with cancer in absence of mammographic findings. (Table No. 14 Ca. A. Cancer Journal for Clinicians – July, August 1982 Vol. 32, No. 4) Table No. 15, same journal showed that in noninfiltrating breast cancer detection with P.E. yield 5.5% detection and when associated with mammography increased the rate of detection to 33%.

We feel that this statistic analysis is sufficient to stress that P.E. is an obligated examination to achieve the highest detection of small carcinomas. It should be made with state of the art technique, including inspection and palpation on sitting and recumbent positions.

IV.

BCDDP again demonstrated that P.E. had a time positive rate of 35–60% combining clinical examinations and mammography produced a time positive rate (sensitivity) of 91%. In our own statistical analysis of 511 visual breast cancers detected from 1973–1984, 26 cases were detected based on P.E. only or 5.08% and when associated with

mammography, permitted detection in 153 cases or in 29.94%.

MAMMOGRAPHY

Before discussing the clinical uses of mammography, we will briefly introduce basic technical information. Perhaps this will help in understanding its uses and limitations. By using x-rays, we can visualize breast parenchyma and define densities in the millimeter, and in certain cases, the submillimeter range. Technical excellence is not only necessary but mandatory in state of the art mammography. This is related to the fact that in the absence of microcalcifications, only a subtle difference in density and/or structure, between normal glandular components and pathological entities can signify the presence of a nonclinical carcinoma. Therefore, the margin of error is more critical in mammography than in any other diagnostic radiographic procedure, because mammographic technical excellence can produce a change in the patient's prognosis.

State of the art mammography has clear requirements: adequate dedicated mammographic equipment with a molybdenum target, filter, phototiming and a moveable grid to decrease scattered radiation, thus improving image sharpness. The use of molybdenum target, filter and phototiming all have enabled us to produce high quality mammography with mean radiation exposure to the breast of 100 m Rem range, not more than a chest roentgenogram, and about 10 times less than what was obtained as recently as 1978 with nonscreen film and xeroradiography (11).

The molybdenum target tube and filter is a dedicated system very useful for soft tissue radiography. The use of a very small source of x-rays (the focal spot 0.1–0.3) and the use of standard/fixed geometry enable us to obtain reproducible high detail, optimal distance of the focal spot from the object and the distance of the object from the film associated with the use of screen film systems, higher film contrast at a low film density and automated film processing technique are absolutely necessary. Fulfillment of these requirements will produce that. The quantity and quality of radiation delivered per unit of time will produce an excellent film diagnostic image in contrast and fine resolution.

With focus to breast and breast to film with distance remaining always the same, coupled with maximum compression and excellent positioning has allowed studies of excellent structural detail and minimal background noise.

Finally, the use of variable focal spots and the advantage of magnification capabilities have increased structural detail and have made mammography by itself, a better screening procedure than the annual physician breast examination and the monthly self-breast examination combined (11).

Radiation dose: The mean breast dose for screen film combination vary between 60–75 mr for 2 views. This dose will raise to 120–180 mr with the use of radiographic grids. Comparing this exposure to the original 3.200 mr for conventional mammography 20 years of age. With the advantage of improved resolution, and decreased radiation it is possible to achieve detection of nonclinical breast carcinomas in a percentage of 40–50%, percentage that 20 years ago were only possible in elaborate dreams. In other words, exposure to the state of the art mammography will in theory cause one excess breast cancer death per 6 million exposed women per year, although, the known benefit from early cancer detection would appear to considerably outweigh the ever proven theoretical risk. Such a "risk" may be equivalent to traveling 70 miles by air, 10 miles by car, smoke one-eighth of a cigarette.

V. MAMMOGRAPHY ACCURACY

Because of the improved accuracy and safety of current mammography technology, the American Cancer Society modified its recommendations to include mammographic screening of asymptomatic women beginning at age 35 years (25).

Statistical analysis of 3,661 breast cancers from the medical literature before 1975 demonstrated a true positive rate of 87% for mammography (10).

In the BCDDP program out of 3,557 detected carcinomas 89% were mammographically identified. Approximately 17% of the group reviewed before 1975 probably were clinically negative, while in the BCDDP group the nonclinical carcinomas were 41.6%. In our statistics out of 1,003 carcinomas detected, 51% were clinically silent.

VI.

The main difference probably is related to the fact that cases from the BCDDP program were asymptomatic while in the first group were the majority symptomatic. In our group 67% were true symptomatic, although 78.9% of the "Symptomatic" patients with small carcinomas, the symptoms were unrelated to the tumor itself.

The use of ancillary imaging modalities, such as ultrasonography, has helped to further increase our accuracy. This would translate into the concept of mammography for screening asymptomatic patients. This use will be discussed in a separate section. We are now detecting subclinical cancer more often than clinically palpable lesions.

Just after the new screening guidelines for mammographic evaluation were publicized in the national news media study was performed on 257 females living in 1983, mostly white. The women were asked to complete a questionnaire regarding frequency of monthly self-breast examinations (SBE), physician clinical examinations and mammography. The research setting was a physician's waiting room. 54% to 69% of the women complied with the recommendations for regular SBE and annual clinical breast examinations. 60% of women 40 to 49 years of age and 51% of women 50 and older had not had a baseline mammogram. 22% of the first group and 24% of the second group have had at least a baseline and one follow-up mammogram (17).

These basic screening recommendations, updated in 1982, have been available by The American College of Radiology (ACR) since 1976, The American Cancer Society (ACS) and National Cancer Institute since 1977.

The data suggests that the underuse of mammography may be more from lack of acceptance by physicians, for the purpose of mass screening than resistance by patients. We hope and feel that since then, health professionals are thinking more of mammography as a screening procedure, a new study is obviously needed (7).

THE VALUE OF PREOPERATIVE MAMMOGRAM

Although a negative mammogram does not preclude the presence of carcinoma (27), a clinically suspected mass lesion should be excised for final histological differential diagnosis regardless of the mammographic report. However, mammography has a preoperative role as to confirm the presence of a malignant lesion, demonstrate multicentricity for exclusion of a clinically negative carcinoma in the opposite breast and for differential diagnosis in presence of a benign mass lesion clinically suspected of a carcinoma, that fortunately can be identified mammographically. This is the case of radiolucent lesions (24) such as lipomas, galactoceles and/or traumatic "oily cyst".

When because a small clinically detected malignant lesion, it is considered the feasibility of local excision and postoperative radiation therapy, it is important to determine the presence of "multicentricity" in the symptomatic breast.

Bilaterability of the breast carcinomas has been demonstrated (39), therefore the presence of a synchronous clinically occult contralateral breast should be excluded. In addition it is possible that the lesion originally suspected as a carcinoma may be benign and be associated with a clinically silent lesion in the suspected and/or contralateral breast. It should be emphasized that mammography should be obtained before invasive procedures, because if it is immediately post-operative, edema and hemorrhage obscure the area in question, and preclude the use of adequate compression to produce a state of the art mammography. Mammographic detection of a carcinoma can be achieved with visualization of a soft tissue mass with irregular and/or spiculated borders, associated or not to malignant type of microcalcifications. If the mass is uncalcified, and clinically occult, there must be surrounded by a degree of fatty background to be entirely visible mammographically, therefore the diagnosis of carcinomas in fatty breast, is simple by all means. Unfortunately if there is minimal or nonexistent fatty background, it is difficult if not impossible the mammographic diagnosis of a noncalcified carcinoma, either palpable or not. Detection of clinically occult carcinoma should be and it is the major goal in breast imaging.

The use of Papanicolaou smear has achieved early diagnosis and cure of *in situ* and minimally invasive cervical cancer. State of the art mammography is also a diagnostic tool, that consistently diagnoses nonclinical small carcinomas.

Despite that mammographic sensitivity and specificity it is higher than in any previous time, not all "early" or small carcinomas are mammographically identified, some are found incidentally during surgical biopsy of other palpable or not, suspicious areas. Eventually some *in situ* lesions are clinically detected despite the general acceptance, that *in situ* carcinomas are not palpable. What may be clinically detected, are connective tissue and/or cystic lesions, produced by perhaps an unknown tumor factor that "excites" the surrounding tissue.

Generally a small nonclinical cancer, can be mammographically detected by subtle indirect radiographic findings such as:

Microcalcifications
Asymmetrical retroareolar dilated duct
Distortion of the glandular architecture
Asymmetrical developing densities.

Breast calcifications are the commoner isolated mammographic findings that reflect the presence of an *incipient* carcinoma, most likely intraductal or lobular carcinoma *in situ*. Sometimes, that small carcinomas are near this microcalcification. We know that the majority of breast calcifications as well as the majority of breast lumps, are nonmalignant. Because malignant microcalcifications are not all clearly visible in dense breast, its identifications and differential diagnosis are directly proportional to the quality of mammograms. Because they are only mammographically visible, this is the major substantial advantage of mammography in comparison with other noninvasive diagnostic existent methods; since they are not capable of detecting this important microcalcification.

Furthermore identification of certain types of cluster or microcalcifications implied a substantial risk of carcinoma. Presence of irregular curvilinear, linear and/or branching calcifications practically always (95%) was associated with carcinoma and should be considered "Typical". Our results are similar to the Sigfusson and Anderson (36) results. After reviewing 501 nonclinical carcinomas detected by mammography only, carcinomas detected by cluster of microcalcifications in absence of a mass, were 64% noninvasive in our cases and 57% in the Swedish group. In the invasive group 10% > 10 mm. 16.5% > 10 mm in our group. Axillary metastases were present in 7% of the invasive carcinomas in the Sigfusson group, 8.5% in our similar group. In our experience the most important incident sign of malignancy the presence of a single or few cluster of calcifications of irregular type and probably linear (ductal) that Sigfusson and Anderson called Risk 1 and 2 should always imply a recommendation of surgical biopsy with preoperative localization and specimen radiography. If for any reason biopsy is not contemplated mandatory follow-up in 6 months and then yearly should be obtained regardless of patient's age.

In addition certain microcalcifications, appearing or increasing during an observation period (median 24 months) implied a larger risk than those remaining unchanged (36).

Another indirect sign we have to consider in our search for small carcinomas is related to asymmetry of breast tissue. In general, the breasts are symmetrical on comparison. If they are not similar in comparable breast regions in both routine mammographic projections, a pathological process should be suspected, in absence of previous abscess, biopsy, blunt trauma or significant congenital asymmetry in size. There are benign and malignant conditions producing asymmetry and should not be considered pathognomonic of malignancy. Previous mammograms will help in deciding if a biopsy should be suggested in our report. In certain borderline cases follow-up in 3–6 months should be recommended to confirm or not stability. Special compression and eventual magnification as well as ultrasonic evaluation of that region may provide valuable additional information as to be stronger in leading toward a biopsy or not.

A developing density is another indirect sign that should be carefully scrutinized since in patients from 35 years and over, their mammary glands are in involution. Exceptions should be if there are clinical information of repeated hormonal medication. The presence of a baseline mammogram is essential in confirming the presence of this sign, that although, it is not pathognomonic of malignancy, a significant number will be developing cancers. Exceptions and reevaluation indications in borderline cases, like when we

Table 3. Accuracy of mammography.

	Literature	*BCDDP*	*Matallana, MD et al.*
Total no. of breast cancers	3661 (10)	3557 (11)	983
True-positive rate for cancer	87.2%	89.0%	91.0%
False-negative rate for cancer	12.8%	8.6%	0.6%
Clinically occult	17.0%	41.6%	51.0%
False-positive	14.9%	–	–
Unknown	–	2.4%	2.5%

consider asymmetrical areas, can also be applied to this sign.

Since most breast carcinomas are ductal in origin and produce invasive duct lesions, the mammographic sign of a dilatated asymmetrical duct of 3 dm or more in length should be regarded with suspicion. Although there are non-malignant conditions such as ductal epithelial hyperplasia with or without atypia, papillomas, or simple intraductal debris can reproduce this sign.

The asymmetrical ductal ectasia is visualized, if there is enough "fatty" background. Obviously in breast of severe density, this sign will be very difficult to detect. Again special mammographic compression with a small compression pad, may help in certain borderline cases. In our experience we use the tomographic water immersed ultrasound with rotational techniques, that provide the best methodology to evaluate the ductal system, in absence of active secreting nipple discharge. However the presence of a progressive dilated duct, after comparison with previous mammograms, warrant a biopsy, because the possibility of an intraductal malignant lesion should be excluded. When we consider these indirect signs while evaluating our daily mammograms, certainly we will increase detection of small breast carcinomas, that have been proven are of good prognosis. There will be a number of false positives, but with experience this will be reduced to an adequate proportion. Referring physicians should be informed of the possibility of benign lesions when recommending excisional biopsy.

OTHER BREAST IMAGING TECHNIQUES

Multiple breast diagnostic modalities, other than mammography, are currently being used. Their value in breast cancer detection is yet to be determined. Other than computed tomography, these modalities are nonirradiating systems. Although it seems reasonable to search for a radiation-free system that would be valuable in detecting nonclinical cancer, it is conclusively evident that none can duplicate the mammographic accuracy, if state of the art mammography is used.

At best, additional nonirradiating systems like echography can be considered complementary to physical exam and state of the art mammography. At worst, these various available techniques, produce a degree of confusion in the general public and certain physicians, as to when these available diagnostic modalities may, if at all, become useful.

It is therefore our intention to scrutinize when they would be applicable.

There is a clear difference between the two well-known applications of breast diagnostic modalities: diagnosis and screening. In the former, we see patients with symptoms and/or clinical findings that are presumably pathological, if not malignant.

When used as a screening modality, breast imaging is performed on asymptomatic patients because of age, family history of breast cancer, or other risk factors mentioned elsewhere in this chapter. The goal is to detect a tumor before it is large enough to be palpated either on breast self-examination or by routine physical exam.

In our experience there is only one possible place for an alternative technique: to complement a questionable clinical and/or mammographic finding or findings.

ECHOGRAPHY

Ultrasonography is based on the utilization of high frequency sound waves to produce images of the area in question and/or the anatomical breast structures. As a non-irradiating system, the theoretic carcinogenesis of this modality is nonexistent at the levels of clinical use.

The main indication of breast ultrasound is to differentiate between cystic and solid lesions that yield an accuracy superior to physical exam and mammography for this particular purpose. It can be argued that cyst aspiration can provide faster, more economic, and no less accurate results in this differential diagnosis, associated with therapeutic capabilities if the lesion is indeed cystic.

Unfortunately if a cystic lesion is associated with malignant microcalcifications in another area of the same or opposite breast, these will not be detected by this aspiration or any complementary modality, other than state of the art mammography.

We believe that the usefulness of breast ultrasound, beside differentiating a cystic from a solid lesion, lies in the multiplicity of sonographic signs available, helping to classify a lesion as "most probably benign" and this obviating unnecessary biopsies.

Echographic search of the area, to confirm, or deny the presence of a mass is of singular importance. Additionally, we can provide information about intrinsic echoes, configuration, and associated sound transmission that can suggest malignancy when associated with mammographic and/or clinical findings. This information is of capital importance in detection of nonclinical carcinoma. Utilization of breast ultrasonography as a complement to physical exam and good mammography certainly enhances our confidence in reporting benign and maligant lesions. This we reported on the occasion of The 4th International Breast Congress in July, 1985, held in Sydney, Australia. We presented a review of 2,031 cases in which ultrasonography was utilized as a complement, yielding an accuracy of 94% in small malig-

Table 4. Comparison of time intervals of persons with breast cancer and control.

Completed years	*Study*	*Control*
2	11	8
3	17	19
4	24	38
5	39	63
6	55	88
7	70	106
8	83	116
9	89	126
10	94	133

nant lesions and 98% in identification of benign pathology (28).

On screening we utilized, after mammography, the water path instrument with tomographic capabilities in evaluating dense "young breasts" in the high risk group for breast cancer due to premenopausal maternae breast cancer, instead of yearly sequence of mammographic examinations. We were able to limit follow-up irradiation to a single medial-lateral mammographic projection every 2 years, looking for microcalcifications. We followed these patients with yearly tomographic breast ultrasound, for exclusion of small solid masses deeply situated in the breast.

The same tomographic, rotational sonographic modality was useful in evaluating patients who have breast implants. These patients generally have a limited mammographic evaluation, because of lack of adequate compression.

Both tomographic and handheld ultrasound modalities have been useful in evaluating patients with mass lesions, seen on only one mammographic projection and for preoperative needle-localization, for nonclinical malignant mass lesions, respectively.

Ultrasonic guidance was also useful in aspirating nonclinical, deeply situated, small, cystic lesions that otherwise would need a biopsy. It was also useful in obtaining diagnostic specimens from breast abscesses with minimal pus content. The latter clinically and mammographically resemble carcinomas when inflammatory signs are subtle.

From our results we feel that ultrasound is the most useful complementary technique to physical examination – mammography in evaluating breast pathology, in diagnosis and/or screening, when indicated.

OTHER IMAGING MODALITIES

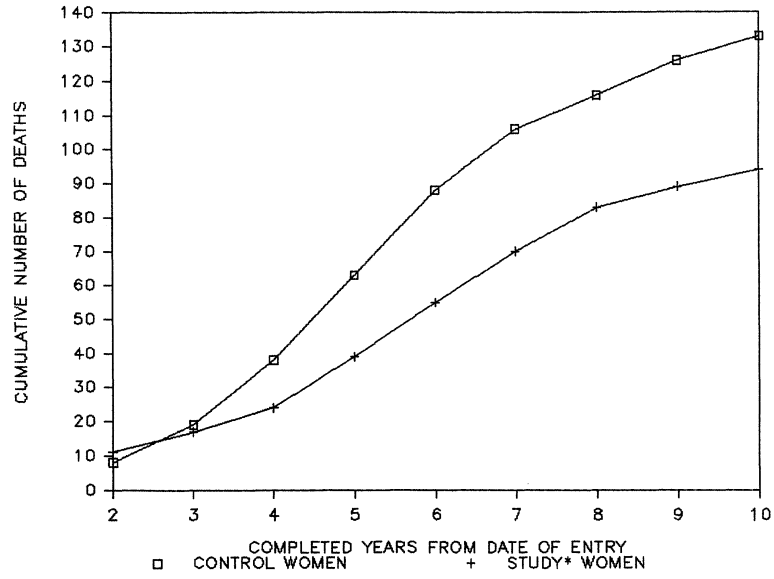
In addition to ultrasound, magnetic resonance is another imaging modality capable of defining cystic changes as such (15). It is much more expensive, takes much longer to perform and, as opposed to ultrasound, its niche in the diagnosis of breast disease is to be determined. T_2 – weighing sequences are being used initially, and T_1 – weighted sequences are subsequently obtained, if needed, through regions of concern (1). Up until the recent advent of simultaneous imaging with dedicated surface coils (44), the total amount of time needed ranged from 60–90 minutes.

Fibroadenomas, the most common solid mass encountered in breast screening, are routinely seen if greater than a centimeter (8). This compares unfavorably with ultrasound.

Recognition of malignant lesions currently is limited to configurational analysis. Signal characteristics do not presently have the gray scale range of low-dose grid-film-screen mammography (15).

Microcalcifications are not seen.

Diaphanography (transillumination) has been used as an adjunct to physical examination in the diagnosis of breast lesions since at least 1929 (7). It is without risk, may detect



* Includes women screened and women who refused screening H.I.P., breast cancer deaths by time interval from date of entry. Study and control groups cases diagnosed within 5 years after entry.
McDivitt, breast cancer

subclinical lesions, and its accuracy is much less than state of the art mammography (12). It is useless in breasts containing hemorrhage, whether iatrogenic or secondary to trauma, the involved area appears indistinguishable from a malignant lesion. Because of its innocuousness and relative low cost, it may have a place (when ultrasound is not available) complementing physical examination and mammography.

Thermography uses the infrared spectrum to image breast pathology. It is more of a prognostic tool than an imaging, diagnostic modality (23).

Muller *et al.* recently compared computed tomography (CT) and mammography. CT yielded little new information. The accuracy of mammography was proven superior in a group of 33 patients. CT may be of value in the evaluation of the patient prior to radiotherapy (33).

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For postscript see end of Volume, p. 296.

3

STAGNATION OF THE TREATMENT QUOTA FOR CERTAIN NEOPLASMS: A CRITICAL EVALUATION OF CONTEMPORARY ANTICANCER METHODS

E.H. KROKOWSKI*

Without any doubt, cancer research has produced a number of important discoveries during the last decades – nevertheless, a decisive breakthrough has not occurred. The events leading to the development of cancer have not been explained, and thus it is not yet possible through elimination of the cause of cancer, to prevent the disease in the early stages. Experimental cancer research using animals has identified many chemical carcinogens. Additional support for the general opinion that 60–90% of all cancer cases can be related to environmental causes comes from epidemiological observations. A number of correlations between the effects of carcinogens, as identified from animal experiments, and cancer frequency in exposed persons have been established. Furthermore, it was discovered that distinct differences exist in cancer localizations/rates worldwide and that the cancer rates prevalent for a particular country will gradually change, if an inhabitant removes to another country having different cancer frequencies. He and his descendents will now adapt to the cancer rates of the new country.

There are two arguments which can be brought against this hypothesis even though it is supported by epidemiological research: First, epidemiological investigations allow drawing correlations between suspected carcinogens and cancer localization, but a causality cannot always be derived from it with a reasonable degree of certainty. Second, P. Koeppe (9) shows the following: If it could be possible to attain, for example, the low lung-cancer mortality in Mexico, the low stomach cancer rate in the United States, and the low rate of prostate cancer in Japan, etc., then it should be possible to reduce cancer incidence by a factor of 8–10. This opinion has to be considered incorrect because the death statistics, standardized by age, of all cancers when considered worldwide, show an extraordinary agreement; in other words, if a certain cancer occurs only rarely in one country, then we find most likely a correspondingly higher rate of cancer of another organ, so that the sum of all cancers of the various organs remains nearly constant. This indicates that cancer prevention is not possible. An extensive investigation by Junghans and Sachs (8) concluded that prevention of cervical carcinoma does not influence the general cancer morbidity! Female patients who had the cervix removed, conized, or electro-coagulated, or had the uterus removed for preventive reasons, were just as endangered later by malignant tumors at other sites as untreated patients of the same age groups.

Thus we should remember that cancer mortality, when corrected for age and numbers, has remained almost constant since 1900. According to recent investigations, this statement holds for all countries. Further if in one country cancer of one site is particularly low or if its rate diminishes, then cancers of other sites are especially high or are increasing, so that the sum of all cancers remains relatively constant. Initially this statement seems to contradict the undisputed fact that cancer has increased greatly during the last decades. But it is essential that the age distribution of the populace be considered for a small change in the age structure can have a considerable effect on the absolute number of cancer deaths. Therefore it is important to discuss the relationship between cancer and age, namely three different associations:

Cancer magnitude. Related to all the people alive, the number of cancer deaths increases due to the increased longevity of the people.

Cancer frequency. Related to a certain number of the people alive; cancer mortality M increases within certain limits with about the fifth power of age A:

$$M = aA^5$$

Cancer danger. Related to the same age group and number of people in it, cancer mortality has remained almost constant over several decades. In the following discussion hematological and lymphatic malignant diseases are not considered, because in the context of age distribution, course of disease and spreading of disease, they differ considerably from the cancers of specific organs and they represent a separate group among the malignant diseases.

Since we have been unsuccessful thus far to prevent cancer significantly, special attention was given to recognize cancer early. This goal is in agreement with the general medical experience that the sooner a disease is diagnosed it can be cured earlier and easier. Without any doubt, this is true for cancer. For example: Pichlmayr, Buettner, and Meyer (17) reported on the results of the surgical treatment of stomach carcinoma. From the actualized survival curves of patients with stomach resection in early carcinoma, it could be seen that the 5-year survival rate came to 77.0%, analogous to those reported from Japan. Nevertheless, this information can lead to a misinterpretation, because in West Germany only about 6% of all stomach carcinoma patients are being

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operated on in the early stage! This means that for 94% of all patients the success rate cited above is irrelevant.

The average 5-year survival rate for patients with stomach carcinoma is about 10 percent which means that within a quarter of a century the percentage of patients cured has not increased appreciably. A similar result can be found in other malignant diseases so that we have to conclude that the 5-year survival rate has remained practically constant over the last 25 years (see further Krokowski) (10). This statement is also supported by the 5th report of the National Cancer Institute. The 5-year survival rate of all cancer patients has increased within the period from 1950 (plus 5 years observation period) to 1973 (plus 5 years observation period), from 39% to 41%. This means, therefore, that within almost 25 years the 5-year survival rate for all types of cancer has improved by only 2%! This result is disappointing because during the same time there was a great improvement in anesthesiology, surgery, and radiation techniques; cytostatic therapy was developed, and numerous organizational efforts were made to decrease cancer danger.*

In summary we must conclude that an improvement and expansion of therapeutic methods did not yield a definite improvement in the successful treatment of cancer. For this reason, attempts were made in the Federal Republic of Germany and other countries to improve the results through cancer prophylaxis in the form of mass screening (3). However, one regretfully has to conclude that the program did not fulfill expectations. Currently, the emphasis is on early diagnostic examinations. But cancer prophylaxis and early diagnostic examinations are not the same! Cancer prophylaxis is intended as mass screening for the early recognition of present cancer, while early recognition pertains to the individual investigation of all organs that can be reached with trustworthy means. It includes, for example, inspection of the skin (skin cancer, melanoma), investigation of the nose-throat area, mammography, investigation of the intestines, gynecological testing including colposcopy, the fingering of the testicles, x-ray and ultrasonic investigation of the kidneys, etc. Such investigations for early diagnosis of cancer must be fully supported. Investigations on early recognition however, cannot be considered as part of the widely advertised cancer prophylaxis program which was initially proposed as mass screening. These investigations on early diagnosis surpass the technical and experience-related capabilities of a family physician and require collaboration by specialists from various disciplines. To put this into practice and to make it beneficial to a broad segment of the populace requires much effort and money. But even if such a program could be realized, one would have to consider the psychological effect on those tested: it could create the idea that this comprehensive testing program would certify that they are free of cancer – an assumption which, naturally, is not true. Thus, we have to search for other means to defuse the cancer problem, which in the mind of the public has become the number one health problem. The present concept of modern cancer therapy rests on two theses, which we must regard as dogmas, because they cannot be derived scientifically and logically:

1. Abnormal tissue that cannot be "made normal" has to be removed from the organism, and

2. The nature of cancer leads to a tumor, therefore a local event, which can become a general disease only in its final phase, namely through metastases or increased cachexia. If both prerequisites, 1 and 2, are correct, then cancer should be curable in most patients, because at present, it is not that difficult in most cases to remove the tumor completely. Nevertheless, at present only about 35–40% can be cured, and about two-thirds of all cancer patients die from the disease. Thus there is a justification to improve the present-day means for removing or destroying the tumor through surgery, irradiation or cytostatic drugs, but also to question the basic prerequisites of the present concepts of therapy.

Is it really necessary to remove abnormal, that is, malignant tissue in each case as quickly as possible from the body? There are sufficient examples showing that it is better to live with the cancer than to remove the tumor from the organism through extensive surgery with subsequent damage like amputation, anus praeter naturalis, ureter transplant, impotence, etc. We refer to dormant cancer, which is known from the prostate, cervix, and mamma; most likely it is present in all organs and tissues.

Furthermore, the results of Gregl (7) show that older women having breast carcinoma will live longer without treatment than after palliative or radical therapy. This leads to the conclusion that therapy in older women with breast carcinoma should be avoided. In addition, most oncologists know of numerous observations where a tumor was present for a long time, sometimes up to a decade or more, without leading to the death of the tumor carrier, or without serious disease symptoms in the patient during the remaining life span.

The second prerequisite also has to undergo scrutiny: is cancer really a local phenomenon or must it be regarded as a general disturbance? On the one hand, there is no doubt that a tumor can be removed in the early stage, with the best chance for complete cure, without causing a general tendency towards other cancers which would make a cure impossible. On the other hand, cancer growth is not completely autonomous, as is often assumed and maintained. Thus, cancer cells in culture can show a tumor doubling time of one day, while cells of the same tumor in a host organism can have a doubling time of 60 days, showing a markedly slower growth rate. The host organism, therefore, slows down the tumor's growth. Is there an analogy to other diseases? As an example: Is pneumonia a local sickness of the lung or is it a general sickness? What would happen if during an early lobular pneumonia the diseased lung would be removed surgically? The lobular pneumonia would be removed, but to conclude that this sickness of the lung is a local event, would be presumptious. The development of pneumonia occurs because of a temporary breakdown of the defense towards pathogens. The causative factors are the pathogens themselves; in addition, it requires a (general) exposure stress ("a cold"). The pathogens as the necessary, but not necessarily sufficient prerequisite for the cause of pneumonia, act organ-specifically; disposition and exposure, however, act on the total body, so that the recognizable symptoms, like changes in the blood count, fever, tendency to perspire, loss of appetite, etc. are a definite sign of a general disorder. Similar situations are valid for other

* In addition to the papers already mentioned see further reports in: *Rheinisches Aerzteblatt; Wiener Medizinische Wochenschrift.*

diseases. When this knowledge is applied to cancer, then it becomes obvious that the question, whether cancer is a local or general disease, is inappropriate. The disposition has to be present; it is possible that every person has the precondition for forming so-called 'malignant cells'. The tendency towards the formation of cancer depends strongly on age for it increases considerably with age. The so-called 'carcinogens' (predominantly chemical substances of varying structure) have the capability for releasing the blocked oncogenes and cancer growth begins – at first unnoticed and unnoticeable, until the tumor can be diagnosed through direct or indirect symptoms, a hypothesis supported by the research of Ladik and co-workers (5, 7, 8, 10–13, 15, 18, 19). Their quantum-mechanical investigation of the electron distribution in the DNA, in the proteins, DNA-protein reciprocity, and the effect of addition of carcinogens have shown that energy band structures can appear in the DNA or the proteins. Therefore, these biopolymers have certain physical properties, as, for example, electrical conductivity. The extent of the DNA-protein reciprocity is determined by genetic control, and it depends largely on whether DNA or proteins are conductors or insulators.

It was shown recently that human tumors contained oncogenes which also appear in normal human cells. In normal cells these oncogenes are generally blocked but this blocking can be repressed through the effects of the chemical carcinogens which attach to the biopolymers. The genetic information present in the oncogene can then be transmitted to the RNA or transferred to the protein. Therefore, the information leading to cancer is normally present in each cell, but it is usually blocked or inactive. Once carcinogens enter, the blockade can be removed and cancer growth can occur. Apparently, this process depends largely on age and predisposition to cancer formation. The concept that carcinogens acting in the submolecular region could explain the observations made. It does not explain why despite all the efforts in terms of money, methods and organization, there has been no definitive change in the results of cancer therapy during the last two-and-a-half decades.

This phenomenon may find its explanation in the fact that intensive therapeutic measures can lead to a complete healing in some cases, but that in others it will provoke metastasis and thus a worsening of the disease.

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4

STAGNATION OF THE TREATMENT QUOTA FOR CERTAIN NEOPLASMS: CHANGE IN CANCER TREATMENT SUGGESTED BY THE ANALYSIS OF METASTATIC GROWTH

E.H. KROKOWSKI*

INTRODUCTION

There is much divergence in the achievements of cancer treatment. Palliative tumor therapy has had remarkable success for in many cases patients suffering from advanced cancer could be kept in comparatively good health, their chance of survival could be extended, and the painful final stage could be alleviated.

The curative results, on the other hand, are not as satisfactory. There are numerous papers presenting positive results, but a careful examination shows that these were frequently based on selected patients. Thus only patients in early stages were treated or those with acute lymphatic leukemia or Hodgkin's disease, which represent only a small percentage of all types of cancer. For a general review of curative results one should consider only statistical investigations based on comparatively large numbers of patients. To judge the success of therapy for bronchial carcinoma, postoperative statistics of 14,927 patients from 16 surgery departments in Germany were analyzed in 1976 (1). The 5-year-survival rate was 23% of the patients who underwent surgery, but due to the fact that two-thirds of all patients were beyond surgery only 7% of all patients reached that 5-year-limit. These figures do not differ significantly from those of Oeser in 1954 (19). A similar study on the statistical material after radiation therapy from 17 German hospitals again did not show any improvement compared with the results of 1954 (10). Only 2% of all patients with bronchial carcinoma reached the 5-year-limit.

In the case of stomach carcinoma, statistics from the surgical department of the University Hospital in Bonn (20) showed a 5-year-survival rate for patients in tumor stage T_1 of 47.5%; in T_2 of 43%; in T_3 of 19%, and in T_4 of 6.5%, respectively. The rates of 47.5% and 43% for the stages T_1 and T_2, respectively, demonstrate the success of early surgical treatment of stomach carcinoma; but one has to note that these early stages contributed only 6% of all surgical treated stomach carcinoma cases. Ninety-four percent of all patients with stomach carcinoma were in stages T_3 and T_4. Taking into account all stomach carcinoma patients, a 5-year-survival rate of only slightly more than 10% results. Furthermore, according to Junghans and Ott (12) during the last 50 years results for stomach carcinoma have not changed significantly.

A similar unfavorable result is encountered with bladder cancer. Improvement in radiation techniques did not result in an improvement of the statistics (15). Although the change from conventional therapy to telecobalt- or betatron irradiatic resulted in a considerable improvement in symptomatic or palliative therapy, no increase in the percentage of cures was achieved. Investigations on rectal carcinoma and other tumors complement these findings (6). A recent report of the National Cancer Institute shows a 5-year-survival rate of 39% for the period from 1950 to 1959 and of 41% for the period from 1967 to 1973. This extensive statistical study points again to the lack of decisive improvements during the last 20 years. The situation is similar for mammary carcinoma. Comparison of the results reported by Kaiser and Karrer (13) with those of Oeser (19) reveals the lack of any noticeable progress during the last 25 years, if the data are correlated as to equivalent stages and sizes of the tumors. The statistics of Benninghoff and Tsien (2) show a 44% average rate for 5-year-survival while the evaluation of our data from 1976 yields 42%, compared to the level of 38% 40 years ago. The slight improvement may be attributed to better informed patients, improved methods of diagnosis, and that more patients enter therapy at a more favorable stage of the disease. There are other investigations which differ appreciably from these 5-year-survival rates. Kaiser and Karrer (13), for instance, find 60% while Seeberg (29) reports 25%.

Is there an explanation for these appreciable deviations? Is one particular method of surgery or technique of radiation more successful than others?

The worldwide studies by Benninghoff and Tsien (2) of 25,879 female mammary carcinoma patients offer one explanation for these differences: Neither the kind nor the quality nor the length of therapy was found to be decisive for the results but only the composition of the sample of patients.

The chance of survival was found to be high whenever the number of patients at an early stage of the disease was high. There was a particularly low probability of a 5-year-survival encountered in groups with a large number of patients having cancer in an advanced stage.

Therefore a noticeable improvement in the 5-year-survival rate with tumors of the same stage has not been achieved during the last two or three decades in spite of the progress made in the techniques of anesthesia, surgery and radiation therapy. There may be essentially two reasons for this lack of improvement.

Early diagnosis of cancer will allow the initiation of

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therapy during a less serious stage of the disease. General prophylactic check-ups, however, were not as successful as anticipated since a relatively small portion of the population made use of this opportunity. Another reason was that the general prophylactic examination was often performed in inappropriate cases. Prophylactic cancer examinations should be directed toward those cancer types for which an early diagnosis and thus an early curative treatment is feasible (taking into account the present state of technically and financially practicable methods of examination). These are, especially, mammary carcinoma, gynecological carcinomas, cancer of the stomach (which is frequently encountered in certain geographical regions), and seminoma.

The second reason is connected with the proliferation of tumor cells eventually leading to metastases; this process cannot yet be controlled. In many cases in which one had presumably totally removed the primary tumor by surgery, or destroyed it by radiotherapeutical treatment, metastases have been observed after a certain time. This development then determined the fate of the patient: development of metastases and healing are inversely related, and a cure is possible despite metastases only in exceptional cases, such as the carcinoma of the thyroid gland where radioactive iodine is accumulated. Eighty to 90% of the deaths of cancer patients are due to the appearance of metastases; only 10 to 20% can be related to the immediate local effects of the primary tumor. The appearance or nonappearance of metastases must therefore be considered as crucial in determining the fate of the patient. Accordingly, we will analyze the conditions for the development of metastases.

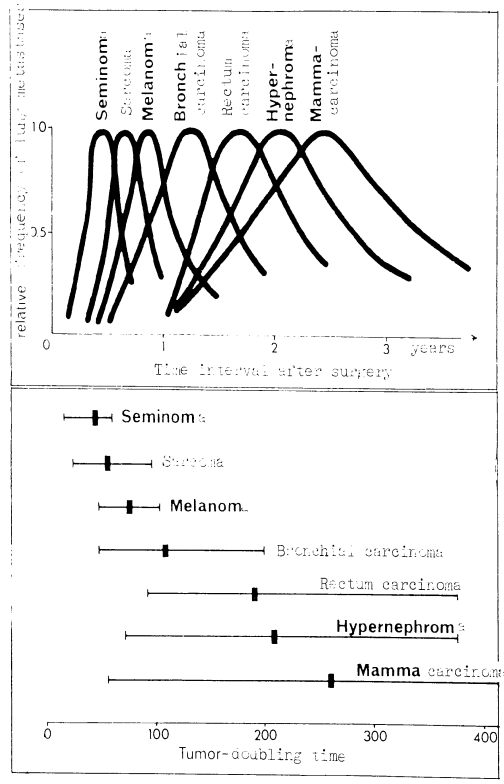

Figure 1. Top, time interval between operation and diagnosis of lung metastases for seven different types of tumors. Bottom tumor doubling time for the same types of tumor. The teratomas indicated in Figures 1, 3, 4 and 6 were testicular teratomas.

OCCURRENCE AND LOCALIZATION OF THE METASTASES

The degree of metastasis formation for different types of cancer has been determined by Denoix (4) from 1,103 autopsies. On the average 20 metastases were found per deceased patient. This figure, which varies only slightly with the proliferative capacity of the tumor, leads us to assume that all metastases originated from the primary tumor. The pattern of localizations of metastases for different tumors shows well-defined characteristics. Detailed descriptions have been made by Walther (23), Bienengräber (3), Hermann (11) and others.

TIME SEQUENCE OF FORMATION OF METASTASES

The time of the first treatment will be taken as a reference point. Since x-ray photography of the thoracic organs is the best chance for early recognition of metastasis, we concentrate here on its appearance in the lung.

For seven different kinds of malignant tumors, the time sequence of the appearances of lung metastases has been determined (Figure 1). In the lower part of Figure 1 the corresponding tumor duplication time is given for every kind of tumor. It is clear from the correlation of tumor duplication time and time sequence of metastases that the time interval between the first treatment and the diagnosis of lung metastases for the fast growing tumors (seminoma, sarcoma, melanoma) is shorter than for the slowly growing ones (rectal, bronchial, mammary carcinoma and hypernephroma). Since, however, the "fast" metastases cross the diagnostic threshold earlier than the "slow" ones, one has to conclude that both have started at the same time. But when did metastasis begin?

GROWTH OF METASTASES

The growth of lung metastases can be determined from a series of x-ray photographs of the changes in size as a function of time. An error analysis of this method has been carried out by Welin (24), Wolff (25) and by Keller and Kallert (14). To be able to extrapolate the measured growth curves of the metastases to the preclinical time with a sufficient certainty, two conditions have to be fulfilled:

1. One has to observe as long as possible the growth process in the particular case to be able to determine the individual growth curve as exactly as possible
2. One has to analyze a great many examples to be able to recognize the principal features of the growth curves.

We have investigated a total of 2,893 metastases in 568 patients who had different kinds of tumors.

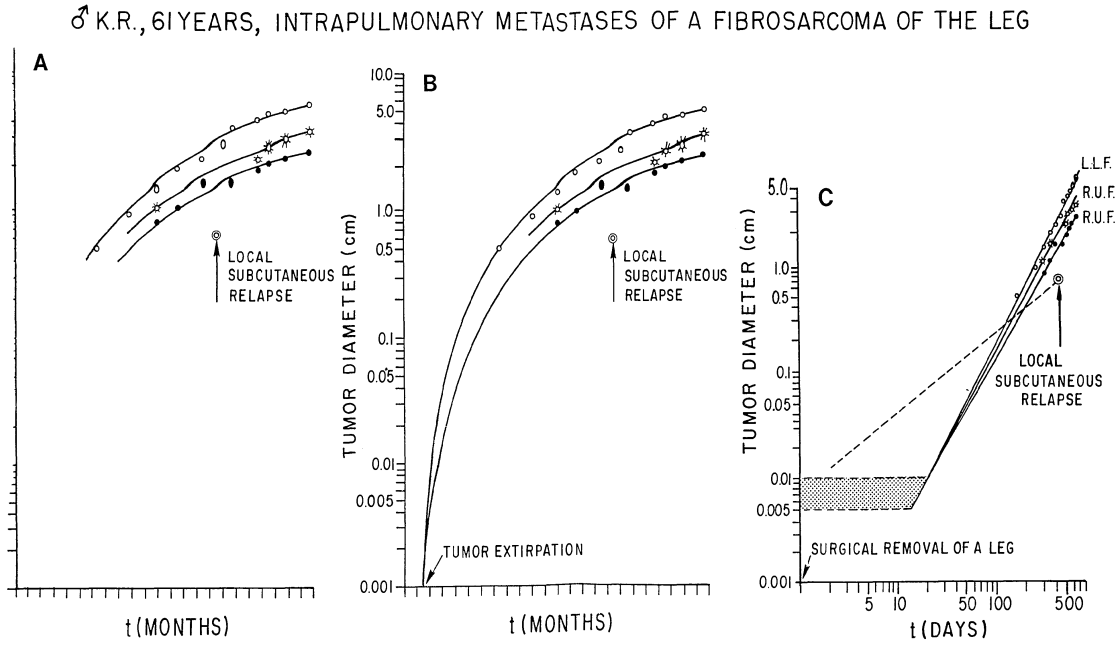

Figure 2. A, progress of intrapulmonary metastases measured from x-rays of a fibrosarcoma of the leg in a 61-year-old patient; inset A, mathematical formula; B, extrapolation of the measured curves; C, transposition of the measured extrapolated curve into a double-logarithmic matrix.

Kind of tumor	Number of patients	Number of metastases measured
Seminoma, teratoma	72	498
Sarcoma	92	736
Melanoma	59	421
Bronchial ca.	28	31
Rectal ca.	8	14
Hypernephroma	112	238
Mammary ca.	191	955

Using the example of lung metastases from a sarcoma of a 61-year-old patient, the main features of the method are shown (Figure 2). The same growth curves can be observed in implanted tumors in animal experiments.

Figure 3 summarizes the growth curves of lung metastases for one kind of tumor but for different patients, where for clarity only part of the analyzed curve is drawn.

After averaging, we tried to show for each of the seven types of cancer under investigation the typical course of metastases and to derive an equation for the growth.

The starting point for this is the experimental observation that for unperturbed growth of tumor cells there is a proportionality between the rate of growth at time t and the amount D existing at that time

$$D - \text{alpha}D$$

The solution of this differential equation gives the equation for the unperturbed growth

$$D = ae^{\text{alphat}}$$

where a is the starting amount and alpha is the proportionality factor of growth. However, if this growth takes place in a host organism the host influences the growth. On the basis of the empirical data one can conclude that this influence can be described by a logarithmic function. Since the process is not independent, the perturbation function has to be combined by multiplication with a general growth function,

$$D = ae\frac{b}{ct^2 + db + 1}$$

To be able to write a functional relationship between b, c

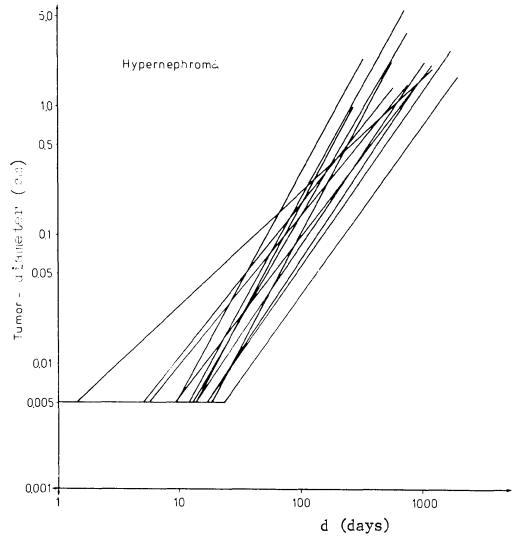

Figure 3. Growth curves of lung metastases in various patients. A, primary tumor; teratoma; B, primary tumor; mamma carcinoma.

and d, we started with the assumption that at time t = 0 the rate of growth is 0. In this way we obtain:

$$D(0) = D_k \rightarrow D_k = ae^b \approx 0{,}006\,\text{cm}$$

(experimental observation)

$$D(t_1) = D_1 \rightarrow D_1 = ae\,\frac{b}{ct_1^2 \rightarrow dt_1 + 1}$$

$$D(t_2) = D_2 \rightarrow D_2 = ae\,\frac{b}{ct_2^2 + dt_2 + 1}$$

$$D(\infty) = D_m \rightarrow D_m = a \approx 7\,\text{cm (clinical observation)}$$

$$D_k = ae^b \rightarrow \frac{D_k}{a} = e^b \rightarrow \ln\frac{D_k}{a} = b$$

$$\rightarrow \ln D_k - \ln a = b = \ln D_k - \ln D_m$$

$$D_1 = a\exp.\frac{b}{ct_1^2 + dt_1 + 1} \rightarrow \ln D_1 - \ln a$$

$$= \frac{b}{ct_1^2 + dt_1 + 1}$$

$$ct_1^2 + dt + 1 = \frac{\ln D_k - \ln D_m}{\ln D_1 - \ln D_m}, \text{ that means}$$

$$ct_1^2 + dt_1 = \frac{\ln D_k \ln D_1}{\ln D_1 - \ln D_m} \text{ and } ct_2^2 + dt_2$$

$$= \frac{\ln D_k - \ln D_2}{\ln D_2 - \ln D_m}$$

that means

$$c = \frac{\begin{vmatrix} \frac{\ln D_k - \ln D_1}{\ln D_1 - \ln D_m} & = t_1 \\ \frac{\ln D_k - \ln D_2}{\ln D_k - \ln D_m} & = t_2 \end{vmatrix}}{\begin{vmatrix} t_1^2 & t^1 \\ t_1^2 & t_2 \end{vmatrix}}$$

$$\text{and } d = \frac{\begin{vmatrix} t_1^2 & \frac{\ln D_k - \ln D_1}{\ln D_1 - \ln D_m} \\ t_2^1 & \frac{\ln D_k - \ln D_2}{\ln D_2 - \ln D_m} \end{vmatrix}}{\begin{vmatrix} t_1^2 & t_1 \\ t_2^2 & t_2 \end{vmatrix}}$$

With a, b, c, and d, we obtain:

$$(\ln D_k - \ln D_m)\begin{vmatrix} t_1^2 & t_1 \\ t_2^2 & t_2 \end{vmatrix}$$

$$D = D_m \exp$$

$$\begin{vmatrix} \frac{\ln D_k - \ln D_1}{\ln D_1 - \ln D_m} t_1 & t^2 + & t_1^2 \frac{\ln D_k - \ln D_1}{\ln D_1 - \ln D_m} t_1^2 & t \\ \frac{\ln D_k - \ln D_2}{\ln D_2 - \ln D_m} t_2 & & t_2^2 \frac{\ln D_k - \ln D_2}{\ln D_2 - \ln D_m} & \end{vmatrix} \begin{matrix} t_1^2\ t_1 \\ t_2^2\ t_2 \end{matrix}$$

This expression for perturbed growth formed the basis for further investigation of the metastatic growths of different kinds of tumors. The amount of tumor is characterized by the diameter D, for which the following values can be calculated. In addition, the scattering regions of the tumors can be determined from the data obtained with the aid of x-ray investigations. Accordingly one obtains the following metastatic growth functions for the different kinds of tumors with the additional assumption that at time t = 0 there exists a critical tumor volume with a diameter of 0.006 cm.

SEMINOMA

$$D = 7\exp\frac{-7{,}06}{0{,}000117\,t^2 - 0{,}00357\,t + 1}$$

$$D_{-s} = 7\exp\frac{-7{,}06}{0{,}00039963\,t^2 - 0{,}016283\,t + 1}$$

$$D_{+s} = 7\exp\frac{-7{,}06}{0{,}000054527\,t^2 - 0{,}00090152\,t + 1}$$

MAMMARY CARCINOMA

$$\frac{-7{,}06}{0{,}0000055\,t^2 + 0.0001\,t + 1}$$

$$\frac{-7{,}06}{0{,}00001336\,t^2 + 0{,}000537\,t + 1}$$

$$\frac{-7{,}06}{0{,}00000276\,t^2 + 0{,}0000099\,t + 1}$$

MELANOMA

$$\frac{-7{,}06}{0{,}000038369\,t^2 - 0.00087757\,t + 1}$$

$$\frac{-7{,}06}{0{,}00013205\,t^2 - 0{,}014052\,t + 1}$$

$$\frac{-7{,}06}{0{,}000018272\,t^2 + 0{,}001357\,t + 1}$$

SARCOMA

$$\frac{-7{,}06}{0{,}000078\,t^2 - 0{,}00248\,t + 1}$$

$$\frac{-7{,}06}{0{,}00018595\,t^2 - 0{,}0011368\,t + 1}$$

$$\frac{-7{,}06}{0{,}000046672\,t^2 - 0{,}0038388\,t + 1}$$

BRONCHIAL CARCINOMA

$$D = 7 \exp \frac{-7{,}06}{0{,}000021277\ t^2 - 0{,}00216\ t + 1}$$

$$D_{-s} = 7 \exp \frac{-7{,}06}{0{,}0000622\ t^2 - 0{,}012646\ t + 1}$$

$$D_{+s} = 7 \exp \frac{-7{,}06}{0{,}000012549\ t^9 - 0{,}00061817\ t + 1}$$

HYPERNEPHROID CARCINOMA

$$\frac{-7{,}06}{0{,}00000655\ t^2 + 0{,}000000859\ t + 1}$$

$$\frac{-7{,}06}{0{,}00001164\ t^2 + 0{,}0001008\ t + 1}$$

$$\frac{-7{,}06}{0{,}000004128\ t^2 + 0{,}000013567\ t + 1}$$

RECTAL CARCINOMA

$$\frac{-7{,}06}{0{,}000007709\ t^2 + 0{,}00003029\ t + 1}$$

$$\frac{-7{,}06}{0{,}0000179\ t^2 + 0{,}0000438\ t + 1}$$

$$\frac{-7{,}06}{0{,}0000043\ t^2 + 0{,}0000232\ t + 1}$$

The time interval between operation and recognition of lung metastases is depicted in Figure 4.

There exists a good correlation for the time intervals between the start of the metastases and the x-ray diagnosis of lung metastases (D = 1 cm), as calculated with the aid of the growth equation on the one hand and the measured values on the other (Figure 5).

Some further results of the growth curve analyses:

1. Metastases grow faster and essentially more smoothly than the original tumors.
2. The growth rates of metastases vary less than the growth rates of the primary tumors of different types and locations.
3. Metastases released at the same time can exhibit, within certain limits, different growth rates depending upon their place of attachment.
4. We were able to confirm that late metastases are formed by the so-called "dormant tumor cells" which grow after activation with the rate specific for the particular tumor. With the help of growth analysis we have succeeded in formally describing the appearance of late metastases and in determining the sequence of tumors of different origin in accordance with the clinical observation.

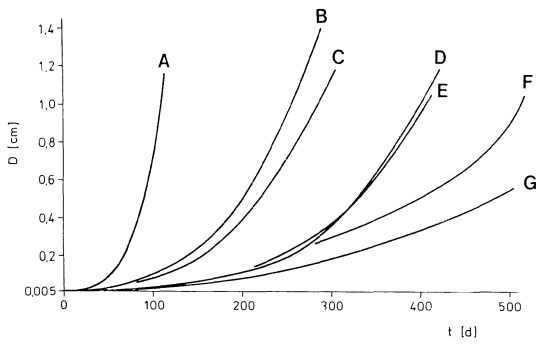

Figure 4. Relationship between the time intervals: Operation on lung metastases formation and tumor double time.

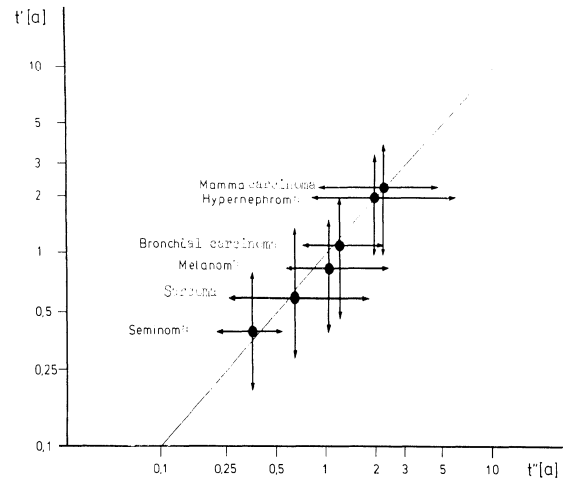

Figure 5. Growth curves of lung metastases in a 20-year-old patient with seminoma.

ORIGIN AND BEGIN OF METASTASES

If we trace the metastases growth curves back to the origin we obtain three important observations:

1. The growth curves show that metastases always originate from primary tumors.
2. Although the scattering of tumor cells happens almost continuously, metastases are usually formed in only one or a few places.
3. Portions of the growth curves (spontaneous metastases) originate before the diagnosis of cancer but other growth curves start at the date of the first treatment.

Thus we have come to the conclusions that they may have been caused through surgery (provoked metastases). Two facts found from animal experiments should be taken into account: The development of metastasis is possible only if a fixed minimal amount of tumor cells accumulates, possibly 500+ cells (26). This critical number of cells (or diameter Dn) varies with aggression and "stickiness" of the tumor cells, on the one hand, and on the other, with the resistance of the affected location and the general defense mechanisms of the patient (age, bioclimatical influences, blood viscosity, etc.). An experiment of Schmähl (21) shows very clearly the different defensive powers of various organs. If one injects tumor cells into the vein of an animal, the injection acts here, to some extent, as the primary tumor and the tumors appearing in the animal are the metastases; one hour after the injection one can recognize active tumor cells in the blood, spleen, liver, kidneys and lungs. Two hours after the injec-

tion there are live tumor cells in all organs except the spleen. A day after the injection only in the lungs are active tumor cells which develop gradually into lung metastases. In the other organs the tumor cells have been killed by local cell defense mechanisms.

If the number of tumor cells from the primary tumor, carried away in the blood and transferred to a distant location, has exceeded the critical value, then the growth of these micrometastases does not start immediately but with a delay which we call implantation time. The tumor cells try to extend their bridgehead. If they are defeated by the local tissue defense mechanisms they perish. If, however, they succeed in overcoming this local defense, further invasion occurs. The metastases start to grow and the fate of the patient is – sooner or later – determined, without the possibility of clinical recognition of the outcome at that time. The metastases may manifest themselves only after months or even years.

One must distinguish between spontaneous and provoked development of metastasis. It can be concluded that the so-called spontaneous metastasis development was also induced in phases of reduced defensive ability of the tumor carrier. It is conceivable that metastases can be provoked by surgical intervention for, metabolic changes are caused by both anesthesia and the operation itself (we could observe, for example, in an ovarial carcinoma the induction of lung metastases by a tumor-independent surgery of the gallbladder), or surgery may cause an increased detachment of tumor cells into the bloodstream. With increasing numbers of circulating tumor cells, however, the probabilities of development of metastases increase. Therefore, the probability of development of metastases increases with the size of the primary tumor and the number of detached cells. A comparison of three different primary tumors illustrates the influence of the transplantability: with a diameter of 3 cm for each species, a bronchial carcinoma has induced metastases in 90 to 98% of the cases, a mammary carcinoma in about 50%, and a hypernephroidal carcinoma in only about 2% of the cases. Since the different tumor species have very different transplantabilities, diagnosable sizes, and probabilities of metastasis development, it is understandable that the ratio of spontaneous and provoked metastases (15) varies greatly for the different tumor species (see Figure 6). This ratio was determined from the mass of the observations as follows:

Tumor species	*Number of spontaneous metastases*	*Provoked*
Hypernephroma	70%	30%
Mammary ca.	46%	54%
Bronchial ca.	40%	60%
Seminoma, sarcoma	15%	85%

As mentioned above our therapeutical measures in fighting cancer may provoke development of metastases leading thereby to failure. Thus therapy can produce a cure but it also can negatively influence the fate of the patient through the induction of metastases. In this light, the two facts which were mentioned in the beginning – the largely constant rate of cures over two to three decades and the time sequence, characteristic for each tumor species, of metastatic development after surgical intervention – find their explanation.

Over four decades animal experiments showed that surgery can favor metastatic development (5), as did the experimental investigations of Fisher and Fisher (7). An example of our assertion is provided by a patient with a sarcoma where it was observed that the first operation and each further surgery of the local recidivism led to additional lung metastases (Figure 7). With the help of X-ray process controls we could simultaneously observe different growth rates for different lung metastases. However, if the lung metastases were related to their act moment of generation, they exhibited the same growth rate and the same implantation time. The investigation of Gregl (9) which shows that old women with nontreated mammary carcinoma live longer than women of the same age who have undergone palliative or radical therapy gives additional indirect evidence.

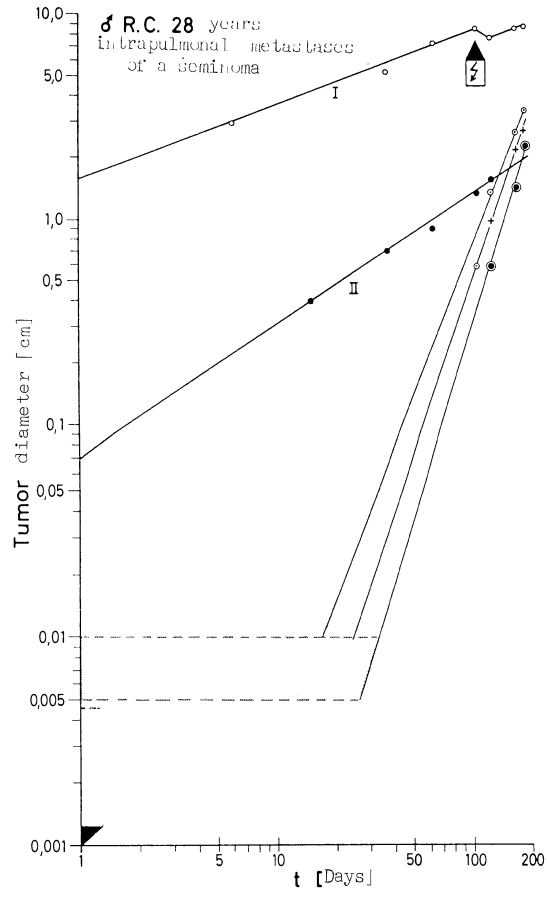

Figure 6. Proportion of induced metastases due to provoked intervention as a function of the total number of lung metastases diagnosed, corresponding to equal tumor stages.

NEED FOR A CHANGE IN THERAPY TO AVOID METASTATIC DEVELOPMENT (16)

To avoid or reduce a provoked metastatic development it is advisable, to undertake prophylactic measures before the

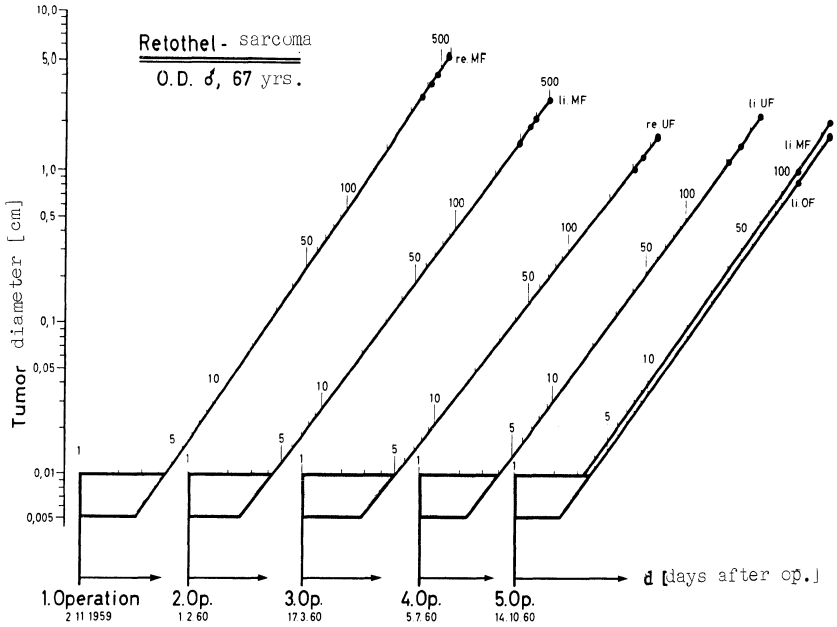

Figure 7. Observed metastastic growth in a 67-year-old patient with a retothelial sarcoma at the right shoulder joint. The operation of the prime tumor and each of its local relapses caused the foundation of a pulmonary metastasis. Related to the point of metastases release, every one of the daughter tumors has the same growth velocity and approximately the same implantation time.

surgical removal of the tumor. Proposed prophylactic measures are:

1. Immunomodulation by means of BCG or Levamisol
2. Application of anticoagulants or aggregation inhibitors (8) (Figure 8)
3. Employment of the radiative protection effect (17, 18)
4. Use of various drugs.

Various techniques whose effectiveness is being tested at this time aim to counteract provoked metastatic development. If this succeeds – in a small group of patients the radiative protection effect seems to have the desired effect – or could overcome the plateau in the rate of cancer cure. This opportunity presents itself, however, only once in the course of a cancer, namely, during the implantation phase. If, however, the metastases have reached a certain size, which might be well below the diagnostic limit, they can no longer be forced to reverse. Then they determine – often after months or years – the fate of the cancer patient. Hidden metastases, however, which are already present at the time of the diagnosis of the cancer, cannot be influenced by these measures.

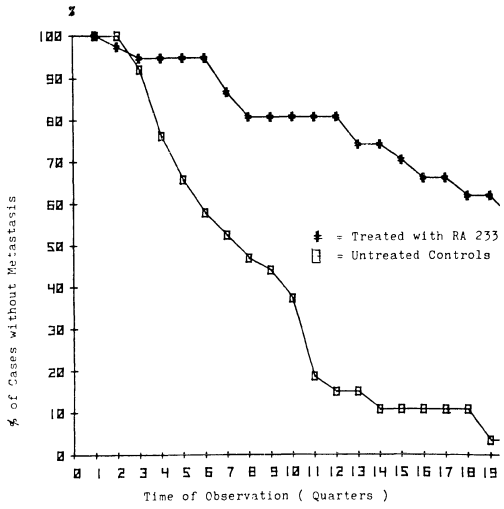

Figure 8. Long-term prophylaxis with the pyrimido-pyrimidine derivative RA 233 after operation, irradiation and the appearance of metastatic or recurrent tumor growth in 38 pairs of patients (after Gastpar, 1979, with permission of Thieme Publishers).

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5

NEOPLASM STAGING

HANS E. KAISER

INTRODUCTION

"Important to both the physician and patient, cancer staging is an agreed upon classification for defining the extent of a malignant tumor." (Henson, 1985) (50).

The development of neoplastic diseases proceeds in stages, in which general and specific aspects of abnormal growth can be considered. The development of a neoplasm from normal tissues up to the final stages of dissemination and death, or temporary or permanent cure by treatment or spontaneous regression, is a chain reaction. The general processes are based on the fact that neoplastic growth is an aberration of normal growth. The concept of staging of neoplastic diseases, in relation to normal development toward an organismic background, runs a common thread through the chapters of these volumes. In man we are able to visualize certain basic phases:

(1) Stage of toxic effects on cells and tissues by chemical compounds, physical agents, viruses, etc.
(2) Precancerous stage
(3) Stage of carcinogenesis which, may be chemically, physically, or otherwise induced
(4) Stage of primary tumor
(5) Stage of secondary tumor, or of dissemination and death or
(6) Temporary or permanent cure, or spontaneous regression.

These six steps vary in the development of the different neoplasms in a single species, such as man, and besides this of the same neoplasm in different species. In the first case, these variations in one species are examined in the intraspecies comparison and in the second, for several species, in the interspecies comparison.

We know that the different neoplasms in our species vary according to their histogenesis, topographical growth pattern, speed of growth, metastatic aggressiveness and reaction to treatment, to name only a few points. Neoplasms of different histogenesis must be considered different diseases, just as infectious diseases caused by different agents. We may learn more if we consider the various specific tumors against the background of general neoplastic development in our own species, and in others. The present chapter deals only with two problems: (1) the practical aspects of cancer staging – a brief review; (2) selected, recent cases of neoplastic staging.

1. THE PRACTICAL ASPECTS OF CANCER STAGING

It has been shown conclusively that neoplastic development is a multistage process. The staging of neoplastic diseases enables the physician to select appropriate treatment methods which vary among conditions which have developed differently. The staging of neoplasms is therefore of great practical significance, especially for prognosis. For example, a cancer *in situ* must be treated differently from the same type of cancer which already exhibits disseminated growth. It is possible to distinguish between stages of neoplastic growth and related therapeutic considerations dealing with the question of treatment. Oncogenes, chemotherapy, adjuvant therapy, the elimination of micrometastases and residual neoplastic cells endangering remission are important questions. Recent findings will be dealt with in the second part of this chapter. These facts can influence the selection of appropriate individualized cancer therapy as outlined in chapter 1 and other chapters.

The description of staging in the neoplastic process presented in the *Manual for Staging of Cancer* by the American Joint Committee on Cancer remains of basic, practical value (13). Another improved staging system which has been developed is that adopted by the Union Internationale Contre la Cancer – International Union Against Cancer – (UICC). The differences in the evaluation of various neoplastic diseases are briefly outlined below.

For practical purposes three main types can be distinguished:

1. Primary tumor
2. Regional spread, or regression
3. Distant spread

ad 1) Primary tumor: Variation in the spreading of primary tumors is given mainly by histologic type, grade of differentiation, topographic location and size of the tumor.
ad 2) Regional spread or regression: The question of direct spread, regional metastasis, topography of organ peculiarities, and vessel supply are important.
ad 3) Distant spread: Exhibits a characteristic relationship between seeding, primary, or secondary neoplasms, and the resulting distant spread to particular organs.

Each neoplasm is a disease in its own right and, therefore, will exhibit specific staging as shown above. As cancer patients survive longer, complications and new follow-up

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stages may increase in the years to come. Another example is the interaction of AIDS and Kaposi's sarcoma. It is noteworthy that AIDS exhibits a development by stages, just as do neoplastic diseases (66).

2. THE VALUE OF STAGING FOR PROGRESSION AND SURVIVAL

Staging permits the marking of segments of neoplastic progression in human tumors. It is therefore an important diagnostic tool. From the stages which have been reached, important clues to the appropriate treatment, or survival of the patient, can be gained. To do this, it is necessary to understand the characteristic development of a particular type of neoplasm. The practical aspects were summarized by the UICC and AJCC. The metastatic potential of a tumor is dependent upon genetic facts and host influence. A local tumor needs different treatment than metastases. In the latter heterogeneity occurs among different cell clones; possibly weakening the reliability of tumor staging. Rat experiments with various chemotherapeutica by Poupon and coworkers (101) showed highly metastatic cloned cell lines, such as subline 6, which were strongly stimulated to proliferate by EGF. They expressed fibronectin, actively degraded the extracellular matrix, rapidly attached to endothelial vascular cells, and resisted natural killer lymphocytes. Inversely, weakly metastatic lines, such as subline 8, were preferentially stimulated by FGF and EDGF, poorly expressed fibronectin, did not degrade the extracellular matrix, attached slowly to vascular cells, and were killed by NK lymphocytes. In malignant Müllerian tumors of the uterus no difference existed in the pattern of recurrence or survival among homologous, heterologous or undifferentiated sarcomatous elements. But 5-year survival of 52% in tumors confined to the uterus dropped to 28% in the case of extension outside of the uterus (118). In this chapter, some pertinent aspects for the understanding of the staging process on a biological basis are offered.

3. VARIABLE STAGES OF DIFFERENT SPECIES

Histologically in various species similar neoplasms are also found in which the staging varies. This is already the case in typical mammalian neoplasms, such as breast cancer, as between man and mouse, mentioned earlier, but this variation increases as taxonomic diversification progresses due to topographic, histologic, metabolic, immunologic, and other changes.

4. VARIABLE STAGES OF DIFFERENT HUMAN NEOPLASMS

In human neoplasms, the progressive pattern and staging are tumor-specific. Each staging pattern of a particular tumor depends not on one but a number of factors which may have a more or less important effect on its development. Malignant melanoma of skin behaves differently than malignant melanoma of the biliary ducts and here, in these examples, the topographic is the distinguishing factor. Other skin neoplasms, such as the basal cell carcinoma, if compared to the above-mentioned melanoma, show that metastatic spreading almost never occurs, whereas metastasis is more pronounced in the squamous cell cancer of the skin.

5. VARIATION IN AGE-AND SEX-DEPENDENT PROGRESSION

Many histologic types of neoplasms in man have a peak occurrence in a certain age bracket. Not only does the number of occurrences vary in certain ages but also frequency and stage progression of the same tumor in different age groups. Thus, a prognosis may be poor in one age group and satisfactory in another. Cancers of the colon and rectum are infrequent in children and young adults, but with a poor prognosis, whereas the peak incidence is in middle and later life, especially in the sixth decade; they occur twice as often in males than in females. The two most important histologic types of bronchial carcinoma, squamous cell carcinoma and oat cell carcinoma, show a pronounced male preponderance but this trend has changed somewhat toward females in recent years. To a certain degree, age distribution is clouded by the effects of life habits especially in middle-aged adults. Rarer cases appear at a younger age.

6. SELECTED CASES OF STAGING IN RECENT YEARS

In the last decade, important improvements in the treatment of childhood malignancies, with increased survival rates, were accomplished due to a treatment strategy in consonance with the characteristics of different tumor types, in which cases the accurate histological evaluation was of great importance. This is particularly true, because childhood tumors are often less well differentiated and may mimick other small cell tumors (126). Surgery and chemotherapy have led to excellent survival rates in children with genitourinary malignancy (65). Against the normal life span of a healthy child, the 90% overall survival in the case of Wilms' tumor is not a normal life expectancy. Computed tomography resulted in a remarkable improvement in the staging of certain neoplasms, but did not do so in others. New evaluations are necessary (56). Neoplastic prognosis needs those which were both qualitative and quantitative (9). A combination of diagnostic and treatment modalities reduced local recurrence and extended patient survival in sarcomas of the extremities (32). New diagnostic modalities may make complete surgical staging unnecessary (28). In its application, the oxygen multistep therapy reflects the staging of neoplastic growth (128).

Stratified squamous epithelium

Recurrent or metastatic squamous cell carcinoma of the head and neck behaves differently, depending upon the fact of localized or systemic recurrence (5). Radical neck dissection when modified, adapts surgery to the stage of disease with reference to lymph node involvement. In greater node involvement, radiotherapy is recommended as well as in the

case of extracapsular spread (88). Tumor size and location of tongue cancer is correctly assessed by ultrasound sonography. Limitations of the method lie in the nonvisualization of the epiglottis, of the retropharyngeal space, and bone infiltration (37). In regard to cervix carcinoma stage I and II 55% of patients with positive pelvic nodes died after various therapies compared to 9% of the patients with negative nodes (3).

Stratified squamous/simple columnar epithelium

Patients with gastric cancer and with uterine cervical cancers showed a wider spreading of macrophages in the early stages of disease, decreasing in the advanced stage. Cytostatic activity of the macrophages was increased in stage I of patients with gastric cancers, and in stage II of patients with uterine cervical cancer. There existed a significant relationship between rate of spreading and cytostatic activities (81).

Computed tomography of chest and abdomen is helpful for the preoperative staging of esophageal and gastric carcinoma (44). For staging of the epidermoid cancer of the anus, see Greenall and co-workers (41). Treatment of carcinoma of the thoracic segment of the esophagus covers three stages: abdominal, radiation and surgical (79). Staging of the malignancy of cervical carcinoma reflects the tumor biology inadequately; cell-cycle cytogenetic and malignancy-grading criteria are important for improvement and upgrading (2). Patients affected with stage I and II cervix carcinoma treated with radiotherapy or radiation-surgery had a follow-up of 6.5 years, with a minimum of 3 years; survival results in both treatment modalities did not differ substantially (68). Squamous cell carcinoma stage IB and IIA showed with postoperative irradiation later relapse and fewer distant metastases but no improvement in survival (51). 57Co-bleomycin scintigraphy is valuable in the staging of carcinoma of the cervix uteri (96). The patients with cancers of the uterine cervix (stage IB) with smaller tumors had a more favorable prognosis. The absolute and determinate 5-year survival rates were 80% and 82%, while the absolute and determinate survival rates in the large, fungating tumor replacing the entire cervix were 56% and 60%, respectively (93). The combination of *cis*-dichlorodiammine platinum, stage II, and radiotherapy offers promising results in the improvement of the local-regional control of advanced cervical carcinoma (22). Of 67 patients with cervical carcinoma, stage IV, treated with radiotherapy, two patients with mobile tumors extending to the bladder died from distant metastases; two patients with regional lymph nodes died within 3 to 4 years; all other patients with distant metastases survived less than 2 years (59).

Simple cuboidal/columnar epithelium

Natural life expectancy of the host, malignant neoplastic potential, extent of the neoplasm, and its response to treatment are important aspects of the staging of prostatic cancer (136). Magnetic resonance imaging (multiple sequences, two orthogonal planes of imaging) is the most accurate diagnostic modality for the local staging of carcinoma of the prostate (54). For the detection of metastatic spread from prostatic carcinoma, bone scintigraphy is most useful, but lymphography should be considered for the demonstration of nodal metastases in 18% of patients (47). Carcinoma of the prostate gland is the third leading cause of death by cancer in American man; adequate treatment depends on the clinical stage of this disease (119). For treatment it is decisive if the disease is confined to the prostate gland or if it extends beyond its capsule (94). Bone metastases and changes in those are critical signs of staging (116). The most frequently used method in staging prostatic carcinoma is lymphography, which shows false positive rates varying up to 58% and false negative rates from 11 to 66% (21, see also 75). Treatment options of D-0, D-1, and D-2 are essential for management of newly diagnosed metastatic carcinoma of the prostate (33); the algorithmic approach, for the staging of renal masses (141). Tumor nephrectomy is the only cure for hypernephroma (103). The survival of patients with cervical cancer treated with postoperative percutaneous radiotherapy shows no evidence of improvement (16).

Simple columnar epithelium

Exact staging of early gastric cancer, recurrence and metastasis together with adjuvant therapies enables a more positive management of this disease which had until recently a very poor prognosis (31, 60, 129). Surgical assessment of staging of gastric carcinoma was correct for tumors in 60% when depth of invasion was assessed; for nodes in 61%; for liver metastases in 92%, but for all aspects in only 21%. Staging errors did not jeopardize conventional surgical management (78). Ultrasound could be very useful in screening patients with gastric cancer for peritoneal dissemination, liver metastases, and lymph node metastases (140). CT is useful for the tomographic staging of gastrointestinal malignancies (104). Staging of local invasion in rectal cancer with endoluminal ultrasound beyond the muscularis propria is predictive with a sensitivity of 97%, specificity of 92% and predictive value of 97% (14).

In endometriosis, the adoption of a satisfactory staging system is the only way to obtain valid comparison among homogeneous groups of patients (20). The receptor status of primary endometrial carcinomas (stages I and II) gives important information in regard to tumor behavior (24).

Luciano and Pitkin (76) list the treatment variations of endometriosis depending on the patients, conditions, in the following groups: young patients with symptoms who wish to delay childbearing; infertile patients with mild endometriosis; infertile patients with moderate or greater disease; patients who remain infertile after conservative surgical treatment; patients who are not desirous of further childbearing; patients with endometriosis involving the organs outside the pelvis. For endometrial carcinoma see Silverberg (115).

Patients with endometrial carcinoma, compared for clinical and surgical staging exhibited a shift to the stage as follows: stage I, 30.4%; stage II, 62.5%; stage III, 60%; and stage IV, 0% (23). The 5-year survival of all stage III patients with carcinoma of the endometrium was 35%; clinical stage III showed a 5-year survival of 8% (40). Stage III adenocarcinoma of the endometrium displayed the asso-

ciation of the tumor grade with increasing pathologic stage, but not the capacity for control of the disease in the pelvis. During therapy higher grade lesions show failure more often at distant sites (42).

Papillary Wilms' tumor is more aggressive than generally believed (63). Anaplastic or sarcomatous histology and involvement of the lymph node are the two most important predictors for metastasis and general relapse in Wilms' tumor patients. A tumor thrombus in the renal vein or internal vena cava is another even more ominous symptom (17).

Pseudostratified columnar epithelium

Computed tomography is controversial in the diagnosis of pulmonary nodules (142); long-term absence of expansion and patterns of calcification are the deciding radiologic criteria (30). Surgical staging methods have against indirect staging techniques the advantage of superior specificity (34). The level of such tumor markers in lung cancer as CEA, hormone peptides and some neurogenic enzymes; in small cell carcinoma (calcitonin, ACTH, ADH, CEA, neurophysin, oxytocin, beta-endorphin, neuron-specific enolase, and CK BB is related to the stage of disease; elevated pretreatment levels decrease with tumor regression. The measurement of CSF and plasma concentrations of ADH, calcitonin, CK BB, bombesin, and neuron-specific enolase may contribute to the diagnosis of CNS metastases, including meningeal carcinomatosis (45). Small cell carcinoma of the lung showed in stage 1, 32% of three year survival, in stage 2, 25%, and in stage 3, 14% (77). A review of the treatment of small cell lung cancer from 1973 to 1983 was given by Morstyn and co-workers (86); see also (87). In squamous cell carcinoma of larynx and hypopharynx no correlation of natural killer cells was found in the extent of the disease according to TNM classification or staging, no change of the lysis produced by natural killer cells, nor alterations with the age process were seen (139).

The extent of metastatic spread is the most important factor in the estimation of survival in patients with lung neoplasms. This is the reason for efforts to develop accurate staging tests for pulmonary tumors, primary and metastatic with special emphasis on the determination of tumor development in the hilar region, and on mediastinal spread. Computed tomography and nuclear medicine instrumentation have made remarkable progress in this direction (49, 131). Additional areas of recent research deal with warfarin, monoclonal tumor antibodies, and chemotherapeutic and biologic response modifiers (95).

In cases of poorly differentiated adenocarcinoma of the lung of which the primary tumor produces both, alpha-fetoprotein and human chorionic gonadotropin. After surgery of the primary tumor, the primary serum level 1039 ng/ml alpha-fetoprotein decreased to the normal range 18 weeks after surgery. The preoperative serum HCG level of 11 mIU/ml, which temporarily decreased to the normal range after operation, soon increased thereafter. The serum HCG level decreased, however, to the normal range after postoperative mediastinal radiation therapy. During relapse, only the serum HCG level increased gradually to 26,000 mIU/ml 7 weeks before his death. The lung cancer was classified histologically as poorly differentiated adenocarcinoma. Immunohistochemically, AFP was detected in the mononuclear tumor cells of the primary tumor in the lung, and HCG was found in the giant cells of the subcarinal metastatic lymph node. The concanavalin A non-reactive fraction rate for AFP was 81.3%, and appeared to differ from those of hepatocellular carcinoma and yolk sac tumor (85). The state of the art in treatment of laryngeal cancer in the RSFSR, comprises complex diagnostic procedures, function saving endoprosthetic procedures, improved modifications of horizontal resection, lower larynx resection, and the great effectiveness of tracheo-esophageal shunting as a postlaryngectomy voice rehabilitation device (91).

Transitional epithelium

The tumor DNA index, as obtained by flow cytometry of nuclei retrieved from deparaffined sections may be especially useful to predict tumor recurrences and perhaps also tumor invasion of superficial bladder cancer. The subjectivity involved through light microscopic evaluation may be reduced (Weinstein, (132); see also Chapter 54). As in prostate cancer, computed tomography can be useful in the staging of bladder cancer (7). Multiple cell markers in bladder cancer have been of value in assessing the prognosis of this heterogeneous tumor (61). The merit of preoperative irradiation for bladder cancer (136), radical surgery for carcinoma of the deep male urethra (35a), and methods for carcinoma of the urethra in women (108) illustrate the specific character of the different diseases mentioned deriving from transitional cell epithelium as entities in their own right, which require a specific staging. The degree of local spreading of bladder cancer is an important part of the staging of the disease (82).

Mammary glands

Breast self-examination is an inexpensive, simple method, which may detect early stages of neoplastic development; the gaining of time for efficacious treatment should not be ignored. The impact on breast cancer mortality is difficult to assess (80). Knowledge of the presence or absence of internal mammary node metastases is of great prognostic importance. Biopsies should be carried out as long as invasive methods are possible (127). Determination of growth fractions by the monoclonal antibody Ki-67 may be useful for the routine evaluation of grading of mammary cancer (71).

Carcinoma of the breast metastasizes to all organs. Early treated breast cancer, stages I, II and III, may be followed by recurrence which can spread locally, regional or distant or exhibit a combined pattern, Table 1 ((70); see also Chapter 8, Volume VI). The pathologic information to stage breast cancer includes tumor size, histologic type, histologic grade, and presence or absence of metastases to the lymph nodes and distant locations (58). Early stage breast cancer requires multidisciplinary cooperation (12). More and more usage of conservative surgery for early (stages I and II) breast cancer has developed in the last decade and combined with radiation has achieved comparable results with 10-year-survival. Recurrence could be kept at least regional. This type of treatment is major progress (29). Sixty-two cases of breast carcinoma developing inside a fibroadenoma

of the breast are known, with an age peak of 42.4 years. All of these combined tumors developed in women (98).

Pure mucinous carcinomas of the breast should be distinguished from mixed carcinomas, especially with regard to the lymph node status and progression (106). Routine screening after mastectomy is valuable in the detection of new stages of the disease, such as recurrences, but it exerts minimal influence on survival (124). A large number of breast cancer patients are encountered in stage I lesions; this indicates that the great majority of patients who are free of disease, 15 years after aggressive primary therapy remain so thereafter (125).

Canine mammary gland tumors

Such tumors are described by Ferguson (36); see Chapter 20, Volume V.

Liver

A new staging scheme for liver cancer, especially encapsulated hepatocellular cancer, has been described by Okuda (90). This tumor is increasingly noted and is most common in Japan, but nearly non-existent in the West. Ultrasound permits accurate measurement of the speed of tumor growth.

Testis

Great progress in the last decade in testicular cancer is a consequence of the wide availability of tumor markers for the assessment of tumor stage; CT scanning for topographic location; and effective chemotherapy, with appreciation of side effects (35).

Staging of testicular tumors is basic for their treatment. From germ cell tumors of this organ, long-term survival of all nonseminomas can be expected in 85%, and in 95% in all seminomas with adequate treatment (27). Eighty to 90% accuracy in clinical examination by computed tomography and radioimmunoassays can be achieved. Ultrasonography may also be helpful (48, 117). Owing to the detailed observability by tumor markers and related diagnostic methods, such as roentgenography, orchidectomy alone, in stage I nonseminomatous testicular germ cell tumors, is sufficient (39). CT is of particular value for defining the exact extent and mass of metastatic disease, prior to and following chemotherapy and radiotherapy; also prior to surgical excision of residual disease following chemotherapy, and for investigating potential sites of disease relapse. For patients with teratoma, CT of thorax and abdomen, with bipedal lymphography for those with normal CT scan, is recommended. For seminoma patients, lymphography and abdominal CT is advised. If either is normal, thoracic CT scan is imperative (121). Vascular invasion is significant in staging and selection of therapy for early nonseminomatous germ cell tumors of the testis (53). Of testicular yolk sac carcinomas in infants (56 patients in Lima, Peru from 1952–1980), 51.8% of clinical stage I survived 3 years without evidence of disease; 14.3% of stage II; none survived at stage IV. It is to be expected that young patients who survive 3 years after diagnosis and treatment will not suffer a recurrence of the disease (102). Of 1124 childhood testicular tumors, 1062 adult cases showed that 29% of childhood testicular tumors are nongerminal, compared to 8% in adults; 49% are yolk sac tumors. The age distribution depends on the tumor histology. Only 9% of metastases occur in children compared to 61% in adults (133). The correlation between pT stage and rate of progression in stage I nonseminomatous testicular tumor appeared significant (95 cases; alpha = 0.01) (134).

Ovary

The stage of ovarian neoplasms must be determined very accurately, using new approaches based on a more difficult separation of tumor types and their connection with the whole body, such as the investigation of estrogen and progesterone receptors, chemosensitivity, and other questions of tumor tissue in its relation to the metabolism of the patient (97). Remarkable progress was made in the understanding and treatment of stage I and stage II ovarian carcinoma in the last decade. Debulking surgery and platinum-containing combination chemotherapy played an important role in the increase of the survival rate from 3 to 5 years (99). Ovarian carcinoma is the only female genital malignancy surgically staged (19). In stages IIb, III and IV of ovarian cancer, chemotherapy is recommended; in stages I and IIa, the use of prophylactic chemotherapy must be evaluated individually (10). About 80% of the ovarian carcinomas have already spread outside the small pelvis and are diagnosed as FIGO-stage III or IV. The stage of the disease is the first parameter for surgery and especially important in younger women who wish to have children despite the disease (122). Such an evaluation can be provided by computed tomography used for staging of ovarian carcinoma (6). In radiation therapy, after careful patient selection, type of radiation, treatment volume and radiation dose, biological parameters, and additional cytostatic agents, depending on the tumor stage are of importance (67). Treatment with single cytostatic agents is not as rewarding (109). In stages III or IV, chemotherapy is the first choice, using alkylating agents, hexamethylmelamine, adriamycin, and *cis*-platinum, which may improve the still poor prognosis (83). Even though fully 100% of women with gestational trophoblastic tumors have a complete remission, debates of the factors playing a role in the progression of a hydatidiform mole via an invasive mole to choriocarcinoma are not completed (113).

Reticular connective tissue

Chronic lymphocytic leukemia is generally a stable disease over months to years, but a transformation of clinical and biological characteristics can occur (38, 105). Rapid responders to therapy for myelomatosis take a much worse course than slow responders (46).

Staging of Hodgkin's disease must be evaluated together with the risk factors (55); see also (100). Treatment of early stage Hodgkin's disease is highly successful but unwanted side effects of radiotherapy and chemotherapy including

infertility, infection and second primary tumors persist (111). Hodgkin's disease, first described in 1832, starts in a single lymph node, expands to other lymph nodes and spreads hematogenically into the parenchymas of viscera. It is not known where the Hodgkin's cells originate; specifically, cellular immunity is affected and the treatment is undertaken according to the stage outlined in the Ann Arbor Conference (130). Conservative therapy of stage IA and IIA of bulky mediastinal Hodgkin's disease in form of a combined modality treatment is suggested due to the failure of radiation therapy alone. Computed tomography is significant in this therapeutic switch and it was suggested to subdivide stage IIIA into substages IIIA1 and IIIA2 (110). Newer approaches of treatment of advanced Hodgkin's disease include combination chemotherapy plus radiotherapy, alternating cycles of two noncross resistant chemotherapy regimens, and hybrid regimens, which combine agents from two different chemotherapy regimens in one cycle. If relapse occurs after combination chemotherapy, the prognosis is poor. Survival cure rates may be extended and toxicity decreased with the new regimens (112). The gonadal function may be impaired by these types of treatment (120).

The Kiel classification was investigated in 1,127 patients with non-Hodgkin's lymphomas. Those with low grade disease had a survival rate of 69.4% and those with high grade malignancy, 30.2%. The histopathology of low grade malignant disease differed from that of high grade malignant disease. The frequency of stages I and II varied between centroblastic-centrocytic lymphoma with 21%, centrocytic lymphoma with 11%, and LP immunocytoma, with 2.5%. Complete, stable remission in stage III of centroblastic-centrocytic lymphoma is due to a prolonged restriction of the lymphoma to the lymphatic system (18). The proper therapy of non-Hodgkin's lymphomas depends on histopathology and staging (43). The childhood non-Hodgkin's lymphomas comprise histologically, immunologically and clinically a heterogeneous group of the diseases (72). Immunomodulation with chemotherapy may be rewarding for treatment of dogs because the animals are immunosuppressed (62). Advances made in therapeutic options resulted of careful clinical staging with the best roentgenographic techniques available for malignant lymphoma. Staging laparatomy may become obsolete due to new techniques, such as magnetic resonance imaging (137). Variable reactions to neoplastic treatment occur in different tumor stages, as in the case of myeloma. In stage I, no superiority of combination treatment was seen in any study, whereas in stage III early mortality is reduced from 45% to 10%. No significant differences in various combinations of alkylators, vinca alkaloids, nitrosoureas, anthracyclines, epipodophyllotoxins, procarbazine and/or steroids were observed (92). The clinical staging system of multiple myeloma is supplemented by the infiltration volume in the biopsy material (11).

Melanogenous system

Stage I thin melanomas have a good survival rate; level III lesions (1–4 mm thick) show 20% of nodal metastases (prophylactic lymph node excision may be beneficial); and level IV and V melanomas with lymph node metastases require dissection of the latter (57).

The staging of cutaneous malignant melanoma which should be individualized for the patient depends on the thickness of the lesion (1). The treatment of choice is surgery; the question of prophylactic lymphadenectomy in stage I melanoma is controversial but indicated for treatment of stage II (84). The matter of risk for recurrence and metastasis is still unresolved (64). For locally advanced melanoma hyperthermic perfusion of the extremity is a useful palliative treatment to prevent amputation (26).

Transverse striated musculature

A pretreatment staging procedure in childhood rhabdomyosarcoma is imperative. Factors of importance include the local invasiveness of the primary neoplasm, tumor size, clinical status of regional nodes, clinical or radiologic evidence of distant metastases, and histologic appearance. The staging value of regional nodes is considered questionable (25, 69).

Neurons of the CNS

Sonography, nuclear scintigraphy, computed tomography are newer diagnostic tools for neuroblastoma staging; also, magnetic resonance imaging and spectroscopy are important (15). Resection of retroperitoneal primary neuroblastoma is only worthwhile if the residual tumor responds to postoperative chemotherapy (89). Primary brain tumors of children are the second most common form of cancer in this age group (4). One of the most frustrating and difficult childhood tumors for successful treatment is the neuroblastoma (74).

Peripheral glia

Postoperative radiotherapy, together with surgery, is superior to surgery alone in the prolongation of therapy-free survival of patients with grade IV gliomas (52).

In animals: The progressive stages of liver cancer in the rat model was investigated by Scherer (114) (see Chapter 16, Volume II). Radiation therapy and hyperthermia and multimodality chemotherapy increase in importance for the treatment of late stage soft tissue sarcomas in the dog. Surgery is still the first choice but these tumors abovementioned are often inoperable or not totally removed (107). Lymphoma occurs nearly everywhere in cats whether infected or not with feline lymphoma virus (73).

As in man, dogs with mast cell tumors and lymphosarcomas which are expected to metastasize should not be treated by radiotherapy alone (123). Canine thymoma, a rare neoplasm in dogs, occurs most often in German shepherd dogs and depends for accurate prognosis and therapy on the staging of the disease (8).

7. SUMMARY AND CONCLUSION

The selected cases in section 6 of this chapter indicate clearly that the stages of the different histologic types of neoplastic diseases are not merely an artificial modality but express the

subdivisions of neoplastic progression. These subdivisions or particular stages are different in the various tumors or even in their subtypes. High malignancy, for example, in a lymphoma is reflected in a different staging and survival. The histology of the tumors is the basic parameter for the expectation of the stages to develop. More detailed knowledge of the particular stage expression in the histologic tumor types, together with the biological background, will lead not only to a more clearly defined prognosis, but especially to better therapy which must be tailored individually and, survival. The staging of neoplastic progression is therefore of utmost practical importance for the patient.

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6

FROM MARKER BIOPERIODICITIES, OVER MARKER RHYTHMS, TOWARD HUMAN CANCER CHRONOTHERAPY

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1. INTRODUCTION

Extensive animal experimentation reveals that many undesired effects of a variety of stimuli, including drugs, can be reduced or eliminated by the appropriate timing of the stimulus. This time-dependent response is not an effect of an unqualified time of day, as such. It constitutes a feature of organismic time structure, synchronized by environmental cycles, the synchronizers, and persists in the absence of known synchronizers, such as the social routine for human beings or the regimen of 12-hourly light and darkness in the experimental animal laboratory (11–13, 22, 33, 44). Desired drug effects are also time-dependent, as demonstrated by a human acrophase-response curve to methylprednisolone (16). Chronopharmacology and chronotherapy (14, 17, 32, 42, 43, 47, 49), developed on the basis of the foregoing evidence of the merits of timing, have demonstrated (24, 34) and confirmed (45) value in experimental cancer treatment, chemotherapy in particular. Furthermore, in a double-blind test of clinical chronoradiotherapy, carried out with a marker bioperiodicity, a gain of 2 from the timing of treatment at the circadian peak of tumor temperature was recorded (6, 26).

For practical reasons, however, and for both diagnostic and therapeutic aims, most chemotherapists, radiotherapists and those in nuclear medicine continue to regard the body as a constant; thereby, often unwittingly, they swell the ranks of those who draw a curtain of ignorance over the physiologic range of variability by subscribing to a temporally unqualified concept of homeostasis. This clinical status quo prevails while, from the viewpoint of basic science, chronobiologic teamwork has already lifted this curtain of ignorance here (26) and there (4). The next task on hand is to render the wealth of basic information clinically useful (15). This task, in health science, revolves, first, around prevention and, only in the face of occult or overt disease, around screening, diagnosis, prognosis and therapy. In all tasks of these areas, chronobiologic monitoring and data analysis are desirable or indispensable.

2. DEFINITIONS

An approach to physiologic change is defined as *macroscopic* when it is based solely upon: 1) the inspection of original data or of averages and dispersion indices plotted as a function of time, and 2) analyses that do not provide inferential statistical point and interval estimates of rhythm characteristics and/or other information as to quantitative time structure.

A *microscopic* approach, on the contrary, objectively resolves quantitative temporal characteristics in biologic data, e.g., by testing the fit of mathematical models to time series, by deriving point and interval estimates for the parameters of fitted models and/or by specifying other quantitative information on time structures.

A *bioperiodicity* is defined as a recurrent physiologic change occurring in animate or inanimate nature, described without inferential statistical considerations and parameter estimation (only macroscopically) (25).

A *rhythm* is an algorithmically formulated periodic component of biologic time series, demonstrated by inferential statistical means, preferably with objectively quantified characteristics (i.e., frequency, acrophase (ϕ), amplitude, midline estimating statistic of rhythm or MESOR, and/or waveform) (25).

A marker rhythm (MR) (or marker bioperiodicity, MB) is defined as a rhythm (or bioperiodicity) in a readily-measured variable, used to monitor a corresponding rhythm in a related but less accessible variable for purposes of prophylaxis, diagnosis and therapy (25). In discussing the procedure of MR monitoring, this term will be used, whether the marker is gauged as a rhythm, in the strict (microscopic) sense of the word given above or only by data inspection, as a bioperiodicity. MRs in basic or applied physiologic and pharmacologic work have been defined and discussed (27). They have potential applications in preventive health maintenance (prophylactic MRs), risk monitoring (risk MRs), for diagnosis (diagnostic or screening MRs), and for timing therapy (chronotherapeutic MRs yielding markers of treatment time), for assessing therapeutic response (response MRs) or for both of the latter purposes (double chronotherapeutic MRs). Such rhythms are used without implying causal relations between the process being monitored for its rhythmic marker, and a given treatment. When such relations exist, in turn, they constitute an advantage and should lead to the preference of a specific MR over an unspecific one.

Apart from any specificity, a multiple chronotherapeutic marker should possess several characteristics, documented by inferential statistical means: 1) the property of being a reference for the timing of treatment leading to an appreciable benefit and/or 2) the property of being an index of toxicity to one or several targets, e.g., to bone marrow, kidney or heart, and/or 3) the property of being an index of the desired effect(s). It is also critical that the index be

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applicable or be rendered applicable with currently available technology on a large scale.

Information on MRs, that indicate the best time for treatment, can also serve to establish reference standards that are time-specified, so-called chronodesms, for the interpretation of time-specified single samples, e.g., to gauge toxicity. The circadian ϕs of white blood cell counts (WBC) and of urinary potassium excretion are both treatment-time markers; the ϕ of the WBC serves in addition to evaluate an undesired response, such as bone marrow toxicity.

Once one considers a potential marker variable, one tries, at the outset, to answer several methodologic questions: What are the distributions of a global index of the timing of the marker variable, such as the ϕ of a time series, as compared to those of a local index, such as a peak in the series, the macrophase, ψ? It can be anticipated that the ϕ is more stable than the ψ, since the latter is more sensitive than the former to random fluctuations in the data series. Concurrently, a global, as compared to a local index, may be less flexible in reflecting a change in best timing. Stability throughout a treatment course may be a disadvantage as well as an advantage. When stability is a lasting advantage, i.e., when it reflects a basic periodic (marker) process, rather than an artefact, the ϕ may be determined only once for a given patient, before the first treatment. When the best timing is variable, the ϕ should be determined accordingly, with some optimal frequency, preferably, yet perhaps not necessarily, before each treatment.

Other questions to be raised concern the sampling span required to determine a trustworthy ϕ and the matter of modulation of a ϕ with a given frequency by rhythms with a lower frequency, such as circannual, circaseptan or other infradian modulations of a circadian ϕ. Since very many potential MRs indeed exhibit about-yearly changes (31), still another question to be examined is the possibility of circannual changes in circadian characteristics, which latter have been explored for the WBC by Halberg *et al.* (27).

3. CHRONOCHEMOTHERAPY WITH DOXORUBICIN AND CISPLATIN: ORIGINAL STUDIES IN RODENTS

The fact that the same anticancer drug has different effects at different circadian stages will be illustrated for doxorubicin and cisplatin. A hypothetical rhythm in the therapeutic index of an anti-cancer drug, Figure 1, was experimentally validated by assessing the doxorubicin-associated shrinkage of a breast cancer in the mouse and the associated augmentation of survival time. In the original study (24), 6 groups of inbred A-strain mice with spontaneous mammary cancer (and 2 controls without palpable tumors, matched by strain, age and sex), feeding *ad libitum* in light from 06:00 to 18:00 alternating with darkness, were given a fixed dose doxorubicin, i.p., at one of six different circadian times, 4 hours apart. Caliper measurements of tumor size and survival times were recorded to find the best timing, if any, of doxorubicin in terms of reducing tumor volume and increasing survival time.

A chrono*t*herapeutic index (CTI) was defined as CTI = PST × TVC, where PST was equated to the individual animal's survival time [expressed as a percentage of the overall mean survival time (in turn, equated to 100%) of all treated individuals irrespective of treatment time] and

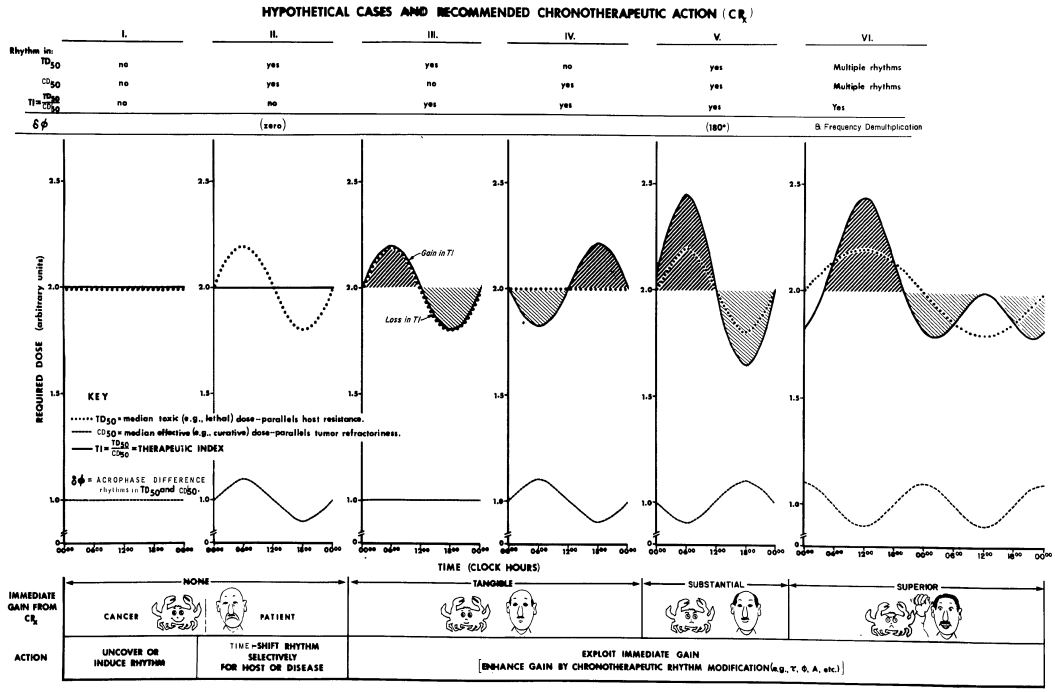

Figure 1. Hypothetical cases of the lack of rhythm in elements of therapeutic indices (extreme left) and rhythms (right half) as the basis for chronotherapy.

TVC was equated to the individual's post-treatment tumor volume change (a) relative to the individual's pre-treatment tumor volume (b) [expressed as a ratio (a/b)]. Results thus analyzed show the highest CTI (= 122%) at 22:00 (i.e., at 16 hours after light on), the second highest CTI (= 104%) at 02:00 and the lowest CTI (= 8%) at 18:00. A 24 h cosine model, fitted by least squares to these several kinds of time series, allowed rejection of the zero-circadian amplitude (no rhythm) assumption for the original mean survival times, for survival times expressed as percentage of overall mean and for CTIs. On the basis of the latter data, the ϕ of the CTI was computed. The time of optimal circadian tolerance of doxorubicin also was assessed, on the basis of follow-up work, by the fit of multiple components, to obtain an orthophase (48). A follow-up study with doxorubicin as part of a combination treatment with phenyl-alanine mustard, on rats with a transplanted breast adenocarcinoma, revealed that timing made the difference between 12.5% and 50% ($\chi^2 = 14.13$; $P < 0.025$) remission (29).

For cisplatin, the original studies (28) were carried out on female inbred Fischer rats, kept on 2 lighting regimens, one of them intended to simulate the 8 h sleep and/or rest span of human beings. The rats were standardized after weaning for 2-week spans, doubly housed, some on a regimen involving 8 h of light alternating with 16 h of darkness, others on 12 h of light alternating with 12 h of darkness. Food and deionized water were freely available to all rats.

After the standardization span, the rats were singly housed, rectal temperatures were measured around the clock at 4-hour intervals (with a thermistor bridge circuit; Halberg *et al.* (19)), body weights were taken, and cisplatin was injected i.p. in a fixed dose of 11 mg/kg to 6 separate groups (each of 16–20 animals, from each lighting regimen). Injections started at 07:10 after 'light on' and continued at about 4 h intervals thereafter to cover a 24 h span. Animals were checked for survival at least twice a day and much more frequently about the time when control animals kept in LD 12:12 reached 50% mortality (the so-called semimortality index; Cornélissen *et al.*, (5)). At that time the experiment was truncated and the data analyzed. For rats from each regimen, a statistically significant circadian time effect was demonstrated for the tolerance of the drug. Best tolerance was found for rats treated at 11:40 from light on, when the mortality of the rats in LD 8:16 was 10% (and that of rats in LD 12:12 ~ 20%). At the peak, 4 h later, the corresponding mortality was 89% in LD 8:16. In view of the non-sinusoidality of the response, the data on tolerance and rectal temperatures, determined prospectively yet used as MRs retrospectively, were fitted not only by a 24 h cosine curve, to obtain a ϕ, but also by harmonic interpolation to obtain a paraphase (8). A long series of follow-up studies on rats provided data on intermodulating infradian changes, with circaseptan (1) and circannual (17) periods.

To carry such information to the clinic, MRs are essential. As a candidate MR, the circadian change in core temperature of the original groups of rats investigated was documented to persist and recommended for further study as a complement and/or substitute for adrenocortical and/or other MRs (28). This suggestion was based on a statistically significant circadian rhythm ($P < 0.05$) demonstrated for rectal temperature of the groups on each of the lighting regimens studied. The extensive data on chronotherapy with cisplatin are discussed elsewhere (17).

4. CIRCADIAN CHANGE IN WHITE BLOOD CELL COUNTS – DOUBLE MARKER RHYTHM

Historically, the count of human and murine circulating blood eosinophil cells constituted a first admittedly indirect and unrefined, yet practical and useful, mammalian marker of adrenocortical changes. This rhythm, as well as rhythmic changes in serum corticosterone, occurs spontaneously (2, 10, 21, 39), and also determines responses to hormones or drugs, including hormone analogues (30, 46).

The WBC, in turn, serves to gauge the dramatic circadian changes that characterize the bone marrow's response to toxic agents, including carcinostatic drugs such as doxorubicin (24, 26), cisplatin (17, 28), and ara-C (3, 24, 34, 45).

The use of the WBC as an index of bone marrow toxicity has to be qualified by the fact that the WBC undergoes circadian and other changes of large extent. These changes are a vexing source of variability when they are ignored, but may be valuable new endpoints when they are exploited by their use as MRs, computed on the basis of around-the-clock measurements.

Six 4-hourly WBCs covering 24 hours constitute an all too sparse sampling schedule for individualized MR use. This sampling scheme for WBC can be used, however, in studies on groups. This was done in data collected in a then cooperative research on circadian-circannual aspects of WBC depression and recovery in patients given doxorubicin and cisplatin (38). These patients of Hrushesky with advanced-stage ovarian and bladder cancer were treated about monthly with 60 mg/m^2 of each doxorubicin and cisplatin, the latter always 12 hrs after the former, in view of studies on rats suggesting that this interval between the administration of doxorubicin and cisplatin was optimal (28).

In a first stage, patients were randomized to receive their chemotherapy beginning either 1 hour before their habitual awakening (at 06:00; schedule A) or 13 hrs after habitual awakening (at 18:00; schedule B), and then crossed over to the other schedule at the following treatment, with a continuing alternation of schedules A and B throughout the treatment span, usually consisting of 9 about-monthly courses. In a second study stage, patients were randomly assigned to either schedule A or B and remained on the assigned schedule throughout the entire treatment span. In these cases, the alternation of treatment no longer confounded the time course of cumulative myelotoxicity associated with a given schedule. When results on schedule A obtained during the first stage of the study were compared with those on schedule B, about one month after each treatment, recovery of the WBC was greater with schedule A in spring, summer and fall, but worse in winter. A 3-way analysis of variance on weekly total WBC, as a gauge of myelosuppression, reveals as main effects the (circadian) difference between schedules A and B and the effect of the day after treatment; both are statistically significant below the 1 per mil level (38). While this same analysis does not detect any added effect of circannual rhythm stage ($DF = 3$; $F = 0.559$; $P = 0.642$), there is indeed a statistically highly significant interaction between circannual

rhythm stage and schedule A vs. B (DF = 3; F = 4.773; P = 0.003).

5. RELATIVE MERITS OF CIRCADIAN WBC MARKER ACROPHASE (ϕ) VERSUS MACROPHASE (ψ)

The circadian ϕ and ψ of the WBC are both candidate treatment-time markers. The question may be raised whether ψ may not serve as well as ϕ for treatment-time optimization. The ψ has the apparent merit of being obvious in most cases when there is but a single highest value. One can construct a simple moving average of 2 or more items when there are equal counts, an unlikely occurrence in the case of the WBC. In using the ψ, one can dispense with the computation of the ϕ or of a paraphase or orthophase (25). The cosinor ϕ is readily and simply computed yet this computation of ϕ is an added step, which might add to cost, although it should not. Another consideration is that the ϕ represents a "complication" for those reluctant to familiarize themselves with new concepts, even if the simplified output of cosinor methods can be interpreted as easily as the face of a watch. Actually, from the viewpoint of cost we shall see from a discussion (below) of the sampling densities required for a ϕ versus a ψ that (to the extent that a bioperiodicity is sinusoidal) the sampling for a ϕ may be more thrifty than that for a ψ. The main concern, in any event, in choosing between an MB and an MR is pertinent. A first question to be asked is then as to whether, if a ϕ is not computed, the average ψ may approximate the average ϕ.

6. SIMILAR AVERAGES FOR WBC MACROPHASE AND ACROPHASE

To compare the location indices of timing, the average ψ and ϕ, the individual ψ and ϕ were identified and calculated, respectively, from six WBCs covering a 24 hr span at 4 hr intervals. These counts were made on each of 59 patients with advanced ovarian or bladder cancer, before each combination treatment (Rx), with up to 60 mg/m^2 of doxorubicin followed after 12 hrs by up to 60 mg/m^2 of cisplatin, in a sequence of up to 11 about-monthly courses. The six values are fitted with a 24 hr cosine curve to obtain the ϕ by the single-cosinor method (23). A computer program for the so-called macro-micro comparison by serial section (36, 37) then serves to compute the mean ψ, found at 17:30, and the mean ϕ, found at 18:30, and to compare them in a first step, for a total of 29 patients who had received 8 or more Rx courses. For these patients, paired t-tests show no difference between the mean ψ and ϕ of the profiles before each Rx course in a given patient, whether one considers all Rxs (P = 0.83) or only schedule A (doxorubicin Rx at 06:00 1 hr before awakening or 12 hrs after the WBC ϕ; P = 0.43), or only B (doxorubicin Rx at 18:00, near the WBC ϕ). The null hypothesis of no difference between the mean ψ and ϕ is not rejected, the corresponding P value being 0.31 (36). The largest differences between the mean ψ and mean ϕ are found for schedule B. A high (angular-angular) correlation (r = 0.52) is found between the mean ψ and ϕ when all 29 subjects are considered, irrespective of Rx timing. The correlation is higher (r = 0.65) for Rx A than for Rx B (r = 0.39). One may conclude that, as an approximation of the average ϕ for larger groups, the average ψ may well be satisfactory. Before this conclusion is extended to the individual, however, the variances of ψ and ϕ also must be compared.

7. GREATER VARIABILITY OF WBC MACROPHASE AS COMPARED TO THAT OF ACROPHASE

By comparing ψ and ϕ variances in all profiles for these 29 patients with 8 or more courses, paired t-tests show a large reduction in variance by the use of ϕ rather than ψ. The smallest, but still significant (P = 0.02) differences between ψ and ϕ variances are found for Rx A. Even in this case, 85% of the cases have a higher ψ variance as compared to the ϕ variance. No statistically significant correlation is found between the ψ and ϕ variances whether one considers all Rxs (r = 0.02) or only schedule A (r = 0.01) or only B (r = 0.08) (36).

The equality of variance in ψ and ϕ is also tested for the set and 2 subsets (Rx A or B separately) of 386 series from 59 patients and for the set and 2 subsets of the 55 means of the series for those 55 patients with at least two series (Table 1). In all cases (except for the means from 28 patients under Rx A) the test of equal variances for ψ and ϕ allows the rejection of the null hypothesis at the 1% level.

Figure 2 shows, on the left, frequency histograms for the 386 WBC profiles from all patients, irrespective of Rx schedule for the ϕ (in the top half of the graph) and ψ (in the bottom half of the graph). The ϕ has a nearly symmetric and unimodal distribution centered at 18:30. By contrast, the ψ-distribution shows a much broader spread along the scale of 24 hrs. The multiple peaks stem largely from the 4-hrly sampling, since there is interpolation in the computation of the ϕ, but (in the cases examined) none in the identification of a ψ.

Figure 2 also compares the mean ψ and ϕ variability for 55 patients with at least 2 profiles (right). In the top third of this figure, on the right, the mean ϕ and mean ψ are compared separately for each patient. The horizontal scale simply lists in a sequence the (number of the) patients studied. Thus, one can compare each quadrangle, corresponding to the ϕ, with the corresponding location, for the same patient, of the ψ; the ψs are connnected by a dashed line to distinguish them from the ϕs, connected by a continuous line. One finds, for the first patient listed, a difference of about 10 hours; for the second patient, the difference is smaller, and for the third and the following few patients, it approaches 0. On the average, the ϕ and ψ are similar. The graph shows, nonetheless, large differences in some cases.

The quasi-unimodal distribution for the mean ϕ shows higher stability, as compared to the mean ψ, with a mode of 21.8% of the total patients at about 18:00. By contrast, the mode in the ψ distribution reaches only 10.9% of the total number of patients. The larger scatter in the distribution of mean ψs, as compared to that of mean ϕs, is in keeping with the larger variance found for ψ as compared to ϕ (Table 1). A similar situation is shown in Figure 3 (right) for the ψ and

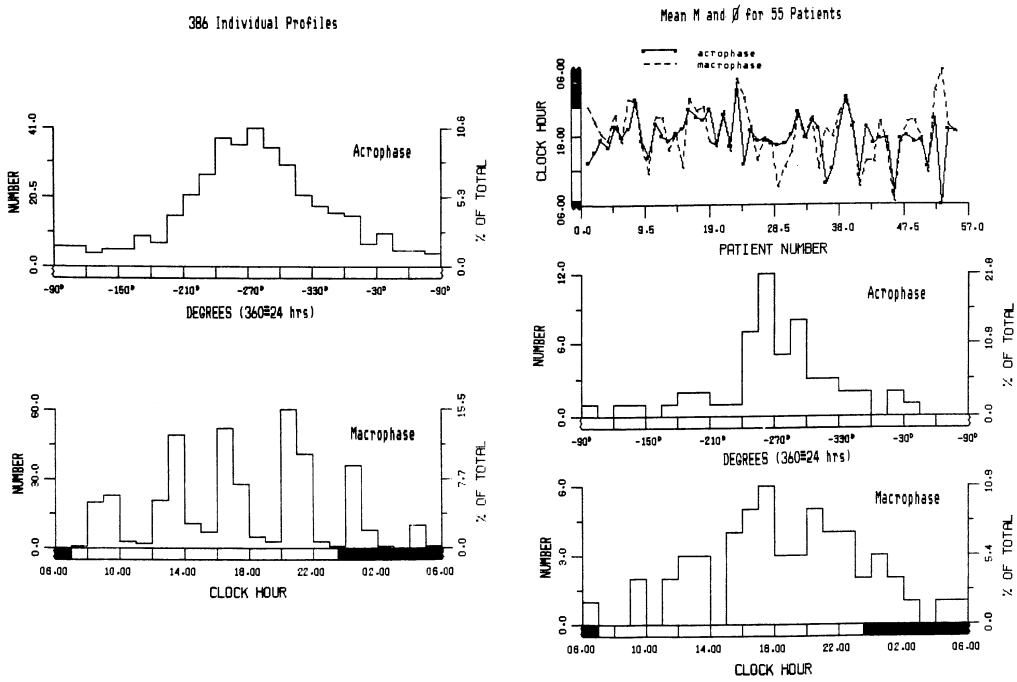

Figure 2. Distribution of circadian acrophases (ϕ) and macrophases (ψ) of total white blood cell counts from all profiles (left) and individual mean ϕ and ψ, suggesting greater stability of ϕ vs. ψ.

ϕ means of 28 patients under Rx A. In this case, the ψ-distribution is rather concentrated around 18:00 and a test of equal variances does not reject this null hypothesis (P = 0.43). Lack of statistical significance notwithstanding, however, a difference in variability between ψ and ϕ can be suspected from Figure 3. Indeed, again the peak class for ϕs is represented by 28.5% of the patients, but the peak class for ψ by only 17.8%. Before one accepts, for Rx A, a lack of statistical significance of the difference in the variances of ψ and ϕ, one has to glance at a summary of the individual A series for which the difference in variances between ψ and ϕ is clearly established.

Figure 3 shows, on the left, similar distribution shapes for the 188 series from 32 patients under Rx A. In this case, one rejects the hypothesis of equal variances in ψ and ϕ (P = 0.009). The higher variability of ψ is apparent in the figure and quantified by the standard deviations in Table 1.

For 198 profiles from 27 patients under Rx B, Figure 4 on the left again indicates a higher variability for ψ as compared to ϕ. Moreover, the ϕ distribution is now centered at 18:30, with a certain delay from the ϕ distribution for Rx A shown in Figure 3. Figure 4 on the right shows the ψ and ϕ means for 27 patients under Rx B. A high dispersion in the ψ-distribution is readily apparent. Most of the mean ϕs (26%), in turn, are concentrated around 19:00.

Table 1. Larger variance for macrophase (ψ) as compared to acrophase (ϕ) of white blood cell count (WBC) profiles of patients receiving timed therapy (Rx)*.

Kinds of series (Rx)	*# of cases*	*Standard deviation of* ψ *(hours)*	ϕ	*For* ψ *versus* ϕ *F*	*P*
Individual – (All)	386	81.64	68.51	1.42	$\ll 0.001$
Individual – (A)	188	83.98	70.54	1.42	$\ll 0.009$
Individual – (B)	198	79.46	66.40	1.43	$\ll 0.006$
Mean – (All)	55	74.54	54.95	1.84	$\ll 0.013$
Mean – (A)	28	50.25	48.59	1.07	$\ll 0.430$
Mean – (B)	27	99.67	60.47	2.72	$\ll 0.006$

*A = doxorubicin Rx at 06:00, 1 hr before awakening or about 12 hrs after the population WBC ϕ, followed by cisplatin 12 hrs later; B = doxorubicin Rx at 18:00, near the population WBC ϕ, followed by cisplatin 12 hrs later.

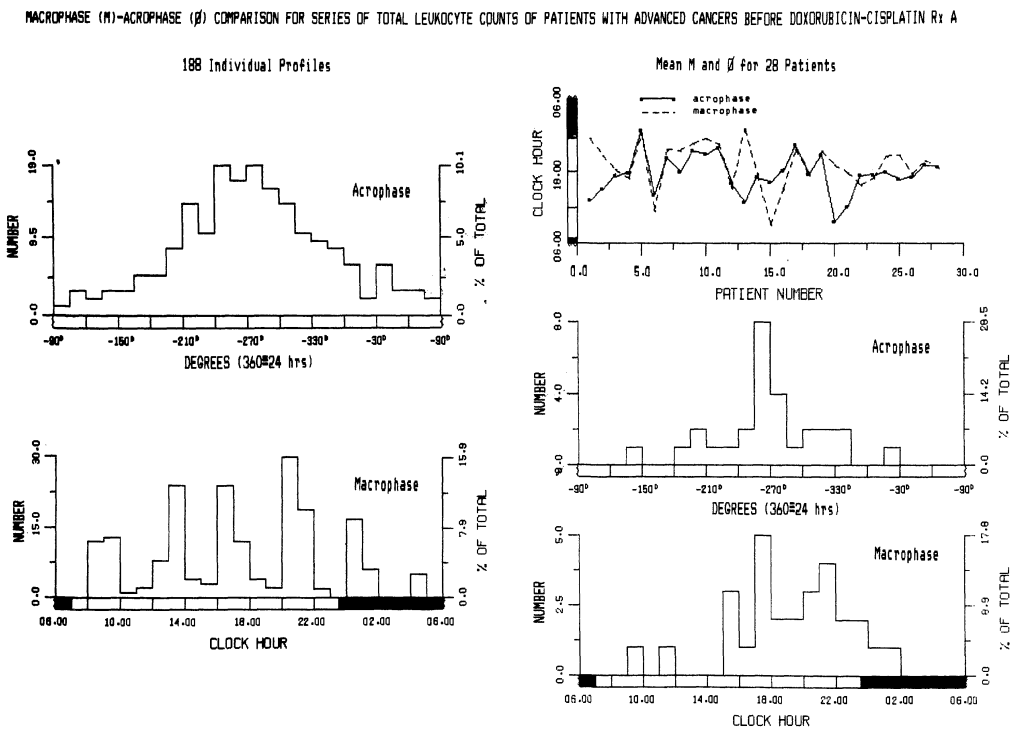

Figure 3. Distribution of circadian acrophases (ϕ) and macrophases (ψ) of total white blood cell counts from all profiles (left) and individual mean ϕ and ψ for patients on treatment A (doxorubicin at 06:00, 1 hour before awakening, or about 12 hours after the population white blood cell count ϕ, followed by cisplatin 12 hours later).

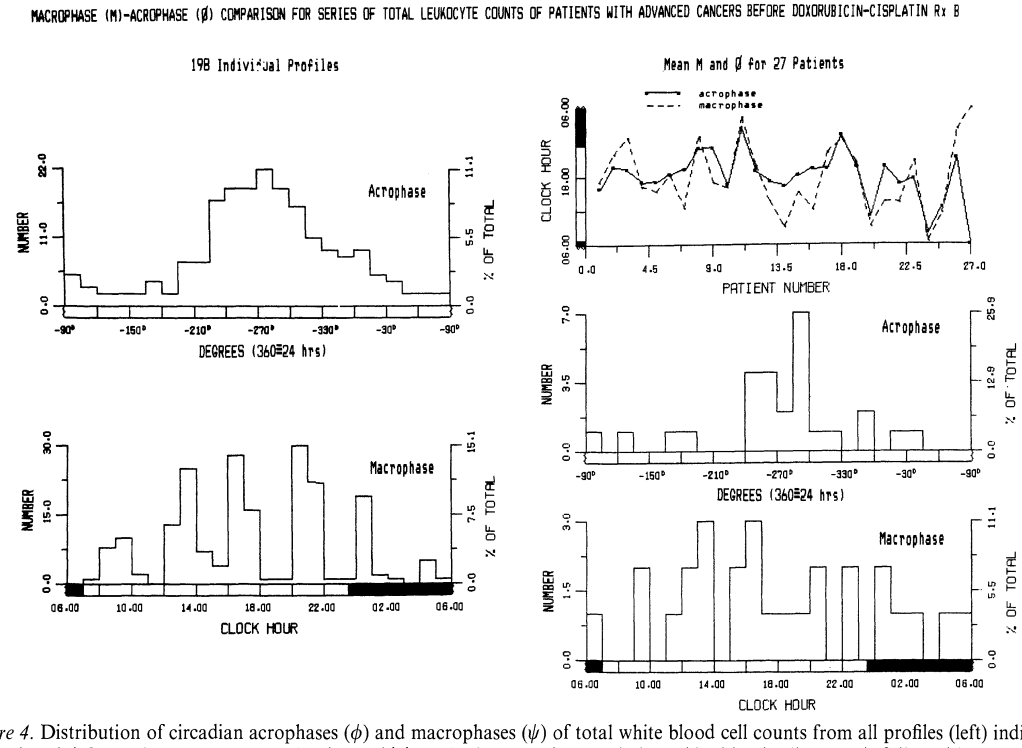

Figure 4. Distribution of circadian acrophases (ϕ) and macrophases (ψ) of total white blood cell counts from all profiles (left) individual mean ϕ and ψ for patients on treatment B (doxorubicin at 18:00, near the population white blood cell count ϕ, followed by cisplatin 12 hours later).

8. URINARY POTASSIUM IN HEALTH

An MR or MB may be needed only once in the theoretical case when a single treatment is administered. One may benefit from a single MB at the outset of radiotherapy given for 5 weeks (6, 18, 26). In chemotherapy, MRs or MBs may be needed for many months. In such cases, information on any infradian, e.g., circaseptan or circannual, changes in a variable considered for marker rhythmometry is desirable and it is indispensable, in the case of long-term chemotherapy. A problem arises when such marker rhythmometry is to be carried out on patients whose treatment is to be urgently initiated. Cancer patients in advanced stages of the disease are cases in point.

In such patients, the time-dependence of treatment effects along the circadian scale undergoes further modulatory changes by rhythms with a lower-than-circadian frequency. Thus, the effect of treatment with doxorubicin at 06:00 is advantageous in some, but not in all seasons. Moreover, effects of cisplatin differ with the stage of about 7-day (circaseptan) rhythms (1). Since the best treatment time exhibits changes that are predictable with an infradian frequency, it follows that candidate MRs for use in chronotherapy must be sought that also undergo an infradian modulation of their circadian characteristics, such as the ϕ.

In the patient with cancer, however, treatment cannot be postponed until infradian, notably about-yearly, changes are evaluated; in other words, treatment must not be withheld for a year in order to assess with serial dependence any circannual change, e.g., in the circadian urinary potassium (K^+) rhythm of patients scheduled to receive potentially nephrotoxic cisplatin therapy. There is no alternative but to analyze data gathered on clinically healthy individuals, with the hope that results may be extrapolated to cancer patients. In so doing, one realizes that any results are at best approximations, since a disease may accentuate or obscure an existing rhythm, otherwise alter one or several rhythm characteristics or may induce a rhythm that is not otherwise observed.

As reported elsewhere (37), we analyzed urinary K^+ excretion in 2,860 samples of an apparently healthy man, 21 years of age when he started collecting 5–7 urine samples on most days for 18 months. Samples at 3–5 hr intervals are more readily collected for long spans covering years than are collections at shorter intervals. The latter are preferred, however, when a ψ or a ϕ is to be reliably estimated. In any event, a 24 hr cosine curve was fitted by the single-cosinor method to consecutive non-overlapping data sections of 1–14 days in length using the macro-micro-comparison by serial section (37). This was done in order to seek the minimal length of a time series (with the given sampling rate) that would allow the rejection of the zero circadian amplitude assumption. Accordingly, a more reliable estimation of the timing of high values in most, though not all, time series available with a given length can be obtained.

The total number of series varied from 513 (for 1-day-long series) to 39 (for 14-day-long series). It can be seen from Table 2 that, as a rule, series of 1-day length do not allow the inferential statistical documentation of a rhythm: of 513 consecutive series on the same subject, only 15% allow rejection of the zero-*a*mplitude assumption ($A = 0$) below the 5% level by the single cosinor method. The resolution of the rhythm, i.e., the % of series that allow rejection of the no circadian rhythm ($A = 0$) assumption, increases as the duration of the data section analyzed lengthens. Up to a length of 6 days, the gain from each added day is large; 80% of series covering 6 days allow rejection of the zero-A assumption. Only relatively small further gains in rhythm description are associated with slightly longer time series of 7–10 days. There seems to be a further gain, however, with spans longer than 10 days, Table 2. A lack of gain from the increment in the length of the urinary K^+ record may be attributed to the obscuring effect of an infradian modulation of the waveform, which largely cancels, in records of a length between 6 and 10 days, gains from the increment in record length.

As noted elsewhere (37) for practicability, the sampling in this study (actually covering 15 years) was sparse, with individual samples (5–7/day) covering, on the average, about 4 hrs. For future work it will be more efficient to increase sampling rate, e.g., from every 4 to every 2 hrs during waking, e.g., for 2 days, rather than to sample longer spans at a lower rate, unless there is a suggestion of desynchronization. Series based on about 2-hourly samples, at least during wakefulness, covering a few days are recommended to render the description of a 24 hr synchronized rhythm more efficient. Longer series are needed for an estimation of the period in its own right, e.g., by nonlinear least squares rhythmometry (25).

As reported elsewhere (37), the cosinor analysis of original K^+ values (mEq/hr) shows not only a circadian rhythm but also a circannual one ($P < 0.001$), $M = 3.15 \pm 0.03$; $A = 0.19 \pm 0.04$ and ϕ at $-27 \pm 14°$ from December 22 with $360° \equiv 365.25$ days). The variability of the circadian K^+ ϕ along the scale of a year is particularly pertinent to treatment-timing, as shown in Figure 5. This figure shows time on the abscissa (from Dec. 16, 1967, to April 29, 1969). On the ordinate, the circadian ϕ is shown with earlier values on top and later ones at the bottom. These ϕs can be seen to extend from nearly 300° from local midnight to 130° from midnight, a considerable variability which is accounted for, to a very small extent, by a circannual rhythm.

In view of the density of the series consisting of circadian characteristics, the zero circannual A assumption can be tested. A 365.25-day cosine curve was fitted to the 240 circadian K^+ ϕs computed separately for consecutive 2-day

Table 2. Relation of series length to demonstrability and quantifiability of circadian rhythm in human urinary potassium excretion in health*.

Series length (days)	1	2	3	4	5	6	7	8	9	10	11	12	13	14
% series with $P < 0.05$	15	37	45	63	66	80	81	85	87	82	94	96	98	97
N series with $P < 0.05$	76	97	81	85	71	72	62	57	52	45	47	43	41	38

* By rejection of zero-amplitude assumption in single-cosinor procedure.

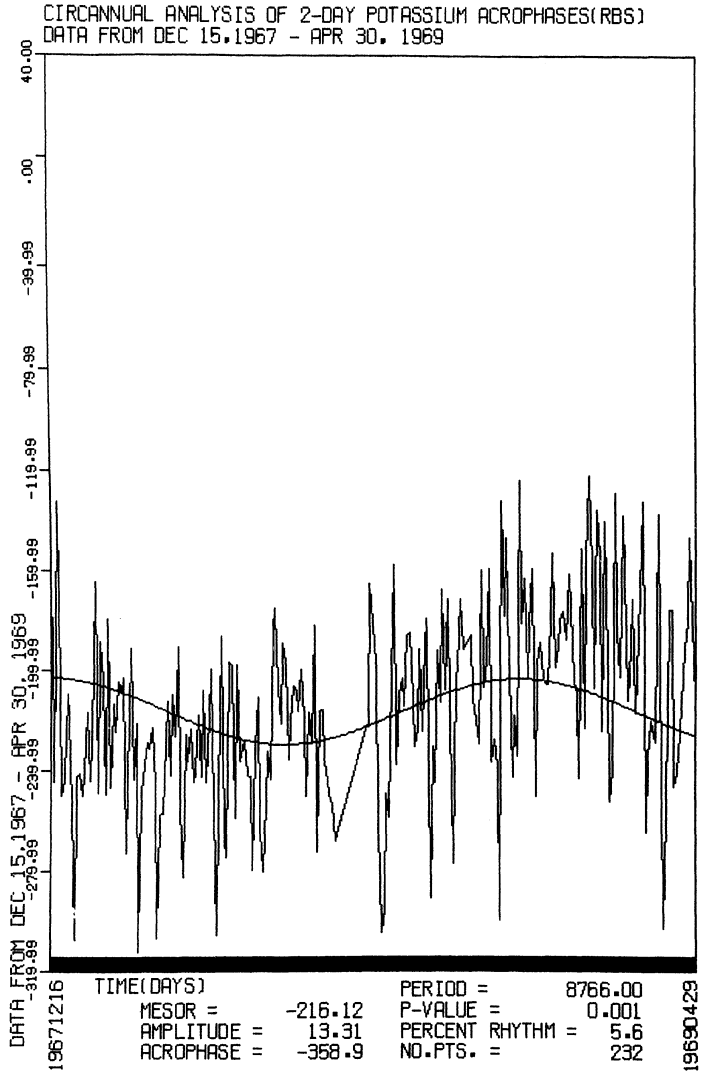

Figure 5. Circannual variability of circadian acrophase of urinary potassium excretion computed for consecutive 2-day spans (by reference to midnight); healthy man, 21 years of age.

spans (by reference to 00:00 and with a 1 hr correction for daylight saving time); ϕs obtained within consecutive 4-day spans were considered as replicates for the sinusoidality test (37). By excluding 8 outliers, a circannual ϕ rhythm was found (P = 0.001; Figure 5) without any violation of the assumptions underlying the use of the single-cosinor model, namely, sinusoidality, the normality of the residuals and variance homogeneity. For at least one serially-dependent longitudinal series in health, a circannual modulation of the circadian K^+ ϕ was thus documented on the basis of dense sampling. Any (possibly larger than 2 hr) circannual change in circadian K^+ ϕ of cancer patients, as a potential marker for chronotherapy, can and remains to be studied only with serial independence.

9. URINARY POTASSIUM EXCRETION BY CANCER PATIENTS

Since a circannual rhythm characterizes the circadian ϕ and other characteristics of urinary K^+ excretion in a clinically healthy subject, it is conceivable that such a rhythm persists and even that it may perhaps be amplified and/or changed in frequency in patients of different kinds, including those with cancer here under discussion. For 53 patients with advanced ovarian or bladder cancer, the circadian ϕ of urinary K^+ before a first 60 mg/m^2 cisplatin treatment (Rx) was compared with the mean ϕ of all profiles in a sequence of up to 10 about-monthly courses (of a combination treatment with 60 mg/m^2 doxorubicin, the latter given 12 hrs before cisplatin, Table 3) (37).

Table 3. Similarity between the circadian acrophase (ϕ) of urinary potassium excretion before a first treatment and mean ϕ of all profiles of patients receiving timed therapy (Rx)[1].

Kinds of Rx (# of cases)		*D = (1st ϕ-Mean ϕ)*	*Paired t-test*			*Correlation*	
		% with neg. D (delay)	*mean D*	*SD of mean D* (hours)	*P*	*r*	*P < .05*
All	(53)	62	−0.33	0.48	0.50	0.39	yes
All*	(50)	60	−0.02	0.48	0.96	0.38	yes
Alternate	(15)	40	1.94	1.07	0.09	0.08	no
A	(18)	61	−0.81	0.67	0.24	0.43	no
B	(20)	80	−1.60	0.64	0.02	0.69	yes
B*	(17)	76	−0.92	0.61	0.15	0.70	yes

[1] A = Rx with cisplatin in the evening (usually near pre-Rx ϕ); B = Rx in the morning ($\sim 180^{\circ}$ from pre-Rx ϕ); Alternate = A and B in alternation, in consecutive courses. * Deletion of three cases with mean calculated by averaging over only two Rxs.

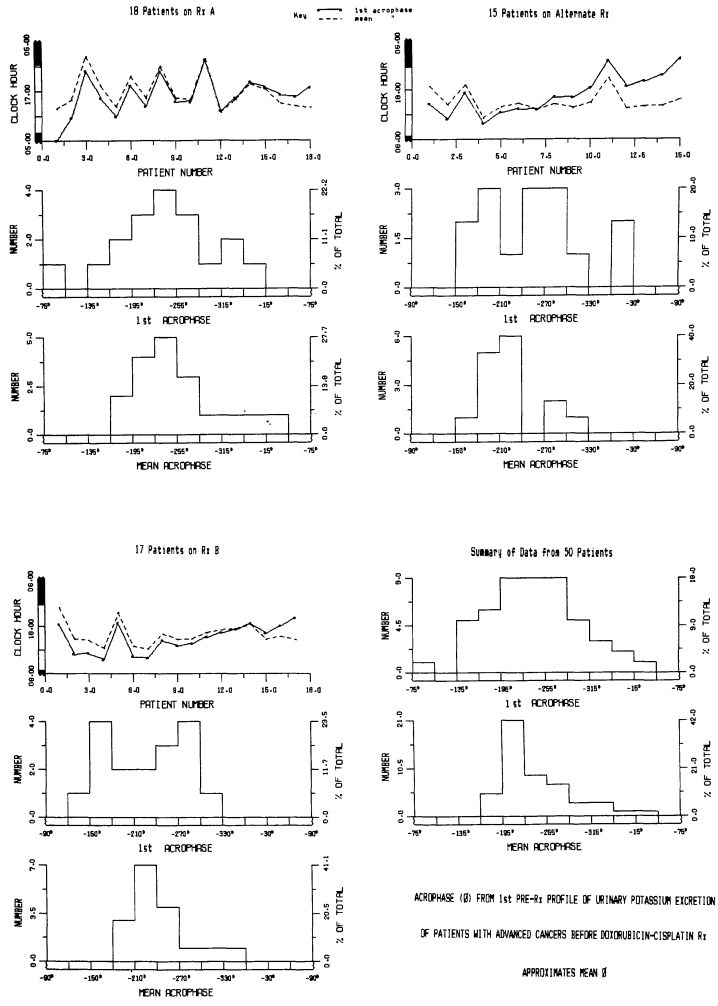

Figure 6. Distribution of circadian acrophases (ϕ) before a first treatment and individual mean ϕ of urinary potassium excretion suggesting no difference between first ϕ and mean ϕ for patients receiving treatment A (doxorubicin at 06:00, followed by cisplatin 12 hours later) or alternating treatment, and a delay in ϕ for patients receiving treatment B (doxorubicin at 18:00, followed by cisplatin 12 hours later).

No difference was seen by paired t-test between the ϕ before the first Rx and the average ϕ of all profiles before each Rx course in a given patient, including the profile before the first Rx. A high (angular-angular) correlation was found between the first and the average ϕs, when all subjects were considered, irrespective of Rx timing. The correlation was maintained for the group on Rx B but not for that on Rx A (35, 37).

The histogram at the bottom on the right in Figure 6 shows, for 50 cancer patients, the average ϕ of all profiles, i.e., the profiles before each of the subsequent Rx courses in a series received by a patient. The histogram just above shows the corresponding ϕ before the first Rx of these 50 patients. In comparing these dispersions for all patients, one can conclude that the mean ϕ is more stable than the ϕ before the first Rx. Indeed, the distribution for the mean ϕ of the 50 patients has a high peak with 42% of the total values around 13:00–15:00.

The graphs in the top rows and the one in the 4th row on the left of Figure 6 indicate the differences between the two ϕs for each patient, classified as a function of Rx mode. A comparison of the two lines connecting the corresponding ϕs for the 18 patients on schedule A (top left) and the 17 patients on schedule B (top left of bottom half) shows no large differences between the ϕ before the first Rx and the mean ϕ. The remainder of the figure consists of frequency histograms for the ϕ before the first Rx and the mean ϕ, respectively, in each of these groups.

The graph on the right on top of Figure 6 shows a similar comparison for 15 cancer patients receiving Rx alternating between schedules A and B. The differences in timing, shown in the top row, are big but not systematic; the mean ϕ randomly appears before or after the ϕ found in the profile before the first treatment. A paired t-test does not detect any statistically significant differences ($P = 0.09$). The peak in the distribution of the mean ϕ of each patient is around 14:00–16:00 (lower third of the upper half in the left part of the graph).

Smaller differences can be observed for the 18 cancer patients consistently receiving Rx A (upper third of Figure 6, left). In fact, the hypothesis of no difference is supported by a paired t-test ($P = 0.24$, Table 3). In this case, a greater variability can be observed for the mean ϕ, with a peak in its distribution of only 27.7% of the total situated around 15:00–17:00.

For 17 cancer patients receiving Rx B, Figure 6, there is again only a small difference between the ϕ before the first Rx and the mean ϕ of urinary K^+. A shift in ϕ after Rx B is suggested by a high (80) percentage of unidirectional differences in ϕ (delays), corresponding perhaps to the organism's endeavor to adjust its ϕ after an inappropriately timed Rx. Conceivably, Rx at the "right" time may not displace the ϕ, whereas Rx at the "wrong" time does so. But at this point, speculation will have to be replaced by data with more definitive outcomes, such as survival.

10. QUALIFICATION

On the basis of the prospective use of a ψ, a clear advantage from timing radiotherapy has already been reported (6). The ψ of tumor temperature was the MB in that case, used prospectively for the timing of treatment. A substantial gain from timing was validated by cosinor analysis of the data (26). In the study with the WBC here discussed (36), however, actual timing was by clock-hour rather than MR. Marker rhythmometry also was done prospectively, but information on treatment effects as a function of rhythm stage was evaluated only retrospectively; hence, the merit of ψ over ϕ cannot be assessed on the basis of outcomes such as the percentage of the cures or times to relapse. The first conclusion drawn is based on evidence in Table 1, showing that the ϕ is the more stable endpoint. Greater stability, of course, does not necessarily imply greater pertinence.

11. PERTINENCE

The problem of pertinence can be examined indirectly. More specifically, the WBC *M*ESOR (M) before treatment can be equated to 100%, and weekly WBCs during the month following treatment can thereafter be expressed as percentages of this M. The area between the horizontal line corresponding to 100% and any depressed weekly WBC is then computed. These areas are assigned, on the one hand, to the time of WBC ϕ ($t\phi$) and, on the other hand, to the time of ψ ($t\psi$). With ϕ, but not ψ, the zero-A assumption of no circadian rhythm in toxic (leucopenic) response is rejected. Moreover, when the areas with treatment within 2 hrs of $t\phi$ are being compared with those obtained with treatment within 2 hrs of a timepoint at 12 hrs from $t\phi$ the difference is statistically significant. This is not the case for the areas in the case of treatment at $t\psi$ and 12 hrs from the $t\psi$. Pertinence of the ϕ can be suggested only on such an indirect basis. Hereafter, primary focus is to be placed upon the behavior of the ϕ.

12. SAMPLING FOR COST-EFFECTIVENESS – HOW DENSELY AND PRIOR TO EACH TREATMENT?

Further work should focus upon the sampling requirements for the determination of the ϕ rather than the ψ. Apart from pertinence, to be assessed in each case, there is the matter of economy. It remains to be investigated whether sampling for a reliable circadian ϕ on a rhythm can be sparser than that for a circadian ψ. If so, the ϕ will be the cheaper endpoint, even if there is a small charge for its computation.

In the case of a strictly sinusoidal rhythm, the number of required samples will depend upon the extent of noise. In the theoretical (never realized) absence of noise, the number of required samples is as small as three. Noise is ever present, however. Moreover, the departure of a rhythm's waveform from sinusoidality also is not uncommon. It seems likely that the interval between consecutive samples for the interpretation of the best timing for the given individual (rather than for search on groups) should be much smaller than 4 hours and, probably, sampling density may have to be higher for the ψ than for the ϕ.

The anticipated (but not yet proved) added benefit derived from the determination of a circadian ϕ before each treatment course, as compared to that derived from MR only prior to the first treatment, also must await the results of pertinent prospective studies on cost-effectiveness inter-

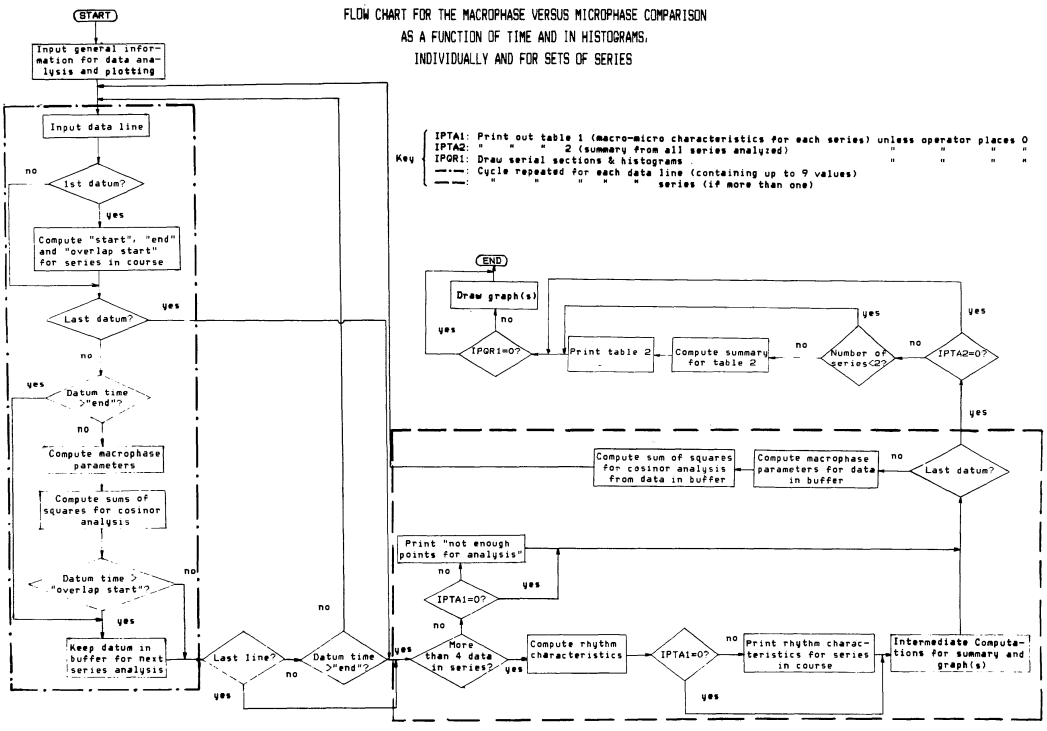

Figure 7. Flow chart for the macro-micro comparison by chronobiologic serial section, allowing the macrophase vs. acrophase comparison as a function of time and in histograms, individually and for sets of series.

preted as increased effectiveness in the light of both financial and emotional costs associated with repeated MR profiles (7). Such questions apply to any MR. It is pertinent that urinary K^+ excretion and the WBC exhibit a more stable circadian ϕ as compared to a ψ (36, 37).

13. GENERAL DISCUSSION

In view of the fact that the results of the two schedules tested, A and B, differ with the seasons, the added condition to be met by a pertinent MR is that it should exhibit infradian changes in ϕ. Since the data at hand do not suffice to demonstrate such a change, with serial independence in cancer patients for K^+, the availability of a longitudinal series on human K^+ was exploited and a circannual rhythm in circadian ϕ, albeit of small A, was demonstrated (37). For the case of K^+, the initial ϕ was compared with the mean ϕ, to explore the possibility that the former could be substituted for the latter. A statistically significant difference for K^+ between the first and the mean ϕ was not established in the available data; a larger sample is needed to ascertain the lack of a difference between the first ϕ and the ϕ based on data over a span approximating a year. If such a lack should be established, this could indicate that a single K^+ ϕ, taken before the first treatment, may be satisfactory for a contribution by this MR in establishing a benefit from treatment timing in cases when infradian modulations of the chemotherapeutic index are negligible. The use of the same MR for gauging kinds of toxicity that change substantially in a circannual or other infradian rhythmic fashion, may not be satisfactory.

Clearly, this chapter on MRs in cancer patients can only introduce methodologic considerations. Figure 7 provides a scheme for programming further research along this line on larger sets of samples on these and other MR variables. The scheme corresponds to the so-called macro-micro comparison (37) by a so-called chronobiologic serial section program (22, 30), allowing analyses on an unlimited number of data and the graphic representation of results (see Figure 6) comparing two of the parameters computed from the data. At this point, the question can be raised as to whether the circadian ϕ suffices as a proper MR, especially in dealing with non-sinusoidal rhythms. Objective methods available to obtain estimates of timing of high values on non-sinusoidal rhythms have already been discussed previously; they include the determination of the paraphase by harmonic interpolation (8) and the computation of the orthophase (48) as well as stacking procedures such as plexograms (25).

An adaptive filtering method also has been proposed (40, 41, 50) to cope with some features that are often present in biologic time series, namely: scarce *a priori* knowledge of the signals; a low signal to noise ratio; the presence of multiple related harmonic components; and above all, the expected occurrence of oscillations with varying frequency. The latter wobble introduces nonstationarities that cannot be treated with classic methods of spectral estimation. Results described elsewhere have shown the ability of adaptive filtering to detect and reconstruct periodic components in noise and to track their changes in frequency. Since this method re-

quires less information about the signal than other methods of spectral analysis, it seems specially suited for chronobiologic applications.

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7

MAMMOGRAPHY

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INTRODUCTION

The clinical and physical examination of the human mammary gland presupposes a knowledge of its post-natal development and its function as a hormone-dispensing organ. It is a paired organ, consisting of adipose, connective, and glandular tissues: localized at the exposed body surface, it is subjected to traumas of all kinds and, in addition, is responsible for the nursing process.

ANATOMY

The post-natal development of the mammary gland takes place in several phases, decisively influenced when a pregnancy has taken place. In addition, there are influences from the endocrine organs. The size, firmness and form of the mammary gland are determined to a large degree by its constitution during specific age periods.

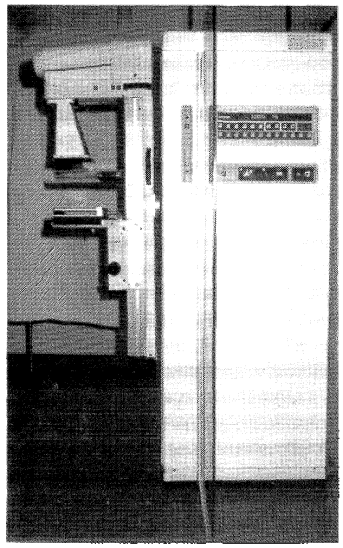

Figure 1. A mammographic unit.
Modern mammographic machine (Xerox Mammography System S 116) - 3 phase H.T. wave rectified. 50 kV 250 mA. KV range 20–50, 1 KV steps.
Automatic line voltage compensation. – Molybdenum, rotating anode with beryllium window. Focal spots: 0,4 mm/0,6 mm, kV range max: 50 kV – Moving grid bucky especially for mammography. Automatic exposure meter especially for mammography. Magnification device. Xeroradiography cassette holder.

In a newborn, the size of the gland varies between that of a hempseed and the kernel of a hazelnut. During childhood, the development of male and female mammary glands is the same. During puberty, however, girls show a more rapid dichotomous branching of the duct system with ramification at the terminals; only at the beginning of menstruation tubular terminals appear which lead to the development of tubular lobules. At the end of puberty, the mammary gland consists of 8 to 15 pori lactiferi with from 15 to 20 exit ducts, lined with squamous epithelium. In the course of further development, only the connective-tissue element increases further. The breast of the maturing woman consists primarily of loose connective tissue immediately surrounding the glandular ducts, as well as of fibrous connective tissue with a few nuclei closely connected with the adipose tissue. At maturity, the coarse connective tissue becomes looser and develops insulariform areas.

The adult mammary gland is composed of an active part (parenchyma) and of a passive part (adipose and connective tissue). The parenchymal portion is proportionally large in all sexually mature women, but the proportion of connective and fatty tissues varies quite markedly.

At time of menstruation, changes take place in the mammary gland which can be recognized macroscopically and microscopically. Rosenburg in 1922 (289) was the first to point out morphologically recognizable changes as a consequence of menstruation and contrasted the Rosenburg mamma curve with the Schroeder uterus curves.

During the pre-menstrual period, a branching of small and very small milk ducts occurs, where the initially solid epithelial mass shows a lumen lined with a single-layer epithelium. Toward the end of menstruation there appear regressive phenomena: the lumen of the alveoli expands, and during the post-menstruation period, remnants of growth and branching and parts of the milk ducts can be detected only microscopically. During the climacteric stage a regression of the glandular as well as of the supportive tissue is observed. The regressed body now consists only of milk ducts. In addition, proliferation phenomena in the epithelium with cystic enlargement of the milk ducts (involutional cysts and growth equipped with a two-layered milk duct epithelium) occur. During the proliferation and involution phases, epithelial islands develop with numerous roundish lumens (open spaces). Every fourth woman beyond the age of 40 exhibits primarily supportive tissue within the periphery of her mammary gland. During the meno-

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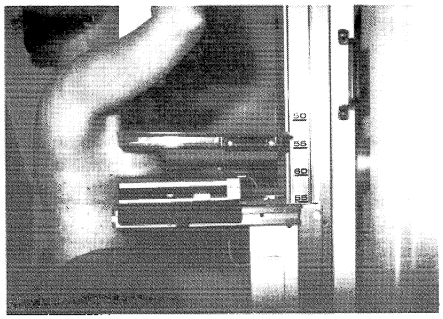


Figure 2a. Patient during cranio-caudal radiography.

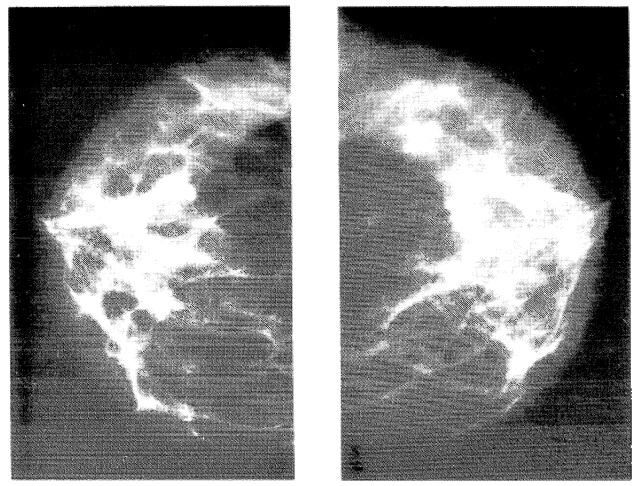


Figure 2b-2c. Mammogram of anterio-posterior radiograph, b right breast, c left breast.

pause activities of growth and regression occur on a large scale. In this period, there is also an involution of the parenchyma; only fibrous septal cells and some individual calcified glandular orifices remain, while the fatty tissue increases in the whole breast.

THE BREAST AS A HORMONE-DEPENDENT ORGAN

In accordance with Spona (333, 334), the mammary gland is under a multi-hormonal influence. During the first two years of a child's life, only a slight secretion of follicle-stimulating hormones (FSH) takes place via the hypothalamus-hypophysis axis. In the pre-puberty phase, there is a sudden increase in FSH secretion, stimulating the synthesis of estrogen in the ovaries, which, in turn, will act upon the mammary gland and encourage development of the milk ducts. In addition, oestrogen stimulates the development of connective tissue.

Following puberty, once the cycle is established, luteinizing hormone (LH) is produced in the hypophysis, in addition to FSH. Also, in addition to estrogen, progesterone is now being synthesized in the corpus luteum, which, in turn, stimulates the development of the acini. During this phase, there is a release of prolactin, a hormone formed in the anterior pituitary gland. While stimulating the female mammary gland and milk secretion, prolactin retards developmental activity in the ovary. During pregnancy, after the eighth week, prolactin is formed in increasing amounts, reaching at the time of birth a plasma concentration of 200 mg/l. The direct effect of prolactin on the mammary

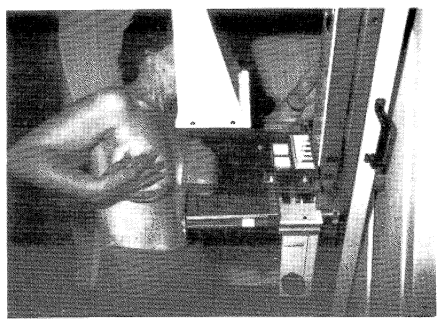


Figure 3a. Patient during oblique radiography.

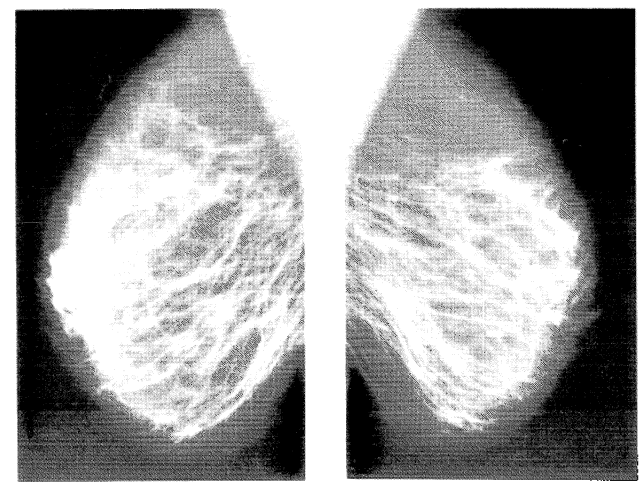


Figure 3b-3c. Mammogram of oblique radiography, b right breast c left breast.

gland is inhibited by the increased synthesis of estrogen and progesterone, since the sensitivity of the acini towards prolactin is inhibited by a vigorous estrogen and/or gestagen secretion of the placenta. After parturition, inhibition of prolactin by estrogen and gestagen ceases. Prolactin is now permitted to act fully upon the mammary gland, encouraging the synthesis of milk and milk proteins in the acini.

Estrogen, gestagen and prolactin represent only a fraction of the hormones acting upon the mamma. There is another series of hormones which also influences its development. In order to understand the effect correctly, it is necessary to consider the multi-hormonal event as a whole.

Hormone	*Effect*
estrogen	ductal, lobuloalveolar structure of mamma
gestagen	acinus-formation, circumtubular connective tissues
growth hormone	stimulation of the duct system and the terminals
adrenocorticotropic hormone	lobuloalveolar differentiation
adrenocortical hormone	increases growth
parathormone	micro-calcification, hypercalcemia
testosterone	alveolar proliferation
thyroxin	proliferation of the mamma
insulin	differentiation and proliferation

Multi-hormonal influence on the breast according to Spona (333, 334)

Changes caused in the breast by menstrual cycles and pregnancy became apparent through changes in the size of the mammary gland and its infrastructure.

We have performed 362 mammograms on 214 women, age 14 to 43 who had been admitted for abortions or delivery. The first mammograms were performed from 4 to 13 days after delivery. Among the 214 women were 21 with interruption cases and 193 with a regular pregnancy (lactation cases). Mammograms were performed in more than half of all these women.

In general, it was found that already in the early stages of pregnancy or at the time of lactation there was a noticeable increase in the size of the whole breast and a distinct solidification of the structures. During pregnancy, the breast is characterized by first, wave-like border contours in the subcutaneous region, a condition which, in part, is caused by tangentially affected blood vessels and secondly, by brightness zones in striped or oval contours of the central portion.

In 90 % of all cases, a normalization of the mammary structure could be observed at the three months in some cases, and in all cases, five months after parturition.

MAMMOGRAPHY

Definition

The greatest challenge to mammography lies in the area of minimal carcinoma: the smaller the cancer, the fewer are the radiographic findings and the more uncertain is the true pathogenesis (164). Classical (historical) factors such as ages at menarche, menopause, or at first child, post-menopausal weight and high parity are not strong determinants of risk factors (45). See also: (23, 48, 171).

Mammography is the production of a radiograph of the breast, using preferably a dedicated mammographic unit suitable to record the morphological structure of the breast and to recognize microcalcifications or nonpalpable mass or architectural distortion.

Mammography remains the most effective, if not the only imaging method for detection of non-palpable breast cancer (28, 29, 30, 37, 60, 115, 243, 346, 57, 338, 88). The widespread use of mammography has led to the detection of a substantial number of *in situ* or noninvasive mammary carcinomas (296). Risks versus benefits of mammography was questioned in 1976 (16), but the risks have declined remarkably since the earlier days due to new dedicated mammographic units. Mammograms are characterized by exposing breast parenchyma to cranio-caudal and medio-lateral irradiation, proper lighting and contrasting, a lack of grain-induced loss of focus, a reduction of blur and a clear film carrier, and a lack of artifacts on the film (22). The decrease of the radiation risk to the mammary gland and the adjacent tissues can be achieved by improvement of the mammographic equipment, strict selection of women for X-ray examination, and increase of the intervals between examinations (294, 261).

Risk factors

Assessment of the hypothetical risk from mammography was first suggested by Bailar in 1976 (16). The question was raised after the observance of excess incidence of breast cancer in several groups of women exposed to high doses of radiation in Hiroshima and Nagasaki atomic bomb explosion. This also was observed in Nova Scotia and Massachusetts in patients who had multiple chest fluoroscopies during pneumothorax treatment for tuberculosis. Similar observation was made in women who were treated with radiotherapy for postpartum mastitis. Swedish women who had radiation therapy for various benign breast conditions and female radium dial workers who ingested radioactive material from paint brushes which they moistered with their tongues.

Whether very low doses of radiation such as current mammographic techniques can cause cancer is unknown. The risk if exists is so small that it has never been observed.

In several respects radiation induced breast cancer in human resemble to animal models. All contain a linear portion which rises with dose above 100 rads. No excess cancer was reported among sanatoria patients in Massachusetts receiving a mean dose of 35 rads or in patients in Toronto exposed to 17 rads. The Japanese women were a larger group and many of them received doses below 100 rads. The highest possible baseline increase was about 25 cases per 100,000 patient years.

For all human cancers in general the BEIR III Committee (32) reports has used a combined linear-quadratic model in which risk is intermediate between a linear and a pure quadratic response. Radiogenic breast cancers in humans do

not appear until a minimum of 10 years after exposure. For younger women latent period is 15 to 20 years. For the years after this latent period, a risk estimate of 7.5 excess cancers per million women per year per rad has been made for the three main groups of western women studied.

Sensitivity is age related. This estimation was derived from populations consisting largely of persons less than 30 years of age.

In Nova Scotia sanatoria patients incidence was minimum in the 30 to 60+ age group; it was highest between 20 to 29 age group. In atomic bomb survivors risk was highest between the ages 10 and 19. Several theories have been advanced to explain why radiation risk depends on age. Moolgavkar (257) postulates that breast cancer evolves in two stages and that the effect of radiation is to act on the second step: to transform intermediate cells to tumor cells. Korenman (208) has suggested that susceptibility to radiation depends on the ratio of estrogen to progesterone in the blood.

In conclusion since there is not a single case in the literature that was reported (in spite of radiation dose of 8 rads in the earlier conventional mammograms with 3 views of each breast taken) it was decided that benefits outdo the risk.

In the United States by recommendations of the American Cancer Society every woman should have a baseline mammogram somewhere between ages 35 and 40 for future reference (242). In the high risk women, yearly examinations should be strongly urged and should be mandatory (246). In 227 women who had mammography malignancy was seen in 14/18 malignant tumors and 4/18 benign tumors and benign changes in 181 benign tumors (340). Mammography performed yearly on postoperative cancer patients (whether had mastectomy or lumpectomy) to detect early, noninvasive recurrences in the same or opposite breast and to improve survival rates (283). Breast carcinoma *in situ* is a nonpalpable but potentially curable lesion it only could be detected by mammography (348). High proportions of breast cancers detected by mammography were noninvasive or locally invasive types (less than 1 cm in size) and a substantial number of cancers were detected in Screening Centres (28).

The American Cancer Society and the American College of Radiology also encourage breast self-examination, since 90% of breast cancers are detected by the woman herself; the clinical breast examination and mammography together (94, 95) gives the highest diagnostic yield. Women at high risk for breast cancer who had mammography at first screening, the detection rate was higher than that among general subjects examined in Miyagi Prefecture, Japan (1, 2). Recent publications have shown clearly that screening by mammography is the only method of reducing breast cancer mortality substantially (118, 198, 35, 298, 219). Mammography is also performed in the follow-up of the patients who have the carcinoma of the cervix or uterus. (74). Mammographic parenchymal pattern have been examined as a guidance of determining woman at high risk of developing breast cancer by Wolfe and others; (52).

The parenchymal patterns are of value as predictors of hormone dependency and survival in breast cancer (166). The concept of minimal breast cancer as a stage of cancer that is 95% curable is a valid one, if minimal breast cancer is defined by strict parameters. Both 0.5 and 1.0 cm have been defined as upper limit of size (399). Carcinomas found by mammography were smaller and less likely associated with metastases to the axillary nodes than carcinomas of the breast which have been diagnosed by palpation during the same time period (279). Interval mammographic abnormalities detect significant pathologic changes in the breast and should be considered a major indication for breast biopsy (396, 397). Screening for breast cancer at an early age should be reserved for those women with high-risk factors (416).

The American Cancer Society recommends annual screening mammography for asymptomatic women over 50 years old. (The risk increases with age). Annual screenings in the United States recently made a great progress also due to efforts made due to reduce cost (as low as 15 dollars), toward educating physicians and patients regarding its value and toward lowering its radiation risks (25, 36, 173).

Improved understanding of breast cancer is necessary (278). At the beginning physicians disagreed with guidelines for mammography, because they feel the cost of the test and radiation exposure associated with its use argue against patients being tested annually, or being tested at all in the absence of symptoms (Survey of physicians' attitudes, 1985 (349)). Diseases suitable for screening are those leading to serious morbidity and high mortality, those with a prolonged preclinical phase in their natural course, and those for which effective therapy is available following early diagnosis (179). It has already been convincingly demonstrated that properly performed mammograms interpreted by a well-trained mammographer can detect breast cancer before it has grown to a stage at which it is incurable (350). Improved survival rate of breast cancer patients in Sweden was apparent in all age groups, but it was of lower magnitude among women younger than 45 years old (5). A similar development was seen after the first 7 years of mammographic screening in Kopparberg, Sweden (351); for Malmo, Sweden, see Andersson (11); Fagerberg (86). The results to the end of 1984 show a 31% reduction in mortality from breast cancer and a 25% reduction in the rate of stage II or more advanced breast cancers in the group invited to screening (350). Mammography is a determining factor in early diagnosis, and at 5 to 10 years contributes significantly to improved 5- and 10-year survival rates (287). In a Canadian study a total of 23,101 women underwent mammography; in 139, breast cancer was detected at first screening; in 20, less than 12 months after first screening; or/and in 47, at second screening (17, 18). From the Edinburgh Breast Screening Project was concluded: Qualitative histopathology may provide a better measure than standard quantitative judgments of size and lymph node status to compare the varieties of cancer detected by screening programs and to understand the biology of the disease (10). Dutch and Swedish trials suggest that the most effective screening strategy may be annual mammographic and clinical examinations for women aged 40–49 years and biennial examinations thereafter (259). After the use of the first experiences with the stereotactic diagnostic device "Mammotest" mammographic follow-up did not yield any false negative findings to date (68). In a rural area near Florence a population-based screening program for breast cancer was started in 1970, offering a mammography test every 2.5 years to all women between 40 and 70 years of age, in which no significant

protective effect was seen in women below the age of 50 years (274, 275). Mammography proved more reliable than clinical examination in evaluating the degree of tumor regression (368). Valid mass screening for breast cancer was also carried out in 13 cities in Osaka Prefecture during the last 15 years (360). Technological advance has made remarkable improvements in image quality in the field of X-ray mammography which ensure to successful management of breast cancer in Japan (6). In general, the breasts themselves of Japanese women are small because their breast fatty tissues are not so developed as those of Caucasian women. Therefore, conventional mammogram tends to show a very dense breast with difficulty in detecting a mass. A compression spot by a small cone may overcome the poor contrast in those women (269). Variation in population groups responding differently to versions of diagnosis and treatment of neoplastic diseases, as well as others may be overcome by better elaboration of the intraspecies comparison. In 50 Chinese patients who had mammograms accuracy in diagnosis of benign breast disease was only 12% and for all breast lesions was 32%. This low diagnostic rate is due to the technical difficulties in examining the small breast by mammography (7). See also (146, 217, 241); Some thing applies to young breast with dense appearance. As a rule carcinoma in women less than 40 years old represents only 7.4% of the total number of carcinomas (55).

Mammography is also performed in inflammatory diseases when there is a palpable mass such as abscess in general and in acute lactation mastitis (343). More aggressive monitoring of the remaining breast by frequent clinical examination, mammography, and selected contralateral biopsy (especially in lobular carcinoma by doing tumor image biopsy) appears to have increased the early detection rate of second breast cancers in patients under observation (311). Through specimen radiography the greatest accuracy of removal was found for lesions which proved to be malignant. (87). Pneumocystography after aspiration may be beneficial in detecting cancers deriving from breast cysts (299).

Method

On the basis of experience gained over the past 40 years in the clinical-physical investigation of the breast, the following procedure has proven successful:

1. In women up to age 35 because of the density of the structure and the fact that mammary (breast) carcinoma is extremely rare in this age group, yearly physical examinations is not performed and mammography should not be performed unless there is a palpable mass. If secretion takes place, and this is not unusual for this group, then determination of the hormonal state (prolactin and stress tests) together exfoliative cytology, should be carried out and evaluated. If there is a bloody nipple discharge then galactography (ductography) is indicated.

2. For women age 35 to 40, it is necessary in any case to determine the cause of breast complaints such as pain, thickening, hardening of lumps or secretion. Clinical examination is followed by mammography using one of the standard techniques (Film-screen mammography or xeromammography (Figures 1, 2, 3) in at least two planes. The oblique exposure including the axillary extensions, is of prime importance. More than 50% of all mammary carcinomas are located in the upper-outer quadrants since the glandular elements are more abundant there.

If a lump, or mass, is present on the mammogram, then a differentiation between a solid mass and a cyst is possible only by additional sonography and/or fine-needle biopsy. When the breasts are very dense but lumpy the additional use of ultrasonography is recommended. If secretion is present, then the color of the secretion is important (serous and amber-colored secretion indicate a carcinoma), bloody secretions tend to indicate rather a papilloma, Considerations of the hormone status and of exfoliative cytology are followed by galactography (ductography).

3. In the case of women past 40 years of age, consideration of possible mammary carcinoma takes precedence. The testing process is similar to that used for younger women. A clinically occult diagnosis (small mass, architectural distortion or microcalcification) is then made: needle localization is performed prior to surgery under sterile conditions. Then the patient with the needle in, together with localization images, is referred to the surgeon.

The steps of a program devoted to dose and quality in mammography are: (a) collection of the working parameters in each unit, (b) dose and image quality evaluation, (c) communication of the results and suggestion for corrective actions (286). Radiation protection in mammography is restricted to the carcinogenic side effect of radiation exposure in mass screening of asymptomatic women (282). The potential usefulness of the ultra-high-strip-density grids was demonstrated in a comparison of mammograms (53).

In medicine, only those methods for examination a patient will remain in use whose results can be reproduced and compared without difficulty, and which, without special instruments, and without excessive expenditures of time and money can be performed routinely by medical personel with time-limited training. Of the physical examination methods of the breast, which must always be preceded by a history, mammography has established itself because of the numerous advantages it offers for a complete medical diagnosis.

The use of all applicable diagnostic methods is justified by the need for recognition of a mammary carcinoma as early as possible, while there are still no clinical symptoms, and to confirm or disprove the finding of the existence of a carcinoma either by the patient herself or by the examining physician. For an accurate mammogram the following optimum voltages were proposed: 28–29 kv for thin breasts, 29–30 kv for average breasts and 33–35 kv for thick ones, (157). Although the number of screening programs for breast cancer has increased in the past decade, real progress has been surprisingly slow and the issues in breast cancer screening have proved to be subtle and complex (19, 20, 43). Ultra-high-strip density grids may be useful in selected cases such as in the performance of adjunctive whole-breast or spot film magnifications and in the radiological examination of surgical specimens (46). The use of a fine microfocus of 0.3 mm for a better study of all fine calcifications is recommended (51). The radiation dose is the same with stationary and moving mammographic grids differences in the design of the two devices may lead to a personal preference in deciding which grid to use (64, 65, 100). Development of xeromammography was the single and most valuable adjunct to mammography (414) and its continued use proves

that it has a superior diagnostic accuracy over conventional film mammography (97, 106, 372).

Direct contact B-scan sonomammography is of considerable value in the radiologically dense breast (145, 149, 155, 156). MRI is presented as a new possible tool for an improved evaluation of breasts with silicone implants (158). MR imaging of breast using Gd-DPTA may be helpful for the evaluation of dense breasts (159, 160, 161, 162). Specimen radiography is a very useful tool in the accurate surgical excision of breast cancer (170). A series of 100 consecutive occult mammographic abnormalities were localized with a curved-end retractable wire system. A true-positive rate of 24% and a failure rate of 4% for initial excision were found (176, 177). The ideal imaging system will have the optimum beam energies of 19 and 68 keV (193, 173); see also (192, 198, 202, 205, 211). Occult malignant lesions are most often found by breast X-rays (mammography or xeromammography) which are done for: the survey of high-risk asymptomatic women; contralateral breast studies: symptomatic breasts without palpable findings: nipple discharge; larger pendulous breasts; and multinodular breasts. Biopsies for occult lesions, based on radiographic findings, are recommended for: suspicious calcifications; stellate-shaped masses; breast masses with ill-defined borders or nodular contours; dominant masses; and areas of increased density or distorted breast architecture (228, 239). Needle localization of nonpalpable breast masses is a safe, rapid, and accurate method for localizing small, potentially highly curable breast cancers with minimal sacrifice of breast tissue (34). See also Zwicker (424). The drill needle biopsy method can be performed easily and accurately on masses less than 2 cm, or even 10 mm or less masses (419). A false-negative result of a mammogram was particularly likely to be obtained in young premenopausal women with small tumors. A negative mammogram should not delay a biopsy in a patient and is dangerous in that it has a significant false-negative rate which carries with it the serious risk of postponing a biopsy (374). For less common questions see (253, 266, 267, 268, 307, 367, 370).

A review of surgical specimens of cancer patients demonstrated at 27% incidence of residual disease at the biopsy side (243). The increased number of mammograms has brought into focus the necessity for radiation dose reduction. Breast thickness and incident half-value layer (HVL) are sufficient to characterize the normalized (mrad.incident roentgen) breast dose (262, 326). The use of film–screen combination or low dose xeromammography techniques reduce the doses to a safe level (320). A significant preclinical radiologic case of abnormalities of the breast is given in borderline lesions from the point of view of histopathology such as tubular carcinomas which originally were missed and called sclerosing adenosis until unproved mammographic methods showed as small as 3 mm lesions (357).

What advantages have these additional physical examination methods in breast cancer diagnosis brought us?

1. The purely clinical breast cancer diagnosis is burdened with an error margin of up to 30% (66, 221).

2. The histological frozen biopsy examination is subject to an error margin of up to 3% (66).

3. The clinico-radiological examination carries an error margin of from 5% to 10%, even after the addition of fine needle aspiration biobsy since the middle of the 1960's with an error margin of below 5%. Thus the total predicative value of the triple diagnosis (clinical, mammographical, cytological) approaches that of the frozen section examination (366).

We tend to forget too often that the additional physical examination methods for the breast have provided us with deeper insight into the hormonal conditions of the mammary gland and into the biological characteristics of benign or conditionally benign disorders. It must be stressed once again that the benign disorders of the mammary gland are four-to six-times as frequent as the malignant.
See also: (234, 235, 329, 331, 336).

BENIGN MASSES

Fibroadenomas

The bilogical behavior of fibroadenoma, next to mammary dysplasia to be seen as the most frequent disease in women, has also been elucidated by mammography. Our knowledge of this tumor, which in most cases can be diagnosed correctly even without sample excision, has advanced to the point where relatively frequently we can safely wait. This is true especially for fibroadenomas, which are accompanied by plaque-shaped calcifications, the case with every fourth fibroadenoma.

Thus, we know today, thanks to the additional physical examinations, that a portion of the adenomas in young girls and young women up to age 25 is hormonally qualified and that often they will regress spontaneously. Solitary cysts, which as a rule appear from the 35th year on, can be diagnosed easily through mammography or sonography in conjunction with fine-needle aspiration. Eighty percent of such solitary cysts will regress completely, following aspiration of the liquid content, so that the additional physical examination also assumes therapeutic importance in the case of cyst diagnosis. Mammography alone or in combination with sonography constitute ideal methods of examination for the further medical control of patients with cystic breasts.

Papillomas

The enormous progress brought about by the additional physical examination methods can be demonstrated best in the case of bloody nipple discharge. Prior to the advent of mammography or galactography, in such situations unnecessary surgery or even mastectomy was performed. Thanks to the latter method, the papillomas, which frequently are the cause for bloody discharge, can be diagnosed easily and removed by a limited surgical intervention (ductectomy).

RISK HISTORIC

Historical review of mammography (214, 345)

The risk from mammography has been stated by Upton *et al.* (362) as "the risk is 3.5–7.5 cases per million age 35 yr to older per year per rad to both breasts from the 10th year throughout the remainder of life (80, 421, 423).

Phase 1 (1913–1930)

First description of microcalcification and of the radiating ramifications in the breast (297). – First x-ray of the female breast *in vivo* (199) – Stereoscopic x-ray screening of the mamma (384).

Phase 2 (1931–1960)

First monographs on mammography by Leborgne (225), and by Ingleby and Gershon-Cohen (181) – First observations of the hormonally and constitutionally influenced changes in the mamma by Seabold (309); Gershon-Cohen and Strickler (111); Bayer (26); Fochem and Narik (93) – Introduction (installation) of gaseous contrast methods into the breast and its surroundings to intensify contrast by Baraldi (21), Hicken *et al.* (62), Bianchi (33), Gros *et al.* (142): precursors of present-day mammography technology; low voltage and foil-less film by Leborgne (223).

Phase 3 (since 1960)

Isodense-technique (immersion of the breast in alcohol solution) by Dobretsberger (72) – Further monographs on mammography by Buttenberg and Werner (47), Gros (139), Egan (76), Baclesse and Willemin (13), Witten (400), Gershon-Cohen (107), Hoeffken and Lanyi (168), Frischbier and Lohbeck (101) – Discovery of the characteristic autoradiation of molybdenum for mammography-technology and beginning of the industrial development of mammography-apparatus by Gros (140) – Introduction of exposure into mammography by Hoeffken *et al.* (167). – First publications on screening tests together with mammography by Griesbach *et al.* (135), Shapiro *et al.* (315) and Strax *et al.*(346) – The first publication on radiation load during mammography by Ewton *et al.* (84), Stanton *et al.* (337) and Siler *et al* (324). First communications on the use of dose-reducing film-foil combinations in mammography by Friedrich (98, 99).

Twenty years ago, Wolfe (401) began the systematic clinical introduction of xeroradiography. This process, instead of an x-ray film, uses an electrostatically charged, selenium-coated aluminum plate as image carrier. As the breast is exposed to radiation, locally differing discharge patterns are shown on the selenium plate, reflecting the passage of the differently weakened x-rays through the tissues. This results in a latent, electrostatic image of the breast, which is fixed through a special development process. A choice is available between a positive and a negative development process. The final xeroradiogram consists of a blue-white paper image. The process requires tube voltages of 40–48 kv. The best results are obtained with a tungsten anode. See also: 175, 192, 193, 198, 202, 205, 212).

ad Phase 1 (1913–1970)

It was during that time that the first, promising treatment results following radical mamma-ablatio with removal of the axillary lymph nodes became established, based on recommendations by Halstead. To this must be added the first subdivision of mamma carcinoma into three clinical stages by Steinthal (341) (Gregl 126, 127, 128, 129). In stages I and 2 axillary surgical clean-up was demanded categorically. From the surgical point of view, efforts were made to find ways to scrutinize the radicality of surgical interventions.

Salomon (297) of the Surgical Clinic of Berlin University had the idea of x-raying the amputated breast following mamma-ablatio and in this way to subject the radicality of surgical procedure to reexamination. In his publication he described for the first time the x-ray evaluations, including microcalcification in carcinomas, with such detail that even today, there is hardly need to add anything basically new to his observations. By chance, and without wanting it, Salomon became the founder of present-day mammography - 20 years later, someone remembered his publication. Even at that time, he had already described three different types of cancer: (1) node-like infiltrations, (2) diffuse infiltrations, and (3) more or less encapsulated cancers which could be moved about within the mammary gland. In some cases, he describes in detail the numerous radiating branches originating at the periphery of the carcinoma. They have a length of 1–2 cm and are from 2–5 mm wide. In another case, he describes small discrete calcium accretions in the centre of a carcinoma the size of an apple.

It was this microcalcification as described by Salomon for the first time which turned out to be the show-piece in radiological examination of the mammary gland and which opened the way to prognosis. The dedicated mammographic units, xeromammography and film-screen methods development of mammography to date serves only one purpose - optimization of the resolution capability of the film and improvement in contrast with special consideration for the recognition of even the finest microcalcifications and the reduction of x-ray dose. During this period the first monographs on mammography appeared: Leborgne from 1953 and Ingleby and Gershon-Cohen from 1960. It must be borne in mind, however, that during that time mammography was restricted to a relatively few centres and to experienced specialists. During this time, too, the first observations were published on the physiologically and constitutionally related changes in the breasts, as revealed on the mammograms. It was possible to demonstrate that the structure of the mammary gland, as shown on the mammograms, reacts as a function of the cycle (27, 93, 111. 309). Technology became more refined, but the mammogram remained relatively poor in contrast. To meet this deficiency, attempts were made to inject contrasting media into the breast. Based on the pneumo-mammography developed by Baraldi (21), Hicken *et al.* (163) developed two methods for the injection of a contrasting medium into the breast: (1) injection into the milk ducts, with subsequent stereoscopic examination, and (2) instillation of CO_2) into the retro- or premammary space. Bianchi (33) instilled pure oxygen into the connective tissue, in amounts ranging from a few to several hundred cubic centimeters. All these "additional methods" did not prevail and are mentioned only for their historical interest, to show that many methods were investigated during that time to amplify the inadequate contrast hitherto of mammograms.

During this phase, there appeared the reports of Leborgne (222, 223, 224) who was the first to work with low voltages

up to 30 kv and foilless films. He prepared his films on two planes, slightly compressing the breast with the imaging tube. The first step towards improving mammography technology had been taken.

Ad Phase 3 (1960–1980)

This phase must be viewed as the most eventful from the standpoint of technical development in the clinical application of mammography in individual practices as well as in the early diagnostic possibilities. The road to progress led from the early tungsten tube by way of the stationary molybdenum anode to the revolving molybdenum anode with high efficiency and two focal point sizes. The first important step was taken by Gros (137, 138, 139, 140) in Strasbourg. It was he who recognized the advantages of the characteristic autoradiation of molybdenum when applied to mammography. Gros and his staff were involved in the industrial development of the first dedicated mammographic unit, at the time still equipped with a stationary molybdenum anode.

Another important step forward was the introduction of automated illumination by Hoeffken, Heuss, and Roedel in 1970 (167). All the mammographic equipment being marketed today contain such illumination. The special characteristic of the mammographic equipment lies in the fact that the ionization chamber is situated behind the film, thus making it impossible to switch to the usual film cassettes. All mammographic equipments contain a tube voltage of from 20 to 40 kv, because this voltage delivers high contrast differences, more exactly, delivers the desired high image contrasts between fat and water-equivalent soft tissues.

At the beginning of the '60s first publications on radiation load during mammography appeared (324, 337). From the middle of the '60s to the beginning of the '70s there were further communications primarily from the Anglo-American literature on how to measure exposures during mammography. The reports spoke of a dose of up to 20 rad/exposure on the skin and a dose in the center of the breast, after 3 exposures, of up to 10 rad.

The first communications on the use of dose-saving film-foil combinations for a reduction of the dosis were published by Price *et al.* in 1970 (280) and in 1973 by Ostrum *et al.* (271).

A comparison of the quality of the images obtained with the various film systems showed that the use of screen caused a certain loss in resolution capacity but that this was compensated in part through an increase in contrast. Thus, the form of the microcalcifications was more often better but on the other hand, could be recognized better on the film-screen systems because of the greater contrast. Through use of film-screen combinations, a 10-fold reduction in the original radiation dose could be achieved. These systems will remain, at least for the near future, the image-recording system possible in mammography. They offer the greatest advantage for technological development with respect to the relationship of those to quality. The classical material test film without screen can no longer be recommended today as the image-recording system for a large-scale screening program, on the grounds of a dose-limiting system of equal quality. Magnification mammography, because of the impracticability involved, cannot lay claim for use as a routine technology, but, in doubtful cases, it can contribute to the improvement of recognition of microcalcifications and to further differentiation of border contours of mammary tumors.

Beginning with the concept that the scattered portion, which reduced the desired recognition-detail considerably, amounts on the average to 44% of the total radiation during mammography, the German radiologist, Friedrich of Berlin, had shown already in his first publication from the year 1975 the need for consideration of a scatter ray grid; in 1978 he demonstrated his grid-film-foil system (98,99). The introduction of the special soft-ray grid in connection with a double-layered material film and a fine-delineation foil represents a decisive advance toward the optimization of mammography.

The mammary gland in the mammogram

Every x-ray image of the mammary gland reflects a certain mammary gland size and structure, which is dependent on age, constitution, and in part, on the prevailing hormonal state of the patient. Therefore, the description of a mammographical finding must also include the influence of size and structure of the mamma.

The size of the breast corresponds to the distance in centimeters measured on the cranio-caudal picture between film border and nipple.

Based on this scheme, in Goettingen the following distribution of breast size has proved the most useful:

1. small breast (up to 5.5 cm)
2. medium breast (6–10 cm)
3. large, super-large breast more than 10 cm).

Asymmetry of the mammary gland occurs rather frequently. In 10,000 women, different size breasts were found in 7.06% ranging from a barely visible discrete hypoplasia to aplasia. When these patients complain about pain in the breast, then it is generally the larger one; this is important to know, on the one hand to calm the patient and on the other, to eliminate possible additional examinations. Of course, these patients must be treated.

Normal mammary structure (Figs. 1–9)

In 1976, Wolfe divided the types of breast parenchyma according to findings of 7,214 patients on one hand, and of 5,284 women over the age of 30 on the other. The lowest risk group had parenchyma primarily of fat (N1). At progressively higher risk were the groups showing prominent ducts in the anterior portion up to one-fourth the breast volume (P1); those in which the duct pattern occupied over one-fourth the volume (P2); and, finally, those with severe involvement plus dysplasia (Dy) (402, 403, 405, 406. 408, 409, 410, 411). High levels of agreement, perhaps the widest one, regarding mammography were found about parenchymal pattern of the gland (42), as proven in the following selected publications: Antalik (12); de Waard (69); Gravelle (125); Grove (143, 144); Massa (247); Whitehead (391, 392); Whitehouse (393).

The structure of the breast in the mammogram depends primarily on the age and constitution of the patient. The hormonal influences have already been discussed in another paragraph (130). The breast structure must be taken into consideration in any diagnosis. Attempts have been made in many places to classify the image of the female mammary gland as it appears in the mammogram in some suitable way in order, in this way, to contribute through statistics to questions of importance in medical practice. Some authors have classified the types with relation to age; others, to age and morphological composition; others, on the other hand, only with reference to morphology (relationship among fatty, glandular, and connective tissues).

Egan (75), Ingleby & Gershon-Cohen (181) classify the mammograms with reference to age, into 5 types: 1. the breast during puberty (here we have a homogeneous, dense parenchyma, separated from the skin by a small fatty strip); 2. the breast of the young nullipara (this image is characterized by some fat deposits within the glandular tissue); 3. the breast of the adolescent and of young women (the glandular tissue, located within the fatty tissue, is fully developed – this breast type also appears in old nullipara, but also in women who have nursed for a long time); 4. the breast prior to and during menopause (slowly progressing involution of the glandular tissue); and 5. the atrophic breast (this breast form represents a continuation of type 4 – the fatty tissue has now increased even more and the trabeculae are often closely together). Occasionally, they are also shown as linearly arranged strands). Witten (400), in his scheme for classification, starts with the structure; he divides the mamma shown in mammography into four types, with a steadily decreasing density in the structure corresponding to types 1 to 4: (1) diffusely glandular, (2) predominantly glandular, (3) predominantly fatty: and (4) fatty.

The classification of the mammographic patterns as developed by French authors, and which is predominant in European literature, is based essentially on the relationship among the individual morphological structures. Gros (141) divided the mammary gland into five types: (1) empty mammo (sein clair) – this type predominated in the postmenopausal stage but also in women who did much breastfeeding; (2) connective-tissue-rich mamma (sein trabeculaire) – it shows enlarged septs between the individual lobes; (3) small-spotted mamma (sein trachete); small, shadows are visible, distinctly separated from each other; (4) cloudy mamma (sein naugeux) – confluent shadows appear throughout the whole body of the gland, and (5) dense-shadow mamma (sein opaque). These are relatively dense and permit only limited penetration by radiation.

In addition, we have a classification of the normal breast structure, independent of the actual morphological image based on purely radiological criteria which satisfy the requirements of a clinical-statistical comparison of the patient-pool on the one hand and daily medical practice on the other (130).

Among premeopausal women, 46.1% had taken an oral contraceptive ("the pill") in the past, and 9.5% were still taking the pill. These patients showed a decreased incidence of P2 patterns and an increased incidence of N1 patterns (227). To compare the advantage of one-view vs two-view mammography screening, films were reviewed for 2500 consecutive asymptomatic women undergoing baseline mammography. Two-view interpretations not only identified more cancers than one-view readings (27 vs 25), they also required fewer additional mammograms to evaluate potential abnormalities (179 vs 642, 7% vs 26%) (318, 320, 321). The discovery of a large number of subclinical carcinomas, the favorable implications for preservation of the breast and the survival justify the indications for surgical biopsy on the discovery of an isolated mammographic abnormality (71). *In situ* cancer was always associated with microcalcification (226).
See also: (49, 332).

I. Dense breast (Figs 4–6)
(diffusely glandular breast, immature breast, sein opaque) densely shrunk, densely stringy, densely cystic, densely atrophic, disk breast.

II. atrophic breast (Figs 9–10)
(predominantly fatty breast; sein clair)
atrophically shrunk, atrophically stringy

III. shrunk – stringy breast (Figs 7–8)
(fibrous, involutional breast, sein trabeculaire)

IV. Cotton wool-like to spongy breast (Figs. 13–16)
(sein trachete)

Statistics

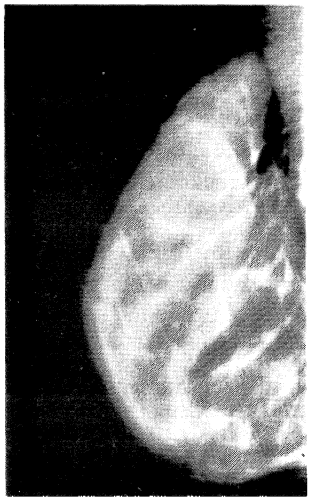
In women up to 30 years of age, predominantly 74.8% exhibit a breast rich in glands (primarily dense structure). Other structures can be found in this group up to 25%. The relatively high number of cases with atrophic structures in young women can be related primarily to the fat-enriched breasts of adipose women. Women past 50 years exhibit an atrophically-stringy and atrophically shrunk structure more frequently than one purely atrophic. A wholly dense structure is found in women prior to menopause in only 30% the cases; after that, it appears in only about 10% of all patients examined.

Due to age, the normal mamma structure undergoes a tendency towards regression steadily directed towards the nipple. With increasing age the glandular, active portion of the mamma regresses with varying speed spreading toward the nipple. Such retro-mammillary parenchymal residuals can simulate a process of a different kind, especially when they occur only on one side.
Of 3,000 women over 65 in the patient pool at Goettingen who were mammographically examined 2.8% exhibited unilateral and 12%, paired, retromammary localized, parenchymal residual structures of various appearance.

Figures 10 to 19 show such a regression-tendency of the parenchymal structures. The dense, elongated oval shadow in Figure 11 can resemble equally well a mastitis, a cyst or an abscess, an adenoma of the nipple, or a colloid carcinoma. Figure 12 shows the final phase of such a tendency spreading toward the nipple.

The retromammary region, from the standpoint of patient and examining physician alike, belongs to the clinically mute regions of the mamma (132).

Thus, in patients with large breasts retromammary



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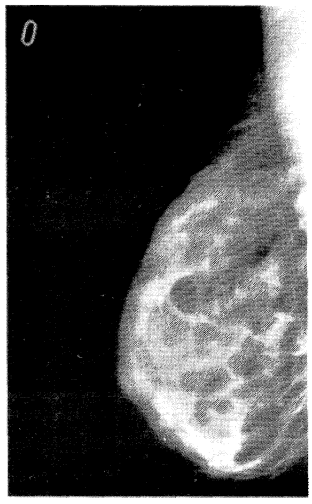

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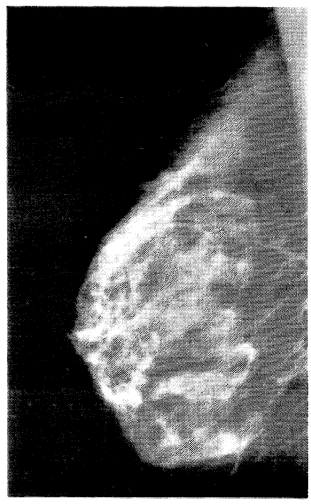

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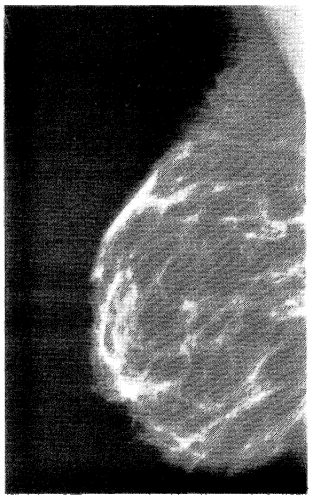

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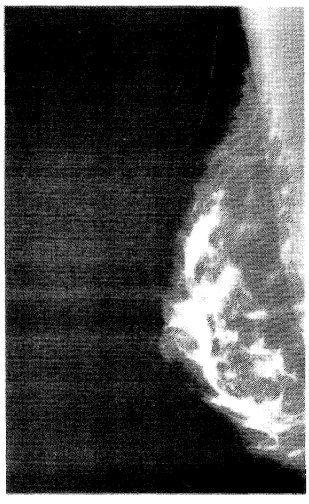

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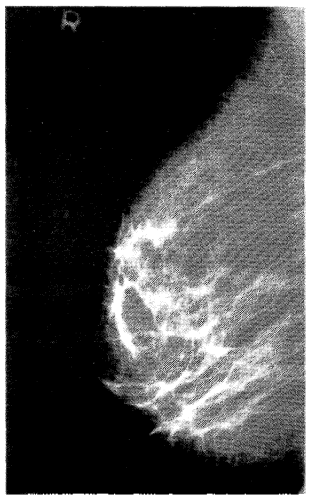

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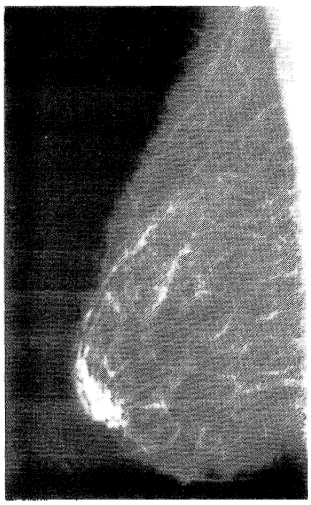

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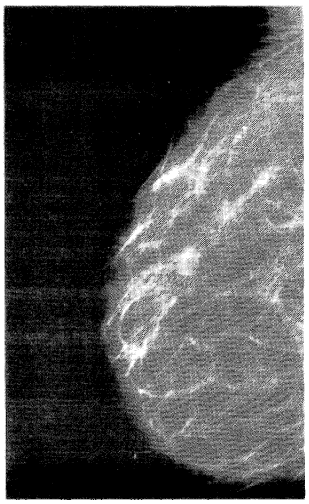

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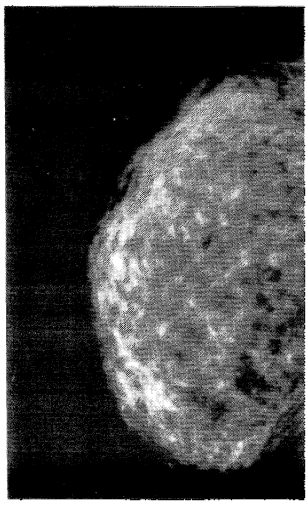

12

tumors up to 10% of breast cancers can be diagnosed only with xeromammography. Luckily, benign processes, such as cysts occur much more frequently than malignant processes in retromammary lymph nodes. During evaluation of the retromammary region, xeromammography is the best method.

I. Age-related regression tendency of the mammary gland

The dense mamma structure (glandular, active portion of the breast gland) regresses with increasing age in direction of the nipple, even if with varying speed. Such retro-mammary parenchymal residuals, especially when they occur asymmetrically, can simulate a mass.

II. The most frequent pathological diagnoses in the retromammary region are of benign nature. These are usually not accompanied with a retraction of the nipple or of the areola. Rather, the nipple appears rather plump and the areola arched. In these benign cases an inflammatory process, an adenoma, a cyst, perhaps even an fibroadenoma, should be considered first. These observations reveal mammographically, in part a specific symptomatic; some of them, as in the case of a mastitis, can be diagnosed in a short time span of from 4 to 6 weeks following frequent mammographic control. Of the malignant diagnoses, only the rare colloidal carcinoma represents greater difficulties, because it seldom accompanied by a retraction of the nipple and it can resemble mammographically a cyst or an adenoma.

1. Non-puerperal mastitis – is frequently accompanied by a clinical symptomatic, but in a mastitis is not always obligatory. Thus, one can frequently find only a solid lump in a mastitis under treatment, which can also resemble a carcinoma. Short-term controls, together with thoroughgoing therapy lead in most cases to a correct diagnosis in about 4 to 6 weeks (132)

2. Typical abscess – mammographically, presents itself in the retromammary region as a two-layered, varyingly dense shadow. The strip of skin, occasionally as thick as a pencil, presents a shadow separating the abscess from the outside. The abscess itself, as a rule, is less dense and can be clearly distinguished from the thicker strip of skin. Larger abscesses, on the other hand, can cause difficulties on a differential-diagnostic basis since they are accompanied by a drawing-in of the nipple, unclear borders, and occasionally, by calcifications as well.

3. Cysts – extremely rare in the retromammary region.

Fibrocystic breast disease or mammary dytsplasia is a distinct clinical entity that requires yearly screening to reduce the incidence of breast surgical procedures, and to diminish the risk of advanced cancer (371).

Statistic

In the patient-pool cited, among the 680 cysts listed, in only 1.84% of the cases was a firm retromammary location. If a cyst is suspected, a follow-up should be taken using sonography or, as the case may be, fine-needle aspiration.

4. Lactating adenomas – do not demonstrate special mammographical symptoms and only through additional examination can be distinguished from a cyst or fibroadenoma or even colloidal carcinoma. They are never accompanied by a retraction of the nipple. Pleomorphic adenoma of the breast is a rare distinctive primary neoplasm and the prognosis comparable to the corresponding salivary gland neoplasms (420).

5. Colloid or medullary carcinomas – as a rule, represent a relatively smoothly bordered round mass independent of their localization, leading more to a protrusion or abnormal plumpness of the nipple rather than a flattening. Correct diagnosis can be made only on the basis of a biopsy.

The normal mammary structure can also be changed through various basic diseases and disorders, developing from an interference with the filtering and waste removal functions of the lymph One of the most common mass lesion seen on mammograms are axillary or intra- or retromammary lymph nodes usually with fat in their hilum and bean-shape appearance (79). These are thromboses of the large veins, heart insufficiencies, nephrotic syndrome, skin-necrosis after administration of anticoagulant drugs, axillary lymph adenopathy of various etiologies, mediastinal tumors, scleroderma, complications following irradiation and surgery, chronic mastopathies, fibrosis caused by the constant pressure of a brassiere, etc. From the clinical standpoint primarily two diseases must be mentioned, non-puerperal mastitis and the inflammatory carcinoma; because, for one thing, they occur frequently and furthermore usually accompany changes taking place in the normal mammary structure.

Non-puerperal mastitis is generally characterized by a limited skin thickening with a greater degree of thickening over the focus of inflammation, along with uniform tapering off towards the periphery. It is usually localized in the region of the nipple, so that the latter is involved in the thickened skin strip. Increased density and concealing of the structure of the breast do not, as a rule, extend over the whole breast. With the help of mammography in most cases an exact diagnosis of nonpuerperal mastitis was made (120). Acute post-partum mastitis may predispose a woman to later developing a benign breast tumor irrespective of whether or not radiotherapy was used to treat this condition (281).

Inflammatory carcinoma is a special form of cancer, marked by the lymphangetic dissemination of a highly un-

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Figure 4. 25-year-old woman – dense, glandular appearance of adenosis. The structure is typical for this age.

Figure 5. 34-year-old woman – less dense, more fatty structure; this structure also occurs frequently in this age.

Figure 6. 30-year-old woman – mainly glandular structure

Figure 7. 48-year-old parous woman with atrophic structure, typical for this age.

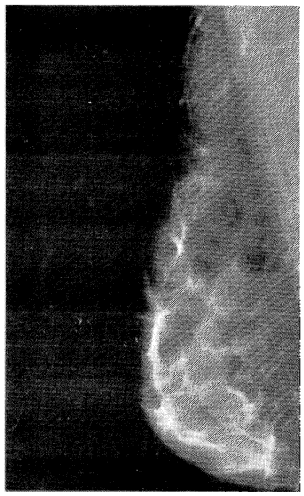
Figure 8. 52-year-old woman – strand-like – atrophic – cystic structure; occurs in this age relatively frequent.

Figure 9. 62-year-old nulliparous woman with prominent duct pattern (P_2).

Figure 10. 70-year-old multiparous woman – predominantly atrophic structure.

Figure 11. 57-year-old woman with subareolar duct extasia (P_1).

Figure 12. 58-year-old woman – diffuse cotton-pad-like appearance of adenosis which occurs equal in all age groups (Dy).



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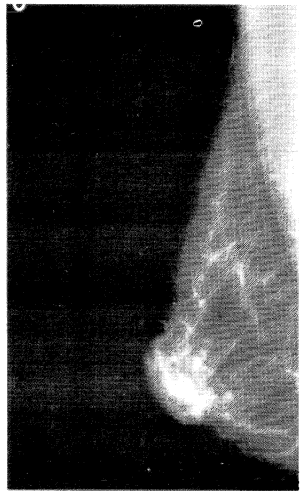

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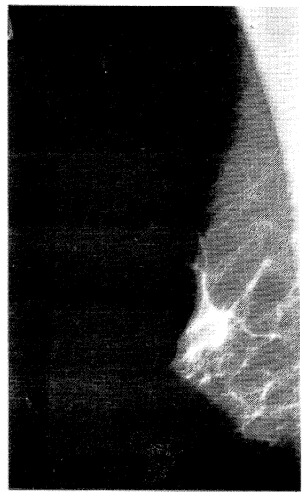

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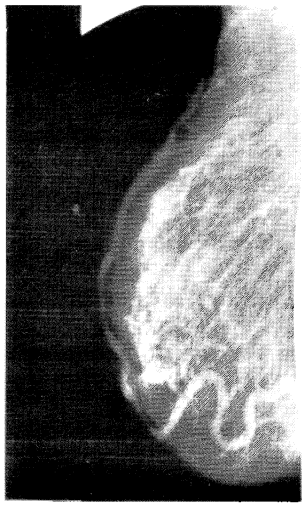

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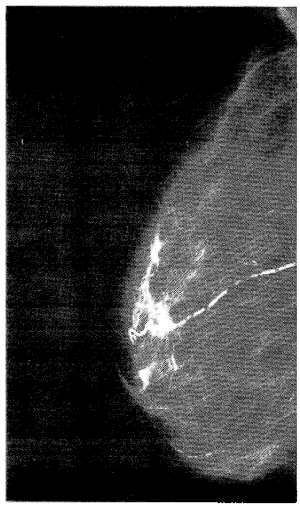

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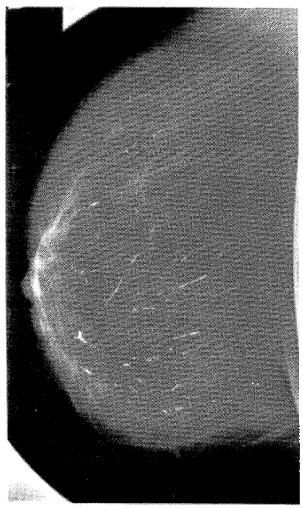

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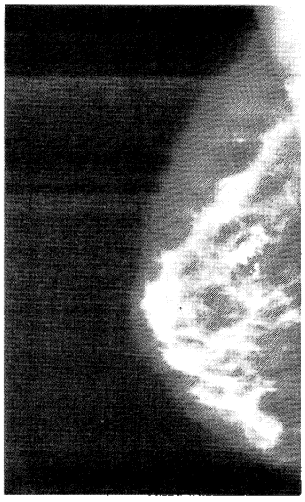

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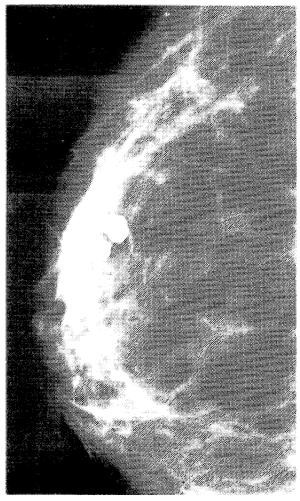

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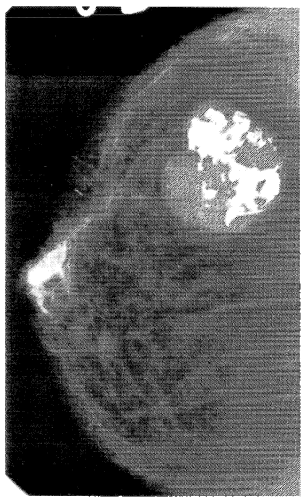

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differentiated carcinoma in the subepidermal connective tissue of the breast. On the mammogram it must be recognized by the following three criteria: 1. thickened skin (normally 2 mm could go up to 1 cm in thickness) sometimes extending over several mm, for the most part, across the whole breast; 2. diffusely increased density of the whole mammary gland structure in comparison with the other breast, and 3. linear increased opacities vertical or oblique to the skin surface along Cooper's ligaments. With verification of the diagnosis of mastitis or, as the case may be, of an inflammatory carcinoma a high degree of clinical symptomatics, but for the manifestation of the two diseases, mammography's contribution is of decisive importance. Rarely tuberculosis of the breast also could not be mammographically distinguished from breast cancer (182).

Before describing the benign and malignant processes revealed by mammography mention should be made of three findings, which can be noted either purely adventitiously or perceived as an accompanying symptom in the presence of pathological processes. These symptoms are: dilated broad veins (Fig. 13), arteriosclerosis (Fig. 14); and typical ductal calcifications (Fig. 15). Broad veins are those with a diameter larger than 2 mm. Some authors assign to the latter the importance of a direct cancer indicator when it appears in a diseased breast. In this author's experience it is also not rare in normal breasts, so it may be regarded as an adventitious observation.

The arteries, which are normally not visible on mammograms, can be seen when calcified. They then resemble dash like calcifications of the media. They are quite frequent in women past 65; arteriosclerosis occurs in one women out of 3 after age 80.

Ductal calcifications, in according with Egan (75), can appear in a number of forms: 1. as ring-shaped, ? rod like 2. as dense, intraductal calcifications, looking as if contrast media were present in the ducts; 3. as parallel linear calcifications in the milk-duct walls; and 4. as thin, linear, interrupted, needle-like calcifications, which also extend intraductally and therefore follow the course of the milk ducts (Fig. 150.

Following a discussion of the normal mammary structure and of the factors which can affect its appearance; size of the breast, and the most common adventitious observations in a mammogram, we may turn our attention to two of the most important pathological findings: the round focus or as the case may be, the round shadowed area, and calcium, at first considered independently of the biological characteristics.

Circular foci (ringlike calcifications)

Cancerous degeneration is rare and is observed only 0.5% to 1.5% of all cases. Calcification, which increases with increasing age, is reported in 15 to 30% of cases of fibroadenomas. Radiologically, a fibroadenoma makes the impression of a smoothly bordered, generally dense round mass, which, not so rarely, can occur also in multiple numbers in one or both breasts. Figures 32–34 show each a fibroadenoma-pea, or cherry-to-apple size.

The less frequent lipomas (Figure 30) or, the lipofibroadenoma adeno-lipomas (Figure 31) can be diagnosed radiologically rather easily. In the case of the lipoma, a structureless radiolucency may be observed, which, with respect to its surroundings, is delimited by a sharply defined border.

The large circular or oval with a rough structure like cotton wool wadding and distinct delimitation from their surrounding areas correspond generally to fibro-adeno-lipomas or fibro-adeno-myxomas; as a rule, they do not require further diagnostic examination. They are extirpated, since they lead to an asymmetry of the breast.

Cysts present homogeneously dense round or oval masses and they displaced the surrounding fat tissue. Therefore there is a radiolucent halo around them. Their delimitations can be made visible radiologically through aspiration and subsequent filling with air (pneumocystography). It is hoped that pneumocystography will make possible delimitation of intracystic carcinoma, which is extremely rare and occurs in only 0.5% of all cases.

The single cysts (Figure 35) regress completely in 80% of all cases following puncture, so that mammography and puncture in such cases also assume a therapeutic importance. Thus, these patients can be spared superfluous surgery.

Cytological examination of the aspirated fluid is important, its color also being important. In 20 single cysts with a purely bloody aspirate carcinoma was found in 6 cases (Gregl).

Other benign tumors, such as osteomas, hemangiomas, hamartomas, mesenchymomas, neurinomas, or infiltrates of malignant systemic diseases such as lymphoma and leukemia are relatively rare. The so-called fat necrosis is somewhat more common. It has a circular calcification of 2 to 3 cm in size, localized mostly subcutaneously and, if they persist for a considerable time, can calcify in a ringlike shape or might show microcalcifications.

Cystosarcoma phyllodes is relatively common. This

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Figure 13. 38-year-old woman – enlarged venous marking without any mass lesion, could be a normal variation

Figure 14. 49-year-old woman – arteriosclerotic vessels in an elderly breast (could be in young women with renal disease also) it is media (Moenckeberg) sclerosis type

Figure 15. 64-year-old woman – continued glandular degeneration

Figure 16. 75-year-old woman – punctate calcifications in a fibroadenoma – usually seen in post-menopausal women

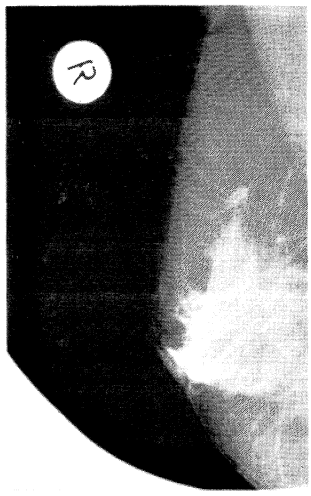
Figure 17. 81-year-old woman – large amorphous calcifications

Figure 18. 68-year-old woman – large fibroadenoma going through hyaline degeneration and amorphous and large calcifications. Note that the mass part is disappearing eventually; only calcifications will remain

Figure 19. 42-year-old woman – diffuse opacity with retraction of the skin and nipple; most likely a malignancy but could be inflammatory process such as abscess or post-traumatic condition with hematoma

Figure 20. 69-year-old woman – nipple retraction and localized mass-like area again could be abscess – note the calcified small fibroadenoma in the upper quadrant

Figure 21. 76-year-old woman – calcification in a fibroadenoma



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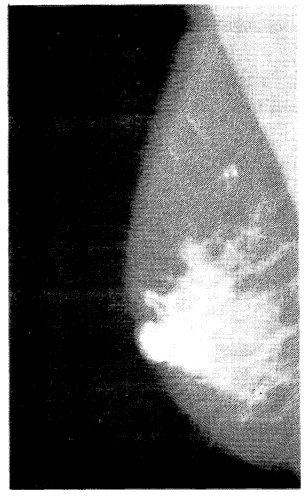

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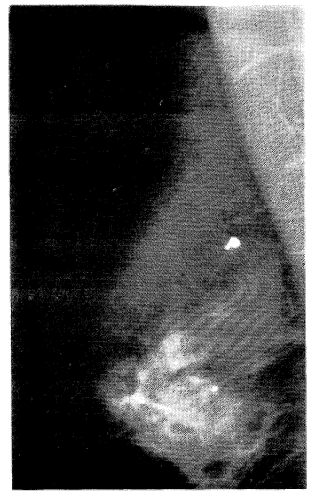

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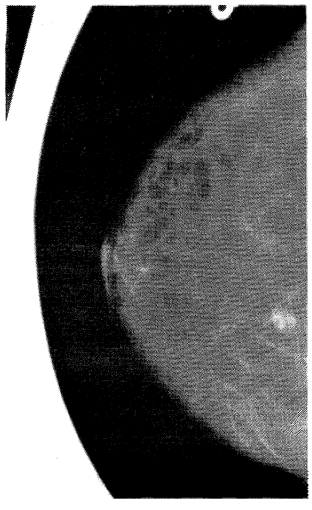

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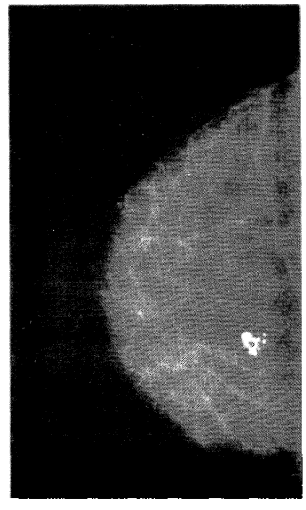

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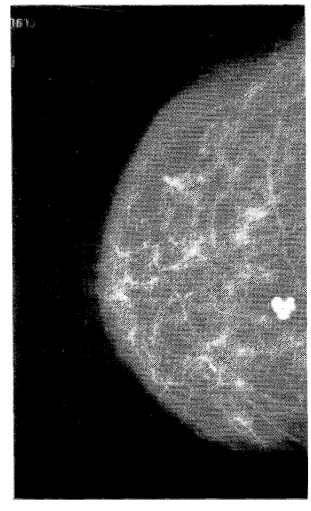

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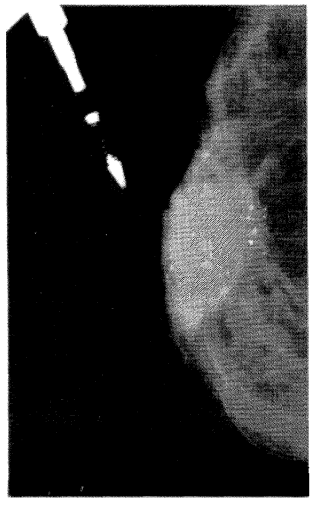

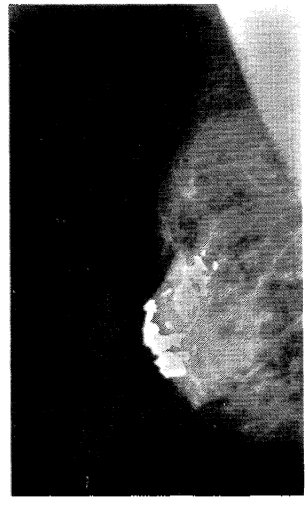

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tumor is usually composed of large, smoothly bordered, round mass, often referred to in the literature as giant fibroadenomas. Their histology is characterized by a phylloid (leaf-like) structure and cystoid degeneration of epithelial-mesenchymal elements.

A diagnosis cannot be made through mammography alone; accordingly it is essential that an additional fine-needle biopsy or tissue biopsy be carried out. In the case of large tumors exhibiting a sarcoma-like appearance, the possibility of a cystosarcoma phylloides must be the first consideration. In some of the cases, one has to reckon with malignant degeneration; occasionally they can be considered as malignant from the start.

In the description of a round mass or, of a demarcated shadow, the following criteria should be observed:

I. Size

1. lentil size: up to 1 cm
2. cherry stone size: more than 1–2 cm
3. cherry to small plum (mirabelle) size: more than 2–3.5 cm
4. plum- to table-tennis-ball size: more than 3.5–5 cm
5. tomato to apple size: more than 5 cm

II. Form

1. Round, oval or lobulated (this also includes the so-called twin bilobed or trilobed mass)
2. Spiculated

III. Density

Homogeneous, non-homogeneous, stringy to striped (typical for fibroadenoma), radiolucent (for example, in lipomas)

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Figure 22. 48-year-old woman – 6 × 4 mm fibroadenoma without calcium.

Figure 23. Same patient with amorphous calcifications in fibroadenoma 8 years later.

Figure 24. Same patient – increased calcifications in fibroadenoma after additional 6 years.

Figure 25. 65-year-old woman – large fibroadenoma with punctate calcifications.

Figure 26. Same patient – increased calcifications in a fibroadenoma 6 years later.

Figure 27. Same patient – increasing calcification in fibroadenoma 5 years later

Figure 28. 62-year-old woman – retromammillary fibroadenoma, 5 × 3 cm in size with calcifications

Figure 29. Same patient – with increase of calcification of fibroadenoma 4 years later

IV. Demarcation

1. less than half of the circumference (difficult to demarcate)
2. from half to 2/3 of the circumference (well-demarcated)
3. more than 2/3 of the circumference (also well-documented)
4. fully demarcated

V. Contour

A round or oval mass may be sharply outlined, nebulous; or lobulated or spiculated.

VI. Halo around mass

The halo around a mass is so decisive a criterium of benignancy that it assumes pathognomonic importance.

VII. Desmoplastic reaction

In the American literature they are called "comet-tail-like extension" if single or it will be like corona radiata if multiple.

VIII. Architectural distortion

Architectural distortions have to be diagnosed.
Some breast lesions may be identified by the mammographic view only (206).

IX. Microcalcifications

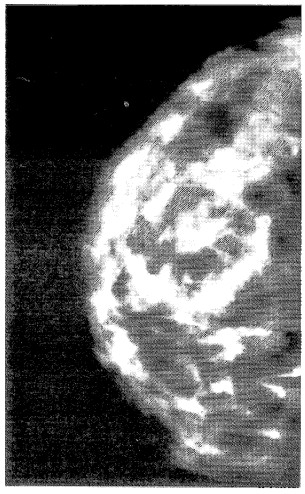
An exact description is required of size, form, localization, but, especially, number of calcium spots (very small specks) observed.

Calcium in the mammogram

Calcification could occur in some carcinomas of other viscera. But in the breast they are found very frequently; 30% to 60% of all mammary carcinoma or specimen.

Black and Young (38) have systematically classified 1,088 surgical specimens of malignant tumors of the mamma and of other organs, using irradiation of 25 to 30 kv. In 95% of the specimens, radiologically discernible calcification was confirmed histologically. They found calcification in mammary carcinoma in 42% of the specimens; in pulmonary carcinoma in 6%; in intestinal carcinoma, in 16%; in gastric carcinoma in 14%; and in bladder carcinoma, in 15.8%.

The microcalcification described for the first time in 1913 by Salomon was rediscovered by Leborgne (122, 123, 124), of Montevideo in the period 1943–1951, later, also rediscovered by Gershon-Cohen and Ingleby (110), and by Gros (139). In the German-language literature it has been primarily Menges (252) and Lanyi (218) who have concerned



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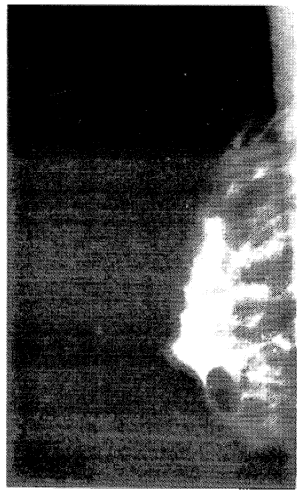

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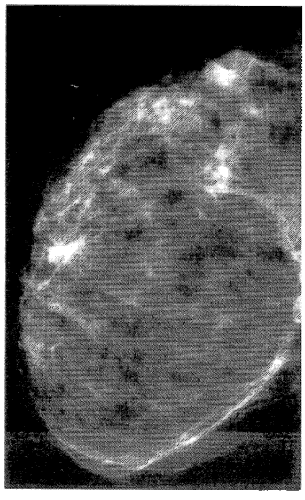

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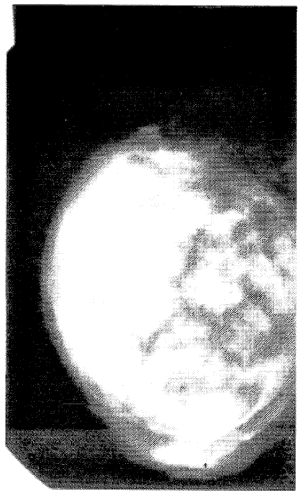

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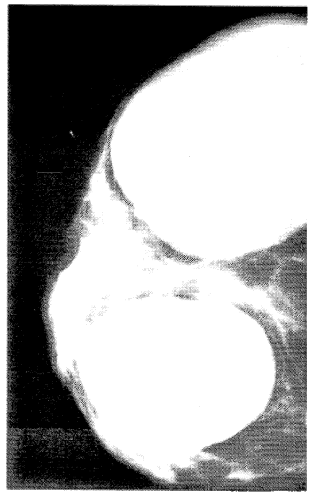

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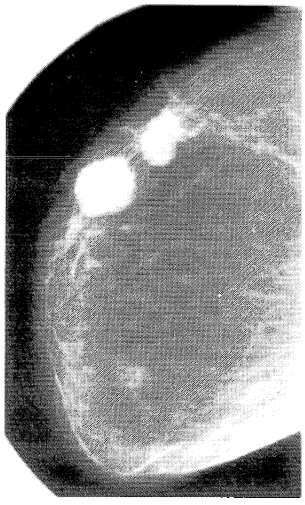

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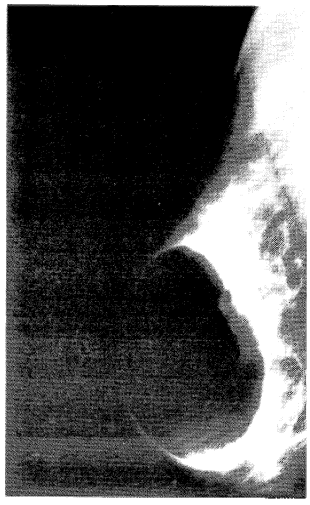

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themselves with the systematic classification of microcalcifications in carcinomas of the mamma. Local excision is essential to ensure histological diagnosis in the nonpalpable breast lesions with microcalcification (184).

The German pathologist, Hamperl (148), together with clinicians devoted himself extensively to the demonstration of calcifications in biopsy materials. he assumed that the calcifications in the mammary gland can be explained in part through retention of the mamma secretion, that contains calcium phosphate, in expanded glandular spaces. The calcification in the necrotic glands and carcinoma cells or in the stroma, cannot, however, be explained by this theory. Hamperl suspected that the strikingly frequent calcifications occurring in the normal and cancerous epithelial cells of the mamma are to be considered only in relation to the secretion. It is possible, he surmised, that the glandular cells themselves may have the capability of absorbing the calcium phosphates and of processing them into secretion. He emphasized that of all forms of calcification, only the so-called granular type assumes pathognomonic importance because this type originates through cancer epithelium necrosis in the milk ducts. All other forms of calcification, in his opinion, can also appear in the course of benign changes.

Leborgne (222, 223, 224) was the first to describe systematically the various forms of calcifications, especially the differentiation between the calcifications occurring in association with a malignant or a benign illness. The "typical microcalcification" of a carcinoma is characterized by innumerable punctate calcium dots which resemble fine grains of salt or sand-like. These calcifications, which usually occur in clusters, can appear in carcinoma mass, but also without a mass. It was to Leborgne's credit that he was the first to have called attention specifically to the occurrence of a calcification in a tumor-free breast. He regarded this calcification as an early stage (*in situ*), or as indicative symptom of carcinoma of the mamma. All the investigations performed following his discovery have been able fully to attest to his observation: the "typical microcalcification" is the most important and, at the same time, the most common indication of the presence of the so-called clinically occult carcinoma of the mamma.

The capability of mammography to make possible the recognition, on film, of these extremely fine calcifications, often only with the use of a magnifying glass, renders it such a superior method in cancer care. The frequency of all microcalcification forms in cases of occult and small carcinomas (carcinomas up to a diameter of 2 cm) is estimated to be from 40% to 60%; of these, the sand-like microcalcifications of only 0.1 mm size amount to 30%. The leading mammography diagnosticians agree that the localized concentration of more than 5 such calcium specks must be regarded as the threshold value for invasive investigation (75, 150).

The calcifications can appear, inside or outside of a benign or a malignant mass focus. Macrocalcification (coarse-layered amorphous calcification) with some few exceptions, can be demonstrated in fibroadenomas of a fat necrosis (Figures 16, 17). As a rule, it then consists of a coarser calcium ring (Figure 17). Less frequently it is associated with very small calcium flecks (Figure 16). The latter findings already present problematic cases, which have to be clarified, either through short-term observation or by excision. Coarse-layered amorphous calcifications in fibroadenomas are pathognomonic for the benignancy so that such patients can simply be followed by observation. We cite three examples for the gradual advance of such a calcification over an observation period of 15 years.

In the first two cases, the initial examination simply revealed the existence of a round mass the size of a cherry (Figures 19–21 and Figures 22–24). Slowly, a layered, dense calcification developed which finally included the whole fibroadenoma. Such progressions are wholly typical for the development of a calcification in fibroadenomas.

Microcalcification, on the other hand, can be present from the start, then mostly in larger fibroadenomas, and gradually increase in size and number (Figures 25–26). Occasionally, such a calcification can exhibit a plaque-shaped, either amorphous or reticulate architecture (Figure 18). During the whole observation period, the coarse layered calcification in a fibroadenoma always remains restricted to the fibroadenoma – in contrast to microcalcifications in malignant tumors.

In contrast to malignant calcifications, the benign are not only larger but, also denser. From case to case they are very dense and resemble a metallic foreign body. All in all, calcifications in fibroadenomas are less common than in a carcinoma. Their frequency of occurring is reported as from 3% to 12%.

From a pathognomonic standpoint, the individual calcium spot is of little significance, whether it can be seen with the naked eye or the magnifying glass. Only the occurrence of multiple calcium flecks similar to grains of salt in a circumscribed area either in a tumor shadow or in a normal mamma structure transform this calcification into an indicator which in 30% to 50% of all cases suggests microcalcification. Microcalcification is discussed only if several calcium flecks are concentrated in a strictly localized area. Based on the findings of experienced authors, a localized accumulation of 5 to 10 such calcium flecks already respresents an

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Figure 30. 38-year-old woman – clustered microcalcifications in the central portion of the breast with dense to cystic structure; histological diagnosis revealed a carcinoma.

Figure 31. 47-year-old woman – diffuse multiple calcifications. The histological diagnosis revealed a sclerosing adenosis.

Figure 32. 50-year-old woman – diffuse microcalcification in the whole breast; histologically a carcinoma was diagnosed.

Figure 33. 49-year-old woman – 12 × 9 cm large lipoma separated from the remaining mammary tissue by a capsule.

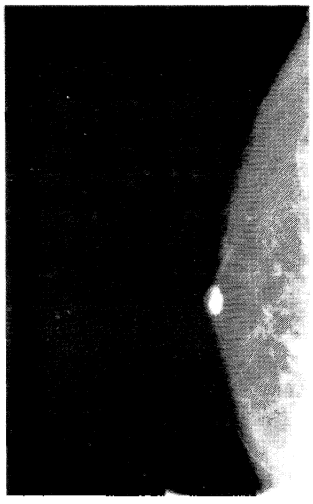
Figure 34. 40-year-old woman – 8 cm large fibrolipoma – separated from the surrounding by a capsule.

Figure 35. 15-year-old girl – two giant juvenile fibroadenomas of 12 × 8 cm and 8 × 8 cm

Figure 36. 68-year-old woman – two fibroadenomas measuring 2.5 cm and 1.8 cm in diameter without calcifications.

Figure 37. 60-year-old woman – two fibroadenomas of 1.5 and 0.6 cm in diameter with amorphous calcification.

Figure 38. 47-year-old woman – 7 × 5 cm cyst filled with air.



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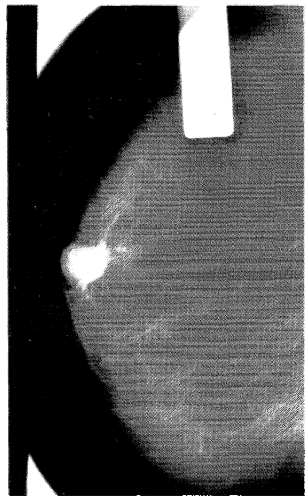

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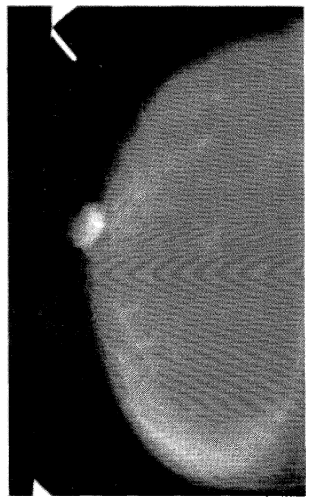

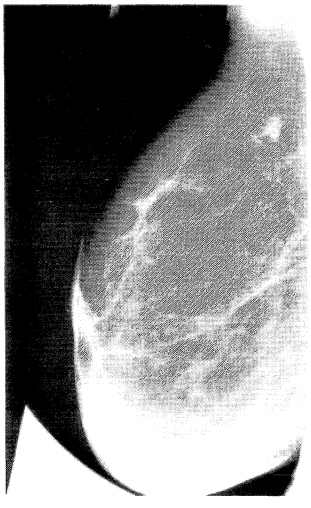

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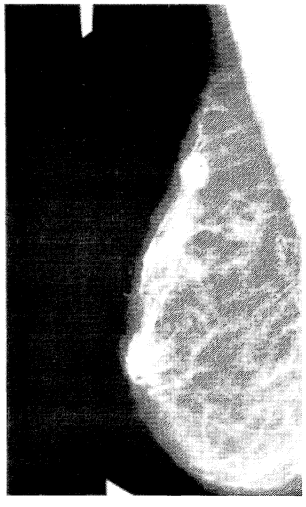

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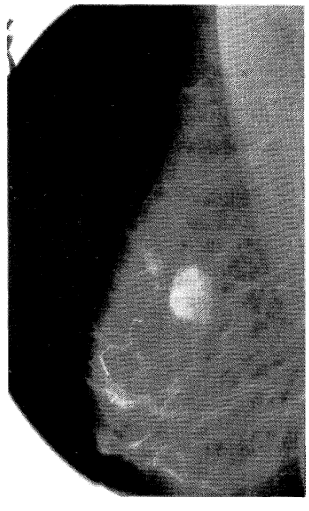

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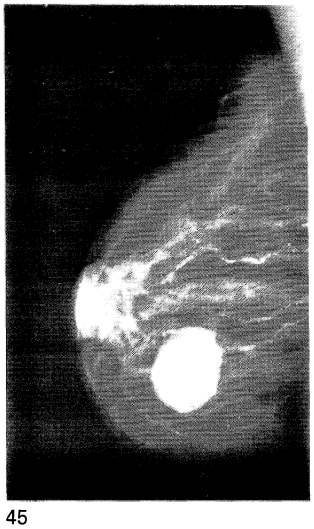

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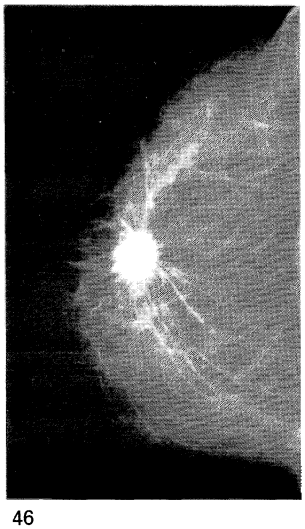

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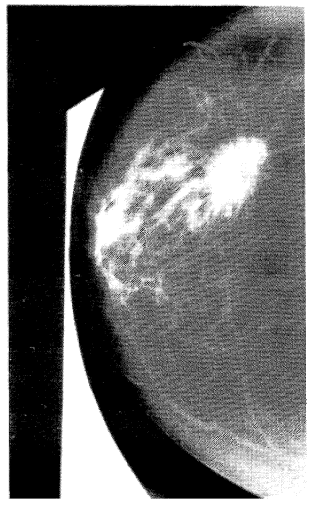

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indication for invasive investigation. If such calcifications are present, in their opinion, there will be found a carcinoma in 1/3 of the cases; 1.3 borderline cases; and in another 1/3, apocrine metaplasia, severe ductal hyperplasia; papillomatosis; or a sclerosing adenosis. An accumulation of 11 or more such calcifications, they feel, is pathognomical with the result, that an invasive investigation must be undertaken without fail. Hassler (150) and Gershon-Cohen (107) stipulate a lower threshold of 9, or, 6 microcalcifications.

How large must a calcification be that it can be recognized radiologically as such?

According to Egan *et al.* (77), it has to be 50 μ large so that it can still be recognized with a magnifying glass; according to Gershon-Cohen *et al.* (111), between 0.25 and 0.5 mm. Kubo (211) assumes that such calcifications can only be recognized from 350 nm on in mammograms, on specimen radiographs histological sections only from 120 nm.

All authors agree that with an increase in the number of calcium flecks, suspicion of the presence of a carcinoma also grows. Increased number of such calcifications during an observation period speaks unequivocally for a carcinoma. Some examples for such a microcalcification are shown in Figures 27–29. Figure 27 shows a group microcalcification consisting of 8 calcium spots; Figure 29, a diffuse microcalcification in the whole still visible parenchymal residual. Such a diffuse calcification, consisting of numerous calcium flecks, but which usually appear in a more regular pattern and less dense, can also occur in benign conditions (Figure 28). Based on all the findings to date, the occurrence of microcalcification in carcinomas is estimated as of the order of 30%–40%. The number of clinically occult carcinomas discovered only on the basis of a microcalcification is estimated by Hoeffgen and Lanyi as 40% (168).

Microcalcifications constitute an important part of non-palpable breast lesions and may be the first sign of a breast carcinoma (356). No correlation was found in breast cancers between the presence of microcalcifications, lymph node condition and histological type (292).

See also: (277, 301, 319, 320, 328, 415, 418)

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Figure 39. 55-year-old woman – medullary carcinoma 1 cm in diameter which condition could imitate a benign fibroadenoma.

Figure 40. 63-year-old woman – medullary carcinoma, 2 cm in size.

Figure 41. 60-year-old woman – subareolar medullary gelatinous carcinoma, 1.8 × 0.8 cm in diameter, could easily correspond to a fibroadenoma.

Figure 42. 56-year-old woman – 1.8 cm large smoothly bordered carcinoma.

Figure 43. 54-year-old woman – carcinoma 1.2 cm in diameter with a round superficial mass with skin retraction.

Figure 44. 62-year-old woman – spiculated and centrally located mass with arteriosclerosis in the same breast. Discrete foggy occurrence of the margins

Figure 45. 64-year-old woman – carcinoma with a diameter of 3 cm. The discrete foggy appearance of the margin speaks for a carcinoma. There is arteriosclerosis in the vessels.

Figure 46. 66-year-old woman – large carcinoma, 2.8 cm diameter and characteristic radiating extensions around it.

Figure 47. 64-year-old woman – typical carcinoma, 3 cm in size, characterized by spiculated margins.

Mammary dysplasia

In the American literature is the term interchangeably used with fibrocystic disease (cystic changes, fibrosis, adenosis, sclerosing adenosis or papillomatosis).

The radiological symptomatic most frequently occurring disease has conscientiously not been illustrated with the appropriate mammograms since they can scarcely not be defined, not only clinically-histologically, but also radiologically.

For example; morphologically, it corresponds to a variegated mixture, which ranges from the physiologically determined involution, processes beyond the limits of the normal condition to certain, pathomorphologically defined, diseases.

Mammary dysplasia could also be termed a possibly hormonally controlled, organ deterioration of the mammary gland, accompanied morphologically by a disequilibrium among the individual building blocks of the mammary gland – in some fraction of the cases, accompanied even by changes in the epithelium (proliferative forms). The condition is so common that one should speak almost of a state of habits rather than pathology (126, 127, 128, 129, 134).

Mammary dysplasia in German literature can be classified into three degrees of seriousness:

1. Mastopathia cystica fibrosa (simplex) without proliferation of the epithelium: no cancer problem. Frequency of occurrence to 70%.

2. Mastopathia cystica fibrosa with proliferations of the epithelium without atypies; in this form, there are included adenoses and epithelioses, that is, intraductal epithelial hyperplasia, whose degenerative risk is increased only slightly or not at all. Frequency of these cases, about 25%.

3. Mastopathia cystica fibrosa with proliferations of the epithelium and atypies; this is a mastopathy in the sense of a precancerosis, characterized by atypical ductal and lobulary hyperplasia. Frequency of occurrence of these cases is low and, for all mastopathy forms, amounts to about 5%.

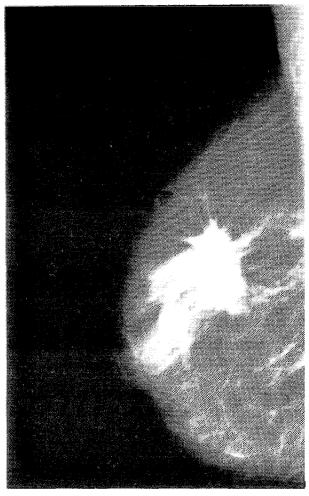
Carcinoma (299)

Based on experience gained over the past 30 years, the criteria for malignancy can be subdivided into primary and secondary; as a rule, in the latter case, a mammography is no longer necessary.

Primary criteria for diagnosis of malignancy:

1. mass
2. microcalcification
3. localized duct ectasia
4. architectural distortion
5. asymmetrically increased density

Of all these criteria, the second-agreement between the clinical palpation finding and the x-ray image – seems to be the most important. The carcinoma lump that can be felt is always found to be smaller in the mammogram. In the



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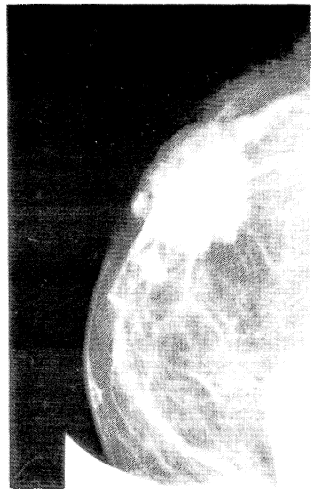

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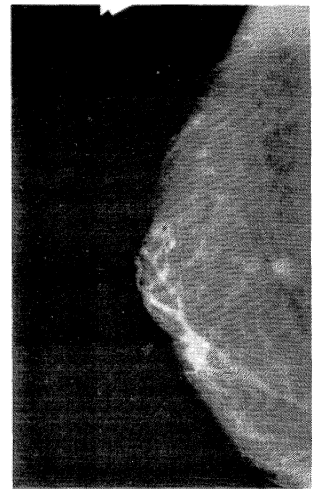

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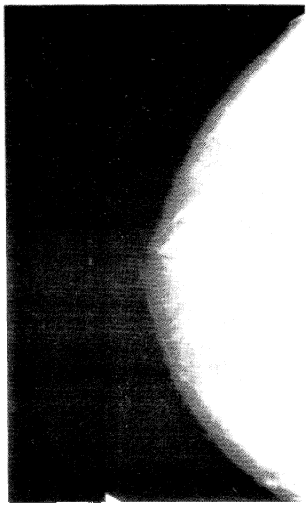

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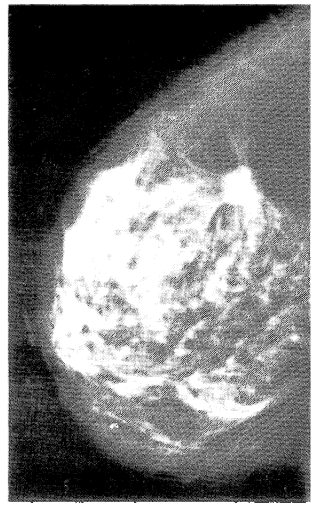

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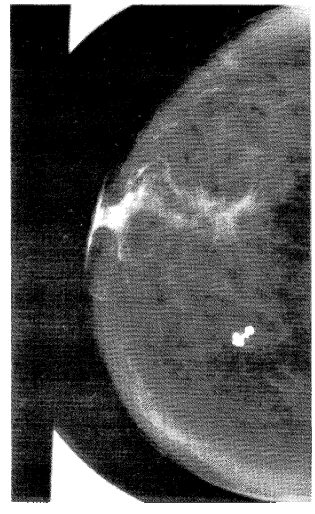

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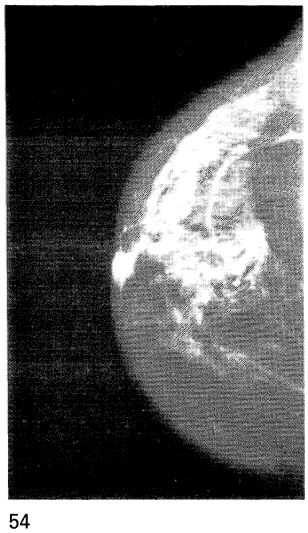

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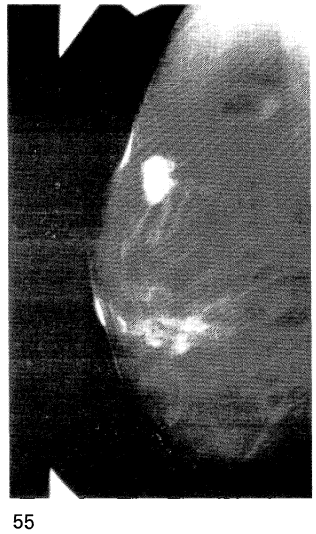

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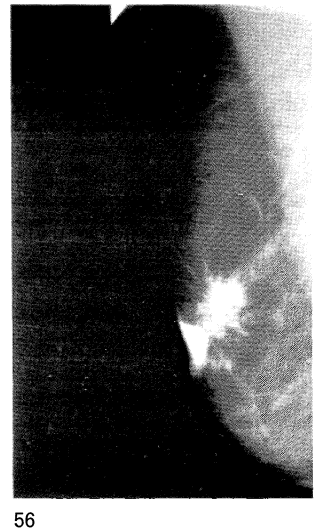

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coarse of retrospective or, alternatively, prospective empirical evaluation of the film-material obtained from the Goettingen clinics, there was developed, in agreement with other authors, a classification scheme of the carcinoma in the mammogram, divided into four major groups:

1. Benign looking round mass, ranging in size from 3 mm to 8 cm
2. Unequivocally malignant round mass, with a blurred margin giving a nebulous appearance to its contour in the same size range as the above
3. Spiculated mass:
   a. without discernible center
   b. small center
   c. large center
   d. corona radiata type of tentacules
   e. comet-tail-shaped single tentacule
4. Opacities of differing degrees of distinctness.

The sharply demarcated, round, oval or lobulated masses (Figures 36–41) cannot be distinguished radiologically from a benign condition. This is especially the case, if they are localized strictly retromammilarily (Figures 36–38). All three of these tumors were colloidal carcinomas. The larger tumors with short-or long-radiating tentacules represent the typical carcinoma. In these cases, as a rule, further diagnosis can be dispensed with (Figures 42–44). Carcinomas, which are accompanied by long- or short-radiating tentacules, represent the second typical group of carcinomas (Figures 45–51).

Of the patient pool of Goettingen, this group represents about 20% of all cases. The indicators of tumors are here the spiculated tentacules of varying length and number which branch out from a not always discernible center. It is not seldom that such images occur in the dense structures of large breasts while the clinical symptomatology remains mute. The cases with an indiscernible center, cases which present a stellar shape, were in terms of frequency, comparable to the cases where in the center a small round-focus-like opacity could be distinguished. Diagnostic examples with large centers and radiating tentacules are less common. Occasionally, such cases with radiating tentacules appear in the image like a feather-ball. Findings which may be distinguished from such cases are those, wherein the carcinoma was located without exception in the upper quadrant, exhibiting a long, thin tail-stretching to the nipple, a tail similar to that of a comet.

Carcinomas with typically long tails are shown in Figures 45–51. In all these patients, a prior specimen excision was not carried out – the diagnosis of "carcinoma" was always confirmed by mastectomy.

Carcinomas of the mamma even if they are not immediately connected with the skin or the nipple, may still cause a thickening of the skin above the carcinoma (Figure 52). Much more frequently a close relationship with the nipple is observed in retromammary localized tumors, the nipple always appears thickened and retracted (Figures 52–53).

Occasionally, such stellar shape masses appearing in the mammogram which ordinarily would lead to cogent suspicion of a carcinoma can be attributed to a benign process such as fat necrosis or sclerosing adenosis. Egger *et al.* (78) have reinvestigated 50 cases with radiating offshoots, pathomorphologically, and in series, in seven cases, determined the cause of such radiating tentacules to have been periductal elastoses accompanied by milk duct obliteration.

Finally, a special form of carcinoma, that of Paget's, should be mentioned. This carcinoma, first described in 1874 by the English surgeon James Paget (1814–1899) (273), concerns a tumor located intraductally with epidermotrope growth, whose structure is characterized by the Paget cell. Diagnosis must first be made clinically, since most patients with a Paget carcinoma show changes in the nipple area, whether in the form of a scab, and eczema or a reddening. Such morphological changes of the nipple are accompanied in some of cases by itching. Mammographically, the Paget carcinoma shows microcalcifications in about 30%–40% of the cases, the calcifications are exclusively localized in the milk ducts. Not uncommonly, such a microcalcification can be observed reaching from a point deeper in the breast along the milk duct to the nipple itself. In such cases mammography is utilized to confirm the diagnosis.

The past 10 years have witnessed a trend in surgical management away from the standard radical or modified for so-called early breast cancer (276, 288). Use of mammography in asymptomatic women does discover breast cancer at an earlier point in development, as measured by size, invasiveness, and nodal involvement. The integration of mammography into routine periodic evaluation of asymptomatic women over 35 years of age should diminish the threat of breast cancer (169). Differentiation of radiotherapy changes from recurrence of carcinoma can be made when a reaction that is normally due to radiotherapy occurs to an inappropriate degree, or with inappropriate timing (44). Evidence that benign breast disease is a risk factor for breast cancer may be misleading if choice of control groups makes no provision for bias produced by inequalities in detection of disease (323). No correction was found between Wolfe's

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Figure 48. 69-year-old woman – carcinoma, 4 × 3 cm in size. Typical finding with spiculated margins and close relation to the nipple.

Figure 49. 68-year-old woman – subcutaneous located carcinoma, 4 cm in diameter with spiculated margins; wide vein in the same breast.

Figure 50. 49-year-old woman – centrally located carcinoma with spiculated margins only 1 cm in diameter.

Figure 51. 63-year-old woman – carcinoma, 1.8 × 1.2 cm in size with spiculated margins and retraction of the nipple.

Figure 52. 57-year-old woman – carcinoma, 2.2 cm in diameter with diffuse radiating extensions to the surrounding tissues and severa P_2 pattern.

Figure 53. 59-year-old woman – carcinoma in the central portion and a second tumor of 1 cm in diameter. In the same breast amorphous calcification in the fibroadenoma of 1 cm in diameter.

Figure 54. 63-year-old woman – centrally located carcinoma, 1.1 cm in diameter, with long tentacules and malignant looking calcifications.

Figure 55. 73-year-old woman – carcinoma of a diameter of 2 cm. Anterior to mass there is localized thickening of the skin.

Figure 56. 76-year-old woman – subareolar carcinoma of 2 cm in diameter. Thickening and retraction of the areola and nipple.

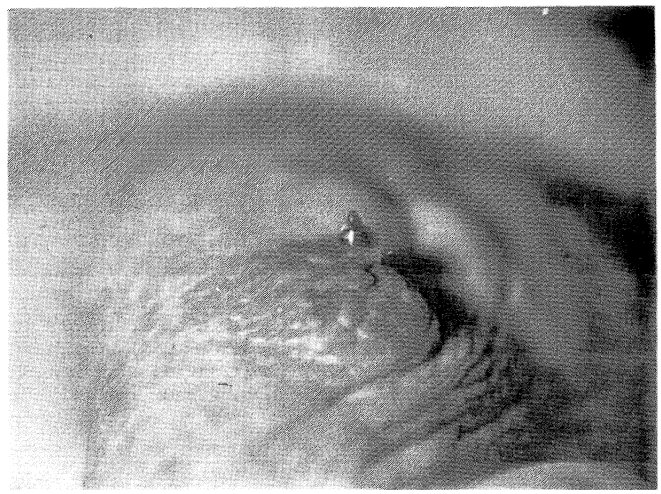


Figure 57. 38-year-old woman – spontaneous water-clear secretion.

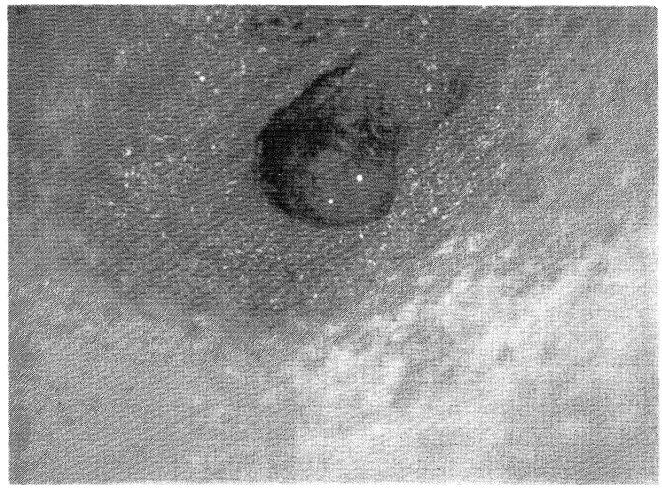


Figure 58. 33-year-old woman – spontaneous water-clear serous secretion.

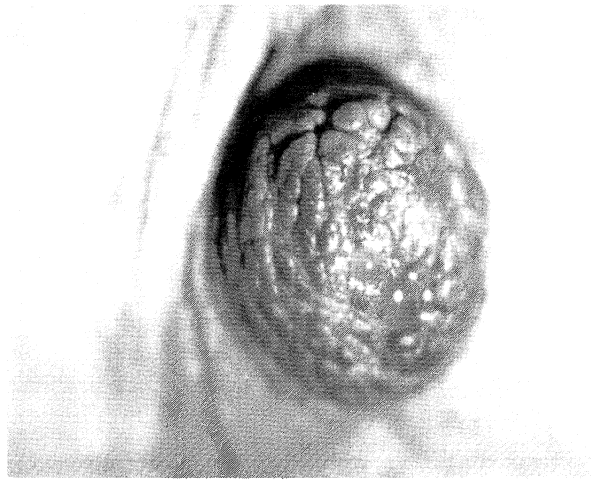


Figure 59. 43-year-old woman – amber-colored serous secretion.

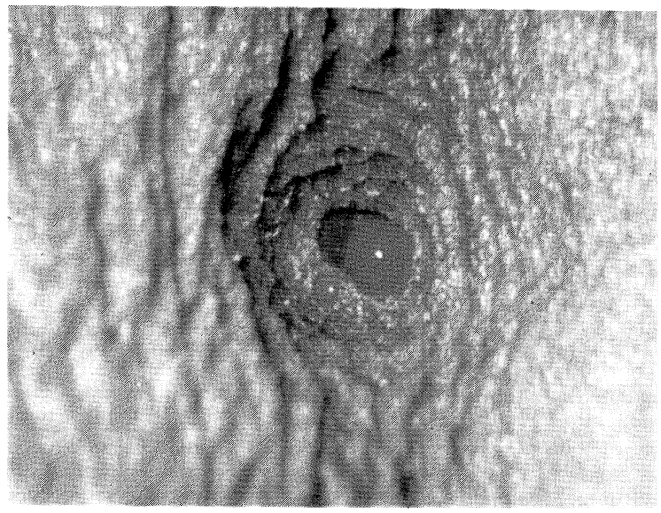


Figure 60. 58-year-old woman – provoked amber-colored secretion.

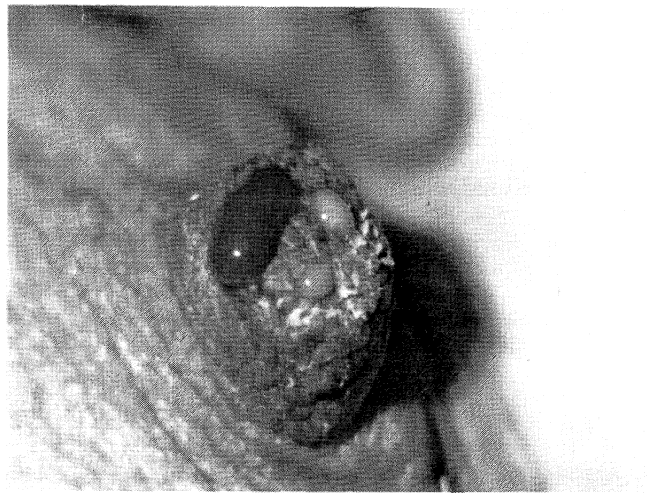


Figure 61. 37-year-old woman – provoked secretion of mixed color.

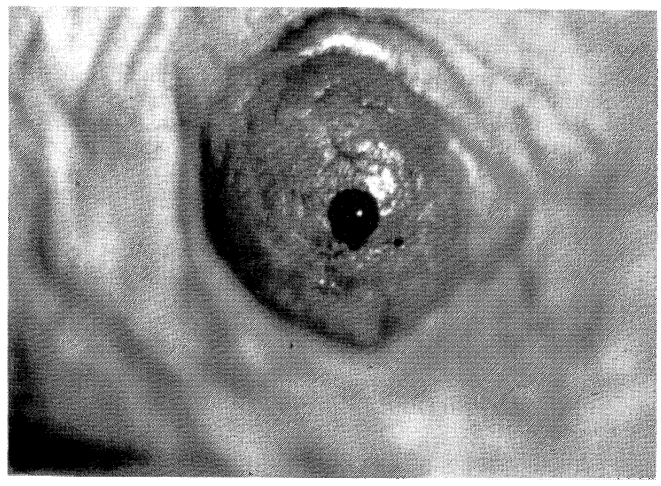


Figure 62. 36-year-old woman – spontaneous bloody secretion.

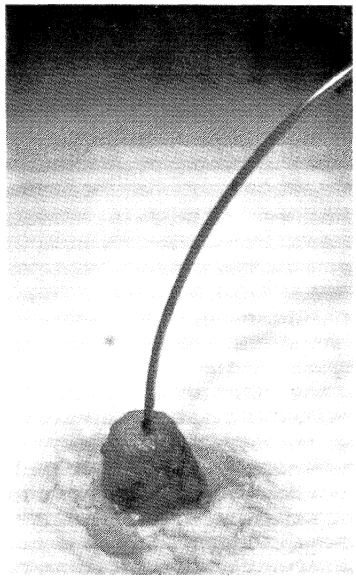


Figure 63. Galactographic technique – inserted catheter in the secreting milk ducts with visible secretory column.

parenchymal patterns and hormonal receptor content, which contradicts a recent English study (265). Recurrence of cancer in the irradiated breast is an uncommon but potentially curable problem. Mammographic follow-up is complementary to physical examination in the detection of local recurrence in women who have undergone radiation therapy for early breast cancer (342).
See also: (210, 61, 62, 121, 185, 191, 205, 233, 258, 388, 417).

Nonpalpable breast lesions in a series of 70 biopsies were detected by mammography with an overall accuracy of 99% (146). Physical examination and mammography are both important for the detection of breast relapses (302). During a previous 8-year period, 37 of 500 primary breast cancer patients (7.4%) developed metachronous (39) or synchronous (4) second-breast primary cancers primarily diagnosed clinically or radiologically (383).
See also: (173, 306).

The mammography in male: (304)

Gynecomastia is by far the most common disease of the male breast. The name originates with Galenus (129–200 A.D.). The literature contains some grotesk and sensational reports about men with enlarged breast who, when the nipple also was enlarged also took part in breast feeding (131). Normally male breast has only one or two ducts.

Clinically, any increase in the mass of the generally quite rudimentarily structured body of the male breast gland is called gynecomastia; morphologically, it designates a hyperplasia and differentiation of epithelial and mesenchymal glandular components. In general, gynecomastia is only a symptom of another, concomitantly existing basic disorder, or it is caused by drug or hormonal therapy. In the last 15 years, the so-called puberty gynecomastia which is also seen as an idiopathological gynecomastia and whose causes are as yet not fully understood, has been differentiated from the systematic. Adolescent gynecomastia signifies unilateral or bilateral swelling of the breast gland in adolescents, occurring generally with puberty, occasionally accompanied with severe pain; as a rule, it regresses spontaneously after 2 years, but it may also persist. The young patients visit the physician for psychological and/or cosmetic reasons; treatment of a retarded puberty gynecomastia consists of surgical removal of the glandular body. Ultrasonography and mammography performed on a male patient with breast carcinoma exhibited mammographic features which were highly suggestive of malignancy and the sonographic findings were subtle (186).

Mammographically, adolescent gynecomastia does not differ from the secondary gynecomastia found in adults. On the other hand, mammography makes it possible to distinguish both of these forms from a pseudogynecomastia which is only an atrophic (structureless) hyperplasia of the fatty tissue (Figure 69).

Gynecomastia is presented mammographically in the form of increased ductal structures; opacities of varying density and size; round-foci-like, usually in homogeneously-dense opacities and in the form of homogeneously-dense, smoothly demarcated round masses.
See also: (264, 270).

From the standpoint of differential diagnosis, the other benign diseases like mastitis, fibroadenoma, papilloma, lipo-

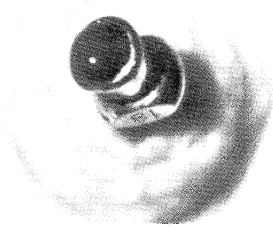


Figure 64. Galactographic technique – inserted cannule into the secreting milk ducts filled with secretion of mixed color.

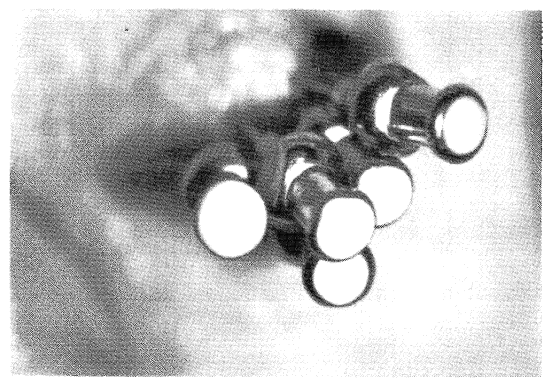


Figure 65. Galactographic technique – inserted cannules into the secretory milk ducts filled with milky secretion.

ma, but also semi-malignant disorders, such as cystosarcoma phylloides, play only a subordinate role because of their rare occurrence (Figures 70, 71).

In malignant conditions, especially when a unilateral gynecomastia in adults is present, a mammary carcinoma must first be excluded. Breast carcinoma in a male can be recognized quite early in its initial stages relatively as a dense round mass with the initial development of a spiculated margin. At that point in time, a differentiation from a gynecomastia is possible only cytomorphologically. Since there are no lobules all male breast carcinomas will be duct carcinoma.

At a later stage, just as in the case of a female mammary carcinoma, all secondary criteria of malignancy can be found: the retraction of the nipple, the thickening and retraction of the skin, and the retraction of the initially circular opacity and enlarged venous markings.
See also: (50, 55).

Additional methods for examining the breast (203)

The following table shows, chronologically, the physical tests employed to date. Some of them (diaphanoscopy, infrared phlebography, phlebography of the mammaria interna, arteriography, direct lymphography) will not be discussed in detail, but have been listed here simply for the sake of completeness. They have not been able to establish themselves; firstly, because they are too complicated; secondly, because they have minor diagnostic value (204).

Galactography (ductography)

See also: (58, 85, 293, 369).

This involves the instillation of a water-soluble contrast-enhancing compound of 0.5 to 0.2 ml into one or more secernent milk ducts; therefore, the test can be performed only on a secernent mamma (126, 127, 128, 129)

Galactography is always combined with mammography and has been used as long as mammography. The injected contrast-enhancing compound as a rule can also be seen with a conventional x-ray machine.

The first galactography, reported in 1930 by the surgeon Ries, was performed by a practical physician in Nebraska. He was consulted by a patient who, six weeks after discontinuing breast-feeding, observed bloody discharge and a lump in the right breast. The family doctor instilled the oily contrast (Lipiodol) into the secernent milk duct of the right breast, using a fine catheter. The left breast, which did not have a lump but was secernent with a brown liquid, was also tested with Lipiodol by the family doctor. Ries (285) saw the patient 6 weeks following galactography, apparently because of an abscess, which could probably have formed because of the galactography.

Galactography, without any doubt, belongs to the aggressive investigative methods because they frequently cause pain and are associated with local complications. (Figures 60–62).

The indicator of a secernent mamma is the secretion which can appear spontaneously or after provocation from one or more milk ducts of one or both breasts. The secretion can be observed once, intermittently, or constantly, possibly over a longer period of time. In the case of a spontaneous secretion, one is dependent upon information provided by the patient. Most of the patients report that in the morning on the nightgown they discovered a bloody spot, or a varicolored stain. A spontaneous secretion comprises a quarter to one-third of all cases of a pathological secretion. A provoked secretion can be induced by the patient herself, during an examination by the physician, or by compression during mammography. To date, over 27 color nuances of a mammary secretion have been described in the literature (126, 127, 128, 129). Basically, these may be divided into a milky and a non-milky group.

The purely milky secretion from several milk ducts of both breasts is more common than all other forms of secretion in the presence of hormonal problems or accompanying hormonal disorders. The secretion colors can be subdivided into four major groups: (1) clear, like water; (2) serous, amber-colored; (3) variegated; and (4) bloody secretion (Figures 57–62). The frequency of a pathological

In order of importance and use

Test method	*Authors*	*Year of appearance*
Diaphanoscopy/diaphanography (transillumination)	Cutler (59)	1929
phlebography of V. mammaria interna	Hollender *et al.* (172)	1956
distance thermography	Lawson and Chughtay (220)	1963
(electronic thermography)	Gershon-Cohen *et al.* (108)	1964
contact thermography	Fergason (91)	1964
(liquid crystal thermography)	Tricoire *et al.* (358)	1970
arteriography	Feldmann *et al.* (90)	1967
direct-lymphography	Kett *et al.* (197)	1972
light-scanning (transillumination)	McIntosh (249)	1983
computed tomography improved MRI	Chang and Sibala (54)	1978
nuclear magnetic resonance	Bovee *et al.* (41)	1980

secretion during a mamma examination is reported by Ulrich (1977) (361) as 6% based on 73,000 cases. It corresponds roughly to the normal population of 6.2%. Among women examined in Goettingen mammographically in the health care program a secretion was found in 5% of all cases.

In some of the cases, a pathological secretion can be caused by a hyperprolactinemia, or in others, by intraductal tumors with an epithelium capable of secretion. More especially, intraductal papillomas and papillary carcinomas are the cause. Of the extramammary causes, there must be included illnesses which are not necessarily accompanied by a hyperprolactinemia; genital diseases; chronic disorders and physical deterioration processes; psychological and mechanical trauma; and secretions following intake of medications, especially of psycho-pharmoceuticas. Intramammary causes may include ductal ectasis which, in association with a retention of secretion, as termed a rebuilding-reaction of the mamma, representing a borderline between the normal and the pathological states because it appears relatively frequent during advancing age as the mamma changes physiologically with aging (14).
This, for example, leads to an explanation for the secretion among elderly and old women, which can be provoked. But since such ductal ectasies can also appear in stenosing tumors of ductal and paraductal origin, such provokable secretions must first be regarded as a serious symptom and require further clarification.

A quarter of all secretion cases can be explained, neither as a manifest nor as a latent hyperprolactinemia, nor as due to an intramammary problem. Therefore, one tends in such cases to speak of an alimentary or idiopathic secretion (126, 127, 128, 129).

In the diagnosis of intraductal processes, galactography is superior to exfoliative cytology for the following reasons: (1) only a few carcinomas are accompanied by a nipple discharge: (2) mammillary secretions are often free of cells, or the cells contained in them have been altered by degenerative processes to the point where an evaluation is no longer possible. The discovery rate of carcinoma by means of exfoliative cytology is estimated to be 1%–2% (39).

Galactography cannot replace histological diagnosis. The results obtained with its use only indicate the presence of the process – generally a recess in the milk duct – which must be cleared up. During the Fourth Mamma Symposium, held 1983 in Goettingen, Ouimet-Oliva (272) expressed the opinion that a carcinoma of the breast must be seriously considered in the presence of certain galactographic indicators: (1) disruption in the milk duct system; (2) extravasations in the region of the malignant process; (3) perforated areas in the milk-duct system; and (4) tapering off of the milk ducts and diminishing of duct diameter.

A galactographic finding includes the following features: (1) localization of the mammary segment represented; (2) width of lumen in the milk ducts represented; (3) tendency of the milk-duct system towards ramification and tapering; (4) course of milk-duct system; (5) presence of paraductal cysts; and (6) localization, size and form of the ductal recesses. These findings are of the greatest importance.

The normal milk-duct system displays uniform tendency toward tapering in the direction of the periphery with a considerable fluctuating size-range in the lumina (Figures 63–65). The recesses in the area where the contrast-enhancing media are found, or, abrupt discontinuities at the terminus of a contrast-medium column indicate most likely the presence of an intra-ductal papilloma (Figures. 66–68). Figures 66 and 67 show a rare example of an extra-ductal papilloma, once following injection of the contrast medium (Fig. 66) and secondarily after injection of air (Figure 67).

The ductal papillomas of the large milk ducts, in contrast to a milk duct papillomatosis, present benign diagnosis, but nonetheless, a definitive differentiation from a malignancy is possible only by histological means.

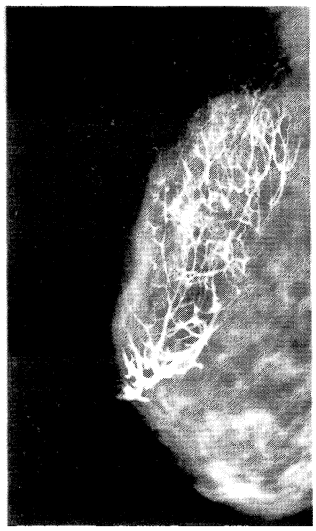
Sonography

The sonography of the breast goes back to Wild (394) and Howry (178). Wild was the first to evaluate the results and to obtain a two-dimensional representation of the breast. Howry *et al.* (178) succeeded in demonstrating a cirrhous carcinoma of the breast that could not be found by palpation. Both authors published the first diagnostic statistics and set up echo-criteria.

Kossof *et al.* (209), by developing the Gray-scale technique, established a milestone in the application of two-dimensional ultrasound images. Through this process, the transformation of variously strong echoes into different tones of gray became possible, leading to a rapid improvement in the image quality.

Based on what we know today, breast sonography has established itself as an adjunct to mammography with reference to the following indicators, differentiation between cystic and solid changes; in guidance for cyst aspirations and fine-needle biopsies; clarification of the integrity of palpable tumors; and the examination of very young and pregnant women. Sonography, disappointing as a primary screening method, has emerged as the single most helpful adjunct to mammography in evaluation of the clinically and/or mammographically abnormal breast (165). Ultrasonography is important in the diagnosis and therapeutic decision in cystic and fibrocystic masses but cannot substitute mammography in early detection of breast carcinoma (290). While breast sonography is frequently a useful modality for breast mass detection, particularly as an adjunct to x-ray mammography, the common overlap in characteristics of benign and malignant masses makes histologic evaluation of all solid masses essential (187).
See also: (92, 123, 136, 152, 180, 214, 216, 230, 237, 250, 295, 305, 308, 317, 344, 354, 355, 373, 389, 390, 422).

Ultrasound as an adjunct to mammography is beneficial in arriving at the correct diagnosis: (1) solid vs cystic mass; (2) lactating breast; (3) equivocal mammogram; (4) multiple breast masses; (5) negative dysplastic mammogram; (6) microcalcifications without mass; (7) postmastectomy chest wall. (215, 327). Ultrasonic parenchymal patterns of the breast can assess tissue patterns defined mammographically and may thus have the potential to serve as a marker of breast cancer risk (194). Ultrasound mammography can also demonstrate the normal involutional pattern of the breast with age, the ductal pattern, the effects of x-rays, and benign and malignant breast disease (26). Breast ultrasonography is a noninvasive, quickly performed procedure that presents no radiation hazard-like mammography and



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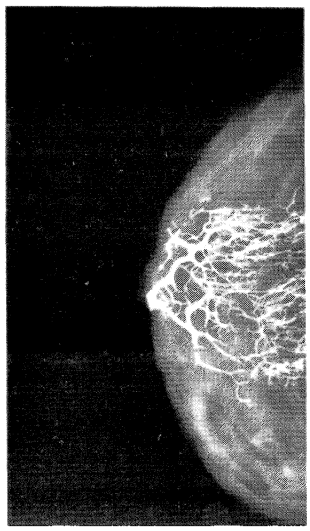

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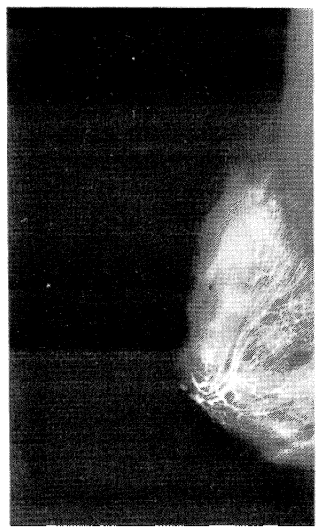

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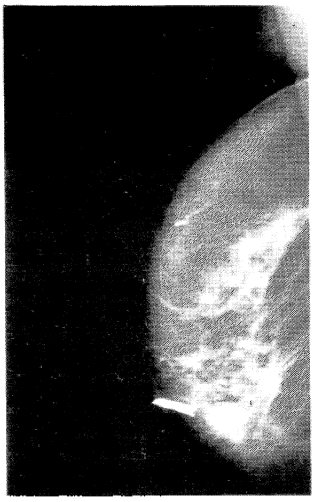

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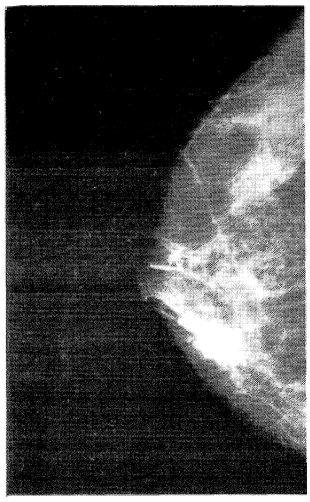

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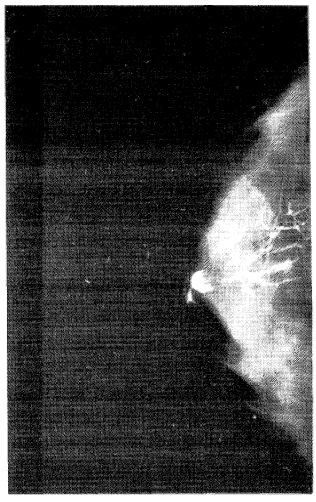

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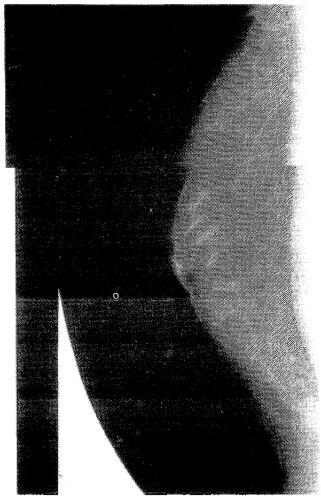

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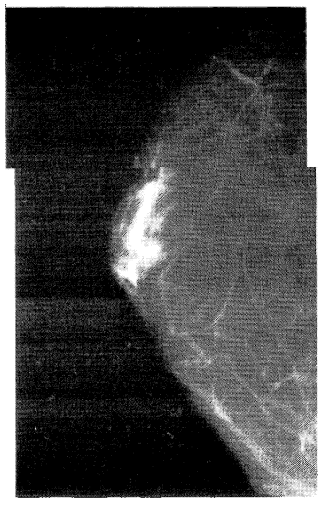

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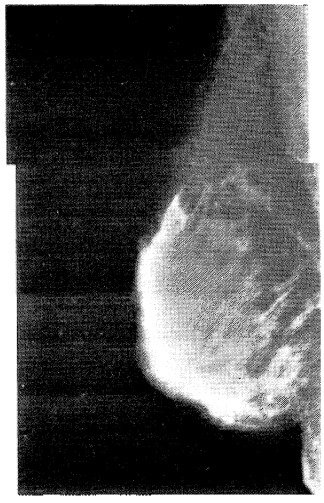

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has an appreciably high diagnostic accuracy of about 85–90% (200). Advantages of combining target ultrasonic mammography with cytology are the possibility of having a more precise puncture aspiration with target ultrasonic mammography and a consequently higher predictive value, compared to each technique alone (232). Hand held target ultrasonic mammography denotes a special procedure to examine a specific area of the breast (231).
See also: (119, 56, 237, 290).
Thermography: comprises either the noncontact (distance or telethermography) or the direct or contact form (the plates are placed directly on the breast), which permits the image representation of the infrared radiation emitted by an object. In the last few years thermography lost some of its value in the diagnosis of breast cancer because of the changeover to X-ray mammography and ultrasound mammography. Nevertheless, it is still a very useful additional method (83). Thermomammography, using proper technique, will permit visualization of cancer in the breast (303). Results from published series of Breast Cancer Detection Centers, in conjunction with calculations of the probability of malignancy based on test results, indicate that thermography was abanded due to high number of false positives and only mammography was used for screening (263). The accuracy rate of thermography is 67.8% for malignant and 70.6% for benign lesions (40). But false positions are as high as 80%.
See also: (3, 58, 70, 105, 230, 240, 259, 312, 339, 365, 425).

Distance thermography uses as detector for infrared radiation generally a semiconductor crystal of indiumantinomide – illumination will change its electrical resistance. To attain the required sensitivity, the crystal must be cooled with liquid nitrogen. The detector converts the heat radiation it has registered into electrical signals, which are made visible on the screen of an oscilloscope.
Contrast- or liquid-crystal thermography is based on the properties of-cholesterin-derivatives to reflect light-determined wave-lengths as a-thermography.

Computed tomography (CT) represents one of the modern, image-producing processes which were applied by Chang (54) and his staff for breast evaluation. several important papers were published by this group at the end of the 1970s, and the beginning of the 1980s on the CT-investigation of the breast. Using a contrast medium, they achieved a hit-rate of 94% in 78 histologically determined carcinomas. On the basis of experience to date, the following indications call for a CT-examination of the breasts: pain and mammographically dense glandular body – no mammary complaints but increased breast cancer risk and presence of breast glands that can be evaluated only with difficulty by mammography – breast cancer following breast conserving measures – augmentation plastic surgery – severe scar-formation following repeated specimen excisions and reconstructive surgery after subcutaneous mastectomy (189). The disadvantages of CT-examinations are radiation load, administration of contrast media, high costs of examination, and long investigation duration. On the basis of these disadvantages, indication for a CT-examination of the mammary gland becomes restricted to clinical suspect, and mammographically occult, lesions. The method has not yet been generally accepted and remains limited solely to some investigative centers.

Bovee, Getreuer, Smitt and Lindemann (41) as well as Ross *et al.* (291) have published the first results of nuclear spin tomography (MRF) of the breast. They examined 128 breasts of 65 patients. The results hitherto obtained with this technique must be reported from the present standpoint as modest when compared to other, potent, investigative procedures. However, nuclear-magnet resonance is still a

Figure 66. 28-year-old woman – normal galactogram; slight ectasis of milk ducts.

Figure 67. 32-year-old woman – normal galactogram; slight ectasis of milk ducts.

Figure 68. 27-year-old woman – normal galactogram, with fine milk duct system.

Figure 69. 60-year-old woman – extraductal milk duct papilloma; ectatic milk duct, filled with contrast medium papilloma showing as a filling defect.

Figure 70. Same patient – air-filled ectatic milk duct with papilloma.

Figure 71. 56-year-old woman – pencil-wide duct, with papilloma showing like a filling defect.

Figure 72. 17-year-old boy – puberty gynecomastia without glandular elements. Radiologically a pseudogynocomastia.

Figure 73. 53-year-old man – with a true gynecomastia. Related to long standing digitalis cut. This shows multiple ductal elements.

Figure 74. 40-year-old man – gynecomastia with female type secondary sex characteristics.

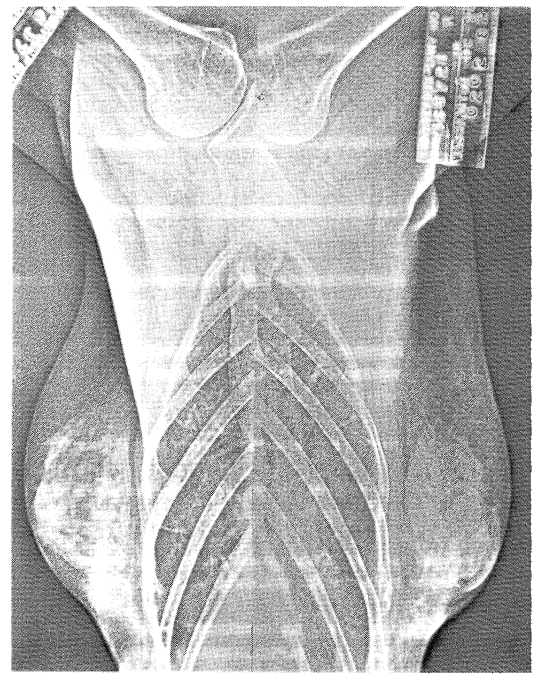

Figure 75. Lumpectomy and radiation therapy on the right side without any evidence of recurrence.

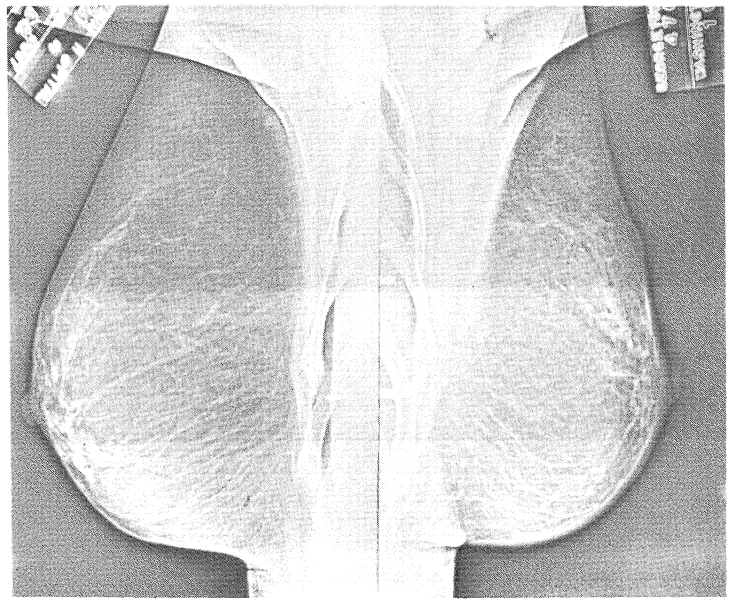

Figure 76. Lumpectomy and radiation therapy on the right with recurrence showing malignant calcifications.

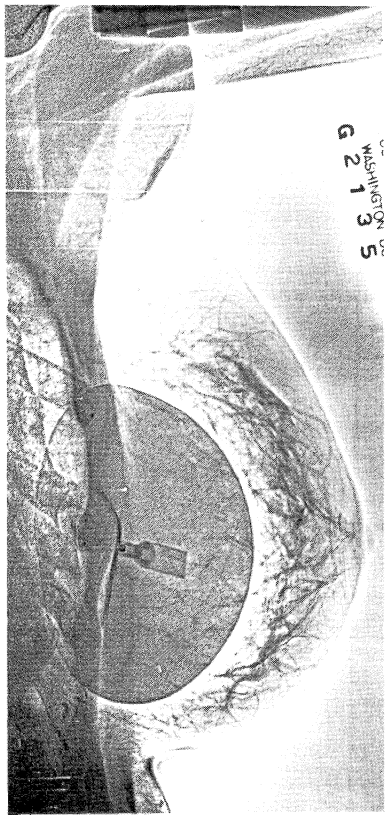

Figure 77. Breast implant.

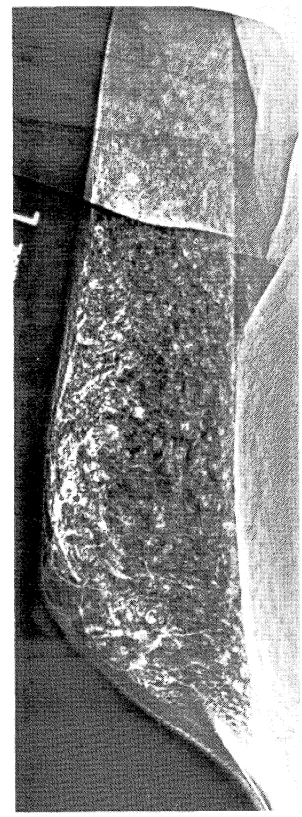

Figure 78. Injected silicone in an oriental (Tai) woman's breast.

very young procedure requiring further development, especially, experience on the part of the investigator. The great disadvantage of the examination procedure lies in the fact that it does not show microcalcifications. Ten malignant lesions exceeding 1 cm in diameter were diagnosed correctly by mammography and magnetic resonance imaging (81). See also: (114, 264, 353).

In the case of video-diaphanography (spectra scan or light scan), the contours of the breast are projected through infrared light by way of a video camera immediately (real time) and in color, after processing by a computer onto a screen. A polaroid camera is attached for documentation, or, alternatively, the images are recorded on discettes and can be reproduced on command. The method must undergo validation by larger examination centers, but it appears likely to recommend itself as an auxillary technique (249).

The use of both mammography and diaphanography reduced the number of false negatives from 11.8% to 5.5%. Diaphanography has been demonstrated to be a useful adjunct to mammography (379). Of 33 biopsy-proven cancers, transillumination light scanning detected 58%, while mammography detectd 97% of the cancers (112). Mammography was superior for detecting malignancy: of the 67 pathologically proved breast cancers, 64 (95.5%) were detected by mammography and 45 (67.2%) were detected by transillumination. Infrared light scanning of the breast has proven effective in the hands of trained personnel and should be used as an addition to breast examination and mammography to increase yield of breast pathology (212). Cooling of the breast should be avoided in diaphanography. A false positive diagnosis, i.e. possibly malignant, probably malignant or malignant was made in 15 cases (9.2%) with OPG, and in 33 cases (10.1%) with M. Use of both M and DPG reduced the number of false positives to 1.8% (376). See also: (73, 82, 175, 346, 377, 378, 379).

Summary

Mammography (film-screen or xerox) is superior to all the methods for detecting malignancy: In one study of the 67 pathologically proven breast cancers, 64 (95.5%) were detected by mammography and 45 (67.2%) were detected by transillumination (113, 207). Investigative uses of breast specimen radiography have received little attention (104). The most important mammographic signs of malignant calcifications are grouped, (clustered), sand-like or filament-like or micro rod-like microcalcifications (15, 151).

With a look to the future, the role of mammography in the early diagnosis of breast cancer and in the confirmation of a clinical diagnosis will remain indispensable. It is the gold-an standard. All other examination methods, including computer tomography (CT) and nuclear spin tomography (MRI), achieve only auxillary importance. The additional application of these methods will depend primarily on the inclinations and the personal experience of the investigators. Another drawback: they will be highly costly and time consuming.

In this connection, sonography assumes a special position, because in the differentiation of solid and cystic tumors it is superior to mammography.

The superiority of mammography in comparison to all other methods lies primarily in the fact that it permits the detection of microcalcifications, (which may occur in 30%–50% of all carcinomas) nonpalpable masses, architectural distortions and localized duct enlargements.

On the basis of medical experience thus far, mammography of the future will be confronted by the following tasks:

1. discovery of clinical occult carcinoma of the breast, which amounts to 10% to 15% of all carcinomas;
2. avoidance of unnecessary tissue excisions as a result of safe diagnosis of fibroadenomas and cysts, concomitant with fine-needle biopsy;
3. exclusion of multiplicity and bilaterality of tumors in the case of fully determined carcinomas;
4. provisions of assistance with needle in localization of clinically occult (smaller carcinomas and microcalcifications) prior to surgery for the surgeon;
5. application in tumor prophylaxis.
6. mammography (film-screen or xerox) is the gold standard, Recommendations of American Cancer Society: Every woman should have a baseline mammogram somewhere between age 35–40 – then even earlier mammograms could be performed in high risk group. After 40 mammograms performed every other year until 50, and yearly screening thereafter.

Check:

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8

THERMOGRAPHY INSTEAD OF MAMMOGRAPHY?

HERMANN-ULRICH LOHBECK

Ray Lawson (45) was the first to observe that the majority of breast cancers investigated radiated more warmth than the surrounding normal tissue. He already suggested at that time that thermometry should be included in the arsenal of diagnostic investigations in breast cancer. As early as 1957, he presented the first thermograms and reported that the skin over a mammary carcinoma is 1–3° warmer than the other skin regions of the breast. By intraoperative temperature measurements in the afferent artery and the efferent veins, Lawson and Chughtai (44) were able to observe that the tumor temperature was higher than the temperature in the blood vessels. In turn, the venous temperature was also higher than the temperature in the artery. These observations allowed the conclusion that the tumor itself produces heat as a manifestation of a raised metabolic activity. By direct heat conduction through the surrounding tissue, the tumor heat reaches the skin surface or it is transported indirectly with the venous blood flow to the skin. Dodd *et al.* (15) showed once more that breast cancers are frequently warmer than the blood in the arteries and veins. On the basis of these observations, the metabolic activity of the tumor itself has a major significance. On the other hand, it has been demonstrated by arteriographic investigations that malignant tumors show increased vascular neogenesis, the growth of which is stimulated by a tumor angiogenesis factor (TAF) named by Folkmann *et al.* (21, 22). This is a protein isolated from malignant tumors, Dodd (16) reported that the temperatures prevailing in the tumor, the arteries and veins reflect the metabolic activity of the tumor.

The increased heat radiation over a tumor which is manifested at the skin surface could also be demonstrated by means of infrared thermography by Lloyd-Williams *et al.* (48), Gershon-Cohen *et al.* (28), Lawson and Gaston (43). Gautherie *et al.* (26) confirmed these observations once more by intratumoral and extratumoral heat measurement *in vivo* by means of thin thermal probes in the thickness of needles. However, Lloyd-Williams *et al.* already reported as early as 1961 that they were also able to measure temperature elevations in some benign processes in the breast.

For a breast cancer to be visualized by thermography, several conditions must be fulfilled. First of all, the neoplasm must produce sufficient warmth. This must reach the skin by heat conduction through the tissue. The tissue surrounding the tumors and the blood vessels of the tumor plays a crucial role in this process. The veins by which the tumor heat is transported away are of great significance here. Special attention must be paid to the venous vascular system in the appraisal of the thermograms. Dodd (16) reported that the heat unevenly radiated over malignant tumors essentially derives from the efferent veins. As a further prerequisite for registration of the thermal signals for the detectability in thermography, the skin must be biologically able to emit the heat, which is not the case e.g. in dermatoses, eczema, ulcerations and scars. However, a vigorously proliferating carcinoma can remain concealed in an adipose breast due to the heat-insulating properties of the fat. At best, it may be manifested indirectly by over-heating of the blood in the efferent veins.

These observations already lead to increased use of thermography in the diagnosis of breast cancer at the end of the 1960s. First of all, instrumental techniques were used which registered the infrared radiation of the human skin electronically by means of detectors. We refer to infrared thermography, which is also known as electrical thermography, distance thermography or telethermography. This is intended to fulfill the task of detecting and visualizing the endogenous heat radiation from the skin from a distance. In contrast to this, plate thermography (see later) achieves this objective by direct contact with the skin surface (contact thermography). In the current infrared thermography systems for medical use, an infrared picture is produced by means of a special optical system. This picture is broken down into electrical impulses line-by-line by a mechanical optical scanning system in order to build up a gray-tone image proportional to the heat distribution and visible on the monitor. Since it is easier for the eye to evaluate the images when they are inverted, warm points and zones appear dark or black. The inversion image has prevailed today. An additional possibility of this apparatus is the electronic representation of lines of equal temperature (isotherms). The use of color filters enables the production by means of isotherms switching of color thermograms in which areas of the same temperature have the same color. The isotherm function enables exact measurement of temperature differences of two points (temperature gradient ΔT), and their specification in absolute temperature. The temperature gradient is of great significance as a criterion of a pathological thermogram in infrared thermography of breast cancer. This gradient is designated as ΔT when a circumscribed region with increased heat radiation or a "hot spot" is found. The temperature gradient is designated as $\Delta T1$ in comparison with the temperature difference of the surrounding region of the ipsilateral breast and as $\Delta T2$ in comparison with the corresponding region of the contralateral breast. A few other characteristics are also accounted as a pathological thermogram: hypervascularization with

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proliferated, dilated and meandering blood vessels, vessel discontinuations, as well as asymmetric hypervascularization. Furthermore, the warm mammilla and global hyperthermia are to be mentioned.

A classification of the thermographic criteria specified above for diagnosis of breast cancer has been suggested by Gros *et al.* (32), Amalric *et al.* (3), Isard (36), Barash *et al.* (6), Lohbeck (49) and Chang *et al.* (12). Draper and Jones (18), Gershon-Cohen and Habermann (29), Gros *et al.* (31), Lapayowker *et al.* (41), Patil (58) and Amalric (3) have attempted classification of the normal thermogram.

Since 1970, and to an increasing extent in the recent years, plate thermography has become more widespread in clinical diagnosis. This is a method developed by Tricoire *et al.* (67) which is also termed "thermographie en plaque", cholesteric thermography, crystothermography and contact thermography. It involves liquid crystals which are applied to a heat-conducting film.

Temperature differences of 0.3°C are rendered visible with different colors. The color differences range from blackish brown over chestnut colors to reddish, orange, green, greenish blue to ultramarine and violet. Such a foil, which is clamped over a frame and which appears black in the cold state is applied on the breast. The alteration of color appears within seconds. It is noteworthy that the areas red or brown (generally regarded as a "warm") correspond to the lowest temperature range in plate thermography, and the intense blue corresponds to the highest skin temperature. By cooling the breast with a current of the air, this picture can be altered. This dynamic alteration with cooling and rewarming of the breast has great significance for clinical diagnosis.

Tricoire *et al.* (67) have described a classification of thermographic findings in plate thermography. The ramifying warm spot, loops of various forms with the vessels penetrating into the tumor, vessels with stellar convergence and occasional vessels of atypical course are regarded as criteria of malignancy.

A compilation of the *results obtained with infrared thermography* for appraisal of the clinical value in the diagnosis of breast cancer (Table 1) shows that there was a suspicion of cancer in an average of 83% of the cases among more than 3,000 cases with breast cancer. At the individual authors, the precision varies between 32.5% and 97%. This scatter does not appear to be remarkable, if the different criteria for evaluation of carcinoma suspicion are considered. The temperature gradient ΔT2 as the most important criterion of suspicion of carcinoma ranges from 1°C to 2.5°C Bjurstam *et al.* (9) showed the significance of the level of the temperature gradient ΔT2 taken to be suspicious for 1°C, 1.5°C and 2°C in 182 carcinomas (Table 2). It can be clearly seen that the "false negative" results rise, the higher the temperature gradient is set for a positive thermogram. Hessler (34) rated 28 out of 34 carcinomas as thermographically positive in application of the critical gradient ΔT2 = 1°C. When the gradient ΔT2 is raised to 2°C, barely more than 50% of the breast cancers could be classified as "suspicious".

Table 2. Positive thermogram in different temperature gradients (after (9)).

Temperature gradient °C	*Normal palpatory finding %*	*Benign alteration %*	*Carcinomas %*
1.0	39	56	82
1.5	33	41	69
2.0	23	20	52

The relatively favorable results with infrared thermography led to the method being widespread at the beginning of the 1970s. In order to be able to evaluate the actual clinical value of the method for diagnosis of breast cancer, it appeared to be of major significance that the rate of false-positive results is known and above all the precision is broken down separately for the individual tumor stages. Table 3 shows once more the percentage results of infrared thermography, especially the relatively high false-positive rates, which reflect the nonspecificity of the thermography technique. Benign, biologically active processes can produce thermograms which are "false-positive" because they are not specific for a carcinoma.

Data on a dependence of the precision of infrared thermography on tumor size are found in the literature only at a few authors. In Table 4, the results from five different groups are

Table 1. Infrared thermography. Results in breast cancer.

Author	*Year*	*Number of carcinomas*	*Positive thermogr.*	*Temperature gradient T2*	*Results %*	*False negative diagnosis %*
Gershon-Cohen et al.	1967	200	184	> 1.0°C	92	8
Melander	1972	232	213	> 2.0°C	92	8
Wallace	1969	195	166	⩾ 1.0°C	85	15
Gros et al.	1971	468	352	> 2.0°C	73	27
Aarts	1972	93	90	⩾ 1.5°C	97	3
Isard et al.	1972	306	218	1–2°C	71	29
Jones et al.	1973	190	156	> 1.5°C	82	18
Pistolesi	1973	108	101	⩾ 2.0°C	93	7
Amalric et al.	1974	1103	1013	> 2.5°C	91	9
Bothmann et al.	1974	132	37	⩾ 1.0°C	32.5	67.5
Lohbeck	1977	103	68	⩾ 1.5°C	66	34
Total		3130	2598	1–2.5°C	83%	17%

Table 3. Infrared thermography. Diagnostic value in breast cancer.

Author	Year	Correct positive finding %	False positive finding %
Dodd	1969	85	11
Lilienfeld	1969	72	20
Forrest	1970	74	40
Davey	1970	73	11
Isard	1972	72	31
Barash	1973	75	22
Stark u. Way	1974	84	16
Jones	1975	81	21
Byrne u. Yeres	1975	81	32
Johansson	1976	72	38
Amalric	1976	74	13
Raskin	1976	88	30
Feig	1977	66	42
Steward	1977	61	22
Egan	1977	29	4
Valdagni	1977	80	33
Clark u. Rideout	1978	84	11
Snyder	1979	62	35
Barrett, Myers u. Sadowsky	1980	99	67

specified in relation to the tumor stage. In 3,772 cases with breast cancer in stage T1 (tumor of a size up to 2 cm in diameter) the thermography result is suspicious of cancer in only 47% of the cases. In the carcinomas of stage T2 (tumors of 2–5 cm size), the correct diagnosis averaged 65%, and in the stages T3/T4 88%. If the precision also varies appreciably in the different tumor stages at the individual authors, the results clearly show that the information provided by infrared thermography is least in small carcinomas. However, since in clinical diagnosis of breast cancer we do not require any additional methods which offer a high precision only in the advanced stages, which can be detected adequately by inspection and palpation, infrared thermography only has a clinical value from its precision in stage T1.

On the basis of the initially favorable results of infrared thermography, this risk-free method has been employed in some centers for mass screening of breast cancer. In Table 5, the results obtained with large numbers of patients are presented. The collected statistics contain the results in 253 breast cancers in which infrared thermography had revealed suspicion of carcinoma in 6 per thousand of the women investigated. The conditions of mass screening with infrared thermography differed in the individual patient groups. Whereas some authors merely used thermography and clinical examination, other authors have primarily combined thermography with mammography. the significance of infrared thermography in mass screening can only be inferred from such a combination, since otherwise the carcinomas not detected by thermography would have remained undiscovered.

In the mass screening for breast cancer in asymptomatic women which we have carried out in Hamburg since 1973, infrared thermography is employed routinely in combination with mammography. Our results from 1973 to 1979 (Table 6) show that among 13,822 asymptomatic women, a pathological thermogram was found in 16%, whereas there was a normal thermogram in 84%. Among all those investigated 62 were found to have carcinomas. In 59 cases which were unequivocally suspicious in mammography, the thermography was suspect in only 11 women. Only three carcinomas were discovered by thermography. However, these displayed further clinical symptoms such as secretion.

A further indication for appraisal of the value of infrared thermography in early diagnosis of mammary carcinoma is provided by Table 7, in which the tumor size is compared with the results of infrared thermography in our patients comprising 62 clinically occult mammary carcinomas. With a tumor size of less than 1 cm, the thermography was negative in six cases and revealed a thermographic result requiring surveillance in only one case. Of the total of seven carcinomas, there was no suspect thermographic result in any case. In the tumors between 1 and 2 cm in size, the thermography was suspect in 19%. If all carcinomas up to 2 cm in size are taken together, then thermography provides a result suspicious of cancer in only 16%. The precision rose to over 20% only in tumor sizes above 2 cm.

The *clinical significance of plate thermography* is rated higher than that of infrared thermography above all by German-speaking authors. Table 8 shows that plate thermography has revealed a suspicion of cancer in 81% of more than 1,000 breast cancers. These results led to a great popularity of the method. On the basis of the favorable figures, hopes rose that a relatively certain method for diagnosis of breast cancer (especially early diagnosis) was now available. However, in my opinion the results are not yet reliable enough to document the clinical value of plate thermography. This can be achieved better via a breakdown of the precision in carcinoma diagnosis in relation to the tumor

Table 4. Infrared thermography. Results in breast cancer in relation to the tumor stage (UICC).

Author	Year	Number of carcinomas	Pathological thermogram 2 cm T1	2–5 cm T2	5–10 cm T3	10 cm T4	T1–T4
Bourjat et Gautherie	1972	486	40%	63%	88%	92%	73%
Bothmann et al.	1974	132	21%	36%		75%	32.5%
Lohbeck	1977	183	48%	70%		90%	61%
Amalric et al.	1978	2198	70%	89%	97%	98%	91%
Hagay et al.	1981	773	58%	67%	86%	80%	73%
Total		3772	47%	65%		88%	66%

Table 5. Infrared thermography. Results in mass screening.

Author	*Year*	*Number*	*Ca*	*Results*	*‰*	*Note*
Davey	1970	1768	15	Combination clinical findings and thermography = 1717. Abnormal thermograms: 197 (11%). These include 11 carcinomas, 2 carcinomas were exclusively detected by thermography	6.4	13 carcinomas clinically suspect
Melander	1972	7200	35	Thermography abnormal – 2100 (29%), combination of thermography and mammography 35 carcinomas	4.8	2 carc. within 10 months
Strax	1973	11000	78	Combination clinics, mammography + thermography, 70 carcinoma	6.4	8 carcinomas by self-exam.
Isard u. Ostrum	1974	4393	36	Thermography: 22 carcinomas Mammography: 30 carcinomas Combination: 32 carcinomas	7.3	4 carcinomas by blind puncture
Stark u. Way	1974	4621	27	Thermography abnormal: 628 (14%) Combination of thermography and mammography 27 carcinomas (22 carcinomas preclinically)	5.8	7 carcinomas within one year
Lohbeck	1982	13822	62	Thermography: 11 carcinomas Mammography: 59 carcinomas Combination: 62 carcinomas	4.5	all cases asympt. women
		42804	253		6	

stage. In the literature, such a procedure is to be found only at a few authors. Table 9 shows these results. With increasing size, the precision also rises with plate thermography (as is also the case in infrared thermography). In stage T1, the precision in our group was for example merely 54%. In the stages T2 to T4, it rose to over 85%. Compared with infrared thermography, it is thus to be observed that comparable conditions are present.

Some authors report that clinically occult breast cancers can also be discovered with plate thermography (10, 24, 35, 57, 67). In addition, Mühlberger *et al.* (56) report that it is also possible to diagnose cancer prestages by means of plate thermography. Our results indicate that the significance of plate thermography in the diagnosis of prestages and early stages of breast cancer is rather encouraging. Table 10 shows a comparison of the incidence of pathologically suspicious findings in these stages and the comparison with control investigations. Criteria of malignancy in plate thermography were found in only 11% of clinically occult invasive carcinomas. Early invasive carcinomas showed suspicious thermographic signs in only 9%. Five carcinomas with noninfiltrative growth did not reveal a suspicious plate thermographic signal. In 80 cases of mastopathy with high degree of proliferation, a sufficiently suspicious criterion was found as a thermographic result in only 5%. It should not remain unmentioned that a positive pathological thermogram was also found in 17% of a normal control group. We cannot confirm the favorable results in the diagnosis of early carcinomas or their prestages by plate thermography. In addition, thermographically abnormal findings in the breast

Table 6. Results of infrared thermography in the context of breast cancer prevention examinations on clinically asymptomatic women by combination of mammography and infrared thermography (n = 13822).

Results of infrared thermography	*n*	*%*	
Suspect	276	2	
Requiring surveillance	1935	14	16% pathological thermograms
Normal	11611	84	
Total	13822		

Table 7. Findings with infrared thermography in relation to size of tumor (62 clinical occult cancers).

Thermographic classification	*Tumor size in the X-ray*		
	< 1 cm	*1–2 cm*	*> 2 cm*
Normal	6	19	7
Requiring surveillance	1	15	3
Suspect	0	8 = 19%	3 = 23%
		16%	

Table 8. Plate thermography. Results in breast cancer.

Author	*Year*	*Number of carcinomas*	*Thermography positive*
Tricoire *et al.*	1970	78	100%
Bothmann *et al.*	1974	132	73.5%
Müller *et al.*	1974	59	78%
Geissler	1974	46	87%
Frischbier	1975	83	84%
Lauth *et al.*	1975	109	92%
Hüppe	1976	146	78%
Barth	1977	198	70%
Lohbeck	1984	287	72%
Total		1122	81%

Table 10. Thermography. Frequency of pathologic or suspicious findings in early forms of cancer and in controls.

	Number	*Frequency of findings*	
		Pathologic	*Suspicious*
Clinical occult invasive cancers	53	26%	11%
Early invasive cancers	11	18%	9%
Preinvasive cancers (comedo-ca; lob. carcinoma *in situ*)	5	20%	0%
Mastopathy, high degree of proliferation	80	19%	5%
Controls	3350	15%	2%

cancer conditions may be situated far away from the seat of the tumor, so that an exact localization is impossible. This gives rise to a special problem which limits the value of plate thermography: it must be accompanied by palpable or radiologically localizable findings, so that it is only of significance for differential diagnosis. It has only minor value for early diagnosis of breast cancer. There is no doubt that the efficiency of diagnosis is improved by combined application of mammography. Application of plate thermography alone in combination with palpation and inspection in clinically normal findings is not reasonably related to its usefulness when the expense and time are considered.

To summarize the results of plate thermography and infrared thermography, their significance in the diagnosis of breast cancer (especially in early diagnosis) can be evaluated as follows: thermography has succeeded in revealing carcinomas which are not detected by mammography, e.g. diffuse mastopathy. As was shown in this contribution, none of the various criteria in thermography is sufficiently specific for differential diagnosis of a suspect, malignant or benign finding.

The apparently higher diagnostic reliability in plate thermography, which is simpler to handle than the very expensive infrared thermography, does not bring about any graduated influence on the accuracy in diagnosis of breast cancer.

It can be seen from the Tables that only half of the palpable carcinomas can be detected by means of thermography in stage 1 of breast cancer.

Tumors far away from the surface which are difficult to palpate and carcinomas less than 1 cm in diameter only very rarely have such a high metabolic activity that a pathological result of diagnostic value can be expected from thermography. The breast cancers detected in a preventive mammographic examination (i.e. in normal palpatory finding in asymptomatic women) show suspicious thermographic findings in less than 20%. Carcinoma prestages can be diagnosed by means of thermography. However, the rating of these suspicious thermographic criteria leads to an unjustifiably high rate of false positive findings. Thermographically suspect findings can be demonstrated far away from the tumor location in malignancies. An exact localization is thus impossible and underscores once more that thermography only has a value when it is accompanied by a palpable or radiologically localizable finding. Thermography is by no means an alternative technique to mammography in the context of breast cancer prevention examinations. Only in combined application with clinical investigation and mammography is thermography of value in the sense of an additive technique. It is possible that thermography is of significance as a prognostic indicator and possible for identification of risk cases. However, this must be doubted on the basis of our experience. Thermography is thus to be rejected as an exclusive method and also as an alternative technique to mammography in breast cancer preventive examinations on the basis of the present results. Thermography is at best an additive technique to mammography in early diagnosis of breast cancer.

Table 9. Plate thermography. Results in breast cancer in relation to the tumor stage (UICC).

			Pathological thermogram				
Author	*Year*	*Number of carcinomas*	*2 cm T1*	*2–5 cm T2*	*5–10 cm T3*	*10 cm T4*	*T1–T4*
Frischbier	1975	83	58%	92%	100%	100%	84%
Hüppe	1976	146	65%	86%	100%	100%	78%
Barth	1977	198	49%	68%	94%	100%	70%
Lohbeck	1984	287	54%	87%	100%	100%	72%
Total		714	57%	83%	99%	100%	76%

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POSTSCRIPT

Since there is still controversy about the effectiveness of current diagnostic methods in pathologic lesions of the breast (1–5), multimodality testing is the logical choice and new improvement are of value (2). In 201 lesions investigated by van Dam and co-workers the sensitivities of physical examination, mammography, ultrasonography and thermography were 0.88, 0.94, 0.78, and 0.49, respectively (8).

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9

THE CHRONOBIOLOGIC PILOT STUDY WITH SPECIAL REFERENCE TO CANCER RESEARCH IS CHRONOBIOLOGY OR, RATHER, ITS NEGLECT WASTEFUL?

GERMAINE CORNÉLISSEN and FRANZ HALBERG*

STATUS QUO

It is customary in biologic and, in particular, in medical research to do a pilot study first. Statements such as '. . . undertook the pilot study to evaluate the methodology for conducting a main study. The pilot study was not designed to yield definitive results, but the figures are suggestive' (22) and often found in the medical literature. Such pilots are indispensable in order to ascertain that a given chemical or biologic method 'works'. 'Work' may also mean that an anticipated effect is found. If timing enters a pilot protocol, it is usually in the form of a suggestion that the study be carried out at one convenient timepoint. If the pilot is encouraging, in cancer or other research, carried out by testing, for instance, a new drug, a 'definitive' approach is practiced. A new treatment is compared with an old one. Usually, the therapeutic arms are specified as to dose, but hardly ever with respect to circadian and other rhythm stage. A chronobiologic assessment of drug effect is also not usually considered. If the 'pilot' is negative, the approach is no longer pursued. The possibility then remains that a 'good' drug is discarded because its time-dependent effect and/or its effect upon time structure were not tested. It is also possible that a major investment is made in the large-scale trial of a bad drug, the time-dependent side-effects of which were tested neither in the pilot nor in the time-indiscriminate definitive study. The choice, at best, of a single convenient timepoint for the test is attributed superficially and, as discussed elsewhere (47), invalidly to the excessive cost of chronobiologic designs. The culprit, however, is a fundamental belief in a homeostatic, if fictitious, 'regulation for constancy', and accordingly the viewing of rhythms as epiphenomena.

Fundamental error

It is tempting to assume that if, in one approach, a given study at 1 timepoint costs x in effort and money, then a study at 6 timepoints (e.g., 4 hrs apart, for the study of a circadian rhythm) will cost 6 × x. This is true indeed if no replications are needed, i.e., if one does not need to assess variability. This hypothetical case is realistic neither in cancer research nor in many other studies. The assessment of variability in 1988 has become a *sine qua non*. A comparison of 2 values without a standard error is hardly acceptable.

The chronobiologic approach provides additional information at no added cost

Once variability is to be assessed and replications are planned, we can illustrate the desirability of a chronobiologic approach. We simply assign, from a set of any 6 experimental units, one to each of 6 different timepoints, rather than testing all 6 units at the same timepoint. But before we do so, let us play the devil's advocate and look at other options. Indeed, given a budget to carry out an experiment, allowing for n repeated measures, one has several choices to design the experiment.

Ignore timing?

One can decide to proceed to the n measurements whenever it is convenient, without consideration of time, in a homeostatic pilot approach. In 1988, this is still a not uncommon and certainly not a rare decision; it deserved comment a few decades ago, when it was realized that the same stimulus has drastically different effects at different circadian times (37). Today, the evidence is available that immunomodulators can enhance or delay carcinogenesis as a function of administration time (32).

N experimental units placed at a fixed time?

The foregoing conclusions relate to a practice which is more common today, namely the stipulation to standardize one's procedure from the start 'simply' by taking all n measurements at the same (conveniently fixed) clock-hour. A better estimate of the mean at that sampling time is anticipated. Whether less variability is found depends on whether any rhythms involved are synchronized by a standardization of the living routine of the individuals investigated. For in-

* This review extends the scope of an earlier paper prepared on the chronobiologic pilot by Halberg (1987 and partly unpublished), amplifying on studies in Halberg *et al.* (66) and adds oncologic focus to this and other such pilot designs, including a jointly developed approach by triangulation, so named by the senior author (GC).

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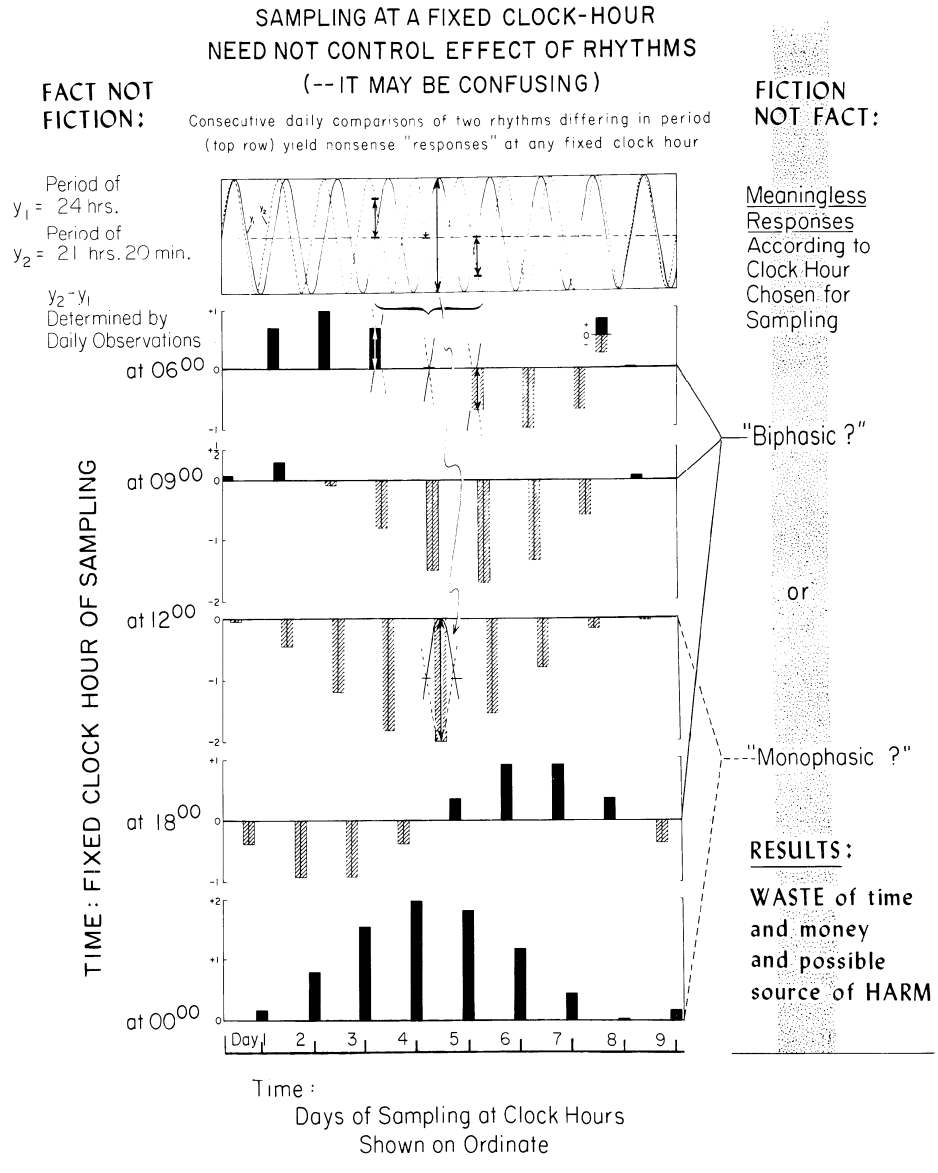


Figure 1. Confusion arising from a difference in period of the rhythms of two individuals or groups, compared at a fixed convenient time of day. The difference between the two groups being compared will undergo drastically different changes with time, simply as a function of the particular clock-hour chosen for observation. More specifically, the figure shows the time-course of an inter-group difference between synchronized controls and desynchronized experimentals, when comparisons are made 24 hours apart, at one or the other clock-hour. A given physiologic function, y, is assumed to be circadian periodic in both groups compared. y_1 could represent the very common 24-hour synchronized case, while y_2 could differ in period from y_1 by 160 minutes in time. On the plot, y_1 and y_2 start out in phase at 06, in each case. The figure models a control, being compared with a subject exhibiting a circadian desynchronization. A similar finding may also occur in a comparison of a 24-h synchronized subject with a subject undergoing a schedule shift. Such patterns may be found in a plethora of publications on functions previously demonstrated as circadian periodic. The value of such responses is questioned, even if everybody samples at the same clock-hour. Thus, if a student working daily at noon records a drop, e.g., in the biochemical value of a patient, a replacement therapy will be advocated. This would be disputed (and should be) by a colleague working at midnight who records a rise and recommends the opposite treatment. Were it not that the more prominent investigators of circadian systems are themselves synchronized by rather similar social schedules, such disputes would be much more frequent. Whether or not a response is contested, however, matters little. Actually, the undisputed 'result' is the more dangerous one, since it can be the basis of unwarranted clinical action.

stance, some laboratories do not institute fixed lighting regimens. It is known that the alternance of light and darkness is a strong synchronizer of circadian rhythms (54) and that, in the absence of this and/or some other synchronizer, in continuous light or in haphazardly illuminated rooms, the choice of a given clock-hour is not likely to involve the same physiologic state on different days or on different experimental units. Investigators who have continuous illumination in their animal facilities may rationalize that they 'randomized' the rhythms of their animals. What in fact is achieved is almost certainly an increase in noise level rather than a rigorous randomization of circadian rhythm stages. Even when not only the lighting regimen but also other synchronizers are used concomitantly – meal timing can be so manipulated – and in addition a fixed time of day is chosen for sampling, it should be realized that this approach completely disregards any rhythmic structure of the variables investigated and can lead to the fallacies depicted in Figure 1 (71). Clearly, polypragmasia and therapeutic nihilism are equally legitimate outcomes of results such as these.

2-timepoint spotchecks?

The chronobiologic approach recommends at the *outset* that the n measurements be assigned to different timepoints in order to test different rhythm stages, since the extent and even the sign of the response to a given treatment may depend on the rhythm stage at which the treatment is administered. If prior information is available as to the times at which the outcome is minimal and maximal, a 2-timepoint approach (at the times of the peak and the trough) can be preferred, since the difference between treatments is then the largest and each timepoint's mean can be more precisely defined (74, 75). The use in the clinic and research of the 8 a.m. and 8 p.m. circulating cortisol test on diurnally active, nocturnally resting subjects is a logical follow-up.

3 timepoints?

When no prior information is available concerning the rhythmic structure of the variables investigated, the 2-timepoint approach may be carried out inadvertently at the midline crossings of a rhythm with a very large amplitude. To avoid this misleading situation, a 3-timepoint approach seems to be reasonable. A cosine curve can be fitted exactly to three (mean) values. The fitted curve provides valuable information whenever the underlying rhythm presents a smooth waveform that can be well-approximated by a cosine curve. If the 3 timepoints are equally spaced, allowance can be made for an 8-hour span of uninterrupted sleep, for studies carried out on (or by) actively involved human beings. At first, such a design seemingly has great merit. Sampling at three equidistant timepoints can, however, be satisfactory only in relatively rare instances, when one deals with a nearly sinusoidal rhythm, with a favorable amplitude-to-noise ratio, usually the result of a large amplitude. Replications at the timepoints tested are also needed to assess the variability involved, mainly when sampling is serially independent. With such replications, in certain cases, one may then explore rhythms with several frequencies. Thus, in a study on corneal mitosis, repeated sampling at three timepoints, 8 hours apart, for a week or two, allowed the assessment not only of the circadian variability involved but also of a rhythmic component with a lower frequency, namely an infradian rhythm, modulating the circadian one (129). The result of the fit of a cosine curve to a single set of three measurements, however, is limited by the fact that an error estimate is not possible. Moreover, once replications at 3 timepoints are being considered, the option of sampling at added timepoints (rather than scheduling replications at 3 fixed times) must not be ignored.

5 or 6 timepoints?

In the majority of designs, it is preferable that the n available measurements, allowed by the allocated budget, be assigned to several timepoints, e.g., to 6 (or at least 5) nearly equidistant sampling times, ~ 4 (or ~ 4.8) hours apart, in the case of a problem anticipated to involve a prominent 24-h synchronized circadian rhythm. The cost will be the same as that of a design with 5 or 6 measurements at a single time. The 5- or 6-armed design, however, is more powerful, mainly if it is accompanied by cosinor methods for rhythm detection (55, 70, 73). In view of the parsimony in particular of the single cosinor model, this type of study design is usually efficient even if, at first, it appears to be demanding of around-the-clock sampling and thus of sleepless nights and overtime pay for skilled personnel. These apparent drawbacks of around-the-clock sampling on rodents were eliminated once it was shown that a host of circadian rhythms at different organization levels can all be changed in their time location by the (cost-effective) manipulation of the lighting regimen (35, 54, 75, 108, 121). Moreover, the amenability of circadian systems to shifts in time location is a rather general characteristic and likely applies to most if not all species, including human beings. It was then possible to stagger 6 regimens of light and darkness, alternating every 12 hours, in 6 rooms or boxes in such a way that lights went on (and off) 4 hours apart. Hence, after an appropriate adjustment span, one could sample 6 circadian stages at the same clock-hour. The amenability to schedule shifts also applies to human beings, but is less readily practicable, except for studies on shift- and day-workers. In human beings, however, studies done on the same day on the northern and southern hemispheres allow at least a concomitant exploration of two circannual stages, if, and only if, it has been shown earlier that these rhythms are about 180° out of phase with each other.

Anticipated sample size

Depending on the question being asked, the number of subjects required to test for a difference in outcome between two cancer or other treatment modes will vary. For instance, results on the radiotherapy of oral cancers obtained in India showed a difference between 30% and 70% tumor regression (and also a gain of 2 in terms of remission in a 2-year follow-up), depending upon the timing of treatment with respect to the acrophase of the circadian temperature rhythm (63). Assuming that such a difference can be anticipated in further studies, the following questions may be asked:

1) Given particular success rates for two groups treated in

different ways, how large must the groups be in order for the difference to be judged statistically and biologically significant? One may worry that with small numbers of patients, quite large statistically apparently significant differences could arise by chance alone. With only two groups, and an anticipated difference between 30% and 70% tumor regression, according to Boag (2), a minimum of ~ 30 subjects would be needed, 15 in each group, in order to achieve significance at the 95% probability level;

2) Assuming particular values for the true success rates, the rates that would be expected with infinitely large groups of patients, how many patients would have to be included in order to make reasonably certain that the trial will reveal the better treatment as significantly better than the other? Again, by chance, the superior treatment might yield fewer successes than expected, too few indeed to yield convincing evidence of an advantage. The more certain one wishes to be that this does not happen, the more patients would be required in the trial. Again, by consulting the curves published by Boag *et al.* (3), in order to be 75% certain of obtaining conclusive results at a $P = .05$ level, about 60 patients would be required, 30 in each group. If one wishes to have 90% assurance of getting a conclusive result, the corresponding number of patients required is about 100, 50 in each group.

In a chronobiologic design, preference is given to studying several rhythm stages, usually in 6 groups treated 4 hours apart in circadian studies. It may appear at first that 'six groups are just too many' (110, 111). We believe we have presented evidence documenting that, for exploiting rhythms in optimizing treatment, 'two groups may be too few' (66). This difference in approach between classical and chronobiologic study designs results in part from differences in their aim. The former places emphasis on a comparison among different treatments, irrespective of time; large changes that are predictable since rhythmic thus enter the noise term. By contrast, the chronobiologic approach has as its very aim what is otherwise buried in the noise term, namely the optimization of treatment by timing.

In chronobiologic study designs, external information concerning the occurrence and importance of circadian rhythms is advantageously used and allows access to refined methods of analysis. Even in the presence of considerable random variability or of a relatively small difference in outcome among the different groups, by testing a circadian rhythmic model, such differences are put to the fore much more easily. Alternatively, as noted above, by ignoring timing, classical study designs unduly inflate the random error term (by including considerable variability in it that would have been rendered predictable by mapping the rhythmic response to treatment), thus leading to the conclusion that large numbers of subjects are needed to pick up a difference among treatment groups. Results from actual experiments support this point, as will be illustrated below.

Cost-effective hormone- and placebo-pilot

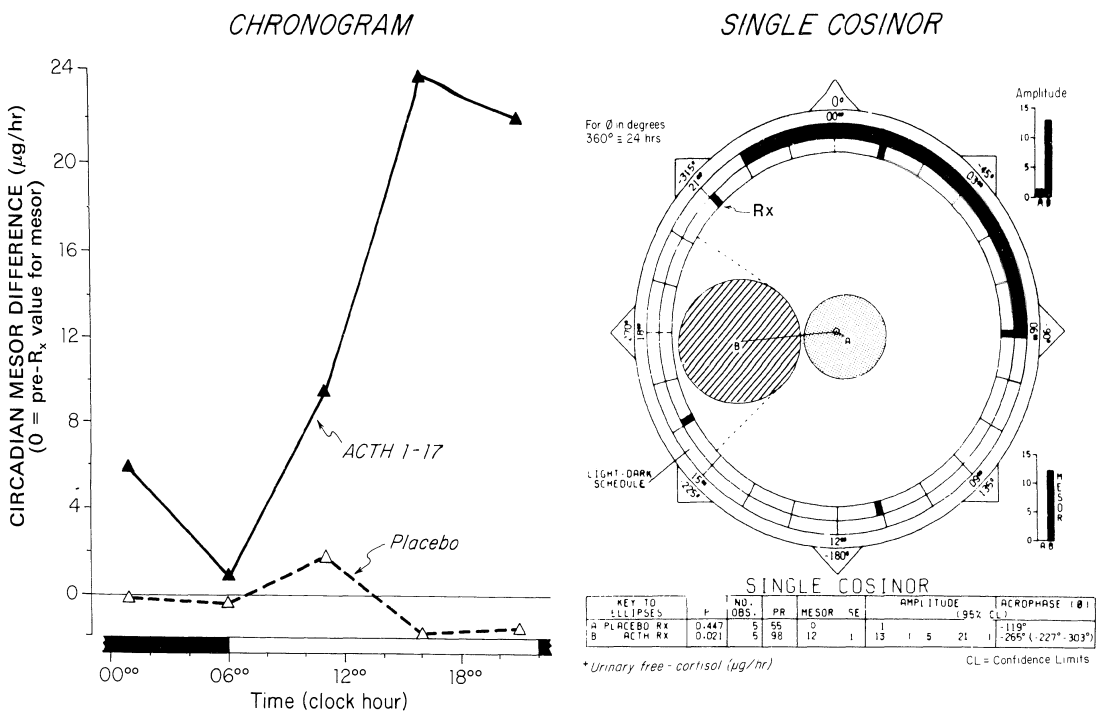
General schemes facilitating the testing of several rhythm stages at times convenient for the experimenter are discussed elsewhere (50). A specific example illustrating the effect of ACTH 1-17 on an individual basis, with studies at only 5 equidistantly distributed times around the clock is discussed by Günther *et al.* (30), Figure 2. The same data are considered further, as is the demonstration of a time-dependent placebo effect by Halberg *et al.* (66), Figure 3. These figures show that with five "replications", a response rhythm can actually be not only suspected, but it is detected with statistical significance. This is achieved in a study of only one subject at each of 5 timepoints, 4.8 hours apart, i.e., with a total of 5 subjects. Figure 3 shows for a placebo that a time-dependent effect upon the circadian amplitude of urinary cortisol excretion can be found as an increase in amplitude when the placebo is administered in the morning or as a lowering of amplitude when it is administered in the evening. If the 5 subjects were all tested at the same timepoint, one, the other or none of these effects may have been detected. The result then likely depends on the convenience of one vs. the other fixed sampling time. While these new placebo effects can be seen by the naked eye, they are quantified as a whole by the cosinor method, as shown in Figure 3.

Such quantification suggests the optimal time for getting a certain magnitude and direction (!) of a response for a fixed dose. Figure 2 suggests, in turn, that the effect of an ACTH analogue, ACTH 1-17, can also be quite different at five timepoints. We are dealing with results of chronobiologic pilots that, of course, await extension to larger samples. It should be emphasized that the Figure 2 test-situation is particularly favorable. The circadian cortisol rhythm is 1) one of large amplitude, its response can be studied as 2) a change in amplitude, in timing, in waveform and in the mean 3) with the same subject serving as longitudinal control. What also appears to be critical is 4) that the adrenal cortex is well-known to respond to ACTH, even if it does so in a circadian stage-dependent fashion (34, 76, 77, 78, 130). These are distinct advantages to the point that, in a chronobiologic pilot, only one subject per timepoint can be meaningfully studied. With this minimal number of 5 subjects, one finds for the given dose not only that it is active, an answer to the major question raised, but also when the dose is most active and by how much it is more active at the best time as compared to the worst.

Let us again turn to Figure 3. The first three just-mentioned conditions hold clearly. The fourth condition holds only to the extent that a placebo injection can also stimulate the adrenal cortex. What is then noteworthy is that a time-dependent response is again detected with the small number of only 5 subjects. In view of this time-dependence, even of a 'placebo-pilot', the chronobiologic design should become the indispensable control in any assay.

Chronobiologic pilots: a *sine qua non* of any test

Before a drug is recommended with a given timing, any time-dependent pilot effects must not only be replicated but extended to large numbers of persons for an exploration of their degree of generality. With this qualification, the simple chronobiologic several-armed design sufficed not only to demonstrate an anticipated effect of the analogue ACTH 1-17, previously known for the parent molecule, but provided at minimal cost much added invaluable information. In other words, an adrenal cortical stimulatory effect



* *10 men, 45-75 years of age, with rheumatoid arthritis, provided 6 urine samples in unequal daily fractions (19^{30}-06^{00}, 06^{00}-08^{30}, 08^{30}-11^{30}, 11^{30}-13^{15}, 13^{15}-17^{30} and 17^{30}-19^{30}) for 24h before and after R_x. One man was treated at each of 5 different circadian stages with ACTH or placebo. Mesor determined from fit of 24h cosine to each man's data before and after R_x.*

Figure 2. Circadian stage-dependent effect of ACTH 1-17 upon urinary excretion of free cortisol by patients with Steinbrocker stage II–III rheumatoid arthritis. Rhythm assessed in data for the 24 hours immediately before and immediately after administration of ACTH 1-17 (or placebo, Fig. 3). Each subject receives the treatment only once, at 1 of 5 different circadian stages. This timing is indicated by black bars on the inner rim of the graph on the right. Note, on the left, an absence of a cortisol MESOR response to ACTH in the morning and the large response to the same dose in the afternoon. Also note, in the left half of the graph, the lack of MESOR response to placebo. These effects upon the MESOR as a function of Rx time are analyzed by single cosinor on the right of the figure (Günther *et al.*, 1980; Halberg *et al.*, 1982). It can be seen that the changes in response to ACTH are not random, but rather constitute a response rhythm, a so-called beta-rhythm (Halberg *et al.*, 1985). (There is, however, a circadian stage-dependent response of the circadian cortisol amplitude to placebo, shown in Fig. 3.) These data from a study carried out in the summer await scrutiny in other seasons. The results of these analyses are limited further by the applicability only of a single (rather than population-mean) cosinor (with the design used). If each patient would be tested at each of 5 circadian stages, generalization to a population (by the population-mean cosinor result) should be possible, another cost-effective feature of a chronobiologic pilot, as yet not implemented in this context. © 1982 Halberg *et al.*

was now extended to another molecule, and the variability of this effect with time, indeed a great one, was determined both in terms of the time location of the best response and the extent of gain from timing (whereas other studies had focused only upon the presence or absence of an effect). A quantification of a time as well as dose response is the true 'pilot' (!); it prevents one from getting no response with a fixed ACTH dose, as at 06^{00} on the left of Figure 2. In other cases, the chronobiologic design safeguards against getting the spurious results of Figure 1.

Opposite effects 12 hours apart

There is no added cost for 1 sample obtained at 5 circadian times as compared to the cost of 5 samples at the same test time, once the circadian times are encountered at the same (convenient!) clock-hour of one's choice. Even at the cellular level, replication at certain different clock-hours does yield opposite responses, as also shown in a study of STH effects upon hepatic mitosis, Figure 4, or of ACTH 1-17 on bone DNA labelling (134), Figure 5. One can pick one's time and may find an inhibition of DNA labelling for 24 hours or a stimulation for 24 hours, with the same dose of the same molecule. At other times, within 24 hours, the same dose of the same molecule will result in stimulation followed by inhibition and, at still another time, by inhibition followed by stimulation. The heuristic value of such results not specified for timing is questionable.

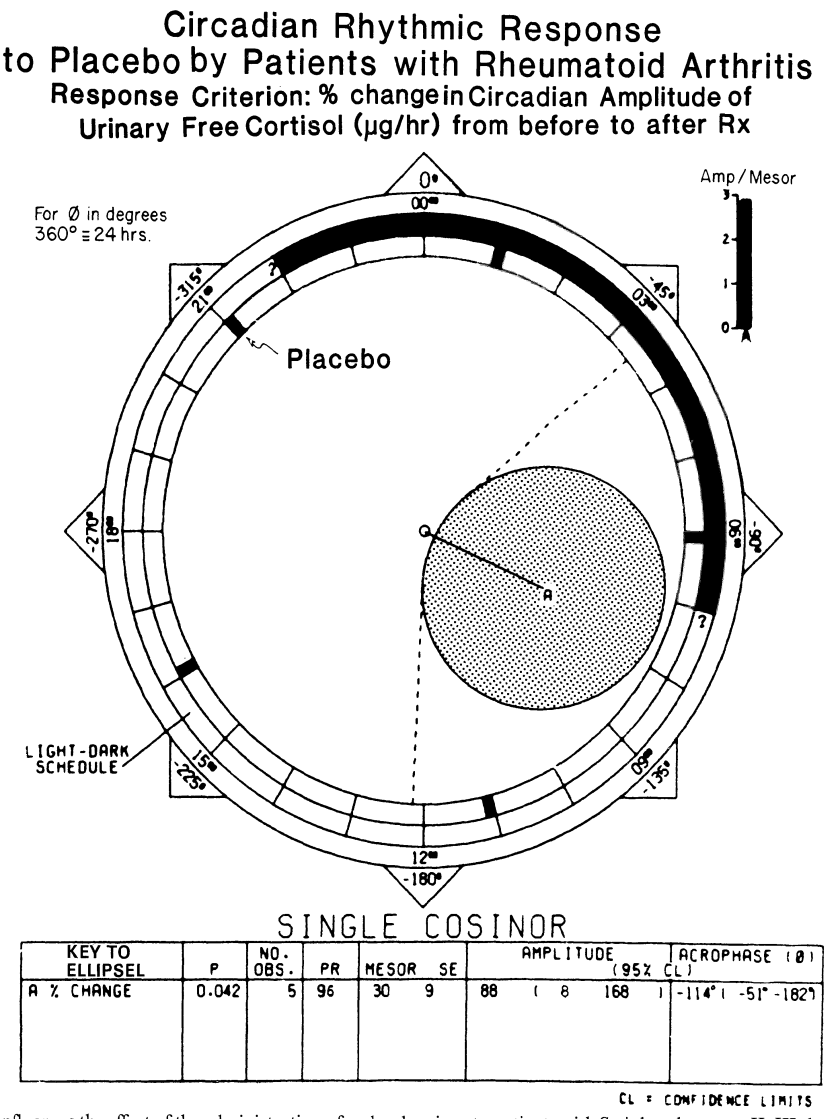


Figure 3. Timing influences the effect of the administration of a placebo given to patients with Steinbrocker stage II–III rheumatoid arthritis, when the response criterion is the percent change in circadian amplitude of urinary free cortisol (μg/h) from before to after Rx. In this case, as seen in Figure 2, the placebo is ineffective upon the circulating cortisol MESOR. © 1982 Halberg *et al.*

Clinical optimization by timing treatment

The experimental designs discussed above illustrate how different treatment modes can be tested while taking into consideration the variability due to timing, resulting from the ubiquity of biological rhythms, and in particular of the circadian system. Exploration of time effects is easily implemented in laboratory animals, which are generally inbred and tightly synchronized by environmental cycles such as the lighting regimen and the scheduling of food intake. Time effects are also easily assessed when screening large populations. Such studies have indicated, for instance, the prominent circadian variation in the frequency of mortality (42, 123) and morbidity (42), recently extended to the objectively documented onset of acute myocardial infarction (104).

When the same treatment is tested at different times on a given individual, different effects again may be obtained. There remains, however, a highly desirable step to achieve, for a full clinical application of timing. Treatment in a given application should be optimized for the given patient, not only for the 'average' individual. We confront a situation similar to that of dosing, however. Some generally applicable timing as well as dosing may be on the label for reasons other than mere compliance. The recommendation that the drug 'Dutimelan 8.15' be administered at 8 a.m. and 3 p.m. presupposes that all patients taking the drug live on similar, if not identical, routines. If some individuals follow quite different, e.g., shift-work routines, the recommended treatment time for them will be quite different from that for the population at large.

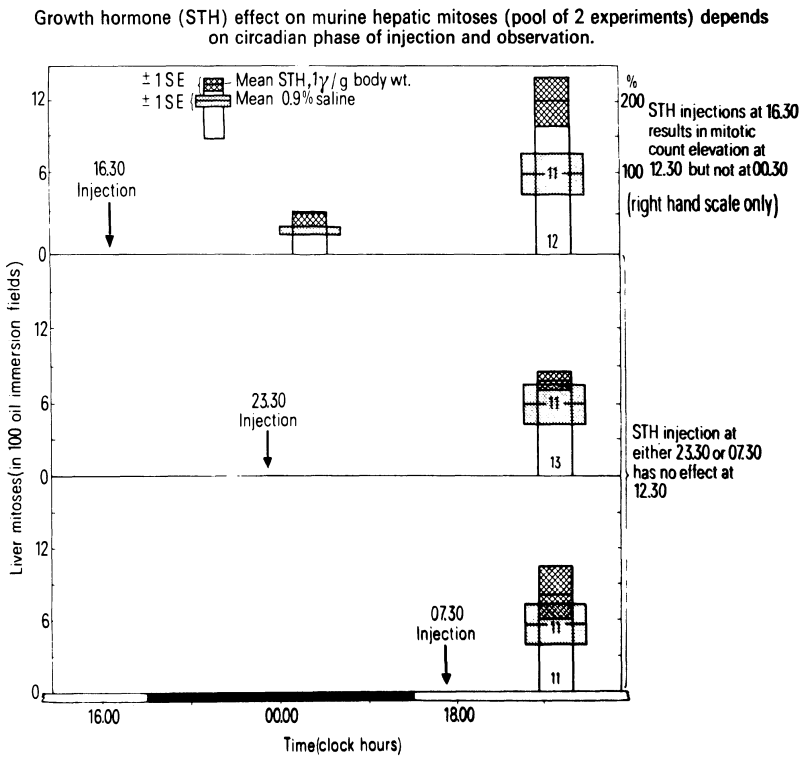


Figure 4. Composite graph summarizing 2 separate experiments. Top row data demonstrate that the organism's circadian phase determines the detection of growth hormone stimulation of cell division in liver parenchyma of growing mice. The effect can be detected at the time of usual mitotic high (mid-light span on a regimen of 12 h of light alternating with 12 h of darkness) but not at the time of the usual low in mitotic count. This experiment involved a series of 3 injections at the fixed time shown by the arrow in the top row; results are graphed only as per cent change from values of saline-treated controls killed concomitantly (right-hand scale only). In another study, the effect of injection at 3 different circadian times (with killing at the 'right time for detection of effect') is investigated (all 3 rows of graph; left-hand scale only). This study is ambiguous in that replicate injections will be needed at different circadian times before the possibility that time from injection may play a role has been ruled out or ruled in. Moreover, a single killing time does not suffice to resolve a phase-shift of mitotic rhythm, also demonstrated for another agent. However, a phase-shift may not only simulate mitotic depression when none occurs, but it may also spuriously indicate a lack of effect when such an effect does indeed take place. With such qualifying restrictions it may be pointed out that the organism's circadian stage at injection time may also determine the occurrence or nonoccurrence of growth hormone stimulation of cell division in mouse liver parenchyma. © 1973 Halberg.

The need thus arises to probe the internal time structure of the patient and to treat accordingly. The timing of treatment now refers to the patient's own rhythmic structure, rather than to mere clock-hour. This approach implies the use of marker rhythms (63), that is of a rhythmic variable that can be easily monitored as a function of time and that reflects the internal time structure of the individual. Temperature has been extensively used as a marker rhythm (35, 38, 60).

Early studies using rhythmically varying doses

Already in 1955, Menzel discussed the roles of frequency, amplitude and phase and recorded temperature in the endeavor to 'guide' body temperature by drugs given not only conventionally but also by the use of a specially-built infusion device. With such an instrument, Menzel administered into the human duodenum, within 12 hours, 2 full cycles of gradually increasing then decreasing doses of a preparation called Gubernal, a mixture of monosaccharides, ethanol and amino acids, recommended as an energizer by Gremels in 1948. Menzel treated by what we may today call ultradian sinusoids. The preparation was administered with the infusion device through a catheter starting at different times with either gradually decreasing or increasing doses. Menzel thus varied sequencing, a feature of treatment deserving particular emphasis in sinusoidal chronotherapy. Although no inferential statistical procedures were used by Menzel, this author reported that in 23 of 30 comparisons on 22 patients, body temperature was lower on the average on nights with Gubernal treatment, as compared to the course of temperature of the same patient on another night in the absence of Gubernal.

Chronotherapy to reduce toxicity and to enhance the therapeutic effect

For chronotherapy, we need the right chronopharmacodynamic concepts, realizing that drugs may act differently at different times. Accordingly, we have to pick the proper

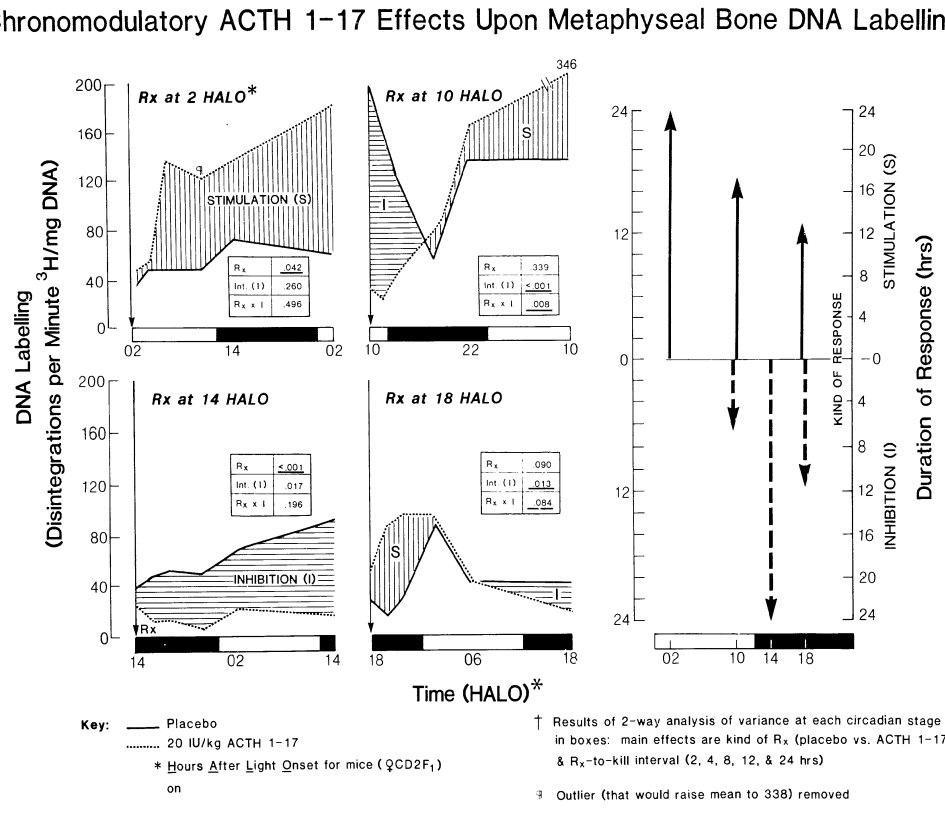


Figure 5. Depending upon the circadian system's stage encountered by the agent tested at administration time, results can be drastically different; the same dose of a given molecule may stimulate or inhibit DNA labelling in bone for the ensuing 24 hrs. At other test times, within 24 hrs, inhibition may be seen first, followed by stimulation or vice versa. These results reveal the importance of a chronobiologic approach to biotechnology (Walker *et al.*, 1985). From Walker *et al.*, 1985.

drug for the administration by the right devices with the right design of the appropriately rational schedules. By the same token, for the objective assessment of the optimal rhythm stage for the treatment of disease gauged by marker rhythms and of the desired and undesired drug effects, we need the right inferential statistical methods.

Exploiting biological rhythms with inferential statistical concepts has great potential value in the treatment of disease, both to reduce the toxicity or side effects of therapy and to enhance the therapeutic effect (37, 48, 49). The most exciting progress being made in chronotherapy – strategically-timed treatment – is in the attack on cancer both by radiation and by drugs. For a given organism under given conditions, there is a time when a drug will be most effective against a certain tumor and another time when the drug will be least useful. Such differences in outcome of treatment dependent upon timing are pronounced when the cell-division rates of healthy cells and cancer cells are governed by distinctly different rhythms. Cells in many healthy tissues divide according to circadian rhythms. Cancer cells, growing and reproducing faster than healthy ones, may show ultradian variation or a circadian rhythm that is out of synchrony with healthy tissue rhythms, or they may multiply with no rhythm at all. In the latter case, it may be advantageous to 'induce' a rhythm 'lost' by desynchronization among cells (which retain rhythms with different frequencies or phases).

Anticancer drugs and radiation disrupt cell reproduction and therefore have their greatest effect on tissues that are growing most rapidly. Drugs or radiation can destroy some cancer cells whenever they are administered; but, depending on the timing of treatment, a certain amount of healthy tissue is also destroyed. The healthy cells most sensitive to the anti-cancer treatment are the fast-growing cells in hair follicles, the lining of the intestine and bone marrow. Hence the usual side effects of anticancer treatment are loss of hair, nausea, and a reduction in the red and white blood cells formed in bone marrow. By charting rhythms of cell division in healthy and cancerous tissue, it should be possible to find the time when chemotherapy or irradiation is least harmful to healthy cells.

Such an approach has already been extensively used in mice and rats. In our laboratory and in the laboratories of Lawrence E. Scheving, mice are kept for 3 or more weeks in light and darkness alternating every 12 hours (LD12:12). In separate rooms, chambers, hives (116) or boxes (122), animals were placed on staggered LD12:12 regimens. This routine, plus regulated room temperature and free feeding,

settled the animals into well-defined circadian rhythms. Each mouse was then exposed to a single dose of whole-body radiation at different times in the course of 24 hours. Eight days after they had been irradiated, all the mice that had been exposed at the middle of the dark span were dead but all the mice irradiated late in the light span were still alive (36, 79, 82).

The amount of radiation required to kill 50% of the mice in a treatment group (i.e., the LD50 commonly used in assessing the safety of a new treatment) also changes with circadian time. This variation in lethal dose was first demonstrated in the 1950s, in experiments seeking the optimal time for an astronaut to pass through the radiation of the earth's Van Allen belt (36). When such findings are extended to people, physicians should be able to shield the healthy tissues of a cancer patient with time, as they now shield them with lead in space.

Timing cancer chemotherapy

Circadian cycles of the benefits and side-effects of drugs used in cancer chemotherapy have been demonstrated in laboratory animals at the Universities of Arkansas, Minnesota and Tennessee. Work in Arkansas with the drug cytosine arabinoside (ara-C) showed the circadian stage-dependent tolerance of mice to this drug (9). In Minnesota, we found the dependence on circadian stage of the toxicity of many other anticancer drugs (43–45, 47, 48), including doxorubicin (67). These agents, some with different putative sites of action, differed in the times of their optimal tolerance by mice. Ara-C, putatively acting on DNA synthesis, had a best tolerance time different from that of vincristine, presumably acting primarily on mitosis. Two antimalignant antibiotics, daunomycin and doxorubicin (which differ by only a single detail of chemical structure), had (statistically significantly) different circadian times of optimal tolerance. Sites of action and drug handling by the organism, which is known to be periodic, will all have to be taken into account for a rational chronotherapy.

In one of the several special series of experiments in our laboratory, 2,677 mice were given a type of tumor that is widely used as a model for studying chemotherapeutic agents. These mice, whose rhythms had been standardized, were inoculated with a million or more (L1210) leukemia cells. Two days later, each mouse received a dose of ara-C every three hours for 24 hours. The total amount of the drug given to each animal in a course was the same. Some mice, however, received eight equal doses, while others had doses that were varied to fit several different sinusoidal curves, each having its maximal dose administered at a different circadian stage. The dosage patterns were repeated every 4 days. In another series of experiments, which was even more extensive, we compared sequencing and circadian time for their respective role and found that both were critical factors. In this series, 270 mice were injected every 180 minutes during 48 hours. The injector was an Olympic runner. It can readily be seen that such experiments are much more readily done by pumps.

In these experiments, control mice, which were not treated, all died within 7–10 days. On the standard course of 8 equal doses, which is similar to the usual regimen for treating people, (on the average) animals survived for much longer than untreated controls and a few were cured. Mice treated according to certain sinusoidal dosage schedules lived even longer and even more of them were cured. Some of the mice treated on the standard (equal-dose) schedule died after only a few days, before any controls died; these early deaths must have been caused by toxic effects of the drug because the leukemia had not yet killed the animals. Part of the improvement in length of survival observed on sinusoidal schedules can be attributed to better tolerance of the drug.

Increased average survival time among the sinusoidally treated mice, however, was due primarily to a higher cure rate. After several series of tests of sinusoidal treatment timed according to body rhythms, we can state with considerable certainty that chronotherapy can approximately double the cure rate of an experimental leukemia in mice. In Arkansas, Scheving *et al.* (121) reproduced the results of the Minnesotan sinusoids (67, 80). Moreover, they increased the cure rate of leukemic mice six-fold by timing the use of several anticancer agents in combination; extremely promising results from combination chronotherapy have been obtained by us for an immunocytoma and two kinds of breast cancer, all used as rodent models of human disease (63).

With respect to both theory and practice, it seems reasonable to pick, for sinusoidal schedules, a cycle length with which the body of the patient might be able to resonate. We have no evidence for resonance with 6-hourly cycles, used by Menzel (101). Indeed, a drastic 12-hourly electroshock schedule results in a free-running circadian rhythm, rather than in a 12-hourly schedule of body temperature or blood eosinophil count (23, 36). Moreover, because of ubiquitous circadian rhythms (7, 36, 57, 58, 63, 64, 66, 84, 93, 102, 114, 115, 120, 126, 127), one might expect that even with a continuous 24-hour infusion, at a constant rate, directly into the blood, one might bring about circadian variations in blood concentration of a drug. This point was actually documented by Patel *et al.* (106), who gave ethosuximide to rhesus monkeys intravenously; during 4 months of approximately constant drug infusion, they collected samples for ~ 1 day/week at 2-hourly intervals, and then determined blood concentration around the clock. When their data were analyzed by the single cosinor method, the zero circadian amplitude could be rejected below the 5% level of statistical significance (48).

A rhythm in the concentration of a drug in blood, brought about unintentionally by interactions within the body and effects of metabolic and excretory mechanisms, may well be timed quite differently as compared to that desired for an optimal treatment effect (19). In the case of a carcinostatic drug, one strives to give a potentially toxic drug at a time which represents the compromise between the times when the drug is best tolerated by the host, insofar as this is compatible with a time when it is most effective for the desired effect. In the case of breast cancer, it is known that a circadian bioperiodicity characterizes DNA synthesis and mitotic activity. Figures 6 and 7 reveal the bioperiodicity of these 2 variables in the breast cancer of the A-strain mouse; Figure 8 summarizes mitosis in human breast cancer (25, 125, 132). Figures 6–8 reject the 'no-rhythm' assumption; Figure 8 does so by cosinor, providing estimates of mitotic

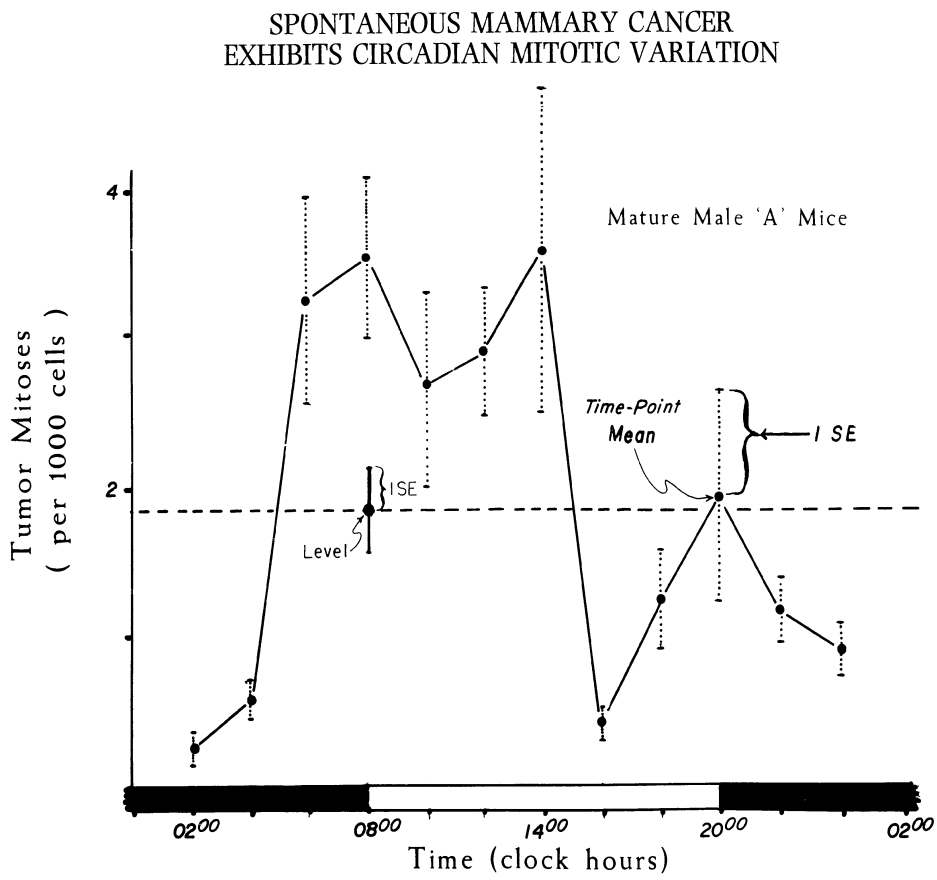


Figure 6. Circadian bioperiodicity in mitotic activity in the case of breast cancer in mature male A mice. © 1973 Halberg.

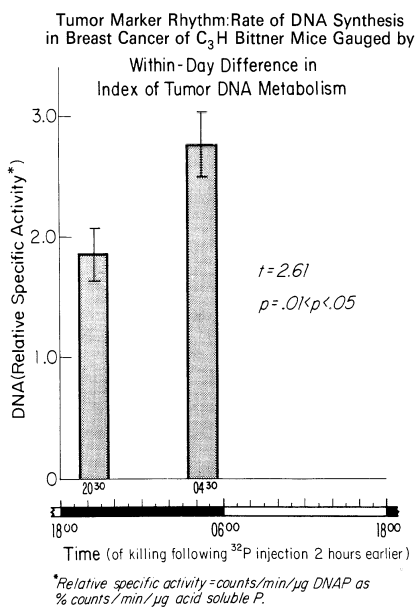


Figure 7. Circadian stage-dependent effect in rate of DNA synthesis in breast cancer of C_3H Bittner mice. © 1973 Halberg.

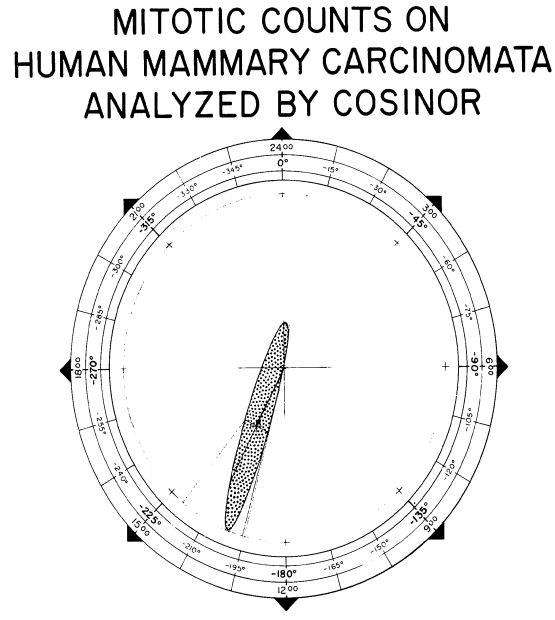


Figure 8. Cosinor analysis of serial mitotic counts suggests that a circadian rhythm might persist in human breast cancer. © 1973 Halberg.

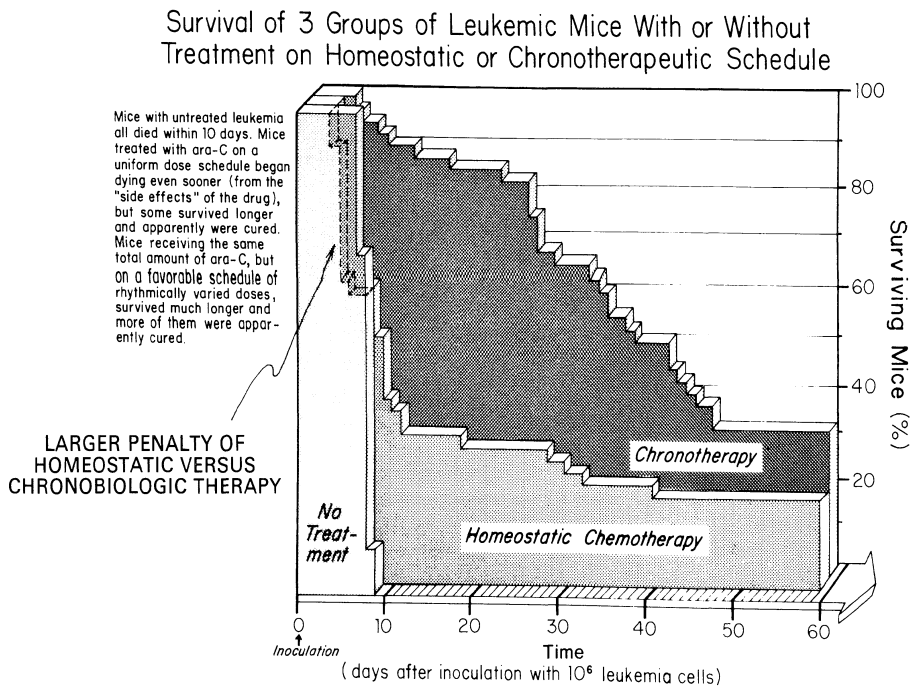
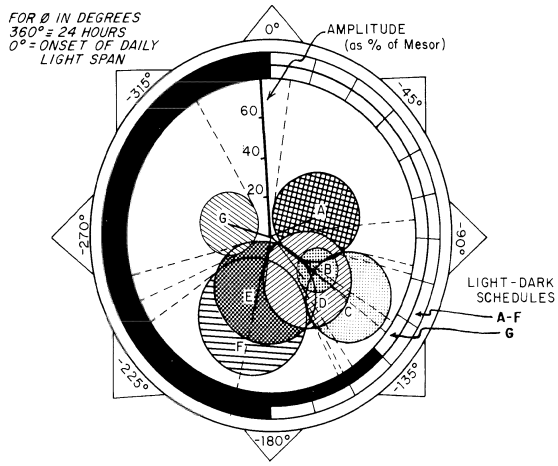


Figure 9a. Survival of leukemic mice on different schedules of treatment with arabinosyl cytosine illustrates the merits of chronotherapy. © Halberg, 1987a.



SINGLE COSINOR[1]

Key to Ellipses	Drug Tested[2]	N Studies	Total N Animals	P[3]	% Rhythm[4]	Amplitude[5] (95 % C.L.)	Acrophase, Ø
A	DAUNOMYCIN	4	690	.021	31	29(4,54)	-67°(-8,-127)
B	ADRIAMYCIN	6	1072	<.001	60	33(21,45)	-124°(-103,-146)
C	ARA-C	2	480	<.001	76	56(32,80)	-126°(-100,-151)
D	MELPHALAN	4	456	.015	33	31(18,57)	-138°(- 83,-193)
E	CYCLOPHOSPHAMIDE	6	826	.027	18	32(3,60)	-185°(-123,-255)
F	VINCRISTINE	2	239	.007	67	46(15,76)	-193°(-150,-235)
G	DDPt	11	1503	.002	18	24(8,39)	-289°(-246,-331)

1) *Results from least-squares fitting of 24-h cosine curve.*
2) *i.p.. All drugs, except DDPt, tested in mice kept in LD 12:12; DDPt tested in rats kept in LD: 8:16.*
3) *P from test of zero-amplitude hypothesis.*
4) *% Rhythm = % of total variability attributable to fitted cosine.*
5) *Expressed as % of Mesor.*

Figure 9b. Circadian rhythmicity in murine tolerance of anticancer drugs, evaluated from data on percent survival as a function of treatment timing. © Halberg, 1987b.

timing in the tumor, which seems to be out-of-phase with mitotic timing in skin during health.

Accordingly, circadian and other rhythm-stage-dependent timing of chemotherapy seems to be more reasonable than a treatment at a fixed infusion rate or in equal doses. Figure 9 shows that a quasi-sinusoidal schedule can be much superior to an equal-dose schedule, even if rhythms with a frequency higher or lower than circadian, i.e., ultradian and infradian rhythms, are not yet taken into consideration. Eventually the entire spectrum of rhythms characterizing human physiology (40, 49, 57, 62) will have to be taken into account for a proper chronotherapy. To achieve such aims, however, even Olympic runners need special devices.

Drug administration devices

The right devices for chronotherapy must be portable, and miniaturized so that they do not interfere with daily life (5, 6, 16, 20, 21, 27, 112, 113, 119, 124). Moreover, the devices should be programmable, so that in a day and age of real problems in compliance, one relies on modern technology rather than on the patient's discipline and memory. Totally implantable devices for drug infusion are becoming available, are replacing the bulky infusion machine and may be better-tolerated than external pumps (124). The implantable miniaturized Medtronic pump, programmable in 10 steps, allows, among other schedules, a quasi-sinusoidal drug administration, the 'quasi-' indicating in this case that the change in rate is achieved in discrete steps rather than continuously (29).

This device has already been very useful in optimizing the administration of cyclosporine in transplant surgery using chronobiologic designs advocated by us (11, 12; cf. also 96, 97). Chronobiologic applications in the field of oncology are, however, lacking. Bertino (1), for instance, discusses the research potential of modern pumps from the viewpoint of cancer, but without referring to bioperiodicity, at a time when not only the importance of circadian stage has been extensively demonstrated to tip the scale between death and survival following exposure to the same agent, documented by Halberg (37), but when the finding has been extended to

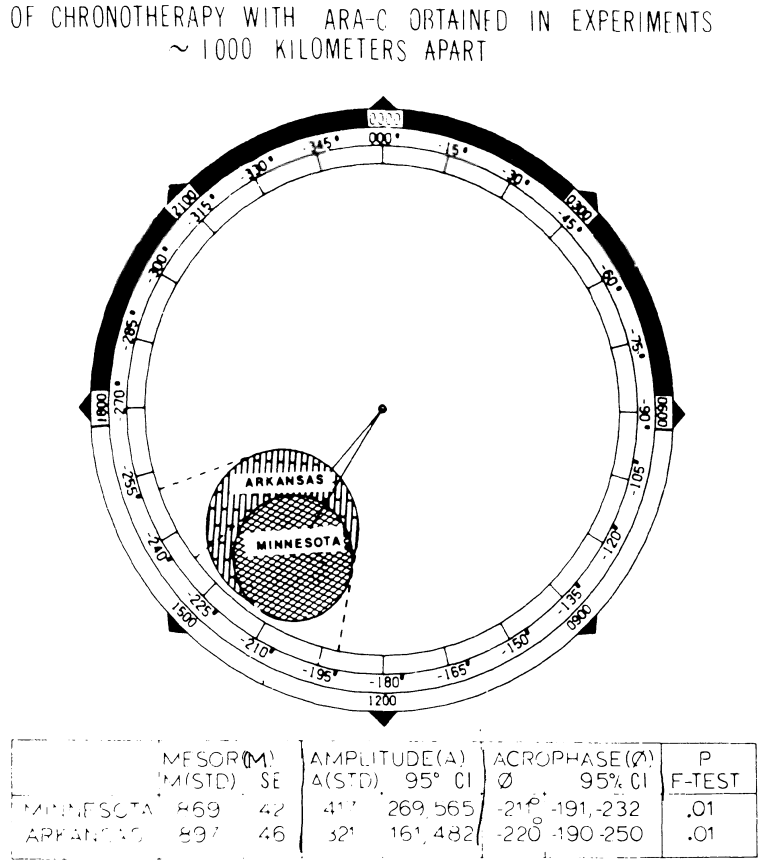


Figure 10. Cosinor summary of data on the reproducibility of chronotoxicity of arabinosyl cytosine (ara-C) in experiments done on the same days in different laboratories in different geographic locations. As can be seen from the large overlap of the confidence regions, the timing of the rhythm in survival time (expressed in hours) is similar for the two locations, the point estimates of timing (acrophases) not differing by more than one hour. In both experiments, $CD2F_1$ mice received ara-C in sinusoidally varying schedules consisting of 4 courses of 240 mg/kg/day, administered in 3-hourly doses. When the same total dose of ara-C is given, certain sinusoidal drug administration schedules are definitely better tolerated by mice than are other sinusoidal schedules or a reference treatment of 8 equal doses over a 24-h span.

a long list of drugs (43, 48, 80, 82, 88, 107, 114, 121), including agents used to treat cancer (63, 67).

Anticancer agents

It has been known for over a decade that the toxicity of doxorubicin in rodents (67) and human beings (89, 133) is circadian stage-dependent. Cardoso *et al.* had shown by 1970 that ara-C effects depend upon the circadian time of administration. Soon thereafter, it was also shown for ara-C that a quasi-sinusoidal drug dosing (with one cycle in 24 hours) is much better tolerated than the administration of equal doses of the drug, Figure 9 (67, 80). The results were so dramatic that a study in 2 geographic locations was set up to check on their reproducibility on a given day, Figure 10 (121). The cure rate could also be doubled in the case of an L1210 leukemia by administering more of this myelotoxic drug at the circadian time when the bone marrow was least susceptible and much less drug at the circadian time when bone marrow was more susceptible (63, 67), Figures 11 and 12. Beyond ara-C, the benefits derived from the timing of drug administration according to the given circadian cycles of host susceptibility and drug effectiveness were extensively documented for many drugs in the past decade and were extended to radiotherapy (33, 48, 49, 65, 82, 88, 107, 121).

By 1973, Halberg *et al.* reported the optimization of the tolerance and of the therapeutic ratio, Figures 13 and 14, of doxorubicin by timing (67). A doxorubicin-cisplatin combination treatment was also optimized by timing, Figure 15;

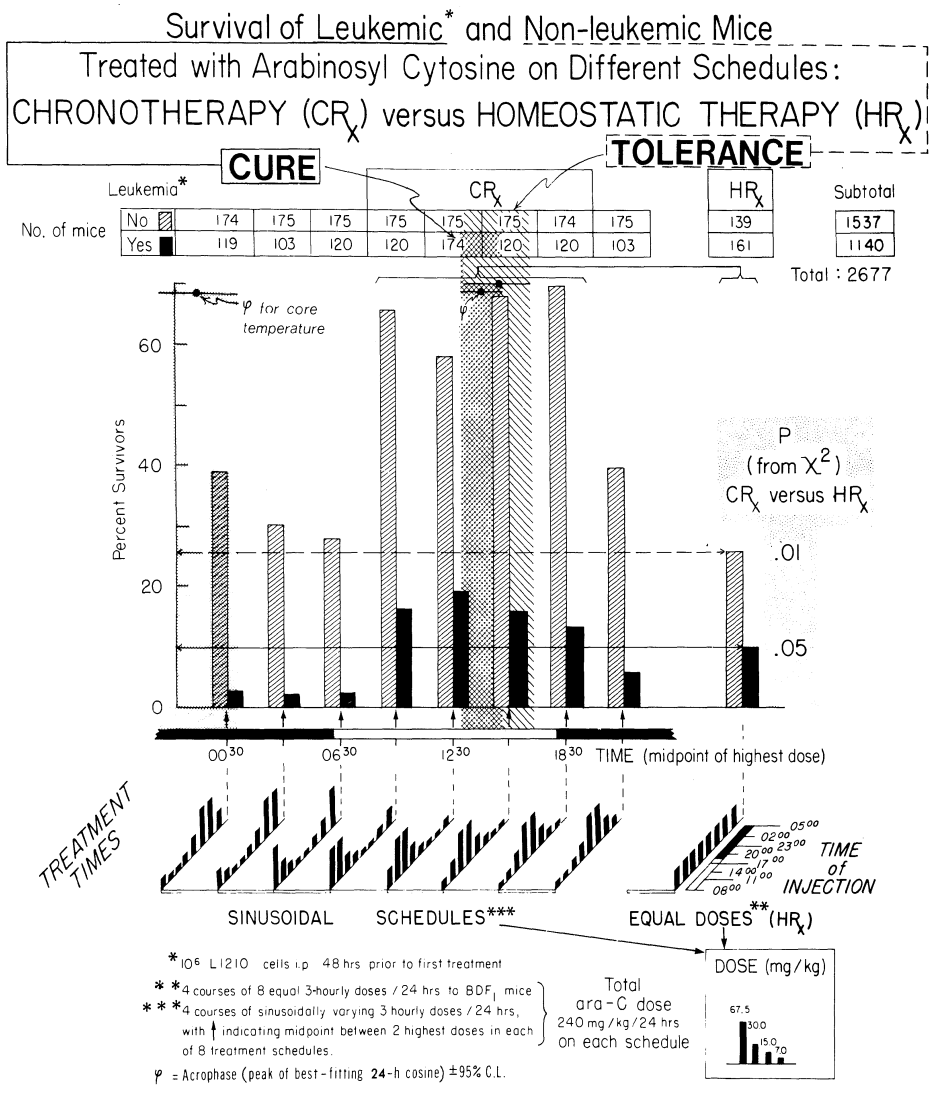


Figure 11. Chronotherapy with arabinosyl cytosine, in sinusoidally varying 3-hourly doses for 24 hours is superior to homeostatic therapy with 8 equal 3-hourly doses per 24 hours, as gauged by the survival of mice in tolerance studies, as well as by the survival and cure of leukemic mice. Note increase in chronotherapy cures as compared to results with homeostatic schedule. © Halberg.

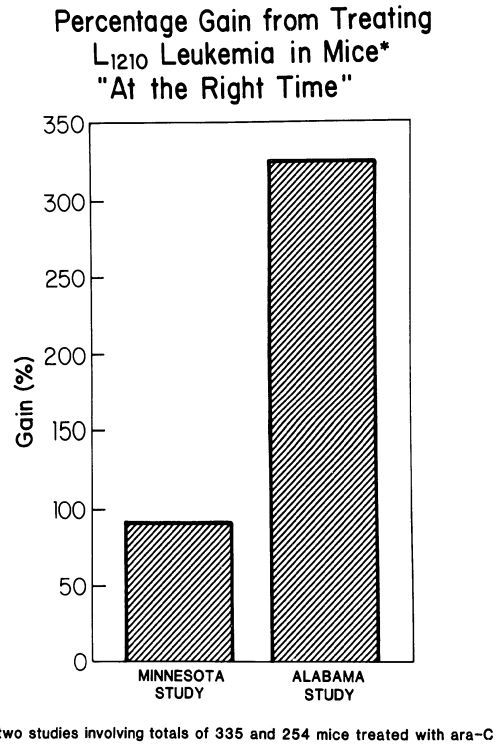


Figure 12. Percentage gain from treating L1210 leukemic mice at the right circadian stage. © Halberg.

in the case of a LOU rat plasmacytoma, over 50% complete cures, unattainable earlier in our laboratory, were obtained by timing the administration of these two drugs (59).

The next step in the clinic focused upon toxicity. Apart from chronobiologic considerations, cardiotoxicity, a major side effect of doxorubicin, reportedly has been reduced by prolonged venous infusion or single weekly doses (10, 14, 26, 94, 98, 99, 117, 128, 131, 135). We, in turn, undertook the monitoring of heart rate to seek an early warning signal for undue cardiotoxicity of doxorubicin (133).

Heart rate monitoring in cancer patients on doxorubicin as a gauge to minimize chronic cardiotoxicity and quantify the risk of congestive heart failure

In a clinical trial of advanced ovarian and bladder cancer at the University of Minnesota, combination chemotherapy consists of ~9 monthly courses of doxorubicin followed 12 h later by cisplatin (88, 89, 90, 133). Patients are randomly assigned to one of two treatment times, referred to as schedule A or B. In schedule A, patients receive doxorubicin at 06^{00}, ~1 h before awakening, and cisplatin in the evening. In schedule B, patients receive doxorubicin at 18^{00}, ~11 h after awakening, and cisplatin the next morning.

With an automatic blood pressure monitor manufactured by Nippon Colin Ltd. (Komaki, Japan), 33 patients of both genders were monitored automatically for pulse at ~10-minute intervals for the 24–36 hours preceding each doxorubicin-cisplatin combination treatment. The first pulse profile was obtained before the first treatment course for 22 patients, before course 2 for 4 patients, before course 3 or 4 for 3 patients and not until just prior to course 5 for one patient. (Three more patients had no more than 1 or 2 treatments and are here omitted from consideration.) Pulse profiles were obtained at approximately monthly intervals for a total of 5 to 9 treatments.

Each individual time series was analyzed for a circadian rhythm by the least-squares fit of a 24-hr cosine in order to obtain rhythm parameters – MESOR, amplitude and acrophase – and thus to quantify any alteration in a rhythm characteristic which might occur during treatment. In order to test for the effect of timing doxorubicin Rx in relation to the circadian stage of the pulse rhythm, two computations were made for each of the patients here considered. First, the difference between the last MESOR available and the earliest was calculated (this ranged from a decrease of nearly 21 beats/min to an increase of nearly 34 beats/min). (In one case, a preexisting congestive heart failure disappeared during treatment; in this case, the lowest of the first 5 pulse MESORs was taken as reference for subsequent change.).

Thereafter, the difference between the early and late pulse MESORs was assigned to a time code representing the time of doxorubicin Rx in hours and minutes from the pre-Rx pulse acrophase. Thus, for schedule B, a time code of 02^{22} indicates that if the pre-Rx pulse acrophase was at 15^{38} local clock time, doxorubicin was given 2 hours and 22 minutes

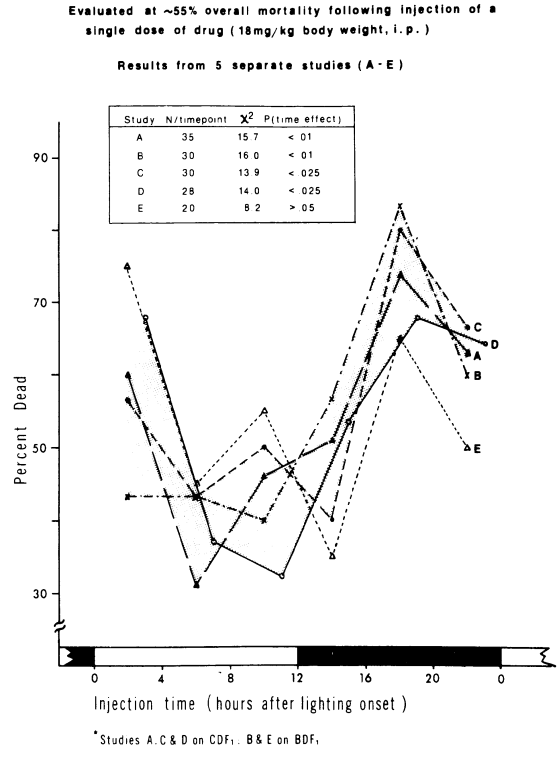


Figure 13. Circadian rhythm in susceptibility of 858 male hybrid mice to doxorubicin. © Halberg.

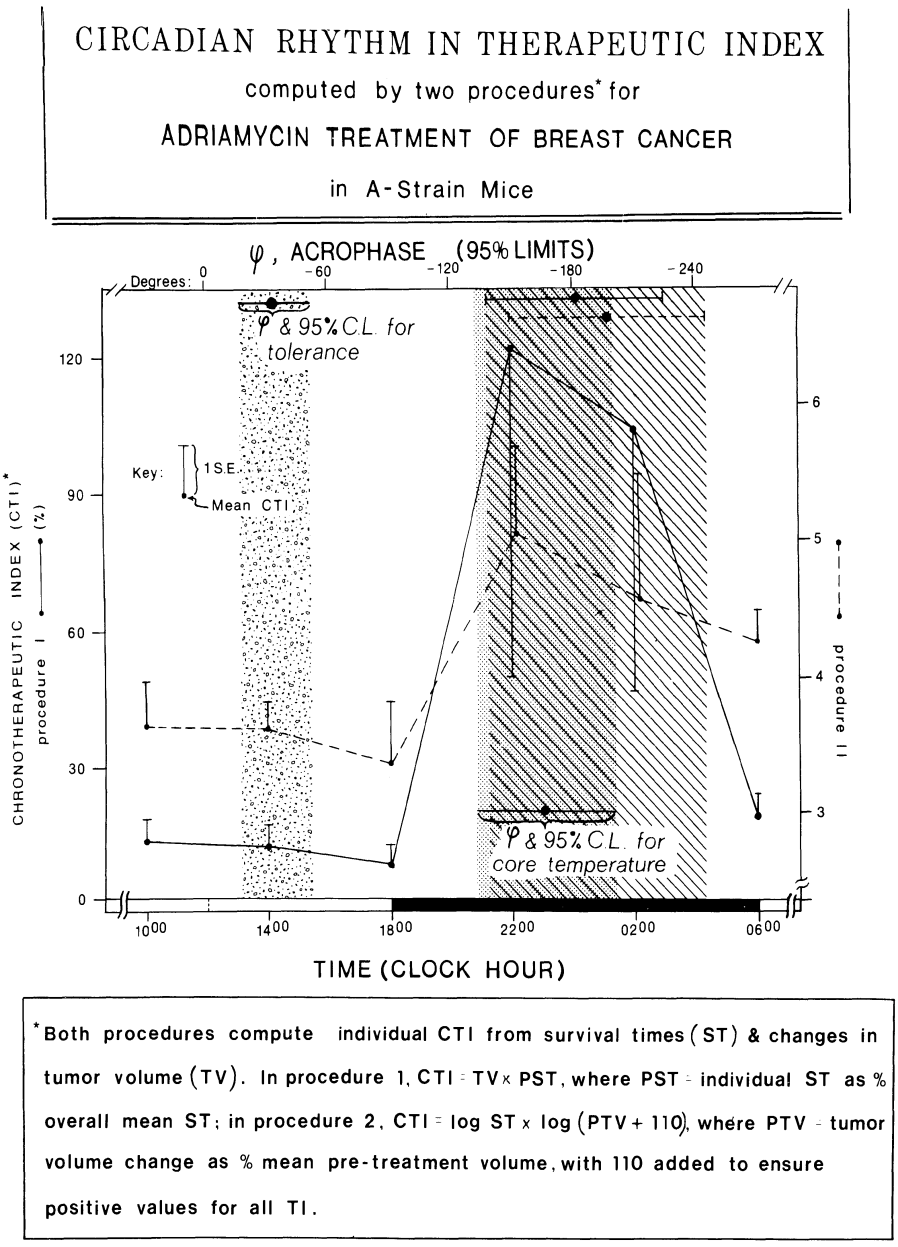


Figure 14. Computation of a therapeutic index (survival time × maximal change in tumor volume) for each of 6 subgroups of A mice with breast cancer, each subgroup treated with doxorubicin at a different circadian stage (4 hours apart) reveals a circadian rhythm with maximal index during the daily dark span. © 1973 Halberg.

later, at 18^{00}. A time code of 16^{44} would represent the pulse-related Rx time for a patient with an acrophase at 13^{20} local time and receiving Rx 16 hours and 44 minutes later at 06^{00} (schedule A). The least-squares fit of a 24-hr cosine to this time series (consisting of pulse ϕ-referred treatment times and the associated pulse MESOR change) resulted in the description of a statistically significant rhythm ($P = .043$), with an acrophase of 10 hours after the pulse acrophase, Figure 16. The patients treated with doxorubicin within 3 hours of this acrophase (between 07^{00} and 13^{00}) exhibited an average pulse MESOR increase of 15.5 ± 4.9 beats/min, while the 5 patients treated within 3 hours of the bathyphase (19^{00}–01^{00}) showed an average decrease in MESOR of 3.6 ± 4.0 beats/min. This difference was statistically significant ($t = 2.93$; $P < .05$), Table 1. Treatment with doxorubicin can be expected to increase heart rate and possibly lead to a higher risk of cardiotoxicity when it is administered 7–13 hours after the pulse acrophase. When the avoidance of cardiotoxicity is the major or sole consideration, the preferred treatment time is not more than 5 hours before or no later than 1 hour after the pulse acrophase. This inference is based first on the observation that in 4 out of 4 cases

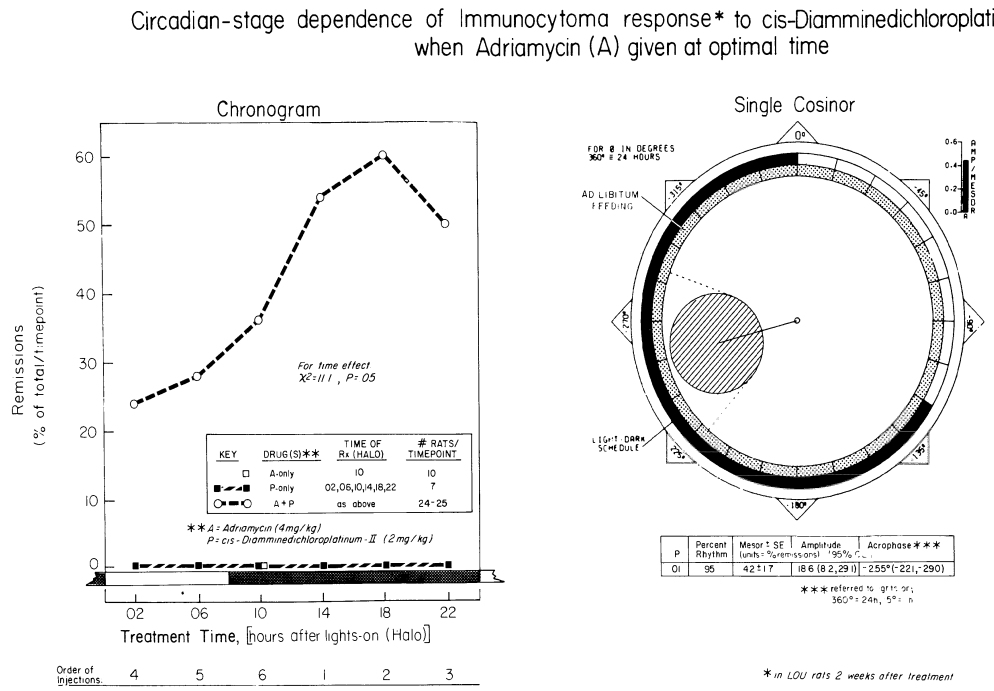


Figure 15. Incidence of remission in immunocytoma-bearing rats given doxorubicin at presumably optimal circadian stage can be optimized by timing (biochronizing) added treatment with *cis*-diamminedichloroplatinum. © Halberg and Delbarre, 1979.

receiving doxorubicin and eventually developing congestive heart failure (CHF), a pulse MESOR increase of over 12 beats/min was noted, with a similar increase occurring in only 5 of 29 such patients who did not develop CHF (P of inter-group difference < .01), Table 2.

This finding supports the possibility that the pulse MESOR increase may be at least an unspecific harbinger, if not an early warning system of heart failure. As a harbinger, it may have the advantage that it identifies those who will develop heart failure, whereas its disadvantage is that it may identify others as well who may not develop heart failure. Thus, the possible merit of heart rate MESOR monitoring is that it can be readily done by automatic instrumentation, that it actually can be done by self-measurement in the case of a motivated patient as well, and that because it is noninvasive and innocuous, it can often be repeated to serve as an indication for additional tests if, and perhaps only if, there is a reliable heart rate MESOR increase established by dense sampling covering at least 48 hours. This minimal span is suggested in view of the great variability encountered in rhythm characteristics based on dense automatic monitoring restricted to 24 hours.

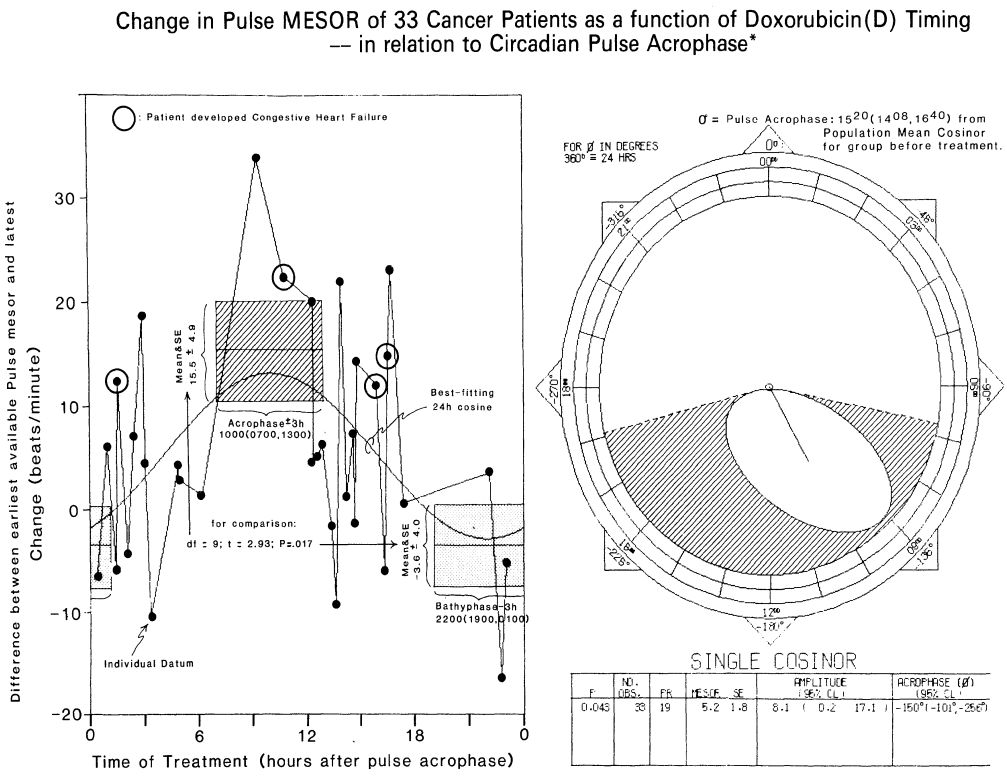
Once one accepts the possible pulse MESOR increase as unfavorable (rejecting the assumption that it occurs randomly as a function of treatment time), one is left with the inference that treatment at the cardiosensitivity chronorisk

Table 1. Elevation of circadian MESOR of heart rate as a marker of doxorubicin-associated cardiotoxicity*.

	Acrophase ± 3 h		*Bathyphase ± 3 h*			*Student*	
Units	*n*	*mean ± SE*	*n*	*mean ± SE*	*Difference ± SE*	*t*	*P*
1. beats/min	6	15.5 ± 4.9	5	−3.6 ± 4.0	19.0 ± 6.5	2.93	0.017
2. percent	6	21.3 ± 6.5	5	−3.8 ± 4.7	25.2 ± 8.3	3.02	0.015

* For each patient undergoing monthly courses of combined chemotherapy, preceded by a 24-hr profile of heart rate monitoring (see text and Table 2), the following computations were made: 1) a 24-hr cosine function was fitted to each profile to determine the MESOR, amplitude and acrophase; 2) the difference in MESOR between the latest and earliest profiles was calculated; and 3) this difference was assigned to a time corresponding to the time elapsed between doxorubicin administration and the time of acrophase of the earliest heart rate profile. At this stage, a time series representing the change in heart rate MESOR as a function of (internal) treatment time is constituted which is fitted with a 24-hr cosine curve to determine the best (bathyphase) or worst (acrophase) treatment times, assuming that an increase in MESOR reflects cardiotoxicity. The difference in heart rate MESOR observed in subgroups of individuals treated at one of these two times (± 3 hr) is here tested by Student's t-test. The 6 patients treated at the anticipated worst time had a statistically significantly greater increase in heart rate MESOR than the 5 patients treated at the anticipated best time.

Acknowledgement: The data here analyzed stem from studies designed and originally summarized with William J.M. Hrushesky, Todd Langevin and Robert B. Sothern, all of the University of Minnesota, among others (cf. references 88–90).



*D at either 0600 or 1800 with cisplatin 12 hours later (at approximately monthly intervals for 5 to 9 treatment courses.)

Figure 16. The change in MESOR of heart rate may be used as a double marker rhythm, first to gauge toxicity of chemotherapy and second to optimize treatment timing. The data here analyzed stem from studies designed and originally summarized with William JM Hrushesky, Todd Langevin and Robert B Sothern, all of the University of Minnesota, among others (cf references 88–90). © Halberg.

(corresponding to the time, ϕ_i, of the anticipated largest MESOR increase) should be avoided. An alternative interpretation is that those individuals who happened to be treated at the circadian ϕ_i time are *a priori* more sensitive to doxorubicin cardiotoxicity. There is no reason, however, to assume such a selection, since the patients were randomized to just 2 clock-hours of treatment timing. In future research, patients should be randomized in relation to their pulse ϕ. The possibility that the pulse characteristics may be used (for the double purpose of timing Rx and of assessing the patient's response to Rx) in chronotherapy deserves further testing.

The use of the same variable, pulse, both for determining the optimal timing of Rx and for analyzing Rx effects, prompts the question of whether independence is thereby lost, when the initial acrophase on the one hand and the MESOR on the other hand both relate to pulse. This objection can be met by the fact that one deals with quite different endpoints based in part on different data, i.e., with a characteristic of timing (ϕ) before Rx vs. a change in a characteristic of overall data location (MESOR). The use of the same variable for dual purposes has the advantage of cost-effectiveness, if it can save multi-functional and hence more costly and more bothersome monitoring.

While a 48-hour heart rate cosinor test on data monitored at 1-hour or shorter intervals is advocated before each (e.g., monthly) Rx course in order to gain stability for an individualized assessment, this does not imply that additional multiple daily self-measurement should not be considered during the entire treatment course. To the contrary, once such measurements are done, they become routine, and their analysis can be carried out on a weekly basis. It is also pertinent to note the demonstrated feasibility of autonomic data collection at home, with prompt data transfer to a personal microcomputer. This approach renders the room-restricted monitoring at home by an instrument such as the Nippon Colin machine (Komaki, Japan) quite attractive, since it meets the merits of practicability, automatic easy data transfer and a turn-around time for analyses of only a few minutes upon completion of the recording. Finally, it must be emphasized that the subjects in this study were randomized according to clock-hour and were studied prospectively, yet the analysis of the data is retrospective. Therefore, a prospective study with circadian pulse characteristics as double markers is overdue. It may allow one to focus upon a minimization of myelotoxicity first, with treatment timing accordingly, until there is a heart rate MESOR increase. A radionuclide test is then done and, irrespective of its outcome, the patient is then assigned to one of 6 circadian treatment times at 0, 60, 120, 180, 240 or 300° from the pulse acrophase.

Table 2. MESOR-tachycardia (MT) as unspecific yet suggestive harbinger of congestive heart failure (CHF)*.

CHF	*Patient; age (yrs)*	*Gender*	*Site of tumor*	*Course (N)* #	*MESOR ± SE* ##	*Course (N)* #	*MESOR ± SE* ##	*Change* ##
Yes	LC59	F	ovary	1A (77)	78.7 ± 2.2	7A (88)	101.2 ± 1.2	+22.5
	YD62	F	ovary	2B (86)	84.6 ± 1.6	10B (51)	97.0 ± 3.2	+12.4
	DL68	F	ovary	1A (90)	78.6 ± 1.6	6A (89)	93.3 ± 1.2	+14.7
	JW55	F	ovary	1A (110)	83.4 ± 0.9	9A (109)	95.6 ± 1.5	+12.2
No	IB57	F	ovary	1B (118)	73.8 ± 0.9	8B (64)	92.7 ± 1.2	+18.9
	IG59	F	ovary	1A (78)	78.8 ± 1.9	8A (91)	101.0 ± 1.0	+22.2
	RJ49	F	ovary	2A (186)	101.3 ± 0.4	7A (109)	115.7 ± 0.6	+14.4
	LG75	M	bladder	2B (64)	79.5 ± 2.0	7B (93)	105.6 ± 1.3	+26.1
	BS71	M	bladder	1A (86)	56.1 ± 1.3	8A (70)	79.3 ± 1.7	+23.2

N = number of observations; ## units = beats/minute.

* Statistically significant ($P < 0.001$ in each case) MESOR-elevation of at least 12 beats/min at completion as compared to initiation of doxorubicin-cisplatin treatment. 29 out of 33 treated patients failed to develop CHF; 5 who developed MT without CHF listed above; all 4 who developed CHF had MT.

Data collected with a Nippon Colin monitor, automatically at 10–15-minute intervals for 24 hours preceding monthly treatment with schedule A (doxorubiciin in morning followed by cisplatin in evening) or schedule B (doxorubicin in evening followed by cisplatin in morning). For these subjects sleeping from 23^{00}–07^{00}, morning treatment was 1 hour before awakening (06^{00}) and evening treatment was at 11 hours after awakening (18^{00}). The following table shows an interim outcome:

MESOR-tachycardia?	*Congestive heart failure?*	
	Yes	*No*
Yes	4	5
No	0	24

$P = 0.003$ (exact method for 2 × 2 contingency tables to test the hypothesis that $p_1 = p_2$, when p_1 and p_2 are proportions of patients with CHF among those who either had or did not have MT.

Acknowledgement: The data here analyzed stem from studies designed and originally summarized with William J.M. Hrushesky, Todd Langevin and Robert B. Sothern, all of the University of Minnesota, among others (cf. references 88–90).

Triangulation of marker rhythms

Thus, marker rhythmometry is developing to a point when it may guide 'triangulation', i.e., the location of a point in circadian time which is associated with best (health)-cost-benefit with respect to both desired and undesired effects. Optimal circadian stages may be evaluated by reference to several marker rhythms, used for a double purpose. First, these rhythms assess the different toxicities of the drug(s) and eventually also allow the evaluation of any benefit from therapy, as in the case of a reduction of measurable tumor size or prolongation of life or, the real goal, an improved cure rate. As such, they provide information concerning the merits and/or demerits of the treatment. Second, the marker rhythms can provide information concerning the timing of treatment by means of their acrophase.

Looking at the first purpose of marker rhythms, drug toxicity is a major health cost item in the cost-benefit relations of doxorubicin. In this respect, changes in the counts of the total number of leukocytes, neutrophils and platelets gauge myelotoxicity by the area representing the extent to which these variables are depressed during the weeks immediately following doxorubicin administration. The change in the rhythm-adjusted mean (MESOR) of heart rate gauges cardiotoxicity. As for the second purpose of marker rhythms, the acrophase of urinary potassium excretion constitutes a marker of potentially more general use as an index for timing drug administration, along with the hematologic acrophases that also serve that purpose.

With such gauges available, the percentage gain in about 500 completed clinical courses with doxorubicin and cisplatin has been impressive (88, 89, 90), as summarized in Table 3 for several variables used for triangulation. To investigate optimization by timing, treatment is not only started either at 06^{00} or at 18^{00}, but circadian acrophases of potential marker variables (heart rate, WBC, neutrophils, platelets, urinary K^+ excretion) are evaluated prior to each treatment. Thus, the patient's responses to treatment (evaluated as an increase in MESOR of heart rate for cardiotoxicity, as the area under the curve of weekly WBC, neutrophils and platelets for myelotoxicity and by creatinine clearance for nephrotoxicity) can be referred as outcomes to the given patient's prospectively determined internal time structure. This approach facilitates the individualization of timed therapy. In order to illustrate this concept, Table 3 summarizes the results obtained for all patients during each monthly course of therapy. Thus, on the basis of the population of diurnally active, nocturnally-resting or sleeping cancer patients sampled so far, the average circadian acrophase of heart rate occurs at $\sim 15^{20}$, of WBC at $\sim 18^{00}$, of neutrophils at $\sim 17^{00}$, of platelets at $\sim 16^{15}$ and of urinary potassium excretion at $\sim 15^{00}$, as shown on the left in Table 3. Optimal (population) response to treatment is assessed in terms of the time elapsed from doxorubicin administration (always given first as part of a combination treatment with cisplatin) to the time of acrophase of each marker variable, determined for each patient individually (Δt_D in Table 3). For instance, on the basis of the population sampled, opti-

Table 3. Elements for 'triangulation' of marker rhythms for individualized time treatment. Doxorubicin optimally administered at $\sim 03^{00}$; $SD1^{45}$¶.

Marker variable (approximate computative acrophase, ϕ) in clock-hours		*Nephrotoxicity Creatinine* clearance*	*Cardiotoxicity ΔM heart rate (last–1st Rx)*++	*Endpoints (percent change)* Myelotoxicity Change in area within 1st month after treatment*		
				Total WBC	*Neutrophils*	*Platelets*
Heart rate (15^{20})	t_D		[13^{20}]			
	Δt_D		22^{00}			
	P		(0.026; < 0.05)			
	L		18 beats/min			
WBC (18^{00})	t_D			[03^{24}]	[01^{34}]	[02^{31}]
	Δt_D			09^{24}	07^{34}	08^{31}
	P			(0.024; < 0.01)	(0.051; 0.02)	(0.038; 0.01)
	L			28.5%	26.8%	36.9%
Neutrophils (17^{00})	t_D			[01^{45}]	[00^{21}]	[01^{28}]
	Δt_D			08^{45}	07^{21}	08^{28}
	P			(0.012; < 0.01)	(0.171; < 0.01)	(0.228; 0.06)
	L			28.8%	28.0%	39.9%
Platelets (16^{15})	t_D			[03^{57}]	[03^{42}]	[05^{45}]
	Δt_D			11^{42}	11^{27}	13^{30}
	P			(0.032; 0.03)	(0.182: < 0.01)	(0.033; 0.03)
	L			33.7%	36.5%	61.0%
Urinary K^+ (15^{00})	t_D	[05^{00}]		[04^{00}]	[06^{36}]	NS
	Δt_D	14^{00}		13^{00}	15^{36}	
	P	(0.052; < 0.01)		(0.164; 0.04)	(0.059; 0.03)	
	L	9%		30.7%	43.2%	

¶ Combination treatment with doxorubicin followed 12 hours later by cisplatin (see text). Since sequence of drug administration is fixed with doxorubicin always given first, 12 hours before cisplatin, timing can be invariably referred to doxorubicin even if, in the case of nephrotoxicity, in human beings, it should be largely attributable to the cisplatin component of the combination treatment.

+ Index of the nephrotoxicity brought about by cisplatin administered 12 hours after doxorubicin administration; results reported relate to time of doxorubicin administration.

++ Difference between heart rate mean before first and last treatment given as such (rather than as percent change).

* t_D is optimal population-based time (clock-hour) to administer doxorubicin as judged by endpoint relative to marker variable; Δt_D is time interval from time of acrophase of corresponding marker variable to time of least toxicity as gauged from corresponding endpoint determined by cosinor rhythmometry; P are P-values from cosine fit, first, followed by that from t-test comparing toxicity around acrophase or bathyphase ($\pm \sim 3$ hours); L is excess toxicity resulting from treating at inopportune time instead of optimal time.

Acknowledgement: The data here analyzed stem from studies designed and originally summarized with William J.M. Hrushesky, Todd Langevin and Robert B. Sothern, all of the University of Minnesota, among others (cf. references 88–90).

mal response to the combination treatment as gauged by creatinine clearance for least nephrotoxicity occurs on the average 14 hours after the time of acrophase of urinary potassium excretion. Since the latter occurs around 15^{00}, it can be concluded that on the average, doxorubicin is optimally administered 14 hours after 15^{00} or around 05^{00}, and a heavier contributor to nephrotoxicity, cisplatin, 12 hours later. Similar reasoning can be applied on the basis of all endpoints and marker variables.

As can be seen from Table 3, on a population basis, and depending on the endpoint considered, the optimal time to administer doxorubicin is $\sim 05^{00}$ (creatinine clearance), between $\sim 01^{45}$ and $\sim 04^{00}$ (total WBC), between $\sim 00^{21}$ and $\sim 06^{36}$ (neutrophils), or between $\sim 01^{28}$ and $\sim 05^{45}$ (platelets). By determining the average best treatment time (triangulation), i.e., by combining the information thus provided, it appears that doxorubicin might best be administered at $\sim 03^{00}$ (SD = 1^{45}) to diurnally-waking, nocturnally-sleeping cancer patients.

In order to further test the benefits from timing treatment according to marker rhythms, the patient's response to treatment was assigned to the time of treatment with respect to the marker rhythm's acrophase and was then analyzed by single cosinor. In most cases, a statistically significant rhythm was thus found, as shown in Table 3 by the first P-value in parentheses. To complement this analysis, a Student's t-test was applied to compare the patients' response (toxicity) when treatment was administered at the best (around the acrophase of the cosinor fit) or at the worst (around the bathyphase of the cosinor fit) time (± 3 hours). Results again show the potential benefit from timing treatment by the second P-value in parentheses in Table 3. Although the above results are summarized for the population, once the information on marker rhythms is available for a given patient, the optimization of treatment can be individualized.

The procedure for triangulation alluded to above, based on the use of multiple marker rhythms, can indeed serve for gauging a given effect on an individualized basis. This approach offers applications for the personalized optimization of the therapeutic ratio and more particularly for reducing toxicity from cytotoxic drugs, but the population-based results are of interest in themselves. Triangulation is based on a collateral hierarchy of estimates relating the anticipated

health benefits to the health risks and health costs (toxicity) and, realistically, also to the financial cost and the effort involved for the patient and the health care team.

When relying on more than one marker rhythm, several indications of optimal treatment time are obtained. These may or may not converge, i.e., they may point toward the same rhythm stage or toward conflicting rhythm stages, as is perhaps the case for timing doxorubicin treatment with respect to cardiotoxicity evaluated by the heart rate MESOR (best time $\sim 13^{20}$, Table 3, whereas otherwise the optimal time is $\sim 03^{00}$). If these indications converge, as in the case of myelotoxicity gauged by total WBC, neutrophils and platelets for a combination treatment of doxorubicin followed 12 hrs later by cisplatin, an average optimal time can be derived from all estimates by 'triangulation'. If they diverge, however, as may be the case between myelotoxic endpoints and cardiovascular or renal endpoints such as the MESOR of heart rate or urinary potassium excretion, optimization may be carried out by allowing more weight to the estimate thought to describe the risk to which the patient is most susceptible.

Thus, with the availability of data on several variables, triangulation serves not only to reduce the measurement error when the different marker rhythms converge in their timing (for instance when they refer to toxicity to the same targetted organ or tissue), but also to determine where most of the damage occurs to assign priority to the corresponding marker acrophase for treatment optimization. Another consideration in the optimization of treatment relates to rhythm scrambling (72a). It appears that one strategy for deploying body defenses at all times is to shift rhythms about in a systematic fashion so that the optimal times for maximal treatment efficacy, compatible with maximal host defense and reduced toxicity, can be identified.

The results shown in Table 3 were obtained by administering bolus doses of both drugs. There is homeostatic evidence (10, 26, 94, 98, 99, 117, 131, 135) that side effects can be reduced and chronobiologic evidence that the desired effect can be ameliorated by administering a drug "continuously" via a pump. Advantages are the anticipated administration of a higher total dose at the tolerance acrophase and/or a better desired effect at the effectiveness acrophase.

Circadian-stage dependence of exposure to external agents affecting carcinogenesis

Potential benefits to be derived from a chronobiologic study design stem from the prominence and ubiquity of biological rhythms, and in particular of the circadian rhythm. The major role played by bioperiodicity in chemical carcinogenesis has been repeatedly studied (24, 39, 53, 69, 91, 95, 103). In several studies, local skin exposure to a carcinogen such as dimethylbenzanthracene (DMBA) or 3-methylcholanthrene induced a different number of tumors (e.g., sarcomas) as a function of the circadian stage in which the agent was applied.

Such a circadian stage dependence of host susceptibility to a carcinogen evaluated after injection of DMBA into the submandibular gland of hamsters is seen macroscopically in Figure 17. In two separate studies, carried out by Anand P. Chaudhry in Minnesota (13), six groups of animals had a fixed dose of DMBA injected into the submandibular gland, one group at each of six circadian times separated by conse-

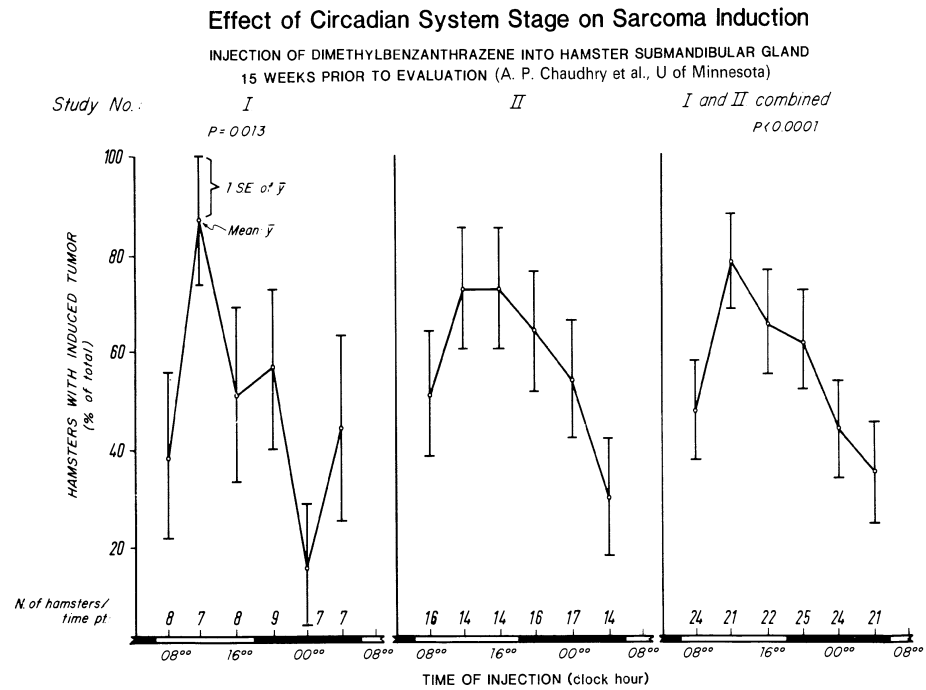


Figure 17. Macroscopic view of circadian stage-dependence of host susceptibility evaluated after injection of dimethylbenzanthracene into submandibular gland of hamsters (Chaudhry and Halberg, 1960) (13). © Halberg, 1964 (39).

cutive 4-hr spans during 24 hrs. Three months later the hamsters were killed and the presence of tumors (sarcomas) in excised glands was determined. Sarcoma was found in 80% of the animals injected at one time – 6 hours after light onset (6 HALO) – and 35% of those injected at another time (22 HALO). In both studies, the stage of the organism at the time of injection was sufficiently important to tip the scale between sarcoma and no sarcoma 3 months after administration of the carcinogen. It overrode the effects of a multitude of factors impinging upon the organism during the 3-month span.

Similarly, a circadian rhythm in DMBA-induced mammary carcinogenesis was demonstrated by Erhard Haus, in our laboratory, in DBA_8 mice implanted with ectopic pituitaries or muscle isografts and orally force-fed with DMBA (2 mg DMBA in 0.2 ml olive oil per gram body weight) at 1 of 6 circadian stages (69). For one week prior to feeding the carcinogen, the animals were standardized on a regimen of light from 06^{00} to 18^{00}, alternating with darkness. Half the animals had been implanted with 3 isologous pituitaries plus 3 fragments of muscle tissue, or with 3 fragments of muscle tissue only. The isografted pituitaries were known to substantially increase the breast cancer incidence. Separate subgroups of comparable animals from both implanted groups were force-fed once at one of six timepoints, 4 hours apart, starting at 12^{00} of one day of study and ending with the last subgroup at 08^{00} of the next day. 24 hours after feeding the carcinogen, the animals were housed 5/cage and observed weekly for the gross appearance of breast cancer. To assure histologic examination, the mice were killed when the tumor diameter exceeded 1 cm. Actual cancers were thus verified histologically in all mice with breast tumors. The highest incidence of DMBA-induced breast cancers in mice and DMBA-induced submandibular sarcomas in hamsters occurred when the carcinogenic agent was administered toward the end of the light (rest) span. It is commonly supposed that a condition for circadian susceptibility rhythms to occur is a short half-life, i.e., the rapid elimination of an agent from the body. In the case of DMBA, the host is exposed to the agent and/or its metabolites for a considerable span following a single administration. Under such conditions, just as in the case of exposure to noise for only a very few seconds, the response of the organism is bioperiodic rather than constant (37). Thus, we can introduce the importance of rhythms to carcinogenesis by showing that the process itself is circadian stage-dependent under controlled conditions (13) as well as under ordinary conditions (103), Figure 18.

Circadian and circannual aspects of prolactin and the risk of human breast cancer

When dealing with rhythmic variables, testing for a difference between two populations also benefits from a

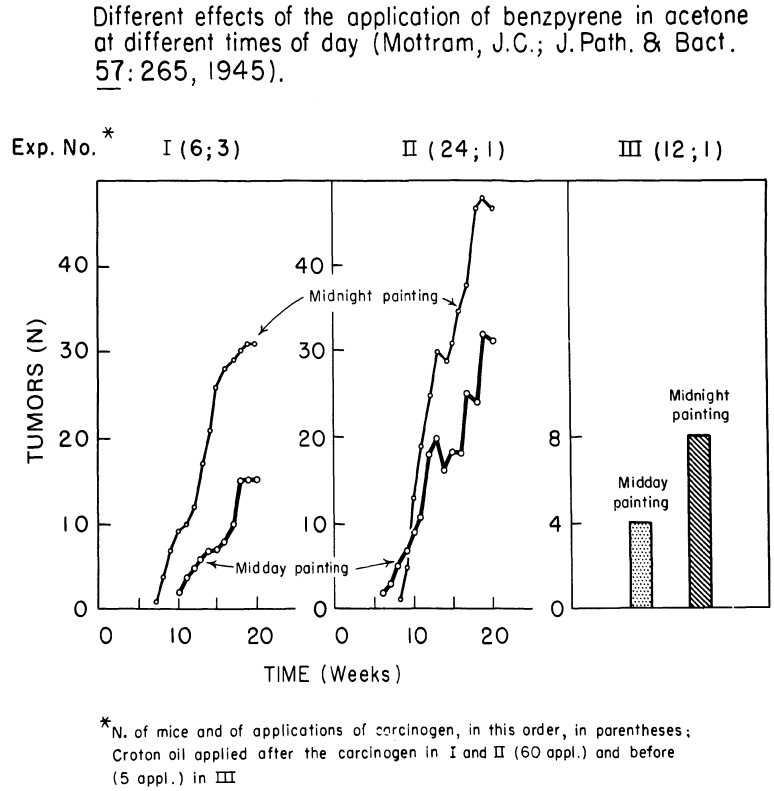


Figure 18. Difference in tumor incidence as a function of circadian stage of application of benzopyrene in acetone in mice treated at midday or midnight. © Halberg *et al.*, 1978.

chronobiologic design. Indeed, differences in one or the other rhythm characteristic among the groups compared may lead to the situation where a positive difference is found at one test time, a negative difference at another test time and no difference at a third test time. This situation is best illustrated in results on prolactin from an international study on the risk of developing breast cancer (57).

Age-specific mortality and morbidity rates attributed to breast cancer (85, 86) are 6 times larger in the USA than they are in Japan. Among the hormones suspected of playing a role in the development of human breast cancer, prolactin has arisen much interest. Prolactin was indeed shown to be essential for the promotion of both spontaneous and carcinogen-induced mammary tumors in rodents (4, 100, 109). The role of prolactin in the pathogenesis of human breast cancer, however, is uncertain (8) and controversial.

The chronoepidemiologic study (57) has demonstrated differences between women from different ethnic-geographic backgrounds in plasma prolactin, with the magnitude and even the sign of some of these differences dependent upon circadian and circannual rhythm stage. The MESOR and amplitude of the circadian rhythm in prolactin and the circannual variations in circadian prolactin MESOR and amplitude are much more prominent in Japanese women than in Minnesotan women. Moreover, the circannual prolactin MESORs in Japanese women are higher than the corresponding values in Minnesotan women. Minnesotan women tend to have lower prolactin values than Japanese women when prolactin concentrations are high, i.e., during the night. This difference is more accentuated in winter and can hardly be discerned in summer.

A large but time-restricted difference in plasma prolactin concentration between Japanese and Minnesotan subjects could be observed during the winter season. The difference

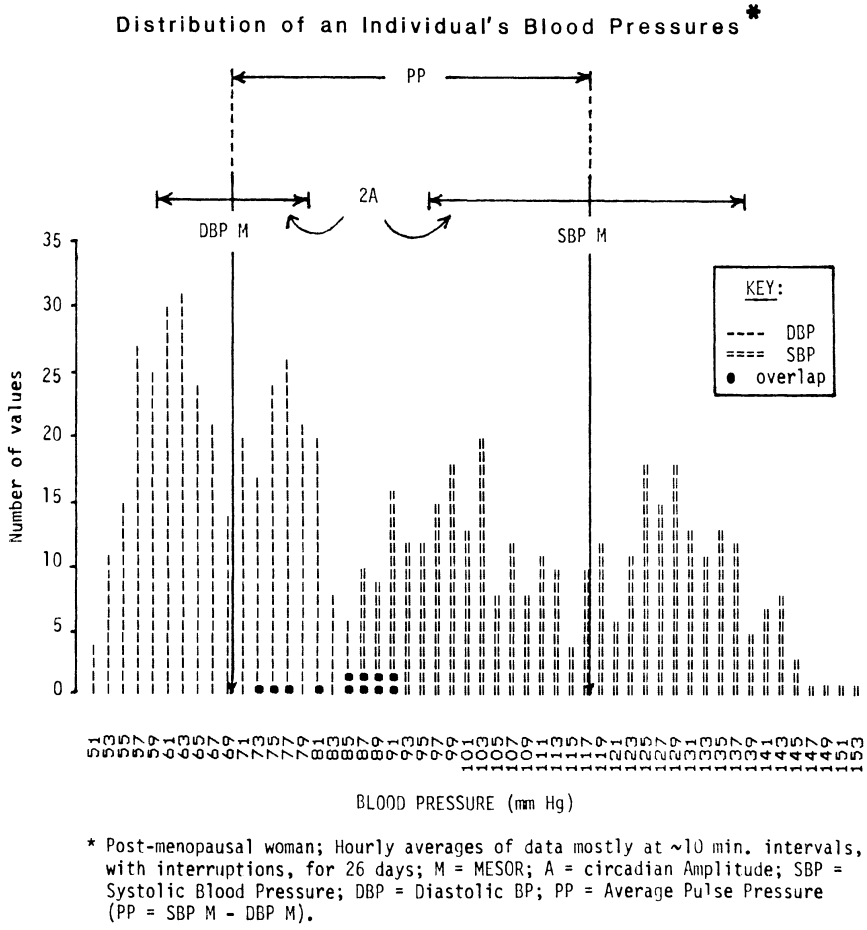


Figure 19. Histogram of systolic and diastolic blood pressures from a single individual. Note two-peaked distribution of each variable, as can be anticipated for a periodic function overlayered by noise. Note further overlap between the highest diastolic blood pressures and the lowest systolic pressures. Today, it is not tenable to ignore the distinction between systolic and diastolic blood pressure. If so, one should realize the implication of even larger differences than those between systolic and diastolic pressure. These differences that may be larger than the pulse pressure occur each day in the vast majority of individuals in either the systolic or the diastolic pressure. Thus, a good many diastolic pressures at some time during 24 hours are actually higher than the systolic pressures at another time. The predictable dynamics that bring about such differences should indeed be assessed; the hardware and software required for this purpose are available, as are chronobiologic concepts for interpreting the variability on hand. © Halberg 1987 (51).

Table 4. Mean and conventional dispersion indices of neonatal blood pressure do not reliably separate groups of 18 neonates with a negative family history of high blood pressure from 16 neonates with a positive family history of high blood pressure.

Kind of index	*Family history of high blood pressure: Index**	*Negative (n = 18) Mean ± SE*	*Positive (n = 16) Mean ± SE*	*Difference ± SE***	*t*	*P-value*	*Does the index 'work'?****
			Systolic blood pressure (mm Hg)¶				
Static (S)	Mean	68.0 ± 1.7	70.1 ± 2.5	2.1 ± 3.0	0.70	0.489	no
	MESOR	68.1 ± 1.7	70.1 ± 2.5	2.1 ± 3.0	0.70	0.486	
Global	Range	45.7 ± 2.8	46.6 ± 3.8	0.9 ± 4.6	0.20	0.847	no
dynamic (D)	90% range	28.4 ± 1.4	32.6 ± 2.8	4.1 ± 3.1	1.35	0.187	
	%change@	97.4 ± 6.7	70.2 ± 2.5	2.1 ± 3.0	0.70	0.486	
	Standard deviation	8.62 ± 0.41	9.58 ± 0.86	0.96 ± 0.92	1.05	0.304	
			Diastolic blood pressure (mm Hg)¶				
S	Mean	41.5 ± 1.1	42.9 ± 1.6	1.5 ± 1.9	0.77	0.444	no
	MESOR	41.5 ± 1.1	43.0 ± 2.0	1.5 ± 1.9	0.79	0.438	
D	Range	30.2 ± 1.6	33.9 ± 2.7	3.7 ± 3.1	1.21	0.236	no
	90% range	19.5 ± 1.0	22.3 ± 2.2	2.8 ± 2.3	1.21	0.236	
	%change@	111.4 ± 6.4	123.0 ± 9.5	11.6 ± 11.3	1.03	0.309	
	Standard deviation	5.76 ± 0.26	6.67 ± 0.66	0.90 ± 0.68	1.32	0.197	
			Mean arterial pressure (mm Hg)¶				
S	Mean	55.2 ± 1.5	57.3 ± 2.1	2.2 ± 2.6	0.84	0.408	no
	MESOR	55.2 ± 1.5	57.4 ± 2.1	2.2 ± 2.6	0.84	0.406	
D	Range	45.8 ± 3.3	46.4 ± 3.4	0.5 ± 4.7	0.11	0.910	no
	90% range	26.2 ± 1.2	30.2 ± 2.9	4.0 ±3.0	1.31	0.199	
	% change@	132.7 ± 10.8	133.6 ± 10.4	1.0 ± 15.1	0.06	0.950	
	Standard deviation	8.21 ± 0.39	9.10 ± 0.80	0.87 ± 0.88	0.99	0.332	

* Range: H–L, where H and L represent the highest and lowest values, respectively; 90% range is range after eliminating the upper and lower 5% tails; % range computed as [(H–L/L] × 100. MESOR = rhythm-adjusted mean: **mi**dline-estimating **s**tatistic **o**f **r**hythm.
** Any discrepancy between these values and the differences computed between the corresponding two means listed here is brought about by rounding.
*** By 'separating' groups with a negative and positive family history of high blood pressure.
¶ Monitored around the clock at 30-minute intervals for 48 hours.
@ Expressed in %. Cooperative work with B. Tarquini, M. Cagnoni, G. Mainardi & C. Panero, University of Florence, Italy.

was less prominent during the other seasons and was statistically not significant during summer. This finding indicates the importance of sampling time in investigations of the role of certain hormones in oncogenesis. It may also explain previous discrepancies and controversies reported in the literature and provides a clue to possible differences in circadian physiological time structure of groups of women with different risks of developing breast cancer.

This large but strictly time-dependent difference between Japanese and Minnesotan women was confirmed in groups of 20 young and 15 adult subjects examined at the one circannual stage (first part of March) used for actual replication in Fukuoka, Kyushu, Japan, and Minneapolis, Minnesota, USA, respectively, the Japanese showing again a higher average circadian prolactin MESOR and a higher circadian amplitude than did the Minnesotans. This observation emphasizes the critical importance of an appropriate timing of the sampling of endocrine functions of potential interest for oncogenesis by single or by a limited number of samples chosen on the basis of chronobiologic

Table 5. Circadian amplitude* separates groups of 18 neonates with a negative family history of high blood pressure from 16 neonates with a positive such family history. While conventional endpoints of data location and dispersion fail to do so, as shown in Table 4.

Blood pressure (mm Hg)¶	*Family history of high blood pressure*					
	Negative (n = 18) Mean ± SE	*Positive (n = 16) Mean ± SE*	*Difference ± SE***	*t*	*P-value*	*Does the index work'?****
Systolic	2.12 ± 0.20	3.98 ± 0.63	1.86 ± 0.63	2.93	0.006	yes
Diastolic	1.48 ± 0.20	2.92 ± 0.51	1.43 ± 0.53	2.71	0.011	yes
Mean arterial	2.06 ± 0.27	3.59 ± 0.56	1.53 ± 0.60	2.57	0.015	yes

* By fit of a 24-h cosine curve with single cosinor method (Halberg et al., Physiology Teacher 1: 1–11, 1972).
** Any discrepancy between these values and the differences computed between the corresponding two means listed here is brought about by rounding.
*** By 'separating' groups with a negative and positive family history of high blood pressure.
¶ Monitored around the clock at 30-minute intervals for 48 hours.
Cooperative work with B. Tarquini, M. Cagnoni, G. Mainardi & C. Panero, University of Florence, Italy.

individual or group reference values. This can only be done, however, once the rhythmic structure has been properly mapped.

Can we do the equivalent of ignoring the difference between systolic and diastolic pressure?

The importance of rhythms relates to all biologic variables in health and disease, beyond cancer. Let us now turn to the analogy of systole and diastole in the case of the circulation. Each day in the overwhelming majority of individuals, some systolic pressures are lower than the highest diastolic pressures. Figure 19 shows this point for an amply investigated individual. The penalty for ignoring the rhythm underlying the distribution in this figure may be akin to ignoring the difference between a systolic and a diastolic blood pressure! The time has come to recognize that in integrative as well as circulatory physiology, among many other areas, rhythms are no longer another factor, like nutrition or genetics. A time structure constitutes the predictable dynamics of life. Those able to assess rhythms dispose of a powerful and cost-effective resolving tool. Those who believe that rhythms can be 'eliminated' by sampling at a fixed clock-hour (cf. Figure 1) may have to cope with a rather confusing and wasteful source of variability, an approach that is hardly warranted in 1988.

We now turn to Tables 4 and 5, summarizing recent results on neonates (56). Table 4 illustrates a circumstance when reliance on the mean value or on conventional dispersion indices is not rewarded, even though the comparison applies to series each consisting of about 96 consecutive cardiovascular measurements from newborns with a negative or positive family history of high blood pressure. Table 5 suggests that at the cost of added numerical analyses, the resolution of an important difference between newborns may perhaps be established. Circadian parameters are more complex than a mean or several kinds of ranges and a standard deviation. Their computation, however, may be worthwhile not only in the case of Tables 4 and 5 (56) but in another documented case as well (105).

Note of caution

The examples discussed above have shown that results obtained with the assumption of homeostasis can be misleading. It can be argued, however, that if certain findings are confirmed under the noisy conditions of casual measurements, they sure must be correct. In order to reconcile these two viewpoints and understand the paradox, let us examine some of the statistical concepts underlying experimental designs. The role of statistics is to solve a problem of induction from the sample (data actually collected) to the population (general inference), by determining (confidence) limits for the parameters being estimated. One of the most important assumptions in applying a test of significance is the independence of the observation errors. It is, however, usually impossible to ascertain whether this assumption is valid or not. Randomization (i.e., drawing a random sample from a population, random assignment of treatments) allows one to proceed as though this assumption is true. Although randomization cannot guarantee independence, it will reduce the correlation that tends to characterize errors associated with observations that are adjacent in space and/or time. Randomization also reduces biases by avoiding treatments to be continually favored or handicapped by unassessed extraneous sources of variation (15, 92).

Provided sampling was truly random, homeostatic findings may be correct, even though the design may not have been the most cost-effective. Misleading or controversial homeostatic findings generally result from the failure of randomization in time, or rather with respect to rhythm stage.

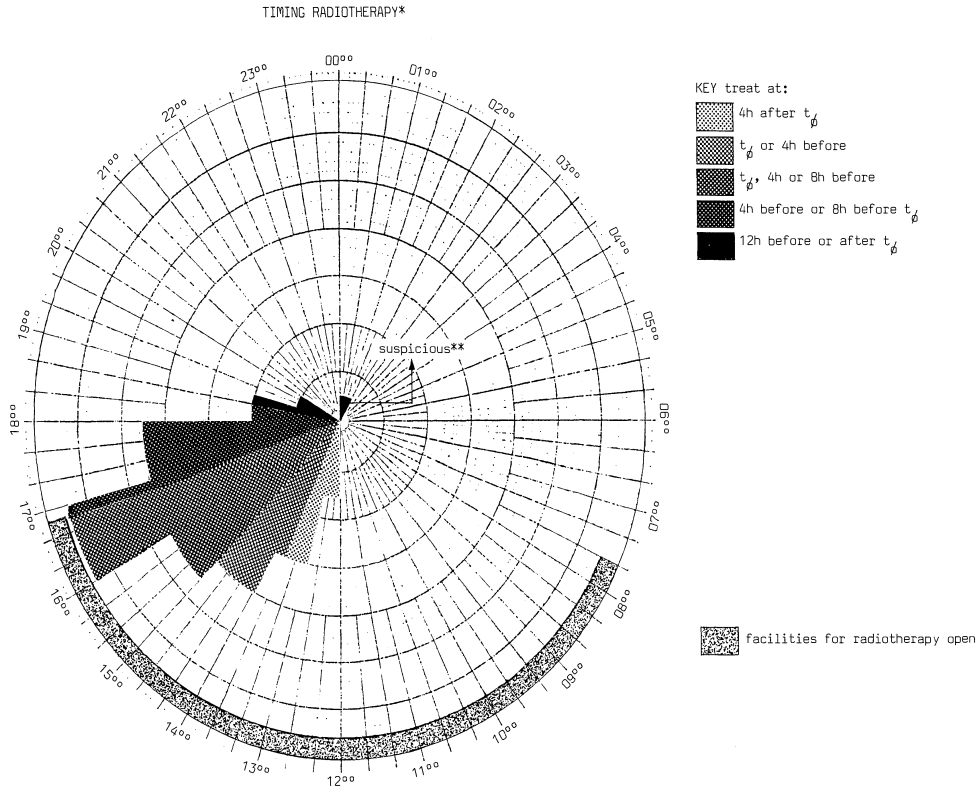
Another argument may be that certain effects that are apparent only at certain ages or certain times of day or year are less amenable to generalization and are less important. Although this may appear to be true, the information provided by chronobiologic designs allows the optimization of treatment. Moreover, notwithstanding the fact that changes in marker rhythm parameters may be small, they can be associated with large differences in clinical outcome. A case in point is the gain of 2 in the outcome of the radiotherapy of perioral cancers treated at the time of tumor temperature peak as compared to radiotherapy at other times. The difference in tumor temperature is relatively small, whereas that in therapeutic benefit is quite large (63).

Patient assignment for therapy guided by a marker rhythm in facilities functioning for only part of the day

For clinical and other chronobiologic trials, it is often desirable to subdivide a set of patients into 6 or at least 5 groups, and to assign consecutive patients at random to one of these groups. Thus, each patient (-group) receives a treatment such as radiotherapy at a different time in relation to a routine of living or the time of circadian acrophase (ϕ) in tumor temperature.

The routine-related treatment may be given, after an appropriate span of standardization at awakening or 4, 8, 12, 16 or 20 hr later, when treatment is possible at any time of the day. In many hospitals throughout the world, however, facilities for treatment such as irradiation are used for only 8 (or at most a few more) hours out of the 24-hr day. In such hospitals, it is difficult, if not impossible, to randomly assign radiotherapy so timed as to cover in a systematic fashion, e.g., 6 circadian stages, 4 h apart, in relation to the patient's awakening.

Another actually preferred approach involves timing with respect to a particular stage of a marker rhythm, such as that of tumor temperature in the case of the radiotherapy of advanced cancers of the head and neck other than brain. For such patients, tumor temperature is a pertinent marker rhythm: by random assignment of patients to 5 groups, one treated at the tumor temperature peak and others 4 or 8 hours before or after the peak, it has been possible to document with statistical significance the importance of timing treatment for very advanced cancers of the head and neck both by inspection of the data (17, 18) and by an inferential statistical analysis (47, 63). It may, however, be almost as difficult to randomize treatment timing according to the stage of a tumor marker rhythm (as it is to do so in relation to awakening) when radiation facilities are not available around the clock.



25 patients; 56 courses (profiles); each patient contributing 1-6 profiles.

*Based on circadian (rectal) temperature acrophases from patients receiving chemotherapy. Each patients provides 1 temperature ∅ at each course of doxorubicincisplatine chemotherapy.
**∅ out of phase with ∅ from same patient at other chemotherapy courses.

Figure 20. Illustration of para-randomization, defined as the allocation of patients to such treatment times that are difficult to assign because of facilities being unavailable part of the day, combined with the random assignment of patients to all times that are more easily accessible. The data here analyzed stem from studies designed and originally summarized with William JM Hrushesky. Todd Langevin and Robert B Sothern, all of the University of Minnesota, among others (cf references 88–90).

A compromise such as a partial or weighed 'para-randomization' then becomes essential. Para-randomization may be defined as the allocation of patients to such treatment times that are difficult to assign, combined with a random assignment of patients to all times that are more easily accessible. This para-randomization should be complemented by the provision of tests for any bias resulting from the specific allocation of treatment times. The patients to be treated should be given a thorough physical and psychological examination, including manility-serality questionnaires (87), in order to consider any and all factors underlying the unusual timing of high values in the marker rhythm. The difference in manility or morningness vs. serality or eveningness is just one as yet not well-defined case in point. On the basis of results from nonlinear rhythmometry applied to the temperature data, but taking into account differences in the daily routine, one could stratify patients before randomly assigning them to similar protocols implemented in hospitals where therapy facilities remain available around the clock.

The reason why certain rhythm stages may be difficult to assign when facilities are open during the day only is because in day-active, nocturnally-resting patients, the circadian temperature acrophase has a relatively tight distribution centered around the late afternoon, as shown in Figure 20, obtained from about 24-hr records of rectal (though not tumor) temperature monitored by TherMolog (Vitalog Corp., Palo Alto, CA) on cancer patients receiving (a combination doxorubicin-*cis*diamminedichloroplatinum) chemotherapy. Each patient contributed one profile per course of chemotherapy, at ~ 1-month intervals. In view of problems related to the short recording span and the fact that patients were also enduring the side effects of chemotherapy at the time their temperature was monitored, not all acrophases were used to draw Figure 20, but only those 29 corresponding to time series satisfying 'quality' criteria, considering the total observation span, the length of the longest gap (missing data), the total number of data collected, and the goodness of fit of the cosine model.

One may question the choice of the circadian acrophase of core temperature as the marker for timing therapy and wonder whether the timing of the macroscopic peak or trough may also be reliable, the more so that the latter approach already yielded a gain of 2 in the therapeutic index for the radiotherapy of oral tumors (18, 46). This question was examined by comparison of the distribution of acro-

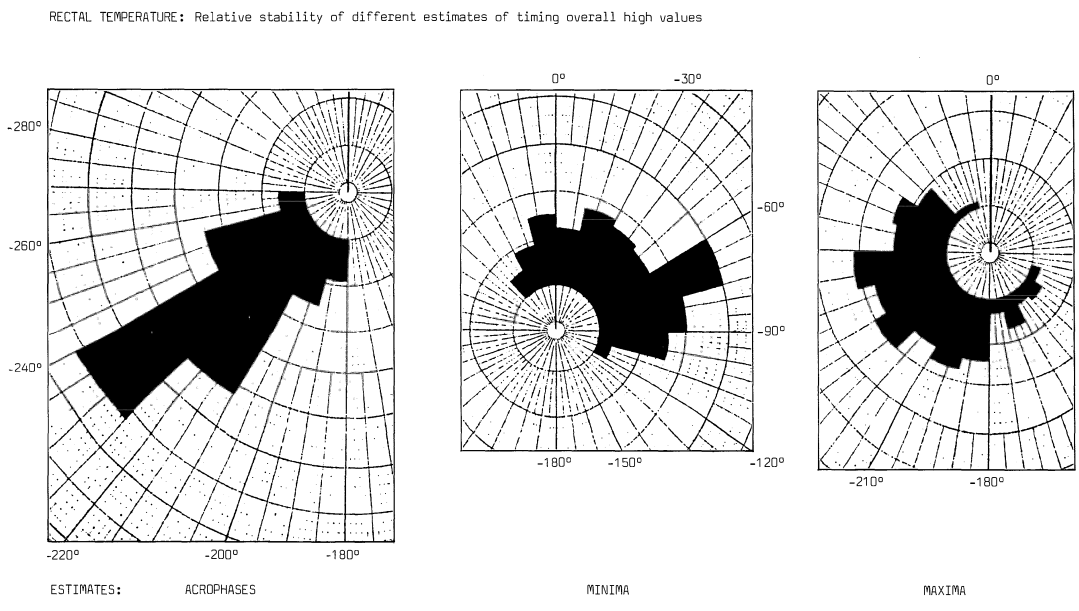


Figure 21. Minima and maxima from daily profiles of rectal temperature show less stability as compared to circadian acrophase.

phases, maxima and minima of 24-hr profiles of rectal temperature in a clinically healthy woman over an about-2-year span. She was also using the TherMolog (Vitalog Corp., Palo Alto, CA) instrument for data collection at 12-min intervals with interruptions. Data over a total of 158 single days with gaps smaller than ~4 hrs and with at least 80 temperatures/24 hrs were available for analysis (31). The respective distributions, shown in Figure 21, indicate a better reproducibility of the daily acrophase as compared to the extrema.

Global versus chronobiologic dynamics

In turning back to blood pressure rhythmicity, it seems interesting to note that at 9 years of age, a chronobiologic index differentiates between those with a negative family history of high blood pressure and those with a positive such family history. The circadian amplitude-acrophase pair of systolic blood pressure separates the 2 groups when the mean fails to do so. Such findings stem from noisy self-measured data (118). It is also clear that a (chronobiologic) static index, the MESOR, which does not distinguish groups with a negative family history of high blood pressure from peers with a positive such history at 9 years of age, does so later in life, notably when it is obtained on systematically and automatically sampled data, at 10-minute intervals.

The point of this paper, however, is to indicate that in 1988, exclusive focus upon a mean of values at a single timepoint or at casual times is hardly warranted. Added focus beyond the mean, at the standard deviation at a single timepoint, is also unduly restrictive (Table 4 versus Table 5). Even when one assesses, in addition to the mean, the global dynamics such as the standard deviation of systematically placed around-the-clock measurements, one may fail to detect a relevant phenomenon, Table 4.

Biologic clocks (i.e., exclusive focus upon period) versus chronobiology

Focus upon some periodicity such as the circadian one may require exclusive attention being paid to its phase or period. This may have to be done with full neglect of amplitude. More often than not, however, concomitant focus upon the amplitude is worthwhile. The amplitude can serve as an added criterion for a better resolution of the period. Whenever possible, the concomitant assessment of the complete predictable dynamics is preferred. These include the extent of change and the timing of overall high values as well as measures of the waveform, and also estimates of the uncertainties of each characteristic.

We can view organisms as structured in space-time and realize that we dispose of the hardware and software for assessing spatio-temporal variability and for isolating its temporal parameters. It is important to carry such tools from the laboratory where they have been extensively used to the citizen interested in preventive health maintenance, e.g., with a view of blood pressure (61), and also in a broader endeavor for self-help for cost-effective health care (41).

Epilogue

Indiscriminate sampling at different times in a circadian and/or circannual rhythm (features of structure in time) is

equivalent to sampling without concern for anatomy, indiscriminately from different organs in space. Sampling at a physiologically arbitrary time, chosen only by convenience, is akin to the choice of sampling, e.g., to assay a hormone, the skin rather than the gland producing it, since the former is more accessible. To carry this absurd example further, suppose that a hormone is to be determined, e.g., aldosterone, which may be contained primarily in the adrenal rather than in the skin. If so, the skin is hardly the most cost-effective source of aldosterone, notwithstanding the fact that it is more accessible (more conveniently located in space) than the adrenal. Without belaboring this analogy, it follows that now-available evidence concerning an anatomy in time (40, 49, 81, 82, 107, 120) in 1988 should be consulted at the start of an investigation. A decision on timing is then required akin to that of choosing an anatomical site. Were we to ignore whether we sample brain or muscle, liver or spleen, lung or intestine in space, our results may be somewhat confusing? Similarly, we encounter dramatic differences in time structure. It is wasteful to neglect such differences and not to explore them now that background information and cost-effective procedures are available to resolve [what we see by the unaided eye (see figures)] by time series analysis on the computer, the equivalent in time of what we resolve by the microscope and the electron microscope in tissues, i.e., in space.

There was a time when controls were not indispensable. Today, we realize that controls are essential in all our actions, medical practice or biologic research. One must also realize that the proper control at the outset, rather than as a follow-up, includes a systematic exploration in time. There is a need for the chronobiologic pilot study. Once a chronobiologic pilot is completed, sampling can eventually be restricted for a given purpose to a given timepoint chosen by pertinence rather than convenience (49), whether we carry out a scientific experiment or decide on the diagnosis and treatment of an individual patient. At this point, chronobiology becomes particularly cost-effective and usually indispensable.

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POSTSCRIPT

When this chapter was written it already started to be clear that histopathology is entering a new era. Any measurements applied in histopathology can now be gathered under the umbrella of Quantitative Pathology, which covers e.g., morphometry and stereology, quantitative histochemistry, static or flow cytometry, and image analysis in histopathology. Quantitative pathology allows for an efficient use of gathered information as was shown e.g. in the the

paper of Baak et al (5, referred to above) on breast carcinoma. Our group has now tested their approach (3), and we could show that the prognostic index based on mitotic count, tumor size, and axillary lymph node status correctly predicted the outcome of infiltrative ductal breast carcinoma in 80% of cases after the intermediate 40% of cases were excluded. Corresponding power of prediction has not been possible by any other approach.

But there are also other benefits from quantitative pathology. Traditional histopathology was theoretically relevant in its classification of disease. The decisions made at the diagnostic situation, however, were left to the intuition of the pathologist. Here the approach of quantitative pathology is helpful because, it will give the pathologist a knowledge of the prognostic probability from which he can make the correct diagnostic decision. This means that the pathologist at the time of decision knows when his prediction will be correct, when there is uncertainty, and to what degree there is uncertainty (1, 2).

Quantitative pathology as a field of pathology has also influenced scientific organizations. The Committee for Diagnostic Morphometry within European Society of Pathology has now changed its name to Committee for Diagnostic Quantitative Pathology. Academic recognition is also at hand: Dr. Jan P.A. Baak has now served several years as Professor of Quantitative Pathology at the Free University of Amsterdam.

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10

MORPHOMETRY IN CANCER DIAGNOSTICS

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Diagnostic histopathology in its traditional form is based on the skillful interpretation of the microscopic image by an experienced pathologist. The results of this process are usually extremely helpful to the clinician who is responsible for planning further investigations and therapy. And for a long time the pathologist has worked with his microscope, eyes and brain – now and then turning to the laboratory for special stains to enlighten the problematic cases. Through the special stains histochemistry has been part of the world of diagnostic pathology. In the last few years we have witnessed the fast development of the immunohistochemical method applying fluorescing antibodies and peroxidase-labelling. This field is still developing fast but another and basically different field, morphometry, has also entered the diagnostic laboratories. This field is now offering methods which are able to predict the clinically relevant aspects of prognosis correctly in a great majority of patients, with an accuracy not seen earlier in medicine. If anything, morphometric approach to histopathology is changing the traditional histopathology from art to science.

Morphometric methods

Morphometry is a concept which covers several lines of development. These developments started from different questions, but when applied to pathology they seem to have a very similar nature. First of them is the interest histopathologists have had for a long time. They have wanted to measure tissue structures, not only to describe them. Such approach is the basis for the traditional type of morphometry. Often the researchers have been satisfied with measurements on the 2-dimensional image seen in the microscope. However, the 2-dimensional section was not enough for all researchers. This is the second line of development: they wanted to know how the measurements on the 2-dimensional section could be converted to apply in 3 dimensions. It was Hans Elias (28, 29) who was especially interested in this aspect in histology and with others (60) started the development of biomedical stereology (a field trying to find correspondence between findings in 2-dimensional sections and the 3-dimensional reality). The third line of development is linked with computers. This field, image analysis, is not interested in biology primarily, but is interested in analysing any image, e.g. the microscopic image, automatically. Much of the work that deals with cytophotometry of nuclear DNA (3, 15, 16) is related to nuclear morphometry, as will be explained later. The morphometric methods are simple in principle but they may be laborious. The latter point has been the main obstacle for their use in diagnostic pathology laboratories. Recent development in image analysers, however, has made the field much more attractive because collection of data is now easier and faster (49). However, numerous parameters can be estimated with extremely simple methods without computers. The benefit from computers is mostly seen when parameters are measured which take much time to measure or cannot be measured at all by simple methodology.

The reason for applying morphometric methods is simple: numerous studies have demonstrated the limited reproducibility of traditional histopathology – especially grading of lesions (18). Morphometric methods, in addition to bringing the element of accuracy into the evaluation of histological findings, are generally more reproducible than the methods of subjective grading. So they give a good basis for diagnostic decisionmaking (19, 20). One should realise, however, that certain simple traditional grading systems may be as good as morphometric grading systems in terms of reproducibility (38). Generally the morphometric approach is superior but it is also exposed to subjective influences (36). In the latter connection, however, one should also remember that morphometry allows accurate measurements on continuous standardized scale whereas traditional grading uses ordinal scale with no independent standard.

Measurement of area and area fractions

If our problem is the measurement of the areas of nuclear profiles on section there are several approaches (8, 25, 51):
- the whole image is photographed and the parts of nuclei in the image are cut out and weighed
- a point grid within the eye piece of the microscope is applied on the image and the points falling on the nuclei. The more points on the nuclei, the large is their total area
- the image is photographed or the images of the nuclei are drawn on paper with a drawing tube and the areas of the nuclei are estimated with outlining the nuclei with a planimeter, an instrument designed to measure areas
- the image is projected on a digitizer plate, or fed into computer memory. The images of nuclei are outlined with a cursor on the screen of the monitor or on the digitizer plate. The results are fed into a computer which can be programmed to create a histogram of the distribution of nuclear areas.

In fact the practical methods that are used may vary

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considerably. E.g. Mehta et al. projected sections mounted on glass slides with a 35 mm slide projector on white board on which a point grid was drawn. In such a system the total of nuclei on the section can be estimated as was done in case of point grid in the eye piece above. Often, however, one is not interested in the absolute values but rather the fraction of section covered by a tissue component. These fractions are called area fractions. The ratios of areas of various types of tissue structures on section are good estimates for the ratios of the volumes of the same structures in the tissue studied (Delesse principle).

Measurement of other parameters

Other features can also be measured from sections. There are various methods for measuring the perimeter of sectioned nuclei. The simplest method applies a computer and a digitizer plate. The measurement is done by outlining the nuclear borders with a cursor. Line grids in the eye piece or on the photographed image can be used for the same purpose. In addition to parameters which characterize the findings in 2 dimensions, on section, also 3-dimensional parameters can be estimated by measurements in 2 dimensions. So volume distribution of nuclei can be estimated (after certain approximations) on the basis of the distribution of nuclear areas on section, the area of nuclear membrane per volume of tissue can be estimated with a line grid etc. (25, 59, 62). One should also realize that directional orientation may be an important diagnostic parameter in certain contexts (50).

So there are numerous parameters that can be measured, and the results can be used to distinguish various types of lesions from each other. The morphological changes, however, do not only have a diagnostic, but also a biological significance, which is best demonstrated by the variation in nuclear DNA in tumors.

Size of nuclei and the nuclear DNA

The dry mass of the nuclear DNA is about 15% of the total dry mass of the nucleus (56). In human cells this corresponds to about 5–7 pg of DNA in a nucleus (32, 39). The mass of histones equals the mass of DNA (35) and changes in the mass of DNA are immediately reflected in the amount of histones in the nucleus. Non-histone proteins are variable, and are most ample in the metabolically active phases of the cell. The mass of non-histone proteins varies from 1 to 2 times the mass of DNA (63). Nuclear volume changes are related to the total amount of material within the nucleus. However, usually nuclear size changes have been associated with changes of nuclear DNA. When we study a large number of nuclei, we can find a general association between the amount of DNA and nuclear size (45). However, nuclear size changes need not necessarily in all nuclei be associated with changes in DNA or histones. Size changes may be associated with changes in non-histone proteins, other organic material, inorganic material, or water. Since Caspersson started the interest in quantitation of DNA (17), nuclear DNA changes have been of great interest in biology. This approach has found special application in the diagnosis of tumors (3, 15, 16).

From the above it is obvious that morphometry and ploidy measurements may give parallel information. This is especially true when computerized image analysis is used with densitometry programs which measure the sum of staining intensities of individual pixels (49) on the nucleus. Results achieved by this means are almost equal to the results of microspectrophotometric measurements of DNA (13), especially when the sample has been stained with Feulgen stain. One is also reminded of the fact that also HE-stained nuclei may give valuable information in this connection (61), even though the staining is not stochiometric. But the morphometric approach allows the use of many other types of measurements that may be valuable in cancer diagnosis. One such parameter is the number of mitotic figures which seems to be well correlated with prognosis in many kinds of tumors. Mitotic figures and labelling index (LI) are two aspects of the same phenomenon, and LI has also shown extremely good correlation with prognosis (57). Other features of importance are the size of nucleoli, cellularity index, area fraction (= volume fraction) of the epithelium, and other parameters related to the amount of tumor tissue present in the sample. The importance of these factors varies in different kinds of tumors. Depending on the site and type of the lesion different kinds of criteria turn out to be meaningful. This very much suggests that morphometric methods cannot be used blindly, without thorough understanding of their special value in each situation. This has also been shown to apply to DNA ploidy measurements, which do not always suggest any general rule to indicate malignant behavior in various types of tumors from various sites (3).

Morphometric classification of tumors

To be able to apply morphometry in tumor diagnosis a morphometric classification has to be created first. At the present status of morphometric pathology we are just starting to create classifications (11). In creating a tumor classification we need to study a large number of tumors, often characterized by their type or histological nature, to find the morphometrical features which best correlate with clinically relevant parameters. So we can estimate the value of the parameters in predicting the prognosis, the therapeutic response, and other type of clinical outcome. Because clinical decisions are basically probabilistic, the results of the morphometric approach should be associated with a probability for a defined outcome (cure, death within a defined period, metastasis at a defined location, specified response to a defined drug etc.)

When we try to create a morphometric classification we have to make measurements. If we want to measure nuclei we are soon confronted with the question "How many nuclei should we measure?" The answer to this question depends on the type of result we are aiming at. If we are interested in the mean value of nuclear area our approach can be as follows (23). First we define our system by measuring a large number of nuclei from one of the samples. We try to do this measurement so that all types of nuclei have the same probability of being measured, i.e. our measurement results contain measurements from all types of nuclei present but more measurements from types of nuclei that are

more common in the sample. Then we define the mean area $\bar{x}$ and standard deviation of measurements. The SD is

$$SD = \sqrt{\frac{x_i - \bar{x}}{n - 1}}$$

where x_i = the value of the ith measurement and n = the number of nuclei measured. After SD has been measured we can estimate the accuracy of our system if we base our result on the measurements of n nuclei. The standard deviation of the mean value based on n samples is called the standard error (SE), and the size of the standard error depends on SD and the number of measured nuclei:

$$SE = \frac{SD}{\sqrt{n}}$$

If n is larger than 30, the confidence limits of a mean value can be estimated with the help of SE and the normal distribution. With 95% probability the true mean value of the nuclear parameter measured will fall within the limits

$$x \pm 1.96 \text{ SE.}$$

With 99% probability the true mean value will fall within the limits

$$x \pm 2.58 \text{ SE.}$$

If n is smaller than 30, corresponding limits can be estimated with the help of the t distribution.

So, it seems that we can answer our question "How many nuclei should we measure?" To get an unambiguous answer we should first decide how accurate we want to be in our measurements. We may decide that the correct mean result should be within 10% of our mean result. On the other hand we have to decide about the probability that the true mean really will fall within these limits, i.e. the number of times of 100 that we want to expect to find the correct mean within the limits we have set. We may decide that we want the correct mean to be within the limits in 95 cases of 100. Now we can estimate the number of nuclei needed:

$$1.96 \frac{SD}{\sqrt{n}} = 10\% \cdot \bar{x}$$

$$\sqrt{n} = \frac{1.96 \cdot SD}{10\% \cdot x}$$

$$n = \left(\frac{1.96 \cdot SD}{10\% \cdot x}\right)^2$$

Example

A pathologist measured 200 nuclear areas and got a result

$$x \pm SD = 45.0 \pm 15.2$$

He wanted to estimate how many nuclei he had to measure to get a good estimate of the mean nuclear area. He wanted the correct result to be within ± 5% on both sides of the mean. Also he wanted to be 99% sure that the correct value will be within these limits. We get

$$2.58 \frac{15.2}{\sqrt{n}} = 10\% \cdot 45.0$$

$$\sqrt{n} = \frac{2.58 \cdot 15.2}{10\% \cdot 45.0}$$

$$n = \left(\frac{2.58 \cdot 15.2}{10\% \cdot 45.0}\right)^2 = 76 \text{ nuclei}$$

Diagnostic morphometry

After measuring several samples the pathologist has data which can be used as basis for morphometric classification. After such classification has been created – and there are such classifications already (11) – individual samples can be studied and their place determined within the classification. However, because this is done by different pathologists in different laboratories, and because samples from individual patients are studied in apparently random order, we are here not dealing with group morphometry, and traditional prospective or retrospective studies, but with diagnostic morphometry and a diagnostic study (23, 54). The latter kind of study differs from the prospective or retrospective studies of group morphometry in that there usually are additional variation sources: pure interobserver and pure interlaboratory variation (23). For instance, we can estimate interobserver variation within a laboratory by letting five pathologists measure the same sample (we take the sample that was studied in the example above). Let the mean ± SD of those 5 measurements be

$$45.6 \pm 2.3$$

after 100 nuclei had been measured. The SD of the five means corresponds to the SE of the original measurements and this relation allows us to determine the SD in a system in which several pathologists study the same sample:

$$\frac{SD}{\sqrt{100}} = 2.3$$

$$SD = \sqrt{100} \cdot 2.3$$

$$SD = 23$$

It is easy to see that the variation is larger in the diagnostic study than in a study in which measurements are standardized and made by the same observer. If we now in this diagnostic system want to work with the same accuracy we had in the example above we have to have a larger number of nuclei measured. The calculation is as follows:

$$x \pm SD = 45.6 \pm 23$$

$$2.58 \frac{23}{\sqrt{n}} = 10\% \cdot 45.6$$

$$\sqrt{n} = \frac{2.58 \cdot 23}{10\% \cdot 45.6}$$

$$n = \left(\frac{2.58 \cdot 23}{10\% \cdot 45.6}\right)^2 = 170 \text{ nuclei}$$

This shows that for the same level of accuracy more work has to be done in the diagnostic situation than in the standardized research on group morphometry.

Morphometric changes in nuclei during preneoplastic and neoplastic progression

Nuclear size changes in the neoplastic progression can be demonstrated in many models. Because neoplastic (and preneoplastic) cells usually divide more actively than normal cells, there are also more cells in the phase of DNA synthesis in tumors. Because nuclear size is correlated with the amount of DNA in the nucleus, neoplastic (and preneoplastic) nuclei are generally larger than normal nuclei. Because the sizes of nuclei in different organs differ under normal conditions, the statement applies to a certain type of cells and to the preneoplastic and neoplastic changes associated with that cell type. But in addition to cells in S-phase there are cells with polyploid DNA values and aneuploid DNA values in tumors. Nuclear size changes even though generally associated with DNA changes, need not be solely defined by DNA changes. Other factors may also be involved.

One of the most dramatic changes in nuclear size (area on section) that we have seen in our laboratory deals with mouse skin papilloma model which can be used to test promotor activity. In this model mouse skin is once treated with a polycyclic carcinogenic hydrocarbon, such as dimethylbenzanthracene (DMBA) and thereafter subjected to weekly treatments of test compounds (36, 41). Four types of changes can be found: normal skin, hyperplasia, papilloma, and carcinoma. In a large experiment which studied the effects of dithranol and butantrone, only normal skin, hyperplasia or papilloma were found (36, 41). Nuclear morphometry was also applied and the results of measurements of nuclear area and perimeter are shown in Figures 1 and 2. The differences were dramatic between normal epidermis, hyperplastic epidermis, and papilloma. In fact the changes are so dramatic that in this model papilloma and normal skin could be distributed by measuring 1 nucleus. Such a clear-cut result is a bit surprising in the light of the fact that the measurements were made from sections. However, in this case a sampling rule was applied which said that the investigator should not select a nucleus in which the borders were indistinct, or which was cut at the pole. The measurements do not reveal multimodal pattern, either. The size distribution is unimodal in all cases and suggests that there is a separate cell clone for each type of lesion. Are we here dealing with clones with different ploidy value (DNA content), or are the size changes due to other factors, remains to be shown. Human tumors do not usually show such clear-cut changes. In grading of human tumors morphometrical results have a tendency to overlap which makes it necessary to use several parameters – those that best distinguish between lesions – in combination (11, 45). Sometimes, however, single parameter values are useful. It is the task of medical research to find the situations in which single parameters can be used, and to find other useful parameters and their combinations to give diagnostic and therapeutic decisionmaking a better basis. A simple example may show the potential of the approach applying a single parameter. The example deals with a distinction between two lesions, seborrhoeic keratosis and basal cell carcinoma, both common skin diseases. In an experiment (23) 800 nuclei were measured from a section of a case of basal cell carcinoma, and 400 nuclei from a section of a case of seborrhoeic keratosis. The results were

	Area
	Mean ± SD
Basal cell carcinoma	57.3 ± 17.0 μm^2
Seborrhoiec keratosis	47.0 ± 15.1 μm^2

It is easy to see that the means are different but this is not enough to distinguish these lesions from each other. Whether they really can be reliably distinguished depends on the number of measured nuclei, as shown earlier. The accuracy of the result depends on the number of measured nuclei and if we choose to measure 100 nuclei the results and their standard error (SE) will be

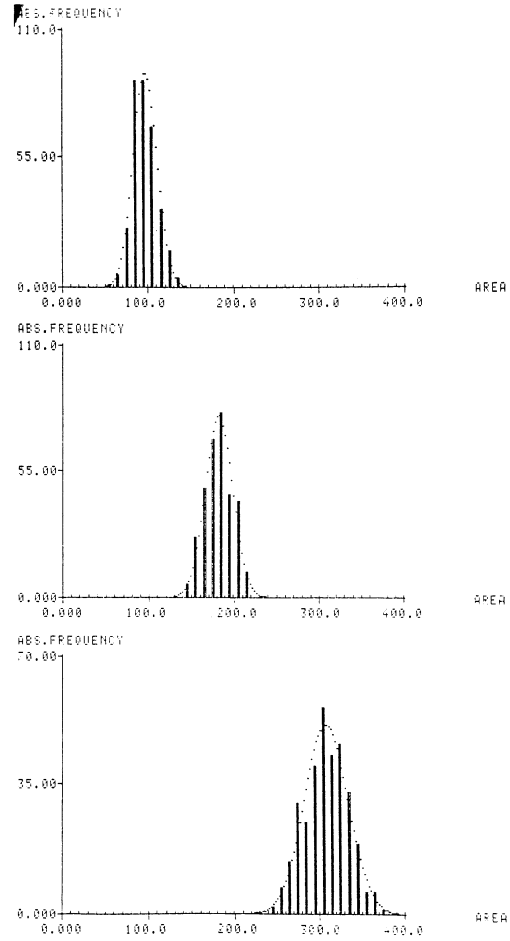

Figure 1. Size distribution of nuclear areas on section in normal mouse skin (above) in hyperplastic skin (middle) and in papilloma (below). The reader is reminded of the important point that the shape and location of the distribution is dependent – in addition to the biological factors – on the sampling rule which is applied when measurements are being made. In this case there is almost perfect separation of nuclei into normal, hyperplastic, and papilloma groups. In many other fields of histopathology morphometric data does not allow corresponding degree of separation. Because the volume of the nucleus would be even more efficient discriminator than the area, it is understandable that stereological methods may be tried to reveal the nuclear volume distribution. However, there is nothing wrong in using the area as a discriminator if it has been shown to be relevant in the diagnostic context.

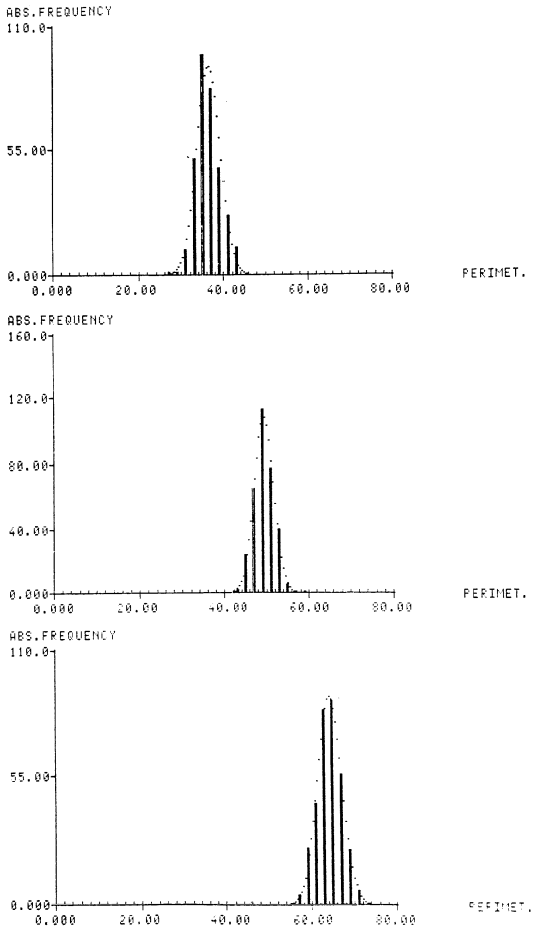

Figure 2. Distribution of perimeter lengths of the nuclei in normal mouse skin (above), in hyperplastic skin (middle), and in papilloma (below). What is said in the legend of Fig. 1 also applies to this figure. In addition one should pay attention to the fact that when there are differences in nuclear size, and nuclear shapes do not change, the area is more efficient discriminator than the perimeter.

	Area
	Mean ± SE
Basal cell carcinoma	57.3 ± 1.70 μm^2
Seborrhoeic keratosis	47.0 ± 1.51 μm^2

We can now estimate how accurate the measurements are. If we take several samples of 100 nuclei, in 95 cases of 100 the results will be within the limits

$$x \pm 1.96\ SE = 57.3 \pm 3.33 \quad (53.97 - 60.63\,\mu m^2)$$

and

$$x \pm 1.96\ SE = 47.0 \pm 2.96 \quad (44.04 - 49.96\,\mu m^2)$$

which shows that we can distinguish these two lesions with nuclear area measurements. This does not mean to say that this is possible in all cases of seborrhoeic keratosis or basal cell carcinoma – again research in morphometric pathology should find out how generally this rule or corresponding rules can be applied in practice.

Human tumors

Morphometric pathology is a starting discipline, but lots of data have been collected to aid in decisionmaking. It has turned out that the practical situation is not often simple enough to be described by one parameter only, or by applying the mean value of parameters. In many situations several parameters are combined, and certain combinations are more effective than others in predicting defined types of outcome (8). In addition to the mean values also the shape of the statistical distribution of measured values and the size of variation around the mean value are important. The latter points are easy to understand in the light of nuclear measurements we described earlier. DNA ploidy changes create peaks at polyploid and aneuploid regions and the shape of the ploidy distribution changes. Area distribution is associated with this change. The occurrence of peaks at certain sites may have diagnostic importance, and these changes can also be described by standard deviation of values. Usually pleomorphic nuclei (great variation in sizes, shapes and staining patterns) tend to increase variation of parameters (e.g. area, perimeter etc.).

For nuclear measurements the most often used parameters are area, diameter (largest, smallest, mean), perimeter, and various kinds of form factors (indicators of shape differences from a perfect circle). Nuclear features are not the only ones that may be of importance. Recently nucleolar measurements have been shown to be important (7, 30). After special stains the number and size of nucleoli can also be estimated automatically with image analysis. Also chromatin pattern can be analyzed (34) and possibly also used for diagnostic purposes. It has turned out that the mitotic index is an extremely important predictor of prognosis. The labelling index, which measured the proportion of cells in DNA-synthesis is also an efficient predictor of prognosis (57). The latter index can be defined by measuring thymidine labelled cells in an autoradiogram. Cellularity index, a measure of cell density, is also important from prognostic point of view in many tumors (10). One of the most effective predictors of prognosis in certain tumors is the volume fraction of the epithelium. This parameter simply tells the space occupied by epithelial tumor tissue in relation to the total volume of the tissue. Inflammatory cell reaction (lymphocytes, plasma cells) in and around the tumor is an important feature to be quantitated and shows a good correlation with prognosis (55).

Many parameters which seem to be associated with epithelial volume fraction, can be shown to be important in certain tumors. Among such parameters there are the amount of glandular surface (inner, outer) per volume of tissue (surface density of inner or outer surface of the glands) or the volume fraction of the glands in the tumor (5).

Morphometry in human tumors: examples

Uveal melanoma

Finally, we will describe three areas of tumor histopathology as good examples on how morphometric measurements can be applied to benefit the diagnostic situation. First of these is based on the study by Gamel *et al.* (30). Their study deals with a rare tumor, the malignant melanoma of choroid and

ciliary body. By applying the traditional histopathologic approach the authors had studied the prognostic factors involved (43). Their traditional study proved the value of Callender's classification in predicting prognosis (31), but they, too, were worried about the low reproducibility of subjective grading. So they decided to take the morphometric approach. They used a microscope equipped with a scanning stage and with a drawing tube facing a digitizer plate. The technician could do the measurements by outlining the nuclei with an illuminated stylus on the digitizer plate. The results were automatically fed into a computer which calculated 18 different parameters. These results were pooled and the mean values of the measurements were counted. In addition to the mean, also standard deviation, ratio standard deviation/mean, mean of the log (logarithmic) value of measurements, standard deviation of the log value, mean of ten largest values, standard deviation of 10 largest values, and the largest single value were calculated and recorded. In addition to the above morphometric parameters the authors used the diameter of the tumor in the evaluation of prognosis. All this may sound complicated but because the calculations were made by the computer, no extra brainteasing was necessary at the measurement session. In principle such results of measurements can be made available immediately after a nucleus has been outlined and again immediately after the next nucleus is outlined, and so on. The technician can concentrate on the measurement, and the results are available in real time, i.e. immediately.

Certain points are of special interest in this study. First of these deals with the sampling rule which was applied. Gamel *et al.* (30) measured 100 nuclei from a single histological slide. These nuclei were selected randomly. In fact they selected the nuclei in two phases; 50 nuclei were selected randomly from the center of the tumor, and 50 nuclei randomly from the periphery. They really wanted to be sure of the random selection of nuclei, and applied a scanning stage which was programmed to select each field randomly (an algorithm that generated normally distributed random numbers controlled the selection of fields). From each field (viewed at the objective magnification of 100×) the technician selected one nucleus, defined as the widest nucleus in the field, and traced the nucleus and the largest nucleolus of that nucleus, and counted the number of nucleoli within the nucleus.

The sampling rule applied by Gamel *et al.* (30) did not aim at a representative mean of all the nuclei in the sample. Instead it tried to find a representative mean value for the largest nuclei in the sample. Although the selection of fields was automatic and randomized, the selection of the nucleus that was measured subjective. In their discussion the authors wondered how much the results measured by different observers could differ. To make this approach generally applicable for diagnostic work in other laboratories one should have estimates on pure interobserver variation, and pure interlaboratory variation (23), which add to the variation sources involved in the study by Gamel *et al.* (30). One could imagine also other types of approaches to sampling. One is selective morphometry suggested by Baak and Oort (11). But there are many others. In fact many sampling rules can work in one diagnostic situation. As an example: for transitional tumors of the bladder four different types of sampling rules were recommended in one single pathology congress (33, 42, 48, 58). It is the task of medical research to find the sampling rules that are the most valuable in the diagnostic context (21). Best sampling rules for predicting different aspects of prognosis (response to drugs, metastasis within 2 years, metastasis after 2 years) may be different.

Another interesting point is how the results were used to benefit diagnostic decisionmaking. The follow-up of 50 patients disclosed that 14 patients died of tumor metastasis within 5 years after enucleation and that 36 patients survived more than 5 years after enucleation. These two groups of patients were analyzed in terms of the results of morphometry. The "dead" group and the "living" group were compared by means of a separate F-test for each variable. Also the influence of the number of nuclei measured on the F-value was investigated. In order to combine the variables for predicting outcome, stepwise linear discriminant analysis was performed (27). Also an F-value was calculated to measure the ability of the pathologists of the Armed Forces Institute of Pathology to distinguished patients in the "living" or "dead" groups by using Callender classification. The latter data was also combined with tumor size to predict outcome.

The results showed that six mean values and seven standard deviations of the variables correlated significantly with patient mortality following enucleation. The mean values that were found to be important were: Nucleolar width, nuclear area, nuclear circumference, nuclear length, nuclear width, and ratio nuclear circumference/nuclear area. The standard deviations that correlated significantly with prognosis were: nucleolar area, nucleolar circumference, nucleolar width, nuclear area, nuclear circumference, nuclear length, nuclear width. Standard deviations of statistically significant nuclear and nucleolar features demonstrated greater correlation with mortality than the means of these features. The latter point seemed to confirm the great value of nuclear pleomorphism for predicting the malignant potential of uveal melanoma. The best correlation with survival was shown by the standard deviation of nuclear circumference. Other significantly correlating standard deviations followed in the order: nuclear width, nuclear length, nuclear area, nucleolar width, nucleolar area, nucleolar circumference. When any of the significant variables were combined with the size of the tumor, the linear discrimination function could reliably predict the outcome in 88 percent of the cases. However, AFIP pathologists and Callender's classification were not much worse: they predicted the outcome correctly in 86 percent of cases. The number of measured nuclei was important when less than 50 nuclei were investigated, but did not influence the rate of successful classification above that value.

The title of this paper deals with borderline cancers. In the light of the above paper by Gamel *et al.* (30) we can see the word "borderline" in new light. The picture that emerges shows that within certain tumor types there are cases with different prognosis. Traditionally we have studied the prognosis as a group parameter. With morphometric investigations a situation approaches that allows one to estimate the prognosis for each individual patient separately. This is to say that uveal melanomas vary in prognosis and that the type of prognosis can be predicted from histopathology. Of course, it is not only histopathology that matters, clinical data gives also valuable information. In individual patients

all this data, of course, should be combined to benefit the patient. Even though histological classification theoretically can give as good prediction of prognosis as the morphometric classification, there is difference between these methods in favor of morphometric approach. The latter operates in continuous scale and can associate more individualistic features than traditional histopathology approach, which uses nominal or ratio scales.

Ovarian tumors

Ovarian tumors are traditionally classified into benign, borderline, and malignant types. Benign tumors are associated with good prognosis. The prognosis of malignant tumors is worse. Borderline tumors are not well defined in this respect. Many of them have good prognosis, but there are tumors which behave as malignant tumors. However, distinction between those two types cannot be made on histological grounds. Baak *et al.* (7, 9) wanted to shed some light into this problem by using the morphometric approach. They (9) studied 10 benign, 10 borderline and 22 malignant ovarian mucinous cystadenomas. They used HE-stained, 5 μm-thick sections. For each tumor 25 epithelial cell nuclei were selected randomly from the section, photographed at a magnification of 1000×, and measured on a digitizer plate by outlining the nuclei with a cursor. The digitized tracing of individual nuclei was fed into a computer. The computer was programmed to calculate and record a total of 32 quantitative features of each nucleus. Area, perimeter, shape factor, longest axis, shortest axis, and nuclear axes ratio were so calculated. Of each above parameter mean, median, standard deviation, minimum value, and maximum value were also determined. In addition, the mitotic activity index, and the volume percentage (i.e. area fraction in percent of total area = volume fraction in percent of total volume of the tumor) of the epithelium were determined. The mitotic activity index was measured in 25 fields at a magnification of 400×; diameter of each field being 450 μm. The index was the total number of mitotic figures in these 25 fields. Only fields with more than 50% epithelial tissue were analyzed for mitotic activity index. The volume percentage of epithelium was measured with a point grid placed on the projection screen of a projection microscope at a magnification of 200×. A minimum of 500 points was counted if enough tissue was available. The reader should pay attention to the fact that in this chapter the sampling rule has not been defined as well as in the report of Gamel *et al.* (30). In fact Baak *et al.* (7) have later explained the approach in more detail (7) and it appears that we are dealing with selective morphometry. Baak *et al.* (7) state: "The areas in which the measurements were performed were carefully selected on the basis of following criteria: (a) highest cellularity, (b) highest mitotic rate, (c) strongest atypicality, (d) avoidance of areas with inflammation, necrosis, or calcification (if present)". This approach selected for measurements the areas which were most suspicious for malignancy. The cells were selected from these areas. No specific rules about how the selection was done from the fields was not given, which may suggest that all nuclei in the fields were measured. As in the study by Gamel *et al.* ((30), see above) sampling may hide sources of variation which might endanger the application of the methodology for diagnostic purposes. Later studies (23) have shown that different observers do not measure the same nuclei from a microscope field. In the study of Baak *et al.* (7) the technicians were carefully instructed before the measurements were made. The authors also stated that the areas selected appeared reproducible between different technicians.

Baak *et al.* (9) used Wilcoxon's two sample test to assess significant differences between groups. When there was an overlap between groups on a single parameter, multivariate analysis techniques were used (26). Stepwise regression analysis was used to select the most promising set of features. The approach, when applied in practice, used two-parameter plots which are very illustrative and helpful in decisionmaking (7).

The results disclosed the following features as useful in the quantitative classification: the mean nuclear area, the mean nuclear perimeter, the mean of the short axis of the nucleus, the volume percentage of the epithelium, and the mitotic activity. The latter two showed the clearest difference between borderline and malignant tumors. The computer was also allowed to estimate the probabilities for each tumor to fall into benign, borderline or malignant groups. Such list of probabilities could be very helpful: e.g. in one case the probability was 0.22, for the tumor to be benign 0.77 borderline, and 0.01 malignant. Quite obviously such a tumor was classified as being borderline in morphometrical classification. Of course one should aim at a model which could make a correct decision about the tumor type. This again would need an extensive study that could correlate the prognosis and morphometrical features, if possible, in a large group of patients.

Baak *et al.* (7) tested the above classification by applying the results on samples sent to the morphometric laboratory from another laboratory. Mitotic activity index above 30, and volume percent of epithelium above 70 were considered ominous features. Further classification help was available through other parameters. There were 20 samples in all. All patients whose tumors were morphometrically graded as having "good" prognosis were alive and tumor-free at intervals ranging from 4 to 14 years. Morphometry identified correctly an adenocarcinoma, which had proved fatal, and two tumors of borderline malignancy that had led to the patient's death, as having "poor" prognosis. One tumor which was thought to have "poor" prognosis was associated with long survival. However, the patient had received adjuvant chemotherapy, the effect of which is not exactly known. The study suggested, by a "mass effect" that the tumor belonged to the group of "poor" prognosis and that chemotherapy had been effective. All in all the study demonstrated that the predictive prognostic power of morphometry was greater than the predictive power of unaided microscopy.

Aalto *et al.* (2) and Aalto and Collan (1) quantitated the PAS staining and immunohistochemical staining for carcinoembryonic antigen in ovarian tumors. The quantitation was done by applying a point grid in the eye piece of the microscope. When a point fell on positive staining the staining reaction was graded. Indexes for staining intensities were calculated from the primary data. By this approach it could be shown that high CEA scores were present only in malignant tumours. The results also suggested a longer survival of patients with abundant PAS positive mucin. These results are most interesting because they show the potential value of

quantitative histochemistry. In fact it was obvious after these studies that good histochemical approach should apply quantitative methods. Through such approach the potentially powerful predictors of histochemistry can be revealed.

Breast cancer

Also breast cancer offers a good example on how morphometric methods, and statistical and follow-up studies associated with them can help us to get rid of the holy dichotomy: tumors are either benign or malignant. In fact, by applying the term borderline lesions the importance of similar conceptual change has been stressed. But there are not three types of lesions, there are probably ten, possibly hundred types of lesions. The prognosis of the lesions seems to be intimately correlated with certain cytological and histological features. Some show very strong correlation with prognosis and can be used to predict the outcome reliably. Best of all, morphometric parameters can be associated with a probability for a certain type of outcome, and such approach will be very helpful in decisionmaking.

Baak *et al.* (10) studied the prognostic morphometric indicators of breast cancer. They were interested in indicators which were most appropriate for determining prognosis in terms of survival. There are also other types of prognosis – prognosis in terms of metastasis, of recurrence, or of type of response to therapy. The authors studied 78 tumors of patients of whom 42 had died from metastasis within 6.5 years (non-survivors) and 36 from patients who had lived longer than 6.5 years (survivors). The results of measurements of tumor diameter, assessment of mitotic and cellular indices, quantitative microscopy of nuclear features, and histological tumor grades were compared between these two groups.

Nuclear and histological grading was done by one observer – to avoid interobserver variation. This was done according to WHO recommendations (52). Cellularity index was measured on a projection microscope at 1000× magnification. Five areas were counted using a square of 10 × 10 cm superimposed on the screen. Only nuclei not in contact with any of the margins, and a mean diameter larger than 4 μm were counted. The latter criterion was used because it was not easy to distinguish between benign cells (such as endothelial cells) and cancer cells.

Mitotic activity index was counted in 10 random fields at 400× magnification. The diameter of the microscopic field was 450 μm. The authors made the measurement twice in each sample. They focused the microscope only once during the measurement of each field. If there was a difference greater than 2 (less than 20 mitoses), 3 (less than 30 mitoses), or 5 (more than 30 mitoses), a third measurement was performed and then the first of the two nearest results adopted.

Nuclear measurements were made from photographs taken at 1000× magnification, a graphic table (digitizer plate) was used for these measurements. 25 nuclei were randomly photographed in the subjectively most cellular areas of the tumor. Nuclear area, perimeter, longest axis, and shortest axis were measured. Nuclear size ($2\sqrt{\text{area}/\pi}$) and nuclear shape factor (4π area/perimeter^2) were then calculated resulting in a total of seven features per nucleus.

Statistical analysis (Wilcoxon's test) compared results between patients with negative and positive lymph nodes, and between survivors and non-survivors. Discrimination analysis after Cooley and Lohnes (26) was carried out to distinguish the survivors from non-survivors, after selection of the most optimal discriminating set with stepwise regression analysis. The results showed that statistically significant difference between axillary lymph node negative and positive patients was found in mitotic activity index and mean nuclear shape factor. Significant differences between tumors of survivors and non-survivors were found in parameters (in the order of significance): mitotic activity index, mean nuclear smallest axis, mean nuclear area, mean nuclear size, cellularity index, mean nuclear longest axis, mean nuclear perimeter, standard deviation of nuclear area, standard deviation of nuclear smallest axis, tumor diameter in centimeters, standard deviation of nuclear diameter. Of the subjective parameters the nuclear grade and the histological grade were significantly different in survivors and non-survivors.

The most relevant data from the clinical point of view, however, was achieved through multivariate analysis. In discriminant analysis six quantitative features (mitotic index, cellularity index, tumor diameter, the mean of the shortest axis, the standard deviation of the longest nuclear axis, and nuclear axes ratio) correctly predicted the outcome of 78% of patients. The corresponding percentages for TNM-classification alone, and for TNM-classification and nuclear and histological grades were 58% and 64%, respectively. The study showed clearly that one can predict the outcome better by using morphometric parameters than by using the traditional grading and staging methods.

Also other researchers have found morphometric results important. Maehle *et al.* (40) showed that nuclear area in metastasis is related to prognosis, and suggested that histological grade and nuclear area measurements should be combined in estimating prognosis. This obviously has been tried by Baak *et al.* (8), but as they also found parameters with even better correlation with prognosis the latter combination was not mentioned in their results.

Stenkvist *et al.* (55) have made an extensive study on predicting breast cancer recurrence. Their study covered 435 cytochemical, cytometrical, morphological, epidemiological, and clinical variables. The 20 variables most strongly correlated with recurrence were analyzed by logistic stepwise regression analysis. It was found that axillary metastasis was correlated with a combination of variables such as mitotic frequency, size of primary tumor, differentiation in primary tumor. There was a strong time dependency in the predictive power of the variables: recurrence during the first 2.5 year period was best predicted by the following combination of parameters: size of axillary metastases and primary tumor, number of lymphocytes around the tumor, mitotic frequency, degree of differentiation. Recurrence between 2.5 and 5 years was best predicted by the following complex of parameters: variance of DNA content among tumor cell nuclei, number of lymphocytes around the tumor, occurrence of multiple tumors in the operated breast, and the occurrence of breast cancer among relatives.

The paper by Stenkvist *et al.* (55) did not include many morphometric parameters from sections. However, in one respect the results parallel the results of Baak *et al.* (10): mitotic index has an important position. Labelling index

(LI) has also been shown to be very important as a prognostic parameter (LI gives the proportion of cells in DNA synthesis phase (S phase) of cell cycle). All this data seems to stress the importance of measuring the number of mitotic figures in prognostic studies.

Later Baak *et al.* (4) studied the prognostic significance of nucleolar morphometry. It turned out that standard deviation of nucleolar area above 2.49 μm was almost exclusively found in non-survivors. This parameter seemed to be independent of mitotic activity index and tumor size. It appeared that the sensitivity of nucleolar morphometry was low among unselected group of breast cancers, but that the method could be used as an additional prognostic indicator. In fact there might be other prognostic indicators of similar nature, indicators which might be able to help to predict correctly the prognosis of those 20% of cases, which were not correctly predicted by the major morphometrical prognosticators.

Baak *et al.* (6) also tested the value of morphometric parameters in 271 breast cancer patients (6). They wanted to test the additional significance of morphometry to the classical prognostic parameters, tumor size and axillary lymph node status. It was shown that factors such as lymph node status, tumor size, nuclear and histologic grade, and morphologic variables: mitotic activity, mean and standard deviation of nuclear area, and cellularity index were significant predictors of prognosis. A combination of three features gave the best prediction: mitotic activity index, tumor size, and lymph node status. Mitotic activity was the most important prognostic feature. The study showed that morphometry added significantly to the prognosis prediction of lymph node status and tumor size.

The above mentioned studies have dealt with prediction of prognosis in carcinoma of the breast. They clearly show that carcinomas are not equal in respect to prognosis, and that the type of lesion that the patient has can be reliably estimated with clinical and morphometric data. Other researchers have been interested in other aspects of breast lesions. One such aspect is distinction between intraductal hyperplasia and carcinoma. Bhattacharjee *et al.* (14) estimated the value of nuclear morphometry in distinction of epitheliosis (intraductal hyperplasia) and intraductal carcinoma. The area of nuclei on the section was the most suitable parameter to measure. The best discrimination was obtained by using the differences between the mean estimated nuclear area of the diseased duct and a normal duct on the same section. Using a difference of 20 μm^2 as a threshold, cases of epitheliosis and intraductal carcinoma could be correctly identified by this criterion alone in 86% of lesions studied. The results of Bhattacharjee *et al.* (14) are very much in line with the results published by Schöndorf and Naujoks (53). These authors used thin needle biopsies (cytological samples) of breast lesions and tissue. Their results were dramatic. The range of nuclear sizes and the samples were the following:

Postmenopausal women 42–58 μm^2
Sexually mature women 54–89 μm^2
Pregnant women 82–135 μm^2
Sexually mature women using hormonal contraception 60–89 μm^2
Sexually mature women with mammary carcinoma 119–280 μm^2
Postmenopausal women with mammary carcinoma 100–260 μm^2

The study shows that under physiological conditions only nuclei of pregnant women can cause distinction problems from carcinoma. However, the mean nuclear area in pregnant women was 118 μm^2, in sexually mature women with carcinoma 185 μm^2, and postmenopausal women with mammary carcinoma 170 μm^2. The study was based on 20 patients in each group. The results seem to show that carcinoma can be distinguished from physiological changes by using area measurement of nuclei in cytological sample.

It is most interesting to compare the results of estrogen receptor analysis and the morphometric results, because estrogen receptors have a place in the selection of treatment. In the study by Mossler *et al.* (47) cells with nuclear area less than 60 μm^2 were associated with significant levels of ER (> 10 fmol/mg protein). Of cells with an area 60–90 μm^2 6/11 showed ER positivity. 5/15 cases with nuclei larger than 90 μm^2 contained significant ER levels. Nuclear area correlated better with ER levels than did the histological grading. Baak and Persijn (12) also tried to find correlation between morphometric results and ER status. Mean nuclear area was the best predictor in patients under 50 years, and elastosis grade in patients above 50 years. Combination of the mean and the standard deviation of nuclear area resulted in 83.3% correct identification of ER-positive samples in patients below 50 years of age. In the older age group a decision process consisting of elastosis grade and mean nuclear area gave the best result. The authors stated that selective morphometry gave considerable enhancement in predicting estrogen receptor in breast cancer by histological means.

Future

The data presented in the latter part of this paper have no doubt been able to show the great clinical importance of morphometric measurements. The most optimal combination of measurements differs in different types of tumors. Because morphometric measurements can be combined with the traditional histopathology routine morphometry seems to be a most promising development in prognostic prediction. Why is it not used more widely? The main obstacle seems to be educational. Physicians, pathologists and clinicians alike do not get good enough working background in statistics and techniques of measurement and prediction. The only way to develop the field is through education, which should be given to undergraduates and postgraduates alike. Steps have already been taken to this direction (23, 25). In addition to the principles and practice of morphometry, and to the results so far available, an important part of education should be on clinical decisionmaking, and how prognostically important parameters should be evaluated in that connection.

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POSTSCRIPT

When this chapter was written it already started to be clear that histopathology is entering a new era. Any measurements applied in histopathology can now be gathered under the umbrella of Quantitative Pathology, which covers e.g., morphometry and stereology, quantitative histochemistry, static or flow cytometry, and image analysis in histopathology. Quantitative pathology allows for an efficient use of gathered information as was shown e.g. in the paper of Baak *et al.* (5, referred to above) on breast carcinoma. Our group has now tested their approach (3), and we could show that the prognostic index based on mitotic count, tumor size, and axillary lymph node status correctly predicted the outcome of infiltrative ductal breast carcinoma in 80% of cases after the intermediate 40% of cases were excluded. Corresponding power of prediction has not been possible by any other approach.

But there are also other benefits from quantitative pathology. Traditional histopathology was theoretically relevant in its classification of disease. The decisions made at the diagnostic situation, however, were left to the intuition of the pathologist. Here the approach of quantitative pathology is helpful, because it will give the pathologist a knowledge of the prognostic probability from which he can make the correct diagnostic decision. This means that the pathologist at the time of decision knows when his prediction will be correct, when there is uncertainty, and to what degree there is uncertainty (1, 2).

Quantitative pathology as a field of pathology has also influenced scientific organizations. The Committee for Diagnostic Morphometry within the European Society of Pathology has now changed its name to Committee for Diagnostic Quantitative Pathology. Academic recognition is also at hand: Jan P.A. Baak has now served several years as Professor of Quantitative Pathology at the Free University of Amsterdam.

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11

PRINCIPLES AND PRACTICE OF SURGICAL ONCOLOGY: THE INFLUENCE OF PRESENT DAY CONCEPTS OF TUMOR BIOLOGY

JOEL LUNDY

INTRODUCTION

Advances in surgical techniques, more sophicticated approaches to anesthesia, and highly skilled intensive care have permitted surgery to develop into the central modality of cancer therapy. In the 1890's, Halsted elucidated the principles of en bloc resection for cancer, the classic example being the radical mastectomy for the Stage III breast cancers that were common presentations for the disease in the early 1900's.

Halstedian principles have been challenged over the last twenty years by present concepts of tumor biology and earlier diagnosis in diseases such as breast cancer and melanoma, which seriously question extended operations for all solid tumors at all stages of diagnosis.

This chapter will look at two specific areas, (1) primary operable cancer and (2) cancer metastatic to the liver or lung. The role for surgery in these settings will be related to our present understanding of tumor biology. In addition, we will constantly refer to multimodal approaches to cancer. Surgical approaches to cancer are modified in certain circumstances because of this ability to balance the weaknesses of surgery with better locoregional control measures, such as radiation therapy, as well as recognizing the fact that many solid tumors require the addition of systemic chemotherapy to eradicate distant micrometastatic disease. Finally, the various indications for surgical intervention in the prevention, diagnosis and palliative treatment of cancer will be briefly discussed.

TUMOR BIOLOGY, SURGERY AND LOCOREGIONAL DISEASE

Appropriate treatment of any disease should be based upon an understanding of the natural history of that illness. Animal laboratory work and clinical trials have served the valuable purpose of giving insight into the mechanisms of spread of malignant disease. These studies have challenged Halstedian concepts and required surgical oncologists to reevaluate approaches to primary operable cancer.

For several decades the following hypothesis formed the basis for cancer surgery: Tumor spread was orderly and mechanical. The primary tumor spread first to lymph nodes. These regional nodes represented a barrier to the passage of tumor cells. The bloodstream was involved as source of tumor dissemination only late in the stage of disease, and the involved lymph node(s) was (were) considered as an "Instigator" of this late spread. Bernard Fisher conducted some laboratory experiments to refute this hypothesis, starting in the late 1950's. He, as well as others, showed that the lymphatic and hematogenous systems are interrelated and not independent routes of tumor cell dissemination (8, 6, 35). Tumor cells can traverse lymph nodes and via lymphaticovenous communications gain access to the vascular system. Indeed, when tumors are slightly larger than 1 mm in size and have developed neovascularity, they have the capacity to shed tumor cells into the bloodstream (20).

Fisher has championed the role of lymph nodes as "indicators", not "instigators", of systemic disease. This modern concept gives the regional nodes importance biologically as indicators of the host-tumor relationship. Early National Surgical Adjuvant Breast Project clinical trials indicated that prognosis was most closely correlated with extent of axillary node involvement, patients with four or more nodes positive having a 90 percent chance of treatment failure at ten years. However, the meaning of positive nodes was better understood by some of the following data. In the NSABP B-04 protocol, patients with clinically negative regional nodes were placed into one of three treatment groups – radical mastectomy, simple mastectomy plus radiation to the axilla, or simple mastectomy and no treatment to the axilla until lymph nodes because clinically involved. It is known that the false negative rate for clinical assessment of axillary involvement is 30–40 %. When followed out to seven years, there was no difference in distant treatment failure or survival among any of the three groups. This protocol has shown that untreated regional nodes did not influence distant treatment failure or survival in patients with positive or negative nodes (9, 11).

This data permits us to support the hypothesis of the biologic importance of lymph nodes as indicators of systemic disease. Treatment failure in many cases is the result of many solid tumors being systemic disease from the time of clinical diagnosis. The patients with positive nodes (tumor overwhelming host defenses) are the ones at highest risk of treatment failure from systemic micrometastatic spread already present at the time of initial operation.

Animal studies suggest that the presence of the primary tumor may slow the growth of metastases. The interesting data of Simpsen-Herren and Skipper indicate that after surgical removal of the primary tumor, micrometastases begin to grow with cells rapidly dividing (28, 29). The above is part of the rationale for using adjuvant chemotherapy, which should be most effective against these actively dividing cells.

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We could summarize the role of the surgery in many primary operable solid tumors as:

(1) Achieving locoregional disease control;
(2) Giving prognostic information as to which patients are at high risk for recurrence (nodal status most important);
(3) By reducing tumor burden, providing the potential for more effective systemic adjuvant chemotherapy.

A better understanding of important prognostic parameters has influenced surgical asppproaches to certain cancers. The MGH-NYU cooperative melanoma group and Breslow have added much to our understanding of the biology of melanoma, and this has resulted in a change in treatment approach (3, 4). Several years ago, a patient with a melanoma of 0.75 mm depth of invasion might have been treated by a wide local excision (5 cm margins), skin graft, and a prophylactic regional node dissection. We now know that the biology of such "thin" melanomas (as determined by the measured depth of invasion of the primary) reflects their less aggressive behavior. We treat these lesions with a more limited local excision, and there is no need for skin grafting. In addition, there is no indication for a prophylactic node dissection because of the extremely low incidence of microscopic nodal disease.

Although one should not compromise survival by inadequate surgery in the cancer patient, we are learning that combining surgery with other treatment modalities may ultimately permit organ or limb preservation. Childhood rhabdomyosarcoma is a classic example of the above. Combining surgery, radiation therapy and chemotherapy has certainly decreased the need for pelvic exenteration in these tumors (19). Adult soft-tissue sarcomas are similar examples of multimodal approaches frequently permitting limb preservation without compromising cure rates (32). Recent NSABP controlled data (mean follow-up 39 months) from protocol B-06 demonstrates that with breast cancers less than 4 cm in size, segmental resection of the tumor and radiation therapy to the residual breast is as effective as total mastectomy, both in terms of local control as well as survival (10). Some uncontrolled studies as well as Veronesi's controlled protocol, which included only T1 tumors, give support to breast conservation for selected patients (5, 34).

The multiple roles for surgical oncology have been expanded, based upon a better understanding of tumor biology, the development of cancer genetics and the availability of serologic markers to indicate tumor recurrence.

Prevention of cancer

A better understanding of the biology of premalignant conditions has resulted in surgery being a primary intervention in several disease states. Perhaps the best known is familial polypsis coli. Fifty percent of patients with this disease will develop colon cancer by age 40 (25). Prophylactic colectomy can alter the natural history of this disease. Multiple endocrine neoplasia, Types II and III, is fortunately associated with a serologic marker, calcitonin. Elevated calcitonins in family members may reflect C-cell hyperplasia (33). This is the precursor lesion of medullary carcinoma of the thyroid. Prophylactic thyroidectomy, before a clinical medullary cancer is detected, is the treatment of choice in these situations.

Surgical diagnosis and staging

There are several methods of establishing a diagnosis of malignancy. Fine needle aspiration (cytologic diagnosis) is valuable only when positive (16). False negatives in this methodology hinder its efficacy. With a competent cytopathologist there should be no false positives. Incisional biopsy should be used only in larger lesions. Excisional biopsy is the preferable approach. It is important in planning biopsy incisions to place them in such a fashion that they do not interfere with planned definitive surgery, such as soft part resection for a sarcoma or a radical neck dissection. Hemostasis should be perfect at a biopsy site to avoid an expanding hematoma with contamination of new tissue planes. In addition, an infected hematoma can delay definitive surgery. There is no experimental or clinical evidence that a reasonable delay between biopsy (excisional or incisional) and definite surgery adversely affects prognosis.

Tumor markers, such as CEA, are used to follow patients for recurrence. In colon cancer, an asymptomatic patient with serial elevated CEA's, even though asymptomatic, may require surgical exploration to confirm the presence of recurrence and an attempt can be made at resection of the recurrence with the possibility of cure. Present data would suggest that at least half of the patients explored can be rendered disease-free and about 30% can be expected to survive 5 years (2, 30).

We talked about staging when we discussed regional lymph node dissections. Another example is the exploratory laparotomy in Hodgkin's disease. This approach was originally used as a research tool to better understand the patterns of spread of Hodgkin's disease (14). At present, it should only be employed if the results will influence the therapeutic approach to the patient (21).

Palliative surgery

This surgical intervention is not intended for cure. The primary aim is to improve quality of life, i.e., relief of pain, restore the ability to swallow or relieve a bowel obstruction. Long-term hospitalization does not improve quality of life. Therefore, approaches must be taken which can keep morbidity as low as possible and permit return as soon as feasible to an outpatient functional status.

Surgery for distant metastases

We will deal here with the two distant sites where surgical resection is most frequently performed with an intent for cure, liver and lung.

Is there any biologic basis for why surgery might succeed where chemotherapy has not proven successful? I believe the work of Fidler and others on the genetic instability of metastatic disease might give some insight into this question (6, 21). The greater a cell's metastatic potential, the less stable its phenotype. Solid tumor metastases reach a state of "phenotypic equilibrium." If cellular subpopulations are lost, such as with less than total cell kill by chemotherapy, a new generation of tumor cell variants arises. This certainly would explain long-term chemotherapy resistance. Any treatment that leaves a surviving subpopulation may trigger

the generation of tumor cell variants maximally refractory to most forms of therapy.

The classic example of the unique role of surgery in the above type situation is the patient with colorectal cancer whose primary tumor has been controlled and now has only a solitary liver metastasis as evidence of distant disease. The advantage of surgery in this setting is that it is the only form of therapy that can achieve total cell kill in some patients. It does this in thirty percent (30%) of patients with solitary liver metastases (11). This must be biologically the most favorable group. In patients in which it fails, it is likely that there is either undetected micrometastatic disease in the liver or other sites, and that this tumor burden is too much for host defense mechanisms to handle and phenotypic characteristics of new variants permit escape from host defense mechanisms and/or other forms of therapy.

Foster has collected a nationwide review, and the role of surgical intervention would seem also likely to benefit patients with slow-growing endocrine tumors (here it frequently gives symptomatic relief, if not cure), and in certain childhood tumors, in particular, Wilms' tumors (1, 11, 22).

Almost twenty percent (20%) of patients dying with pulmonary metastases have no other evidence of locally recurrent or metastatic disease foci at autopsy (19). Although chemotherapy and radiotherapy play a significant role in the treatment of pulmonary metastases, we will attempt to define those situations in which surgical intervention may be indicated.

There are many criteria for selection of patients, some reflecting good common sense and others based upon observed tumor biology. As with resection of liver metastases, the primary tumor should be completely controlled. There should be no other evidence of distant metastases. Potential should exist for complete resection of disease. If the disease is potentially controllable by chemotherapy, many would advocate that this approach be tried with multiple metastases, prior to thoracotomy. Most studies have indicated that the longer the disease-free interval from treatment of primary to presence of metastases, the more favorable the results of surgical excision (22, 25).

Joseph *et al*, using tumor-doubling times, found a good correlation between growth rates and prognosis (18). Sixty-three percent (63%) of patients with TDT of greater than forty days survived five years after pulmonary resection. Obviously, one must take into account the histology of the tumor as well as its biology. A carcinoma of the esophagus or melanoma patient would almost never be a candidate for this type of approach. On the other hand, patients with osteogenic sarcoma, testicular cancers, soft tissue sarcomas, and perhaps certain head and neck cancers may be candidates for pulmonary resection (14, 20).

In summary, select patients with isolated metastatic disease to the liver or lungs may be candidates for a curative attempt at surgical resection. Rigid criteria should be set up to define the above patient populations, and other non-operative modalities are a first choice for the majority of patients with distant metastatic disease.

This chapter makes no attempt to cover the entire spectrum of surgical interventions for malignant disease. The major purpose of this chapter is to attempt to integrate known tumor biology with some of the surgical approaches used in the management of cancer. In 1985, it is exceedingly difficult to look at surgery in an "isolated" fashion when attacking a cancer. Better understanding of host-tumor interactions and a more thorough knowledge of how the above can be favorably manipulated by integrating multimodal treatment approaches will lead to more effective therapy.

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12

RADIATION ONCOLOGY

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INTRODUCTION

Radiation Oncology is a clinical medical discipline in which patients with cancer are treated with ionizing radiation. It has evolved over the past decades as a distinct specialty from both surgery in whose numbers were the early practitioners and from diagnostic radiology. As emphasized by Kaplan (84) the practitioner is concerned first with oncology and only secondarily with the technical aspects of treatment. The implication is that the radiation oncologist is available for consultation to the patient with cancer and participates in his or her management without an implicit or explicit commitment to radiation therapy in the individual case.

With surgery and chemotherapy, radiation is one of the primary modalities for cancer treatment. It is estimated that one-half of all cancer patients will receive radiotherapy sometime during the course of their illness (23, 93). Clinically, radiation is utilized for localized disease either alone or in combination with surgery and/or chemotherapy with curative intent. For disseminated disease it is used for the palliation of symptoms associated with advanced disease and more recently as an adjunct to chemotherapy at sites of bulky tumor.

While many radiotherapeutic techniques have evolved empirically, to fully understand the current practice requires a familiarity with radiation physics and biology as well as past clinical experience. As a prelude to these, some brief historical introduction is required.

HISTORY

The beginnings of radiation therapy have their origin in the 1895 discovery of x-rays by Wilhelm Conrad Roentgen at the University of Würzburg. While a more complete review of the history of radiotherapy is available elsewhere (22, 27, 96, 125, 145) a few salient points bear repeating.

In short order following the discovery of x-rays, Becquerel described radioactivity (1895) and the Curies isolated radium (1898). However, following some initial enthusiasm which saw the treatment of patients within one year of the discovery of x-rays, disappointment began to prevail as it was appreciated that this new modality was difficult to work with. It was with refinements in radiation dosimetry and the development of improved equipment that the early progress was made.

Sources for therapeutic radiation gradually improved from the invention of the hot-filament Coolidge tube in 1913 and the 200 kvp orthovoltage machines of the 1920's. Over the ensuing decades the limitation of low output and poor beam penetration were overcome with new technologies. In the post World War II era, the availability of intense neutron fluxes from atomic piles generated the production of Co^{60} and Cs^{137} as a substitute for "radium packs". The early low megavoltage Van de Graff generators, developed in the late 1930's, were supplanted first by betatrons and later by the linear accelerators used today.

Concomitantly, the development of radiation dosimetry allowed for the accurate measurement of treatments. From the crude measurement of "skin erythema dose" or the use of Sabouraud-Noire pastille, refinements were made with the adoption of the roentgen (R) in 1928 as a measure of exposure and the rad in 1954 as the unit of absorbed dose. In keeping with the international system, the gray (Gy) was introduced in 1976 (100 rad = 1 Gy).

As practiced early in the century, the effect of radiation depended on the delivery of one or a few large doses to eradicate tumors. As Buschke notes (22) radiotherapy was effective "through its caustic effects – a slough in lieu of excision."

It was with the contributions of Claude Regaud that biologic studies began to have an impact on the practice of radiotherapy. With his colleagues at Foundation Curie in Paris in 1919, he demonstrated that spermatogenesis in the testis could be eliminated by the administration of fractionated doses of radiation. By contrast no single dose of radiation could achieve the same effect without damaging the overlying skin (121). In 1922, Regaud, Coutard and Hautant presented on the application of this technique of fractionated radiation in laryngeal cancer (122).

The development of atomic energy gave new impetus to the study of radiation biology and with it further understanding of clinical radiation therapy. Puck and Marcus (116) exploited *in vitro* culture of mammalian cells and developed a clonogenic assay to quantitate radiation effect. The survival of cells following irradiation was determined to follow an exponential function with an initial shoulder. Use of their technique led to the understanding of physical and biologic factors that modify radiation response following single and multiple exposures.

In current practice the advances in physics allow us accurately to deliver cancericidal doses to tumors where earlier workers were limited by skin tolerance. Linear accelerators and Co^{60}, now standard equipment in radiotherapy departments, provide high energy radiation from x-rays and electrons or gamma rays at high dose rates. As will be discussed,

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the maximum dose of radiation with such megavoltage equipment is deposited beneath the skin surface. Thus, the physical constraint of skin tolerance is circumvented. In addition, penumbra of lateral scatter of such equipment is minimized, allowing for a sharp beam edge capable of treating both small lesions and those in proximity to vital organs. Furthermore, the high output of such units, in contrast to orthovoltage equipment, allows for treatment to be given at an increased distance. At distances typically of 80 cm to 120 cm, larger fields can be employed to encompass multiple sites of tumor involvement in contiguity.

Similarly, the advances in radiation biology have provided a rationale for empirically derived practices and a basis for subsequent modification. As the factors that modify radiation response have been elucidated, new approaches have been suggested. Included among those under study are the use of radiation sensitizers and protectives; hyperthermia and particulate radiation. The use of centigray (cGy) has replaced rad in most recent publications (1 cGy = 1 rad).

RADIATION BIOLOGY

Cellular effects

Following the deposition of energy from the incident beam of ionizing radiation, a sequence of events is touched off which culminates in a biologic effect at the cellular level. The manifestations of this initial damage may take from seconds to decades to be manifest within the organism. For a full explanation of these events the reader is referred to one of the standard texts (7, 9, 68). However, to simplify this complex question, there are three principal kinds of cellular damage: Mitotic Inhibition, Mutagenesis and Cell Death.

Mitotic delay

Mitosis, studied through the light microscope, for many years was the only observable portion of the cell cycle. Notwithstanding, Strangeways and Oakley (135) were able to observe that ionizing radiation delayed cell division.

With the development of tritium-labeled thymidine and autoradiography, Howard and Pelc (76) were able to observe the cell cycle more closely. During S phase DNA was synthesized. Following mitosis and synthesis and again between synthesis and the subsequent mitosis were time gaps referred to as G_1 and G_2 respectively. Doida and Okada (40) determined that the mitotic delay is caused by a transient interruption about $\frac{1}{2}$ to $\frac{2}{3}$ of the way through G_2.

The duration of the delay depends on the dose received and upon the cell cycle position when irradiated (105, 138). In asynchronous cultures 100 rad induces a delay of about 60 minutes. Cells irradiated in G_1 are ultimately delayed in G_2 for a more brief period than S phase or early G_2 cells.

Partial cell synchrony is induced by this period of mitotic delay both because of the G_2 block with the accumulation of cells and because of the differential effect secondary to cell cycle position. This partial cell synchrony can influence radiation sensitivity to subsequent exposures; however, this feature has not been exploited in clinical radiotherapy.

Mutagenesis and chromosome changes

Following exposure to radiation, both chromosome and chromatid abnormalities are observed. In cells irradiated prior to S phase, damage is reflected in chromosome abnormalities; following DNA replication, in the chromatid. Among the alterations noted are included acentric fragments and dicentric chromosomes, translocations, deletions, inversions, and ring forms. However, as is often the case with a break on one arm of a chromosome, the damage may be repaired and the chromosome restored to its pre-irradiated status. At mitosis chromosomes may also clump so that they do not separate properly to daughter cells. All these aberrations carry the risk of defective genetic transfer to the progeny of the irradiated cell.

At a finer level, specific mutations have been studied extensively, both in *Drosophila* and mice. The available human data to predict risk of mutation have been presented by the BEIR Report (13) and UNSCEAR Report (147) and summarized by Brill and his colleagues (18). While radiation is unquestionably carcinogenic and mutagenic, at low levels and low dose rates it is difficult if not impossible to establish reliable risk estimates (165).

Cell death

Within the context of therapeutic radiology, the most significant radiation effect is cell death. The lysis of a cell prior to mitosis, interphase death, is a relatively uncommon occurrence but may be seen in all cell types and is the predominant mechanism for death in lymphocytes. The second and major form of cell death is mitotic or reproductive death. Following one or more cell divisions, the clonogenicity is lost; such a loss of reproductive capacity is of obvious significance to clinical radiotherapy and is used synonymously with cell death. With mitotic death it should be remembered that the irradiated cell may remain metabolically active and morphologically normal until division is attempted.

With the development of *in vitro* cloning techniques (116), our understanding of mitotic death has advanced. By examining the survival of single cells by colony formation, it is possible to generate plots of survival fraction *vs.* dose and to study biologic and physical influences that modify the radiation response. *In vivo*, similar clonal assays have been established for bone marrow stem cells (142), intestinal crypt cells (159) and epidermoid cells (156).

On a linear plot of survival *vs.* dose, the form of the lethality response is sigmoidal. In the more commonly used and more useful form, where fractional survival is plotted on the log scale and dose on a linear scale, an initial shoulder is followed by a straight line (Figure 1).

Having described the relationship between dose and survival graphically, a number of equations and theories have been advanced to relate the two.

While considerable evidence exists that nuclear DNA is the target for cell lethality, it has not been conclusively proven. For the sake of clarification, it is usual to assume that DNA is the target and proceed from that point.

In the simplest case, for example bacteria, the survival curve is exponential and one would predict a single critical target per cell. The implication of this model is that for a

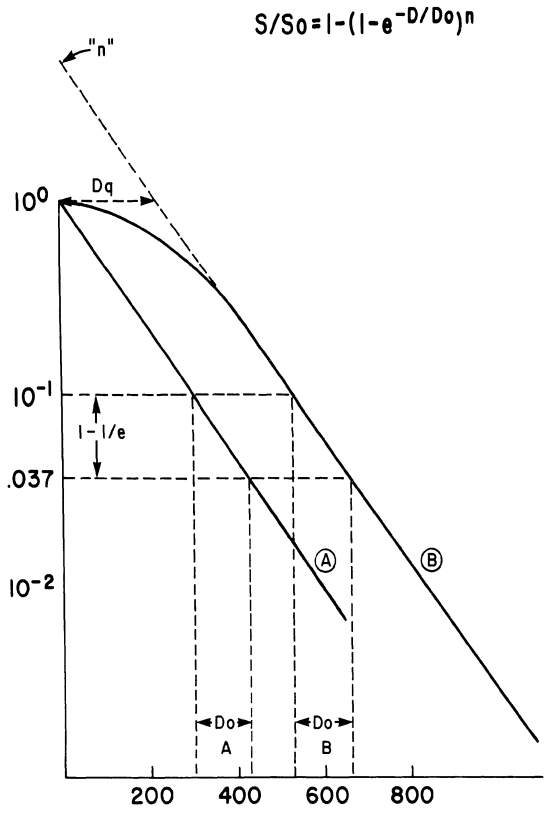

Figure 1. Survival curve for mammalian cells exposed to A. high LET radiation B. low LET radiation $S/So = 1-(1 - e^{-D/Do})^n$
For curve A: Do = 130 rad; n = 1
B: Do = 130 rad; n = 5; Dq = 220 rad.

given increment in dose a constant fraction of cells are killed. Since the lethal radiation event is randomly distributed in the cell population, if there is an average 1 lethal event per cell, then some cells will have no lethal events and some more than 1. The proportion of cells surviving under these circumstances, *i.e.*, those with no lethal events, is 1/e or 37% as predicted from a Poisson distribution. The dose required to reduce the surviving population to 37% is referred to as D_0 or 1/slope.

In the more complex situation posed by mammalian cells, in the low dose region there is a shoulder to the survival curve, reflecting a reduced efficiency to cell kill. Under these circumstances the terminal portion of the curve is described by D_0 and the initial portion by D_q or quasithreshold dose and "n" the extrapolation number (Figure 10).

Utilizing models to describe this situation, it is postulated that there are a finite number of targets, "n", all of which require a single hit for inactivation and all of which must be inactivated for cell lethality. Given such a situation it is postulated that low doses will be less lethal because of the low probability of hitting all targets. As targets are saturated by increasing doses of radiation to the point where n − 1 targets are hit, conditions of exponential cell kill will accrue.

The survival fraction $S/S_0 = 1 - (1 - e^{-D/D_0})^n$ where S/S_0 = fraction of cells that survive, D = dose delivered and "n" is the number of targets or the extrapolation number. D_q, the quasithreshold dose, is the dose at which the origin intersects the extrapolation of the exponential portion of the curve: $\log e^n = D_q/D_0$.

The limitations of the single hit, multi-target model are most apparent with mammalian cells with doses of radiation of less than 200 rad. Under these circumstances, particularly relevant to radiotherapy, killing is underestimated.

To accommodate this variation, a variety of hypotheses have been advanced. Bender and Gooch (15) described two independent mechanisms: one for single hit kinetics and one for single hit/multi-target. $S/S_0 = e^{-k_1 D}[1 - (1 - e^{-k_2 D})^n]$ where k_1 is the rate of cell kill by single hit mechanism and k_2 the exponential rate of cell kill at high dose. Unfortunately, experimentally it is difficult to specify the 3 parameters k_1, k_2 and n. A linear quadratic model has also been advanced, $S/S_0 = e^{-(\alpha D - \beta D^2)}$ where α = rate of cell kill by single hit mechanism and β = rate of cell kill by double hit mechanism. This model was proposed by Sinclair (130) on an empiric basis and later by Kellerer and Rossi (86) and Chadwick and Leenhouts (28).

The dual radiation action theory of Kellerer and Rossi (86) argued from microdosimetry that the passage of one densely ionizing particle is capable of inactivating the cell, while for low LET irradiation a lethal lesion requires the interaction of two independent sublesions induced by electrons. As a consequence, high LET radiation kills cells in direct proportion to the dose; low LET in proportion to the square of the dose. At low doses of low LET irradiation, it is postulated that delta rays (a high LET component present as a small proportion of all low LET beams) account for cell kill.

Chadwick and Leenhouts (28) also propose a linear quadratic model based on the lethal lesion being unrepaired double strand DNA breaks. Such a double strand break could result from a single event occurring simultaneously in both strands or from two adjacent single strand breaks.

In a fourth model, "repair saturation", discussed by Alper (7, 8), it is postulated that the shoulder represents a repair process operating at the outset but becoming less effective as dose increases, until inactivation of the target occurs without concomitant repair. The implication of such a model is that the basic lesion generated is induced by single hit kinetics.

Finally, a "repair-misrepair" model has been proposed (143) in which the initial lesion is uncommitted, i.e., not necessarily lethal. Following enzymatic repair, eurepaired cells survive while misrepaired cells die or yield mutants.

From the variety of models proposed, it is clear that no single model is universally accepted. Nevertheless, the usefulness of models is rooted in the two functions which they serve: 1) models allow us to make comparisons among the parameters of several cell lines or a single cell line under different experimental conditions; 2) the success of a model in fitting the data provides a basis for judging the hypotheses of radiation action upon which they are based.

In the former instance, a comparison of the parameters, D_0, D_q and n, from the single hit, multi-target model reveals that for most cell lines there is narrow range in sensitivity (Table 1).

With respect to the data fit, in the clinical range of < 200 rad per fraction, the single hit, multi-target model is less satisfactory than the linear quadratic model (alpha/beta). At that dose level, Chapman (29) estimated that 70%

Table 1. Radiosensitivity of cell lines.

Type	*Assay*	*Do*	*n*
Bone Marrow Stem Cell	*in vivo*	95	1.5
Intestinal Crypt Cell	*in vivo*	130	–
Epidermoid Cell	*in vivo*	135	12
Osteosarcoma	*in vitro*	145	1.8
Medulloblastoma	*in vitro*	131	1.6
Breast Carcinoma	*in vitro*	134	1.3
Melanoma	*in vitro*	150	2.5

Derived from Withers (156); Till and McCulloch (142); Withers-Elkind (159); Weichselbaum, Nove and Little (153).

of cell kill is by the linear (α) mechanism, 30% by the dose^2(β) mode.

Thus, D_0, which approximates $1/\sqrt{\beta}$, may be an inappropriate measure of clinical sensitivity. However, the α/β model is more difficult to apply and consequently, while the parameters n, D_0, D_q are used, their limitations should be appreciated.

Factors influencing cell survival

The cell survival curves are generated under specific conditions of irradiation. A variety of physical, chemical and biological conditions will modify the dose response relationship.

Physical Modifiers: As implied earlier, the quality of the radiation beam will influence the dose survival curve by virtue of the density of the ionizing events. Thus, equal doses of two types of radiation do not necessarily produce the same biological effect (Figure 1).

If target theory is invoked, one might imagine a finite spacing of the target within the cell. At low LET (less than 10 KeV/μ) the likelihood of a sparsely ionizing electron producing damage to inactivate all targets is remote. Cell kill would result rather from the inactivation of the target by multiple ionizing events. In a sense, cell kill is inefficient because a portion of the dose is "wasted" on non-lethal damage (Figure 2).

As the density of the ionizing events increases (in the range of 10 KeV to 100 KeV/μ) the density of the ionizing event approximates the spacing of the target.

Beyond this optimal LET, the density of the ionizing event exceeds the spacing of the target. In this "overkill"

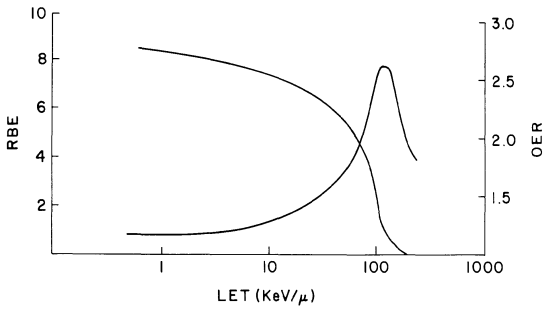

Figure 2. Influence of LET on RBE and OER (Modified from Barendsen (11).

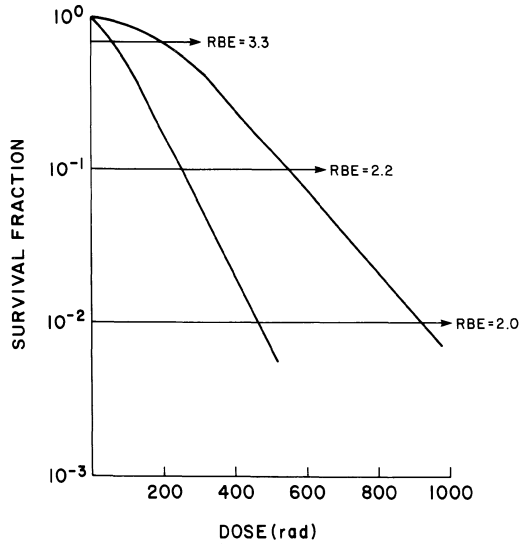

Figure 3. Influence of high LET radiation on survival. Note the variation in RBE with dose.

range, the efficiency of cell kill declines. In a sense, a portion of the dose of radiation is "wasted" on superfluous ionizations within the target volume.

To quantify this relationship, the term Relative Biologic Effectiveness (RBE) is employed. The RBE is defined as the ratio of the dose of 250 KeV x-rays to the dose of that irradiation being tested, which is required to produce the same biological effect. Thus, the RBE will depend on the quality of the radiation and how it is delivered (dose rate and fractionation), as well as the test system and the end point employed.

In a typical situation, RBE will vary little with LET in the range of less than 2 KeV/μ (e.g., 250 KeV, Cobalt^{60} and linear accelerators), being approximately 1. Beyond 10 KeV/μ the RBE increases rapidly to a maximum of approximately 8 and a range of 100 to 150 KeV/μ (e.g., alpha particles). At LETs greater than 200 KeV/μ, RBE declines. Neutron beams with an intermediate LET have an RBE of approximately 2.5. The ranges of RBE given above are approximations since, as noted, the RBE will vary as a function of the end point examined (Figures 2 & 3).

Examining the individual cell survival curves as LET of a beam increases to 100 KeV/μ, the effect is toward an increased slope (smaller D_0) and a smaller shoulder ("n" approaches unity) (11, 19).

Dose Rate: Again, reasoning from target theory, lethality following low LET radiation would depend upon the interaction of sublethal lesions. While repair of such sublethal damage will be discussed in detail later, it is sufficient to note that in the face of ongoing repair within the cell, a temporal relationship must exist for the interaction of such sublethal lesions. Lajtha and Oliver (95) were able to demonstrate that as the dose rate is decreased, the slope of the survival curve decreases. Hall (67) has demonstrated that for dose rates between 1 rad/minute and 100 rad/minute, the dose rate effect is dramatic.

However, clinically most megavoltage units operate at outputs of greater than 100 rads/minute, where changes in

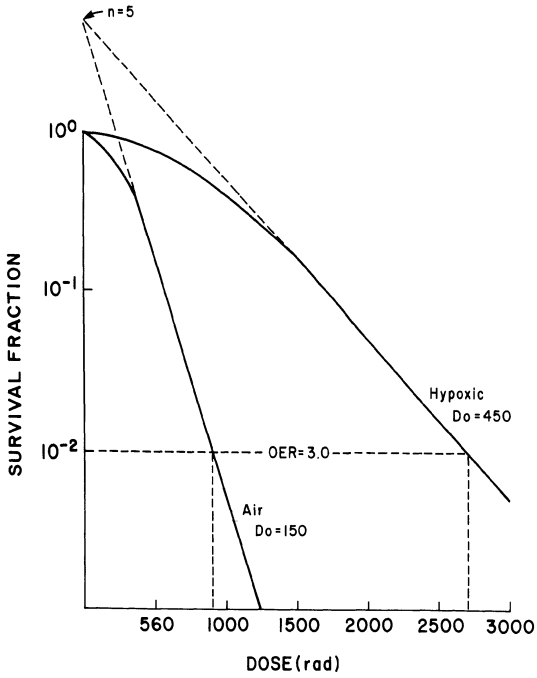

Figure 4. Influence of hypoxia on survival. While the "n" is 5 in both cases, D_0 = 450 rad with hypoxic cells; D_0 = 150 rad with cells in air.

dose rate are less dramatic. Nevertheless, when low dose rate therapy is employed (e.g., brachytherapy), this dose rate effect may be important, both in terms of its effect on slope and also as it affects the shoulder. Furthermore, at very low dose rates the cell population may actually proliferate while being exposed to radiation.

Chemical Modifiers: A variety of chemicals have been found to modify the radiation response. Both radiation sensitizers, including oxygen and misonidazole, and radiation protectors with a sulfhydral moiety, including WR-2721, have been studied. It should be noted that a variety of cytotoxic drugs also interact with radiation, either additively or synergistically, but are beyond the scope of the current presentation and the reader is referred to Sokol and Maickel (133).

Of the sensitizers to low LET radiation, none is more potent and ubiquitous than molecular oxygen. While the influence of oxygen was appreciated as early as 1921 by Holthusen, it was not systematically studied until the 1930's by Mottran (106) nor generally appreciated until the 1950's (64, 118, 119, 120).

With low LET radiation, a detectable sensitization occurs at oxygen concentrations of 100 ppm. At concentrations in excess of 25,000 ppm or 20–40 mm Hg, maximum effect is reached (47).

When the dose/effect curves derived with and without oxygen are superimposed, the sensitization of O_2 can be graphically appreciated. This relationship can be quantified as the Oxygen Enhancement Ratio (OER) (Figure 4).

The OER is defined as the ratio of the dose under hypoxic and well oxygenated conditions to achieve the same biologic effect. For low LET radiation, typical OER values are in the range of 2.5 to 3.0. With increasing LET the oxygen enhancement ratio decreases to unity. With particles such as neutrons, the OER is approximately 1.6, while for alpha particles the OER equals 1 (Figure 2).

Oxygen must be present, for all practical purposes, at the time of irradiation to sensitize the cells. This was suggested by the early work of Wright and Howard-Flanders (164) and Howard-Flanders and Moore (78). In an elegant experiment, Michael and collaborators (103) resolved the question of the interval between irradiation and reaction with oxygen. A monolayer of bacteria was irradiated by a two microsecond electron pulse. The oxygen was released into the oxygen-free space by "explosion" from a reservoir within 1 millisecond of the irradiation. From this work they found that there appeared to be two reactive species with which oxygen interacts; in bacteria the half-life of the species was 0.4 and 4 milliseconds (104). Similar results were obtained with mammalian cells (151) with a half-life of the reactive species of 1 millisecond.

While the mechanism whereby oxygen acts is not fully understood, there is a consensus that it interacts with free radicals. It would appear that oxygen mediates fixation of the radiation-induced molecular damage produced by free radicals (77, 87). Molecular oxygen with two unpaired electrons acts as a strong oxidating agent. The organic free radicals ($R\cdot$) form an organic peroxide ($RO_2\cdot$) precluding restoration of the organic free radical to its undamaged state ($R\cdot + H\cdot \rightarrow RH$). Oxygen then "fixes" that damage which the radiation-produced free radicals have effected.

The impact of tissue hypoxia for clinical radiotherapy was suggested by Thomlinson and Gray (141), when they correlated the capacity of oxygen to diffuse from the capillary with the presence or absence of necrosis in a tumor system. In respiring tissues, oxygen diffusion is negligible at 150 to 200μ; histologically, this correlated with the finding of necrosis in all tumors with a radius of greater than 200μ, and the limitation of the radius of actively growing tumor cells to 180μ.

The implication of these findings is that if hypoxic cells are a significant proportion of a human tumor, and if they are relatively resistant to low LET radiation, they may contribute to the failure of conventional radiation therapy to eradicate tumors. This has led to a variety of strategies to circumvent the problem posed by hypoxic cells.

Among those strategies advanced was the use of electron-affinic compounds that would mimic the effect of oxygen by fixation of the DNA damage of free radicals. As proposed by Adams (2, 3, 5), there are two rationales for the use of these modifiers. Since normal tissue is already well oxygenated, such compounds would selectively sensitize the hypoxic cells of tumors. Secondly, in contrast to oxygen, such compounds would not be metabolized by respiring tumors and should be able to penetrate beyond the limits of oxygen.

Adams (4) was able to demonstrate that there is a good correlation between the electron affinity of compounds and their ability to sensitize cells. With the added constraint of tissue permeability, the most extensively tested compounds to fulfill the criteria have been the 5- and 2-nitroimadazoles: metronidazole (Flagyl) and misonidazole (R0-07-0582).

Nitrofurans have also been studied but have been disappointing because of their metabolic instability and toxicity at doses required for sensitization (3). With metronidazole,

Foster and Willson (57) and Chapman, Reuvers and Borsa (30) were both able to demonstrate that CHO cells *in vitro* were sensitized, although less so than with molecular oxygen. Denekamp (37), with artificially induced skin hypoxia, was able to demonstrate *in vivo* sensitization by a factor of 1.6 in hypoxic cells and the lack of sensitization in normal tissue. Subsequent studies with tumors *in vivo* have confirmed these studies (12).

However, since large doses were required to yield this sensitization, 2-nitroimadazoles were introduced (10). On a concentration basis, misonidazole is about 5 to 10 times more efficient in sensitization than metronidazole (10, 70). Furthermore, the extent of its sensitization more closely approximates oxygen, with a sensitizing factor of 2.5 (6).

The utility of misonidazole has been amply demonstrated *in vivo* and *in vitro* (reviewed by Adams, Dawson, Stratford (5)). It should be noted that in addition to the direct radiation chemical mechanism via free radical fixation, misonidazole may also be directly cytotoxic to hypoxic cells (56, 136).

Currently, clinical studies are under way to determine if this sensitization will result in improved tumor control with acceptable morbidity (39).

An alternative to the sensitization of hypoxic cells is the modification of the dose response relationship by protecting oxic cells.

Patt, Tyree and Straube (110) determined that cysteine protects against the damage of low LET radiation. Subsequently, other sulfhydral compounds were demonstrated to exert a similar effect.

In the late 1950s, the United States Army began an ambitious project to develop further protectors. Among these were several of the triphosphate class (167), in which the active sulfhydral moiety is attached to an aminopropyl group. WR-2721 appeared to be the most effective of this class (168). Subsequent work has demonstrated this protective effect in a variety of normal tissues (169), and that normal tissue is preferentially protected over tumors (168). The selective effect on normal tissue is the result of better uptake in normal tissue (166) and that WR-2721 is less effective in protecting hypoxic cells (73).

The precise site of action of such compounds is not clear, but the favored hypothesis is hydrogen donation from the free sulfhydral as a reaction competing with oxygen fixation (1).

As with hypoxic cell sensitizers, clinical trials are underway to evaluate whether, with the protection afforded by WR-2721, better tumor control can be achieved.

Biological Modifiers: In addition to the extrinsic factors such as physical and chemical modifiers cited above, the dose response curve is modified by cellular processes. Among these are the position of the irradiated cell in the cell cycle and the repair of radiation damage.

Cell Cycle: As early as 1906, Bergonie and Tribondeau (16) appreciated that mitotic cells were more sensitive to radiation than interphase cells. Subsequently, the delineation of the phases of the cell cycle with autoradiography and methods of cell synchronization allowed for the refinement of this observation.

Cells in mitosis tend to round up and are less firmly attached to the culture plate. Exploiting this, Terasima and Tolmach (138) "harvested" cells in mitosis by agitating the cultures. These cells can be replated and will move through the cycle as a synchronous population.

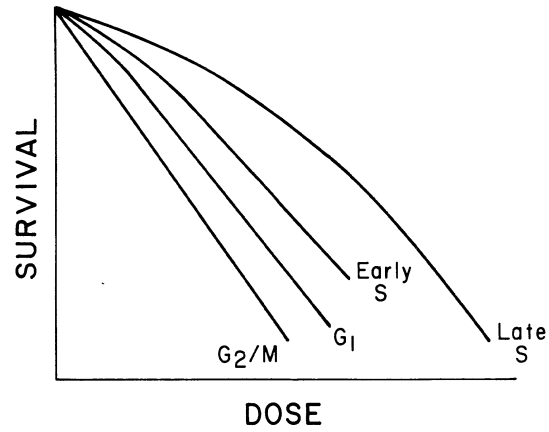

Figure 5. Influence of cell cycle position on survival.

A variety of cell lines have been characterized, but in general G_2 and M are the most sensitive phases, while late S tends to be the most resistant. If G_1 is long, early G_1 also appears to be resistant (Figure 5).

These differences may be due to either "n" or D_0 changes, or both. In V_{79} cells (132), most of the effect is due to a change in the shoulder, but the slope of the resistant phase, late S, is also less steep. For HeLa cells the effect is predominantly on the slope of the survival curve (139). As pointed out by Withers and Peters (162), there is a five-fold difference in survival after 200 rads between the most resistant (late S) and the most sensitive (G_2/M) portions of the cell cycle.

With high LET irradiation, qualitatively this effect is similar, but quantitatively much less (62, 66). Consequently, for tumors with a high portion of resistant cells by virtue of the cell cycle position, or tumors wherein cells of the resistant portion tend to accumulate by virtue of poor redistribution between successive doses of radiation, Fowler (58) has predicted that the therapeutic gain by utilizing neutrons may be more significant than the gain anticipated with respect to hypoxic cells for neutron irradiation.

Repair of Radiation Damage: The damage which occurs following irradiation of cells may be lethal, sublethal or potentially lethal; thus, the effects of radiation may be modified by the repair of radiation damage either between doses of irradiation (sublethal damage repair) or by exposure of the irradiated cells of various post-radiation conditions (potentially lethal damage repair).

We have seen that the mammalian cell survival curve following low LET irradiation displays a shoulder. In simplest terms, this indicates that the efficiency of cell kill increases up to a certain point beyond which cells are exponentially killed. Arguing from target theory, this phenomenon could be accounted for by the accumulation of damage until a critical number of targets are inactivated. Others (7) would argue that this threshold represents the saturation of a repair mechanism within the cell.

Whether or not one accepts target theory, it has been shown by Elkind and Sutton (44, 45) that when two sufficiently large doses of radiation are delivered, separated by

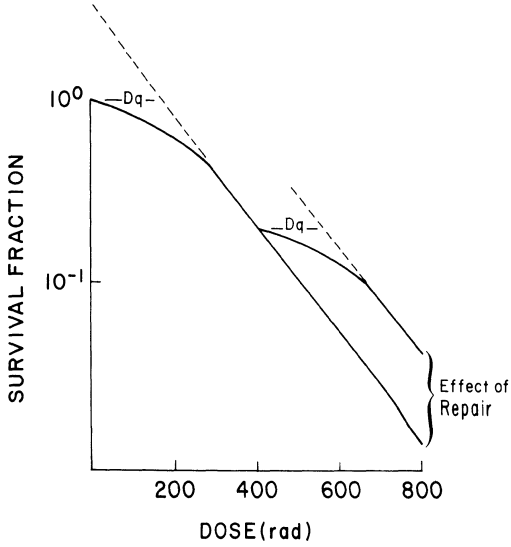

Figure 6. Influence of SLD repair on survival comparing 400 rad × 2 vs 800 rad in 1 fraction.

a time interval of several hours, the shoulder will be reproduced and survival enhanced. Such an enhancement of survival is interpreted as the sublethal damage (SLD) repair or "Elkind-type" repair. In these experiments the first fraction of radiation reduced survival to the point where further increments should produce exponential kill; *viz.* all surviving cells would be expected to have (n − 1) hits. A delay before the second dose increased survival such that maximum survival was reached at two hours. With a further delay, survival decreased, then rose to a plateau level, comparable to that expected if the cells had not had a prior exposure. The explanation of the initial decrease in survival is the partial synchronization of cells by the first dose of radiation (48, 131). The full recovery of cells indicates that the sublethal damage has been repaired during the interval between doses, typically a four-hour period (160), but partial synchronization precludes precise measurement.

As indicated earlier, D_q is the measure of the width of the shoulder of the dose response curve. Thus, it is equal to the difference of doses required to achieve the same effect when given in two fractions versus one fraction (Figure 6).

The work of Elkind (47) suggested that the repair of SLD was only weakly influenced by cellular metabolic processes, leading to the tentative conclusion that recovery was a nonenzymatic process (43). Subsequent work by Bryant (20, 21) with algae and Koch (90, 91) with V_{79} cells indicated that SLD recovery does require the presence of oxygen, although low concentrations may suffice (69, 137).

Repair can be suppressed by metabolic inhibitors; *e.g.*, Actinomycin-D and severe hypoxia. Thus, the protective effect of a hypoxia for tumor cells may be partially offset by decreased repair capacity (71).

Less clearly understood is the role of protein and nucleic acid synthesis in SLD repair. 5-Fluorodeoxyuridine, a specific DNA synthesis inhibitor (88), 5-FU (17) and the protein synthesis inhibitors, puromycin (17, 43) and cycloheximide (17) did not inhibit repair. Actinomycin-D which prevents RNA synthesis by binding to DNA does inhibit repair (46). This effect may be due to "distortion" of the DNA structure rather than inhibition of RNA synthesis *per se*; puromycin at concentrations sufficient to inhibit RNA synthesis was not an effective repair inhibitor ((7), p. 178).

As would be expected from the magnitude of the shoulder with high LET radiation, there is little SLD repair. Thus, Ngo, Han and Elkind (108) concluded that V_{79} Chinese hamster cells displayed recovery following neutrons and x-rays qualitatively consistent with differences in their extrapolation number to single dose experiments.

Distinct from the cellular recovery following split dose irradiation (Elkind or SLD repair), cell survival may be enhanced or diminished by modification of the post-irradiation conditions. It is postulated that a fraction of the lesions induced are only potentially lethal; thus, under favorable conditions following radiation, they may be repaired, or conversely under unfavorable conditions, the damage is expressed, i.e., becomes lethal. The enhancement in survival by modification of the post-irradiation conditions is termed potentially lethal damage (PLD) repair.

This phenomenon was first noted in mammalian cells by Philips and Tolmach (113). In general, suboptimal conditions for growth favor the repair of PLD, and the magnitude of the effect increases with increasing doses of radiation, resulting in a change in the slope of the survival curve.

Among the conditions reported to enhance PLD repair are: holding in nutritionally deficient media (14), growth inhibited plateau-phase cultures (98, 99) or balanced salt solution (14, 65). In addition, Little *et al.* (99) have suggested that large tumors with noncycling cells, analogous to density inhibited plateau-phase cultures, are more proficient in PLD repair than small tumors.

With high LET radiation, PLD has not been observed (61, 128).

The clinical impact of PLD repair has not been studied in the detail that SLD repair has been studied. However, it has been suggested that it may influence both tumor control and also the complication rate in slowly normal tissues (152).

PHYSICS

The physics of ionizing radiation will be presented only briefly in this section; for a fuller explanation the reader is referred to comprehensive texts (80, 126). As implied above, the specific type of radiation employed for therapy produces its effect by virtue of its capacity to produce ionization. Energy is transferred from the incident beam to the biologic material through atomic ionization to fast charged particles.

Two basis types of ionizing radiation are of interest: electromagnetic and particulate. In the former case, photons of electromagnetic radiation (X-rays or gamma rays) interact with electrons or the nuclear coulombic in field of the atoms of the target material. In the latter instance, the particle may be charged (electrons, alpha particles, etc.) and interact directly, or uncharged (neutrons) and create secondary charged particles via nuclear recoil or reactions.

Electromagnetic radiation

Both x-rays and gamma-rays are electromagnetic radiation;

Table 2. Electromagnetic spectrum.

Type	*Frequency (cycle/sec)*	*Energy*
Radiowave	1×10^{5}– 3×10^{10}	4.13×10^{-10} eV– 1.24×10^{-4} eV
Infrared	3×10^{12}– 3×10^{14}	0.0124 eV– 1.24 eV
Visible Light	4.3×10^{14}– 7.5×10^{14}	1.77 eV– 3.1 eV
Ultraviolet	7.5×10^{14}– 3.0×10^{16}	3.1 eV– 124 eV
Soft X-rays	3×10^{16}– 3×10^{18}	124 eV– 12,4000 eV
Diagnostic X-rays	3×10^{18}– 3×10^{19}	12.4 keV– 124 keV
Therapeutic X-rays	3×10^{19}– 3×10^{21}	124 keV– 12.4 MeV

along with light and microwaves, all travel at the speed of light. X-rays and gamma-rays are distinguished from other forms of electromagnetic radiation by virtue of their wavelength (or frequency) (Table 2). X-rays and gamma-rays share several common features: the ability to penetrate matter, exponential absorption, and the production of secondary charged particles.

Because of the overlap in frequency between x-rays and gamma-rays, there is a corresponding overlap in the possible energies of x-ray and gamma-ray beams as described by: Energy = hf where h is Planck's constant and f is frequency. Consequently, the distinction between x-rays and gamma-rays is (more by convention) based on how the radiation was produced.

Gamma-rays are the product of the decay of natural or artificial radioactive material. As an isotope decays to a more stable form, part of the energy (or mass) of the parent compound's nucleus is given up as a gamma-ray or rays produced will be discrete and depend on nuclear energy of the isotope.

In current practice, the external source of gamma-rays used is ^{60}Co; for intracavitary or interstitial use ^{137}Cs, ^{192}Ir and others are employed.

X-rays are produced extranuclearly in a man-made generator by the deceleration of electrons in a media. The energy of the x-rays produced will form a spectrum from zero through the maximal energy of the incident electron. Electrons used for the production of x-rays acquire their energy by acceleration across a voltage gap in a vacuum tube of a linear or circular accelerator. The targets used to stop these electrons are typically heavy metals with a high melting point, since the generation of heat accompanies the production of x-rays. The higher the energy of the incident electron beam, the higher the energy of the photon beam.

In common practice, x-rays are described as a function of the energy of the electron beam from which they were produced: kilovoltage 10–300 KeV; orthovoltage 220–300 KeV; supervoltage 1–3 MeV; megavoltage $\geqslant$ 1 MeV but typically > 4 MeV. The unit of energy, electron volt (ev) is the amount of energy released when an electron falls through a potential difference of 1 volt, hence 10^{6} ev = 1000 KeV = 1 MeV (Table 3).

Table 3. Typical therapy units.

Kilovoltage	10–300 keV
Orthovoltage	220–300 keV
Supervoltage	1–3 MeV
Megavoltage	$\geqslant$ 1 MeV

Charged particles

A variety of charged particles are emitted by radioactive decay or are produced in an accelerator or nuclear piles. Among these, some have been used for therapy, including electron or beta rays, protons, alpha particles and pi-mesons.

The major limitation in their usefulness is the difficulty obtaining suitable energies to penetrate tissue. The difficulty of accelerating particles depends on their mass; furthermore, higher energies are required for charged particles to obtain equivalent tissue penetration. Consequently, the routine use of charged particles is limited to electrons produced by clinical linear accelerators.

Uncharged particles

Neutrons are produced by a variety of reactions including radioactive decay (252-Californium); the bombardment of a suitable target with a charged particle beam (*e.g.*, deuterons or protons); by the fusion reaction during the bombardment of a tritium target with deuterons or in nuclear reactors.

Neutrons have no charge and are not directly ionizing. Rather, during their interaction in matter, secondary charged particles are liberated which in turn cause ionization.

While neutrons are not part of routine clinical practice, all of the methods noted above have been employed experimentally as neutron sources.

Dose

The Roentgen (R) (Table 4) is the unit of x-ray or gamma ray exposure measuring the number of ionizations in air. As defined it is equal to 1.61×10^{12} ion pairs per gram of air.

Table 4. Common physics terms.

Currie (Ci): unit of activity used for radioactive isotopes; one curie is the quantity of a nuclide in which the number of disintergrations per second equals 3.7×10^{10}.

Electron Volt (eV): a unit of energy; 1 eV is the equivalent of the energy gained by one electron passing through a potential difference of 1 volt.

Gray (Gy): international unit of absorbed dose; 1 Gy = 100 rad = 1 Joule/kg.

Milligram-hour (Mg-hr): dose of radiation delivered by 1 mg of 226 Radium or its equivalent.

Rad: dose of radiation absorbed in a mass; 1 rad = 100 erg per gram.

Roentgen (R): unit or radiation exposure required to produce 1.61×10^{12} ion pairs per gram of air.

Rem: unit of dose used in radiation protection; for most x-rays and gamma rays 1 rad = 1 rem; for other ionizing radiation the rem dose equals the rad dose × RBE.

Sievert (SV): international unit of radiation dose used in radiation protection; 1 SV = 100 rem.

While the Roentgen represents what happens in air when exposed to a radiation beam, the rad represents the quantity of energy absorbed in tissue. One rad (not abbreviated) indicates the absorption of 100 ergs/gram tissue. A new SI unit, the gray, had been introduced: 1 gray = 100 rad = 1 Joule/kg.

Within the range of energies commonly employed in therapy, the dose in rads to soft tissue is approximately 0.96 times the exposure in roentgens.

Linear energy transfer

In addition to describing the type of radiation as electromagnetic or particular and the dose of radiation delivered in rads, radiation may also be described in terms of the submicroscopic density of the ionizing events produced or linear energy transfer (LET). LET is the energy transferred per unit track length.

X-rays, gamma-rays and electrons are sparcely ionizing; i.e., give up relatively little energy to ionization per unit tract length and are referred to as low LET radiation. By contrast, neutrons and alpha particles are densely ionizing.

LET measured in KeV/μ is a complex function increasing with mass and charge and decreasing with increased energy. For Co^{60} and 250 KeV X-rays, the average LETs are 0.3 and 2 KeV/μ respectively. Some heavily charged particles have average LET's in the range of 100–1000 KeV/μ. Neutrons, occupying an intermediate range, have an LET of approximately 10 to 50 KeV/μ. There are limits to the usefulness of LET, since as an average, it may ignore a biologically significant high LET fraction of a low LET beam.

Interactions with matter

The photons of both x-rays and gamma-rays travel in a straight line until they interact with matter by one of three processes: photoelectric absorption, Compton scatter, or pair production.

The photoelectric effect is the predominant process for the absorption of low energy photons. In this interaction, bound electrons of the K, L, M, shells are ejected and most if not all of the energy of the incident photon is transferred to the electron. The vacancy in the shell from which the electron was ejected creates an unstable state. An electron from the next highest level drops down to fill that space. Because it occupies a ring of lower potential energy, the excess energy is given off as a characteristic x-ray specific to that atom and that shell.

The probability of an interaction in the photoelectric range is proportional to the cube of atomic number (Z) of the absorber. There are two practical implications of this phenomenon: Diagnostic x-rays operate in this range to take advantage of the differential absorption in biologic material as a function of Z^3. Secondly, for patients treated with orthovoltage beams of < 100 KeV, the photoelectric effect results in a higher absorbed dose in bone. With photoelectric absorption, the bone will absorb about six times as much energy, gram for gram, as soft tissue.

At energies greater than 200 KeV, photoelectric absorption is negligible and the dominant process is Compton scattering. In this process a portion of the energy of the incident photon is imparted to the outer orbital electron or a free electron, which then causes the secondary ionizations. The photon, although reduced in energy, continues on at some angle to its initial path.

In contrast to the photoelectric process, Compton interaction is independent of atomic number. Consequently, the dose absorbed in bone and soft tissue per gram is nearly the same. Co^{60} (1.25 MeV), Cs^{137} (.667 MeV) and linear accelerators operate in the Compton range.

Finally, photons of energy greater than 1.02 MeV may interact with matter by pair production. In this process, the photon is converted into an electron and a positron (e^+) when it interacts with the nucleus. The electron may continue to cause subsequent ionization while the positron undergoes an annihilation reaction with electrons in the material producing two photons of .51 MeV.

While pair production is dependent on atomic number and consequently yield a higher dose in bone, for clinical radiotherapy this process is relatively unimportant at energies less than 20 MeV.

Charged particles interact in a manner significantly different from photons. Because they have a charge, they tend to ionize throughout their tract length. The tract is characterized by a natural tendency for linear projection, deflection upon interaction with matter and an increased density of ionization as the particle is slowed. Because of this last feature, as the particle is slowed by past interactions toward the end of its tract length, large quantities of energy are given up at this point, the Bragg Peak (Figure 7).

The Bragg Peak for heavier charged particles such as protons and alpha particles is quite pronounced, since, by virtue of weight, they continue to travel in a straight line. By contrast, electrons (1/1836 the mass of protons) are more likely to be deflected and scattered. As a consequence, there is no apparent Bragg Peak and energy is deposited more uniformly.

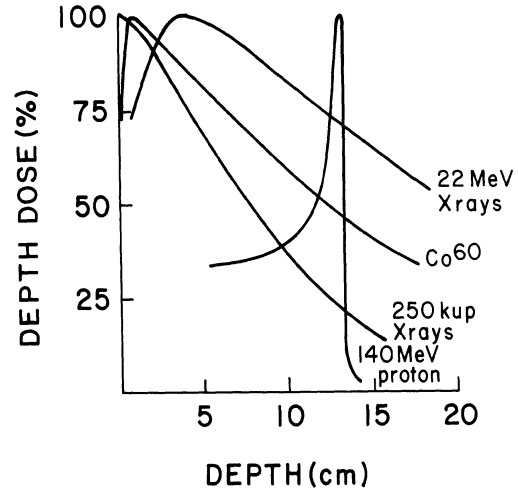

Figure 7. Percent depth dose for 250 Kvp x-rays; CO^{60}; 22 MeV x-rays; and 140 MeV protons. Note that the depth of maximum dose is deeper and the attenuation less with increasing energy among the photon beams.

Neutrons share with X-rays the exponential deposition of energy, however, the process is markedly different. Neutrons interact randomly with the nucleus by either elastic scattering, inelastic scattering, nonelastic scattering or capture of low energy neutrons. The most common process in biologic material with beams of clinical relevance is elastic scatter. The products of these reactions, protons, or particles and nuclear fragments, are densely ionizing in contrast to the sparcely ionizing electrons set in motion by photon beams. Like photons in the photoelectric range, the energy is not transferred homogeneously. However, in the case of neutrons, the probability of an interaction in matter and consequently the deposition of energy is related to the hydrogen content. Thus, bone absorbes somewhat less than soft tissue while fatty tissues absorb somewhat more.

Dose distribution

As noted above, each of the various beams has one or more means of absorption in tissue and consequently different patterns of penetration.

Charged particles all have a finite range in tissue or water. Except for electrons, all have a pronounced Bragg peak, the depth of which is determined by the energy of the particle (Figures 7 & 9).

The net direction of the electrons scattered by Co^{60} and other megavoltage beams is in a forward direction. As a result at some distance beneath the surface, dependent upon the energy of the photon beam, the maximum dose is deposited. The clinical consequence of this feature is that the skin and the superficial tissues receive less dose; this is referred to as the skin sparing effect.

Neutron beams are absorbed exponentially, like photons, however, the skin sparing is considerably less.

In all cases as the energy of a beam increases, the penetration in tissue increases (Figure 7).

Chemical products of radiation interactions

In the foregoing section we have been concerned with the initial transfer of energy from the incident beam to the absorbing media. The initial chemical event, over a time scale of less than 10^{-10} seconds is the production in the target molecule of an electron and a positively charged molecule. Within the cell the initial ionization may occur in the DNA itself – direct effect –, or in other molecules principally water with the DNA being secondarily involved – indirect effect. (The direct and indirect effects of radiation on DNA should not be confused with directly or indirectly ionizing radiation).

Since the cell is composed of 80% water, interactions with it account for the majority of cell injury by the indirect pathway. In the radiolysis of water H_2O forms H_2O^+ and e^-. The ion H_2O^+ reacts with a second molecule of H_2O to form H_3O^+ and $OH\cdot$ or hydroxyl free radical. The initial electron may react with water forming H_2O^- which in turn forms $OH-$ and the $H\cdot$ free radical. Though subsequent recombination H_2O_2, H_2O and H_2 may be formed (Table 5).

The hydroxyl free radical can diffuse over 20 Angstroms in its brief (10^{-5} S) life and accounts for 70% of cell damage.

Table 5. Radiolysis of water.

H_2O	$H_2O^+ + e^-$	
$H_2O^+ + H_2O$	$H_3O^+ + OH\cdot$	
$e^- + H_2O$	H_2O^-	$H\cdot + OH^-$
Products: $H\cdot$		hydrogen free radical
$OH\cdot$		hydroxyl free radical
H^+		hydrogen ion
OH		hydroxyl ion

With X-ray beams the DNA so damaged by the indirect production of a free radical within it may be fixed or repaired as will be seen in the earlier Table 4.

CLINICAL RADIATION BIOLOGY

The clinical practice of radiotherapy represents a more complex situation than that present during the *in vitro* and *in vivo* generation of survival curves. With the goal of uncomplicated cures in mind, it must be appreciated that the concern is not damage of the tumor *per se* but rather the damage to the tumor relative to that of the normal tissue. In particular, four principles of radiation biology apply to the clinical situation: repopulation, reassortment, repair, and reoxygenation.

Repopulation

Repopulation is the increase in the number of clonogenic cells during a course of fractionated radiation therapy by either cell proliferation or migration. An increase in the number of such cells will decrease the effect of radiation.

Repopulation by migration might be expected of circulating stem cells such the marrow or from the margins of the mucosa and skin. Repopulation or regeneration by cell division may occur during a fractionated course of irradiation, since the mitotic delay following a typical dose of 200 rads is on the order of 2 to 4 hours, while 24 hours typically elapse between fractions.

Normal tissue mucosa responsed to radiation injury with recruitment of noncycling cells and a shift from stem cell maintenance to differentiation and regeneration.

It is not clear if all tumors exhibit such a physiologic response to radiation. However, in a mouse carcinoma, repopulation may in fact exceed the normal volume doubling time (94). Similar results have been suggested in human squamous cell carcinoma such that with prolongation of the treatment times, lower cure rates were observed despite comparable nominal standard doses (NSD; see below) (100).

Consequently, unnecessary prolongation or interruption of therapy should be avoided, for while repopulation may offer an advantage in terms of acute reaction, deleterious tumor repopulation may also occur.

Reassortment

As indicated earlier, both normal and tumor cells vary in sensitivity over the cell cycle, such that late S and at times

mid G_1 are more resistant. Theoretically, the partial synchrony produced by radiation can be exploited to deliver the subsequent dose of radiation during the tumor's sensitive phase. However, there is no practical way to take advantage of this property.

Nevertheless, fractionated radiation offers some advantage over single dose irradiation. Fixed post-mitotic cells or those chracterized by slow turnover in such tissues as the central nervous system or heart will reassort poorly and have a relative resistance conferred on them. By contrast, tumors, as well as certain normal tissues with rapid renewal (e.g., skin and intestine) will reassort within the cell cycle from a relatively resistant phase to a more sensitive one.

Reoxygenation

As discussed previously, hypoxic cells are relatively more resistant to radiation than well-oxygenated cells. In studies of animal tumors hypoxic cells may represent one to twenty-five percent of the tumor population (68). In the absence of reoxygenation, as would be observed with a single dose irradiation, prohibitively high doses of radiation would be required for tumor control.

With fractionated doses of radiation, hypoxic cells may reoxygenate (81). Reoxygenation may involve several processes including: a reduction in the total number of tumor cells relative to the capillary bed, particularly those that are initially well-oxygenated due to their proximity to the blood vessel; an effective increase in the oxygen diffusion capacity by virtue of decreased consumption of oxygen in the lethally irradiated cells; changes in tumor vascularity secondary to variations in the shunting of blood either cyclically or due to the reopening of compressed vessels as the tumor shrinks.

While tumor cells reoxygenate and are sensitized during a fractionated course of radiation, from a radiobiologic point of view, normal tissue is already fully sensitized and thus not affected by reoxygenation. Consequently, with regard to reoxygenation, fractionation promotes tumor control without affecting the normal tissue response.

Repair

As indicated, most normal tissues and tumors have a survival curve characterized by a shoulder and can repair sublethal damage over a period of a few hours. With fractionated doses or irradiation, higher total doses are required to achieve an effect comparable to a single exposure. Withers (157) felt that there was no systematic differences between tumors and normal tissues with respect to their ability to repair SLD. However, Field (51) and Philips (114) felt that tumors had a reduced capacity for repair. Subsequently, Denekamp and Stewart (38) measured the repair capacity of six mouse tumors and skin. With one exception, the repair in tumors was lower than in skin.

Two additional features favor the use of fractionated irradiation: hypoxic cells such as in tumors may have a reduced capacity for SLD repair as indicated earlier; late injury in normal tissue, which frequently limits the dose, is less frequent with multiple small doses than with a few large fractions suggesting an increased capacity for repair of these critical tissues (140).

Moreover, the choice of the typical fractionation scheme, while empirically derived, has a basis in radiation biology. Reoxygenation of hypoxic cells by fractionation clearly offers an advantage in terms of tumor control. Similarly, the repair of sublethal damage with fractionated radiation appears to favor normal tissue over the tumor, but the process takes place in both. Against the possible advantages of increased fractionation must be weighed the risk of tumor repopulation, which some feel proceeds more rapidly than normal tissue repopulation (144).

Nominal standard dose

A complex relationship exists among various factors that determine the response to a fractionated course of radiation. Strandqvist (134) introduced a concept of a mathematical formula to relate time-dose and effect: dose $\propto T^x$ where dose = total dose, T = overall time, x = slope of the isoeffect curve. Subsequently, modifications were introduced to account for the volume irradiated, number of fractions, and interruptions in treatment. Ellis (49) defined the nominal standard dose (approximately equivalent to the dose delivered in a single fraction for a given level of injury) as $NSD = TD/(T^{.11})(N^{.24})$, where NSD is measured in rets (rad equivalent therapy; T is the overall time; N is the number of fractions; TD is the total dose.

Despite the desirability of such a formula, its application for tissues other than skin is clearly inappropriate, and even for skin it is probably an oversimplification (54, 161), as is the TDF (109) a mathematical derivation of the NSD formula. Subsequent modifications in this formula (31, 32), utilizing the cell kinetic modification program have introduced additional parameters to provide some guidelines for an isoeffect of normal tissue tolerance.

Therapeutic index

The relationship between the desired effect (tumor control) and the undesired effect (normal tissue complication) is described as the therapeutic index. Since the cell survival response is described by an exponential function of dose, the probability of either is an exponential function dose. Under favorable circumstances, these curves are widely separated and there is a favorable therapeutic index (Figure 8).

However, as an exponential function, it is inappropriate to speak of a tumoricidal dose or of a tolerance dose. Rather, the probability of control or the risk of a complication is more appropriate terminology, since there is no threshold for either. In the case of tumor control, even when the probability of viable clonogens is some fraction (i.e., 0.01), there is still a finite risk of recurrence (one case in a hundred treated). A second implication of this exponential relationship is that over a limited range of dose, at the inflexion point of the curve, the probability of a given effect is sensitive to small changes in dose (Figure 7). Thus, from a 10% probability of control, a 200 rad increment dose, increases survival to a 43% 400 rads to 73%; and 600 rads to 89%. However, the corresponding increase in complications

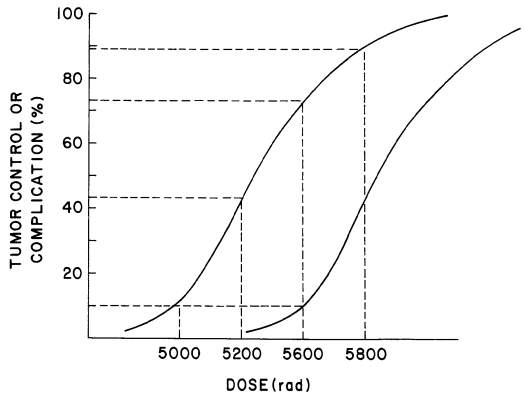

Figure 8. Percentage of tumor control or risk of complication as a function of dose.

for the final increment in dose may alter the therapeutic ratio such that an increase in control by 16% (73% to 89%), the risk of complication increases four-fold from 10% to 43%. While the example presented is an idealized case, it is in reasonable agreement with experimental (42) and clinical data (82, 129). It should be noted that in clinical cases the results may be further modified by unequal slopes to the curves for normal tissue and tumor.

Normal tissue effect

The effects of radiation on normal tissue has been studied in detail by several authors (50, 124, 154). This topic, while of importance to both the radiation therapist and the pathologist, will be covered here only in general terms.

The distinction must be made between the early or acute affects of radiation and the late and often more serious complications. The former arise during or shortly following treatment reflecting damage to the actively proliferating cell population. In most cases these acute effects will subside within a few weeks of radiation. By contrast, the late effects may evolve over a period of months to years and reflect damage to the organ's parenchymal elements either primarily or secondarily due to changes in the stroma. The pathogenesis of this damage is not precisely known. Some have suggested that the lesion is predominantly an effect on the vasculature, effecting the arterioles and small arteries (155) with an endarteritis or an increased capillary permeability (146). Others have suggested that the damages observed in blood vessels only reflect the depletion of slowly dividing stromal and parenchymal cells (163).

The risk of developing a complication in 5% of the patients treated to a given dose at a given tissue site over a 5 year period of observation has been defined as the tolerance dose 5/5 ($TD_{5/5}$). Employing this concept Rubin and Casarett (124) and Fajardo (1982) have developed approximate ranges of tissue tolerance. However, these figures are only approximate since an addition to the effects of fractionation and dose, tolerance depends on the volume irradiated; the end point by which tolerance is defined; and environmental factors.

The influence of volume (or area) has been demonstrated for skin by von Essen (149) such that there is a 3% risk of necrosis following 1500 R for a 100 cm squared field, while for a 1 cm squared field 3000 rads is required to approximate a similar risk. Similar effects have been demonstrated for other sites such that tissues (e.g., lung or liver) may be taken to a high dose provided the volume is kept small.

The end point by which tolerance is defined will also influence our determination of the risk of a complication. To illustrate this point, Shalet *et al.* (127) examined 32 children irradiated for Hodgkin's disease. Clinically all were euthyroid; however, chemically 16% were hypothyroid and when subjected to still more elaborate testing, only 9% were truly euthyroid. A similar point is made by Peylan-Ramu et al (112). Brain CT scans of asymptomatic children who had received 2400 rad to the cranium for leukemia revealed abnormal findings in 53%. Thus, the definition of tolerance is not unambiguous, rather it is defined clinically, radiologically or chemically. It should also be noted that tolerance will depend upon the "stress" to which the irradiated tissue is subjected. Thus, while irradiated skin may tolerate fractionated doses in excess of 6000 rads at most sites, when such doses are delivered to areas (e.g., the foot) which are subject to trauma untoward sequelae may occur. Similarly, in most patients the mucosa and mandible tolerate high dose radiation well. When subjected to subsequent dental extraction, almost $\frac{1}{2}$ of the patients so treated developed mandibular complications (63).

Thus, both the acute and late effects on normal tissue are influenced by the total dose, the number of fractions and overall time, the volume irradiated and how one defines a complication.

CLINICAL RADIATION PHYSICS

As we have seen, there are a variety of sources for ionizing radiation. Based upon the method of their delivery in clinical practice, they may be divided into two categories: brachytherapy and teletherapy.

Brachytherapy

In brachytherapy, the distance from the source to the tumor is short. The source is a radioactive nuclide which may be implanted permanently or a removable implant which is inserted for several days. Such sources may be interstitial, intracavitary or a surface mold. The advantage of this technique is that the dose falls off rapidly, largely determined by the inverse square law. Consequently high doses may be delivered to limited volumes while the dose to the surrounding normal tissue is minimized. The use of brachytherapy is restricted by the accessibility of the tumor and the tissue's ability to tolerate high doses given over a short period. The isotopes commonly used include: 137-Cesium, 192-Iridium, which have supplanted Radium and Radon in clinical practice; the advantage of Cesium and Iridium lies in their improved safety i.e., they are easier to shield and there is no risk of radioactive gas if a source leaks. Permanent implants are usually performed with 198-Gold or 125-Iodine.

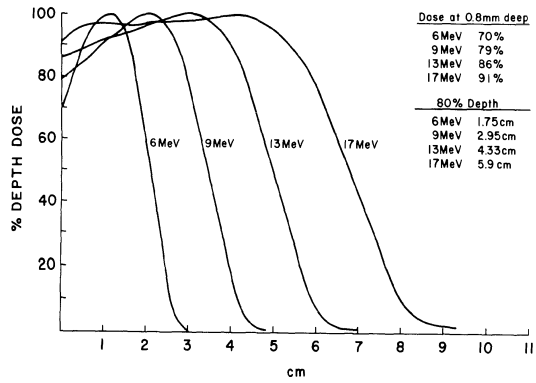

Figure 9. Percent depth dose of electron beams.

Teletherapy

Teletherapy employs radiation sources located 80–100 cm from the patient. Among the treatment beams commonly employed are: Photon beams from the Cobalt 60, X-ray units or Linear Accelerators; and electron beams from either Linear Accelerators or Betatrons. X-ray units delivering kilovoltage or orthovoltage photon beams are largely restricted to superficial tumors or low dose palliative situations because of their poor tissue penetration. Most modern teletherapy employ Cobalt 60 sources or Linear Accelerators.

The choice of treatment unit will depend upon the skin sparing and the tissue penetration of the unit relative to the depth of the tumor and the dose to be delivered (Figures 7 & 9). Consequently Cobalt 60 or 4 MeV Accelerators with 0.5 and 1 cm depth of maximum dose are suitable for head and neck and breast cancers while they may be less suitable for deep seated tumors. With higher energies, better penetration and depth more skin sparing and a sharper field edge are offered. Thus, for pelvic and abdominal malignancies there is an advantage to using a high energy linear accelerator.

Electron beams produced by linear accelerators afford little skin sparing but after the maximum dose is reached, fall off rapidly. Clinically they are most useful for relatively superficial tumors or to deliver a boost dose in conjunction with photon therapy since the deeper structures will be spared by virtue of the rapid fall off of electron dose. Caution must be exercised with the clinical use of electron beams since their distribution of dose may be effected by tissue inhomogeneity such as bone or air cavities.

Dose

As described, the dose prescribed will be influenced by the tumor burden. For subclinical disease such as microscopic deposits in lymph nodes, 5000 rad will eradicate 90% of these deposits. For gross disease higher doses of radiation are required. The final choice will depend upon the therapeutic ratio, i.e., tumor control versus normal tissue complication. Nevertheless the dose response curve can be described from clinical results which demonstrates more effective control with increasing doses and decreasing control with larger deposits of disease.

Although for some sensitive tumors such as seminoma and lymphoma, a low dose will suffice; typical doses for epithelial tumors will equal or exceed 6000 rad. When the goal of radiation therapy is the palliation of metastases, not permanent control, doses of 3000 rad are often sufficient. The dose of radiation is typically delivered at a rate of 180–200 rads per fraction, given 5 times weekly. Other fractionation schemes such as accelerated fractionation of hyperfractionation are now being evaluated in clinical experiments.

CLINICAL RADIATION THERAPY

Introduction

It is the intent of clinical radiation therapy to achieve local tumor control while avoiding untoward normal tissue reactions. The implementation of this goal requires an understanding of the physical behavior and biological effects of radiation as discussed above, in addition to an understanding of the natural history of the malignant process itself.

Without minimizing the significance of an understanding of biology or physics, the crucial step in the treatment of the patient with cancer is the initial evaluation of that patient. This evaluation includes a complete history and physical examination, as well as a review of the radiographic and laboratory studies, including the pathology of the tumor.

Based on this evaluation, the intent of therapy, palliation of disease or an attempt to cure the disease, is made. From the intent of therapy follows the choice of therapeutic modality or modalities to be used.

Treatment planning

When radiation therapy is to be employed, the actual treatment is preceded by tumor localization and treatment planning (Figure 10).

The localization of the tumor volume may in fact define several tumor volumes, distinguishing areas of subclinical involvement from gross disease, and within the former, areas treated electively from those treated for microscopic involvement. The volume or volumes to be treated is predicated on a knowledge of the anatomy and natural history of the disease; pathological evaluation; physical examination; and radiographic studies. The natural history of a given tumor suggests its routes of spread and consequently areas that will be treated electively although not clinically involved.

Such extension occurs by local infiltration of adjacent tissue, seeding within body cavities, lymphatic pathways and by hematogeneous dissemination. While the features of such spread will be covered in greater detail elsewhere in this volume, the propensity for spread will be governed in part by the site, size and histology of a given neoplasm.

These features will be taken into account by the radiotherapist in the treatment planning process. To illustrate this point, one may consider how the radiation portals vary

Clinical evaluation	History Physical examination Laboratory studies Radiological studies Pathology Review
Therapy decision	Palliative or Curative Choice of modalities
Tumor localization	Patient contour Tumor volume determination Critical organ determination
Treatment planning	Immobilization Simulation Treatment Plan Fabrication of compensators and blocks Dose calculation
Treatment	Dosimetry check Verification films Clinical evaluation of response Supportive care
Follow-up evaluation	

based on the patterns of spread for neoplasms arising in the larynx/hypopharynx and in the central nervous system.

Owing to the relative paucity of lymphatics, small squamous cell carcinomas of the glottic larynx seldom metastasize to the cervical lymph nodes. A suitable field would be delineated by the arytenoids posteriorly, the epiglottis superiorly and the cricoid cartilage inferiorly. With a similar lesion in the adjacent supraglottic larynx larger fields would be required because of the rich lymphatic network and the attendant risk of subclinical disease in the cervical nodes. For such malignancies fields would extend from the mastoid to the clavicles and posteriorly to the posterior cervical triangle. Lesions of the pyriform sinus, which have few barriers to local infiltration in addition to access to lymphatic plexi may extend submucosally along the pharyngeal wall, involve the post-cricoid area and the oropharynx. The radiation portal then extends still farther superiorly to include the oropharynx and the retropharyngeal nodes.

Within the central nervous system, while there are no lymphatics with which to be concerned, the radiation volume is still determined by the patterns of spread. Thus low grade astrocytomas are irradiated with a limited margin around the tumor. By contrast, high grade astrocytomas (glioblastoma multiforme), which infiltrate more extensively, are typically treated with whole brain irradiation and a subsequent boost to the gross lesion. Tumor such as ependymomas and medulloblastomas arising in or near the forth ventricle may gain access to it and spread via subarachnoid seeding. Consequently, the field of irradiation includes the entire neuroaxis.

The pathologic evaluation, in addition to determining the histology and grade of the lesion, also addresses the adequacy of any prior surgical excisions. Finally, physical examination and radiographic studies determine the extent of the disease and its location relative to critical normal structures.

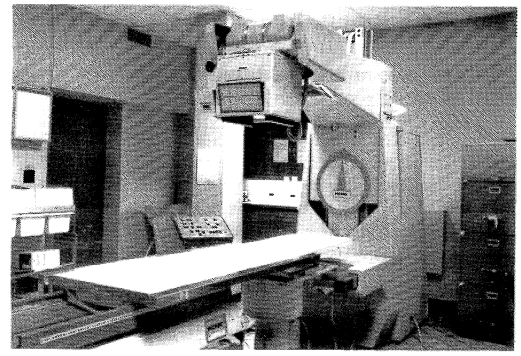

Figure 11a. Treatment simulator.

Having localized the tumor, the minimum requirements of treatment planning would be to indicate, typically with skin marks, the portals to be treated, the depth of the tumor within the field of radiation, and the dose and treatment unit to be employed.

Often, treatment planning requires a more complex process for optimal therapy, beginning with simulation.

Since the patient will be treated five days per week for several weeks, the treatment position must be reproducible. This requires that the patient be immobilized in a comfortable position. To this end, head or limb holders, restraining devices and custom molds may be made for the individual patient. Wall and machine mounted lasers allow for the accurate positioning of the patient on a daily basis. The volumes to be treated, while taking into account superficial landmarks, often require radiologic localization. The simulator, (Figure 11a) a diagnostic x-ray unit often with fluoroscopic capability which duplicates the geometry and capabilities of a treatment until is employed to assist in the radiation field placement. Thus, the path of the treatment beam with respect to the tumor volume and the critical normal tissues is visualized.

In conjunction with the actual simulation, CT scans and patient contours may be obtained. Used with treatment planning computers, a three-dimensional representation of the radiation dose to the tumor and normal tissues is obtained, allowing the radiotherapist to select an appropriate plan (Figure 12).

In selecting the optimal plan, the radiotherapist may choose from among one or even several beams for therapy

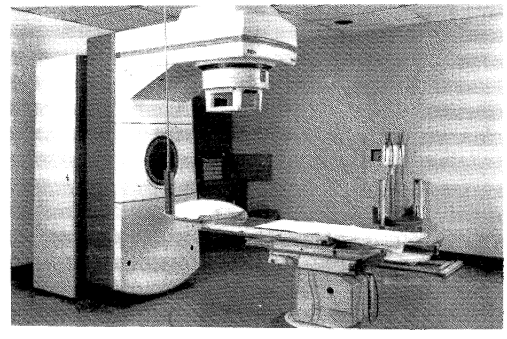

Figure 11b. 6 MeV Linear accelerator.

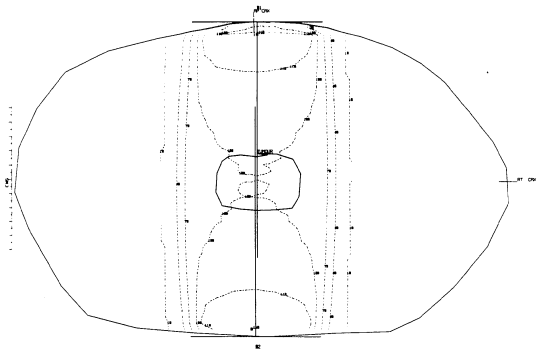

Figure 12a. Opposed AP/PA fields with Co^{60}

(Figures 7 & 9) and from multiple beam arrangements. Among the techniques for beam arrangement include simple opposed AP/PA fields, a four-field "box" technique used often with pelvic tumors; an anterior and lateral field with appropriate compensating wedges; rotation of the beam about the tumor in either a 360° or in discontinuous arcs. A variety of factors determine the choice of the field arrangement, but in general the aim is to minimize the dose to the normal tissue while providing uniform coverage within the tumor volume. In addition, secondary blocking with cerrobend, a low-melting-point alloy, may be fashioned for irregular fields. Finally, compensating filters may be inserted in the beam path when variation in the patient's contour would result in a relative overdosage of the thinner portions of the patient.

Finally, the dose of radiation to each of the several volumes is specified and calculated. The determination of the dose is based on a complex relationship between the tumor burden, its sensitivity to radiation, and the normal tissue tolerance. It is not unusual to employ a series of "shrinking fields" or boost volumes to distribute the dose in accord with the number of tumor cells present. This, the initial volume which includes subclinical sites of involvement, are not treated to tolerance. Rather, only the final treatment volume which includes the gross disease is treated to tolerance.

As the actual treatment begins and proceeds, port films are obtained in the treatment position. While such films lack the clarity of the original simulator films, they serve as a quality control on the daily treatment.

It should be clear that the delivery of radiation therapy is not performed solely by the radiotherapist. While the individual ultimately responsible for treatment is the physician, the medical physicist, dosimetrist, and technologist are part of that team. The physicist is responsible for assuring that treatment units are accurately calibrated and performing to specification. The dosimetrist, under the physicist's direction, assists in treatment planning and dose calculation, checks treatment records for accuracy, and fabricates treatment aids such as the blocks and compensators alluded to above. Radiotherapy technologists, registered by the American Registry of Radiology Technology, by virtue of their special training are able to deliver the actual treatment according to the treatment plan. Oncology nurses, along with the physician, monitor the patient's progress during treatment and assist in the care of the acute side effects of therapy.

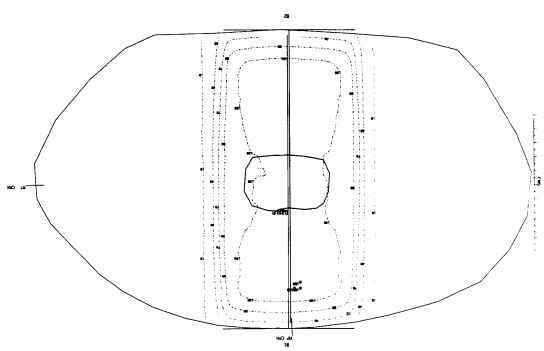

Figure 12b. Opposed AP/PA fields with 18 MeV

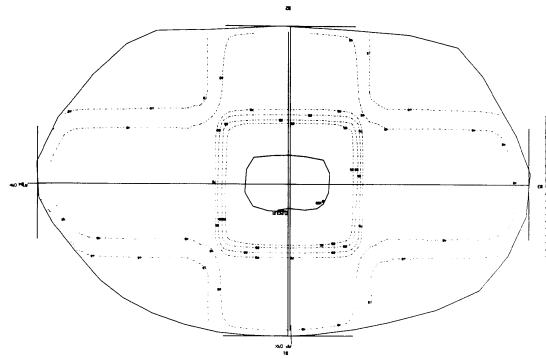

Figure 12c. "Box" technique (AP/PA and lateral fields with 18 meV. Notice that with Co^{60} (12a) the dose to the subcutaneous tissue is greater than that to the tumor. While the opposed AP/PA 18 MeV field is better, with the "Box" technique (12c) the dose to the tumor is more homogeneous and the high dose volume more limited.

Results of therapy

In 1983 it is estimated that there will be 855,000 patients diagosed with cancer in the United States (Cancer Facts and Figures 1983); if skin cancers are included, this figure increases to over 1.2 million. Radiation, for either palliation or cure, will be utilized in over half of these patients (Committee for Radiation Oncology Studies 1981).

Improvements in the treatment results with improved equipment and greater knowledge of behavior of the disease, are reflected in the contrast between the survival statistics drawn from 1955 and 1970; Hodgkin's disease, 30 to 35% survival in 1955 vs 70 to 75% survival in 1970; prostate cancer, 5 to 15% survival in 1955 vs 55 to 60% survival in 1970; bladder, 0 to 5% survival in 1955 vs 25 to 35% survival in 1970 (35).

Further refinements in therapy are anticipated to further improve results. Already the Patterns of Care Study's survey of radiotherapy facilities suggests this with disease-free survival of 75 to 97% for early cervix, Hodgkin's disease, testicular tumors, oral cavity malignancies, cancer of the larynx and prostate (72).

The choice to use radiation therapy in the management of cancer is ultimately determined by the results of the therapy. A full discussion of the indications and results of treatment

are indicated in most standard text books (53, 101, 107) and beyond the scope of this chapter.

In general terms, one may speak of the strategies behind the application of radiation. While not comprehensive in scope, these strategies with some clinical examples may provide an introduction to the clinical experience with radiation therapy.

For localized disease radiation is used alone or in combination with surgery or chemotherapy.

Radiation alone is employed for localized malignancies particularly sensitive to radiation, resulting in a favorable therapeutic ratio; malignancies which while not uniquely sensitive relative to normal tissue, can be boosted with a shrinking field technique or with brachytherapy; limited tumors where resection would result in unacceptable cosmetic or functional impairment.

Among malignancies characterized by a particular sensitivity to radiation are Hodgkin's disease and seminomas. The sensitivity of Hodgkin's disease was appreciated by early radiotherapists (60, 111), although the medical profession remained generally pessimistic until the 1960s. Kaplan (83) was able to demonstrate that modest doses of 4000 rad controlled local disease in 95% of the cases. Subsequent work (92) suggests that the plateau for control may be still lower. To be effective, however, the radiotherapy technique employed must encompass large irregularly shaped fields, the mantle and the inverted "Y" or the spade (85), to treat multiple lymph node chains in continuity. Among patients with disease and without systemic symptoms (Stages IA and IIA) five-year survivals of 84 to 100% have been reported (75, 123).

The sensitivity of seminomas exceeds that of even Hodgkin's disease. With a preoperative dose of 1000 rads in 10 days, Friedman (59) was able to demonstrate complete histologic destruction of the tumor on pathologic review. With the current practice of orchiectomy followed by irradiation of the draining nodes (2500 to 3000 rads for subclinical disease, 2500 to 3500 rads for most gross disease, and up to 4000 rad, using a shrinking field technique, for large masses) excellent results are obtained. Survival of patients with Stage I disease approaches 100%, and in Stage II 80 to 90% (25).

In contrast to the examples of seminoma and Hodgkin's disease, to which one might also add some non-Hodgkin's lymphomas, which display a unique sensitivity to radiation, carcinomas of the cervix and prostate do not. To improve the therapeutic ratio in these diseases, the use of shrinking fields or brachytherapy boosts are used.

In the case of advanced, inoperable, cervical tumors, 4500 to 5000 rads are delivered to the pelvis, followed by a boost, ideally with intracavitary sources within the uterus and vagina, or with a reduced field. With the use of intracavitary applicators and the rapid fall-off in dose which they provide, the dose to the rectum and bladder are minimized. Utilizing this technique, even with patients whose disease extends to the pelvic wall or lower vagina, cures may be expected in 35 to 45%, and with lesser degrees of extracervical extension, 60 to 85% (102). Employing careful tumor localization and treatment planning of the primary and boost fields megavoltage equipment has also provided good results in prostatic cancer. Ray and Bagshaw (117) have reported 71% actuarial survival when disease is limited to the prostate, and 41% when the disease has extended beyond the gland.

Finally, radiation may be recommended for limited tumors such as those of the lip or larynx where surgery might give an unacceptable cosmetic or functional result. As in other sites, attention must be paid to careful fractionation of the dose and treatment planning to minimize the risk of normal tissue damage. In laryngeal tumors, where vocal cord mobility is normal before treatment, cure rates of 90% are expected, with useful voice in 95% of the patients treated (74, 150).

Combined therapy with surgery and either preoperative or postoperative radiation is an alternative to the approach of using radiation alone for localized disease. In this approach, radiation in modest doses, 5000 to 6000 rads, is used to control subclinical disease while the surgical procedure removes the gross disease (52).

In the case of early breast carcinoma, a "lumpectomy" followed by radiation to the breast and regional nodes gives results comparable to radical surgery and a better cosmetic result (i.e., an intact breast). Survival at five years was 91% in stage I, and 60% in stage II patients (115). A similar approach has been employed in the so-called radiation resistant soft tissue sarcomas in lieu of amputation (97) and in epithelial tumors of the head and neck (41, 55).

In certain tumors such as small cell carcinomas of the lung and acute lymphocytic leukemia, multiagent chemotherapy is the major treatment modality. Nevertheless, radiation therapy may play an adjuvant role even for these diseases. In both cases it is appreciated that the blood-brain barrier provides a sanctuary site from chemotherapy. When whole-brain irradiation (3000 rads) is added to the management of small cell carcinoma, the incidence of subsequent brain metastasis declines from 30% to 5% (36). In leukemia, the CNS irradiation reduced the frequency of relapse from 50% to 10% or less (79). Chest relapses were also a problem with chemotherapy for small cell carcinoma, occurring in 60 to 70% of the patients (33). When moderate doses of radiation were added, the thoracic failure rate declined to less than 20% (24).

Thus, radiation may be used as the sole modality of therapy, or as part of the integrated management of malignancies along with surgery and chemotherapy.

SUMMARY

The concern of the radiation oncologist is the patient with cancer. To provide that patient with optimal therapy requires the clinical acumen to evaluate the patient and alternative modes of treatment. In the exercise of radiation therapy, the practitioner is assisted by clinical physicists, radiation therapy technologists and a nursing staff. Innovations in therapy treatment units, tumor localization and dosimetry offer the hope for improved survival and quality of life. An understanding of physics and radiation biology of radiation therapy provide not only the rationale for current practice but also the framework for future improvements.

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POSTSCRIPT

The failure of radiation to control a malignancy may reflect either a geographic miss of the disease, the inherent resistance of the tumor to radiation relative to normal tissue(s), and/or a large tumor burden. Research efforts in radiation oncology have sought to overcome these obstacles.

Computerized tomography (CT) and magnetic resonance imaging (MRI) are used increasingly to better define the radiation field and avoid the problem of geographic mass.

In clinical trials, hyperthermia, neutron radiation and unconventional fractionation are being studied in an attempt to address the factors which may contribute to tumor resistance.

Data on the effects of heating suggest that hyperthermia may preferentially damage hypoxic cells, cells with a low pH and cells in S phase and preferentially damage tumors over normal tissue (20). Meyer (17) has summarized the results of clinical trials of radiation with or without heating. With radiation alone, the response rates ranged from 7 to 42%, while combined radiation and hyperthermia resulted in responses from 47 to 87%. In a randomized trial, Arcangeli and colleagues (1) achieved a 75% tumor control with the combined treatment vs. 40% with radiation alone.

Neutron radiation reduces the kinetic and hypoxic gain of tumors relative to normal tissue and eliminates repair as a cause of failure. For squamous cell carcinoma of the head and neck, Catterall (2, 3) found an apparent advantage to neutrons; this was confirmed in a small trial of the RTOG (8). However, subsequent studies from Edinburgh (5) from the RTOG (10) failed to show a similar improvement except in a subset of patients with positive nodes (9). For inoperable salivary gland carcinomas the advantages of neutron radiation are more apparent with an improvement of the control rate from about 25% photons to 67% with neutron (19). These findings have been confirmed in a prospective trial (11). An advantage to neutron therapy was also appreciated in patients with advanced prostate cancer. Patients treated with a combination of photons and neutrons (mixed beam radiation) had significantly fewer local failures and longer survival than patients treated with photons (14).

Peters and Ang (18) have recently reviewed the status of unconventional fractionation schemes. Typically, radiation is given in fractions of 180–200 cGy five times per week in hyperfractionation, the total dose and the number of fractions each day are increased while the dose per fraction is decreased and the overall treatment time is unchanged. Theoretically this should allow for an increased the total dose, a better change of tumor sensitizing by redistribution and less dependence of the presence of oxygen. Promising preliminary results have been reported for selected cancers of the oropharynx (12). In accelerated fractionation, the overall treatment time is reduced and the dose per fraction is unchanged but two or more treatments are given daily. The rationale is to minimize tumor regrowth between fractions without affecting normal tissue tolerance. In a comparison to historical controls, there has been some improvement in the local control of head and neck cancers (13, 22).

To reduce the tumor burden, radiation has been employed with both surgery and chemotherapy. Cytoreductive chemotherapy has been given prior to definitive local radiation to reduce the tumor

burden in advanced breast (6) or head and neck cancers (23) although the efficacy of this approach is still uncertain (21).

Alternatively, chemotherapy has been given concomitantly with radiation to sensitize the malignancy; this approach has met with favorable results for anal cancers (4, 15). Radiation has long been used as an adjuvant to surgery in advanced head and neck cancers (16) rectal cancers (7) and malignancies traditionally felt to be resistant to radiation such as sarcomas (24).

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13

HYPOXIC CELL RADIOSENSITIZERS

GEORGE H. HARRISON and JEREMY WRIGHT

INTRODUCTION

The possibility that the enhanced radioresistance of hypoxic tumor cells may be the limiting factor in the local control of some tumors treated in clinical radiation therapy has been a focus for research and clinical study for the past 30 years. Several approaches to this problem have been investigated, including the use of densely ionizing particle radiation therapy and hyperthermia discussed elsewhere in this book. Oxygen is a potent chemical radiosensitizing agent which happens to be present in most normal tissues. This realization led to the trial of hyperbaric oxygen combined with radiation, and to the use of radiation sensitizing compounds designed to diffuse into hypoxic tumors and then mimic oxygen as a radiosensitizer. The concept of hypoxic cell radiosensitizers has led to a comprehensive systematic program in basic and clinical research over the last decade. As the first topic in this chapter, the oxygen effect itself will be reviewed, and evidence for tumor hypoxia as a limiting factor in the local control of some human tumors will be outlined. Then, the history and current status of preclinical research in hypoxic cell radiosensitizers will be presented, including the development of non-nitro electron-affinic compounds. This review of the underlying research in sensitizers is designed to clarify the trends in a future sensitizer development, as well as the clinical manipulations undertaken to improve the response to current sensitizers. Finally, the clinical use of hypoxic cell radiosensitizers over the past 10 years will be reviewed.

In addition to hypoxic cell radiosensitization, another method of differential radiosensitization is to supply radiotoxic thymidine analogs during DNA synthesis in rapidly dividing tumor cells. The use of bromodeoxyuridine for this purpose has attracted renewed clinical interest, and has recently been reviewed elsewhere (55).

Review of the oxygen effect and evidence for hypoxia as a limiting factor in radiation therapy for some tumors

The early history of the discovery of the oxygen effect has been outlined by Hall (47). In independent experiments over the period 1910 to 1935, evidence for the oxygen effect was observed in various test systems, but misinterpreted as being related to metabolism (77), rate of cell division (49), respiration (27), or blood flow (61). The first correct interpretation was probably that of Petry (66). The first quantitative estimates of the oxygen effect were published by Read (74). Following the first publication of a quantitative dose-response relationship (survival curve) for mammalian cells irradiated with X-rays (70), Dewey appears to have been the first to publish what is now considered a typical description of the oxygen effect on the cellular level (30). Typically, oxygen potentiates the cytotoxic effects of X- or γ-irradiation by a factor ranging from 2.3 to 3.0. That is, 2.3 to 3.0 times less dose under oxic conditions is required to bring about the same cellular lethality observed under hypoxic conditions. The factor of 2.3 to 3.0 is known as the oxygen enhancement ratio (OER). This effect is often observed to be independent of the level of lethality, so that the shape of the dose-response curves under hypoxic and oxic conditions is the same, and oxygen is said to be a dose modifying agent. The concentration of oxygen required for potentiation is low; 50% of the total achievable potentiation occurs at a partial pressure near 3 mm of mercury (42). Oxygen must be present during irradiation, not before or after, in order to produce the oxygen effect. The most accepted model of the mechanism of the oxygen effect was advanced almost 30 years ago (88). The model is based on the idea that radicals produced by ionizing radiation events near critical targets within the cell competitively either undergo restitution by intracellular hydrogen-donating compounds or are permanently fixed as sites of radiation damage by oxygen or other electron-affinic agents.

With a few exceptions such as cartilage and testes, normal tissues are sufficiently well oxygenated to experience the full oxygen effect. Radiation therapy has found successful applications in spite of the fact that normal tissues experience the oxygen effect, whereas many tumors are suspected of harboring viable but hypoxic cell populations not experiencing radiation potentiation by oxygen.

What is the evidence that tumor hypoxia may be a limiting factor in the radiation therapy of some tumors?

A pioneering paper (88) attracted the attention of radiation therapists by showing histological evidence of necrosis in the central cores of human bronchial carcinoma. Tumor tissue was in the form of cylindrical cords surrounded by vascular stroma so that nutrition and oxygen were supplied to tumor cells only by diffusion from outside the tumor. It was observed that for cord radii exceeding about 200 micrometers, there was always a necrotic central core.

Since these dimensions were consistent with calculated diffusion lengths of oxygen in tissue, the histological observations were interpreted as evidence for hypoxic foci in tumors, with hypoxic but viable cells on the edge of necrotic areas being potentially radiation resistant.

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An extensive body of radiation research literature has been published on experiments designed to elucidate the role of hypoxia in animal and human tumors. While a few of these reports involved direct measurements of oxygen tension within tumors (36), most of them give only indirect evidence of tumor hypoxia or are based on experimental tumor lines which are cited as being hypoxic, but the citation provides only indirect evidence for tumor hypoxia. Typically, a tumor is assumed to contain hypoxic cells; then external modifications such as asphyxia, high-pressure oxygen, or tumor clamping are applied, and alterations in radiation response of the tumor are noted to be consistent with the hypothesis of the role of radiation-resistant hypoxic tumor cells. More recently, however, detailed studies (without radiation) in the field of tumor microvasculature have clearly demosntrated tumor hypoxia in animal systems by direct measurements with micrometer-diameter polarographic probes in sufficient detail to provide oxygen tension profiles with adequate resolution and statistics (93).

Accepting the evidence for hypoxic cells affecting the radiation response of tumors, radiation experiments of the type developed by Powers and Tolmach (69) yield quantitative estimates of the proportion of hypoxic cells as surveyed in various experimental animal tumor systems reviewed by van Putten and Kallman (92). In this survey, hypoxic cell percentages ranged from 1 to 50%, with typical values around 15%. If these results were applicable in human tumors, they could imply that hypoxic tumor cells can be a limiting factor in the radiation therapy of some human tumors. However, an important additional factor needs to be introduced before the role of sensitizers can be evaluated: the phenomenon of reoxygenation in solid tumors. According to the radiobiological assays for determining the proportion of hypoxic tumor cells as a function of time post-irradiation, it was found that a significant proportion of hypoxic cells surviving irradiation became oxic in a matter of a few days or in some cases hours after post-treatment (53, 92). If this phenomenon did not occur, then one would expect the proportion of hypoxic (radiation resistant) cells to increase after treatment. Instead, the proportion of hypoxic cells usually remains approximately constant through a fractionated radiation delivery sequence. This means that the clinical problem posed by hypoxic tumor cells is less serious in fractionated treatment than in the case of single-dose therapy, and the efficacy of proposed hypoxic cell radiosensitizers must be evaluated taking radiation treatment schedules into account.

Several alternative approaches to overcoming the radiation resistance of hypoxic tumor cells have been attempted in the clinic. An early and obvious approach was the use of hyperbaric oxygen during irradiation. In a careful evaluation of the effect of hyperbaric oxygen in one animal tumor system, oxygen at four bar was shown to eliminate hypoxia in all regions of the tumor (62), although it must be pointed out that intercapillary distances within the tumor did not exceed 180 micrometers. Extensive clinical trials involving long-term evaluation of more than 1500 patients have been reviewed (32). For radiation therapy of head and neck tumors, studies have indicated a statistically significant benefit from hyperbaric oxygen both in terms of tumor control and survival over periods from one to four years (48). Other head and neck trials have shown benefit from hyperbaric oxygen, but not at statistically significant levels. Radiation therapy of bladder cancer in 236 patients was not improved by hyperbaric oxygen, while carcinoma of the cervix responded equivocally to this therapy (32). Although it seems that definite clinical benefit could be derived from the use of hyperbaric oxygen for some tumors, little work in this area has been initiated recently, perhaps due in part to the cumbersome and ominous-appearing facilities and techniques required. More enthusiasm seems to have been generated for hypoxic cells sensitizers, a more convenient but still unproven modality.

The use of heavy particle radiations (neutrons, protons, and other charged particles) in place of x-rays and γ-rays was promoted partly in response to the challenge of tumor cell hypoxia, since most of the particle radiations are more densely ionizing than conventional radiation, leading to a reduction in the OER. This topic is considered in more detail Chapter 14, Volume IX. The use of hyperthermia alone or in conjunction with radiation or chemotherapy is currently undergoing clinical trials and development. The expected therapeutic benefits of hyperthermia are based on several rationales, including the elimination of differential cell sensitivity as a function of oxygen tension when heat alone is used as the treatment modality. These topics are described in more detail in the Chapter 15, Volume X "Hyperthermia".

Preclinical studies

In this section, we shall review the basic mechanisms of action of the electron-affinic compounds which are currently being developed as hypoxic cell radiation sensitizers. In addition to aiding the development of new sensitizers, a better understanding of mechanisms has led to more sophisticated treatment strategies including the use of altered drug delivery sequences and added pharmaceutical agents to enhance sensitizers efficacy. We will then review past studies *in vitro* and *in vivo* which set benchmark standards now exceeded in current (1980–83) studies. These current studies will be treated in some detail in order to show possible directions for clinical progress.

Mechanisms

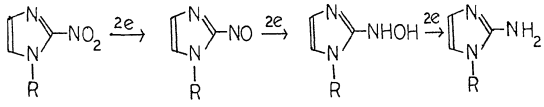
The mechanism of action of a group of drugs can give valuable information to researchers who intend to develop new drugs which are improvements over the existing compounds. For this reason we include brief discussion of the research leading to the present theories of the action-mechanism of nitro compounds which are hypoxic cell radiosensitizers. Since this is not intended to be an exhaustive review, the reader is referred to comprehensive papers (85, 96), for more detailed discussions.

Electron-Affinity

It has been known since the early 1960's that a number of compounds had the ability to mimic the effect of oxygen in overcoming the radioresistance of hypoxic cells in tumors. As early as 1963, Adams and Dewey (1) hypothesized that the sensitization of resistant cells depended on the electron affinic properties or redox potential of active compounds. In 1969, Adams and Cooke investigated the radiosensitizing

properties of a large number of chemicals, and concluded that those compounds which were the most effective in the radiosensitizing process were those which were the most electron affinic. Later, Adams *et al.* (5) were able to correlate the one-electron reduction potential of a number of nitro-compounds with their radiosensitizing efficacy. This work was expanded by Adams *et al.* to incorporate the partitioning properties of nitroimidazoles as well as the electron affinities (3). A study of partition coefficients was also reported by Brown *et al.* (17, 19). A number of authors agree that although the physical properties, including the partition coefficients, are important for transport to the site of action, the primary mechanism involved in the radiosensitizing action of the nitroimidazole and related compounds are the redox properties manifested by their electron affinities (19, 39, 96). Although a number of other parameters have been used in attempts to show structure activity relationships (Hammet correlations (71), polarography (43), electron spin resonance (58)) the one-electron reduction potential gives the most useful correlations in *in vitro* systems. It seems reasonable to assume, therefore, that the reduction of nitro compounds is involved in the radiosensitizing action. The effects of partition coefficient on the *in vivo* action will be discussed later.

Addition of electrons is a process of reduction, and in organic systems, the stepwise conversion of a nitro group to an amino requires the acceptance of two electrons at each stage: For 2-nitroimidazoles the sequence is:



Overall, the conversion of a nitro compound to an amine is a six-electron reduction.

The process does not necessarily involve the addition of two electrons at a time, but goes via one electron intermediates. The first step of the reduction may be represented by:

$$ArNO_2 + e^- \rightarrow ArNO_2^{\cdot -}$$

The resulting nitro radical anion cannot be isolated but is detectable by electron spin resonance. The generation of one-electron reduction products by pulse radiolysis, and the measurement of the unstable reduction products has proved to be a powerful tool in the quantitation of one electron transfer equilibria (58, 94, 95). In addition, it provides a clue to the mechanism of action of the radiosensitizing nitro compounds. The radical intermediates are unstable and highly reactive species. Although conclusive evidence is not available to identify specific areas and reactions which take place in test systems and *in vivo*, it is likely that the efficacy of radiosensitizing agents is governed by electron-transfer reactions between the drug and target areas in susceptible cells. The exact nature of these target areas has yet to be determined.

Depletion of cellular thiols

Some recent studies have focused on the effects of pretreatment of hypoxic cells with electron affinic radiosensitizers (12, 47). Prolonged incubation of cells with misonidazole or other nitro compounds prior to x-ray exposure results in an "Extra Ratio" (47). The sensitizers are more effective after prolonged pre-irradiation incubation of hypoxic cells than if the radiation is administered immediatedly after drug exposure. The efficacy correlates well with the depletion of non-protein sulfhydryl compounds (NPSH) and with the time of incubation (47). In contrast, compounds such as N-ethylmaleimide or diamide deplete thiols under both aerobic and hypoxic conditions (14, 21). This suggests that misonidazole and similar nitro compounds, under hypoxic conditions, are reduced to reactive metabolites which can combine with NPSH and interfere with the ability to repair cell damage post-irradiation. Under aerobic conditions, the unaltered nitro compounds are unable to react with NPSH. Evidence to support a metabolic action is provided by Anderson and Patel who utilized strains of *E. coli* which differed in their nitroreductase ability (9).

The enhanced effectiveness of these drugs following preincubation is due to the fact that two different mechanisms of cell destruction appear to be operating. This phenomenon is even more pronounced in compounds which have good chemical leaving groups present in the molecule in addition to a nitro group (85). The result of the dual mechanism of action is that these compounds are much more effective as radiosensitzers than their electron affinity would predict. Electron affinic alkylating agents appear to be particularly effective (85), and may lead to clinically useful compounds.

Importance of partition coefficient

The current limiting factor in the clinical use of sensitizers is neurotoxicity (24, 33). This toxic effect is probably due to the exposure of neural tissues to the 2-nitroimidazole, which in turn is governed by the ability of the drug to cross the blood-brain barrier. Some current reasearch on the nitroimidazoles has, therefore, been directed at compounds which have similar electron affinities to misonidazole, but lower partition coefficients. Brown *et al.* (17, 19) have shown that the octanol/water partition coefficient (P) has little effect on the efficiency of compounds over the P range of 0.05–0.40, but that below 0.04, the sensitizing efficacy was markedly decreased. They concluded that the lipophilicity of a radiosensitizer can be decreased to only 1/10 that of misonidazole without losing effectiveness, since transport into cells is much slower at low P values.

Presently, therefore, efforts are focused on the synthesis of compounds which are much more electron affinic than misonidazole, and which have much lower partition coefficients. Two promising compounds resulting from this research are Ro 03–8799 (81) and SR-2508 (16).

In cell culture, Ro 03–8799 has been shown to be 5–10 times more potent than misonidazole. *In vivo*, however, this compound is not so effective (100). Since Ro 03–8799 possesses a basic group and is ionized at physiological pH, it is probable that distribution into the hypoxic region of the tumor may be adversely affected, and thus results in the striking difference between *in vitro* and *in vivo* effects.

The importance of ionization and of pharmacokinetic studies in assessing the ability of nitroimidazoles to penetrate into the hypoxic areas of tumors in sufficient concentration to produce the desired effect has been stressed by several authors (86, 98).

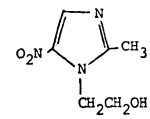
Preclinical results

In the original paper suggesting electron affinity as a mechanism for hypoxic radiosensitization (1), N-ethylmaleimide, diacetyl, and benzophenone were among the first compounds to be identified as electron-affinic radiosensitizing agents. Numerous additional hypoxic cell sensitizers were then listed, along with an elaboration of the electron affinity hypothesis (2). These compounds, including unsaturated diesters, diketo compounds, indanetrione, quinones, and several others, were found to be hypoxic radiosensitizers of the bacterium *Serratia marcescens*. Bacterial response constituted a convenient screening assay for new sensitizers, but the ability to sensitize mammalian cells without undue cytotoxicity is a more relevant test; in 1971 para-nitroacetopheneone (PNAP) was shown to increase the radiation sensitivity of hypoxic V-79 cells (Chinese hamster lung) at drug concentrations not affecting colony forming ability (Adams, 1971). To obtain a given level of cellular lethality under hypoxic conditions, 1.7 times less radiation dose was required in the presence of PNAP. By analogy with the definition of OER, this dose ratio is designated by the sensitizer enhancement ratio (SER). In 1972, Chapman *et al.* demonstrated mammalian cell SER values ranging up to 2.2 for nitrofurans in clinical use: nitrofurantoin, nitrofurazone, and nifuroxime. However, *in vivo* efficacy of these compounds was precluded by their poor solubility and low drug concentrations achievable in tumors (73).

Meanwhile, PNAP was found to be too water insoluble for *in vivo* use, but the related soluble compound p-nitro-3-dimethyl-propriophenone hydrochloride (NDPP) was effective in sensitizing hypoxic mouse skin to radiation (28). This was probably the first *in vivo* demonstration of hypoxic cell radiosensitization. In 1974, Berry and Asquith (11) reported an SER of 1.6 for NDPP injected directly into P388 cells growing intraperitoneally in ascitic suspension. But investigations of NDPP ceased after it was realized that intraperitoneal or intravenous injections of NDPP produced little solid tumor sensitization in the test systems under study, due in part to the short biological half life of the compound (80). By this time in the development of radiosensitizers, investigators were beginning to focus the pharmacological requirements of chemical and metabolic stability, of solubility in water or lipids, and of the ability to diffuse adequately from capillaries to hypoxic cells.

2-Methyl-5-nitroimidazole-1-ethanol, metronidazole (Flagyl) was the first sensitizer used clinically. It had a previous clinical history as a trichomonacide, so that its pharmacology and toxicology were well known. In 1973 Foster and Willson (38) first published an *in vitro* result for metronidazole. The SER was 2.0 compared with an OER of 2.8. Subsequent studies with various mouse tumor systems yielded SER values from 1.2 to 1.8 with drug concentrations ranging from 0.1 to 2.5 mg/g body weight as reviewed by Fowler and Denekamp (39). Metronidazole was not considered to be a particularly potent sensitizer and was less electron-affinic than other compounds such as misonidazole, but the proven low toxicity of metronidazole was taken as an indication for clinical trial. The results of clinical use of metronidazole will be reviewed later in this chapter.

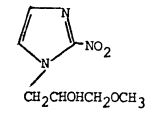
Metronidazole was soon superseded by misonidazole (1-(2 - nitro - 1 - imidazolyl) - 3 - methoxy - 2-propanol),



Metronidazole

MISO, which was recognized as a potent radiosensitizer with seemingly acceptable levels of toxicity. This compound has been studied extensively during the past 10 years, and a summary of preclinical findings is useful in evaluating current progress with sensitizers.

Single-dose SER values and toxicity: Early papers indicated that *in vitro* single-treatment ER values approaching the OER could be achieved using drug concentration below cytotoxic levels (10, 45). In aerated cells, MISO produced no detectable effect alone or in combination with radiation. Single-treatment *in vivo* studies, mostly with murine tumor models, indicated somewhat lower but still encouraging SER values typically between 1.5 and 2. Many of these studies have been evaluated and compared in a comprehensive review paper (39). The single-dose results seemed encouraging since even a modest increase in SER could theoretically lead to a large therapeutic gain factor in the treatment of some tumors; however, it was also recognized that sensitizer efficacy had to be evaluated in the context of clinical multi-fraction therapy, which was expected to lower the SER for several reasons discussed below. At this point in the investigation of MISO, the LD_{50} in mice had been measured to be around 2 mg/g, or about half that of metronidazole, and investigators in radiobiology were aware (29) that repeated high doses of metronidazole and MISO caused central nervous system damage in dogs (79). However, the potent and selective action of MISO on hypoxic cells stimulated further research leading to clinical trials.



Misonidazole

Pharmacokinetics of MISO

The concentration of MISO as a function of time post-injection has been measured in serum, tumors, and critical normal tissues. The tumor concentration at the time of irradiation determines efficacy as a hypoxic cell sensitizer, while the cumulative dose to critical, limiting normal tissue was originally thought to govern toxicity.

Most of these data refer to mice, and the extrapolation to man has not been straightforward. For example, the serum half life in mice is 1 to 1.5 hours versus 10–18 hours in man. The maximum tolerated single dose is in excess of 1 mg/gm of administered MISO in mice, but less than 1/10 of that in man. A more reasonable intercomparison of sensitization results would involve measurement of tumor levels at the time of irradiation. Polarography can be used to detect the total level of nitro products, but does not distinguish between MISO and some of its metabolites. Spectrographic and chromatographic techniques are used to identify MISO

concentrations in plasma or tumor more accurately. Tumor levels have been found typically to be somewhat lower in tumor than in plasma in the mouse and in man. The ratio of drug concentration in brain and in tumor has been suggested as a therapeutic index for prospective sensitizers if neurological damage is the limiting toxicity (18). This approach, while reasonable for CNS effects, may not be predictive for peripheral neurological damage.

Neurotoxicity of MISO

Neurological symptoms in patients in the early clinical trials triggered a concentrated effort in animal-based research to understand this problem. Several specialized assays have been developed to study neurological damage from radiosensitizers (24, 25, 26, 57, 75). Such testing is considered necessary in the evaluation of newly proposed nitro-based sensitizers; however, it is not clear that neurotoxicity will necessarily be the dose-limiting factor in future non-nitro sensitizers.

Hypoxic cytotoxicity

MISO seems to be typical of many nitroimidazole sensitizers in causing hypoxic cell toxicity after several hours of exposure. This effect with MISO was clearly demosntrated in 1975 (45). Following an original suggestion by Sutherland, interest has developed in exploring the clinical possibilities of the cytotoxic properties of hypoxic cell sensitizers alone or in combination with other chemotherapeutic agents (87).

The hypoxic cell cytotoxicity of MISO has itself been found to be enhanced by ionizing radiation, so that the shoulder of *in vitro* survival curves as a function of incubation time in MISO decreases as a prior dose of radiation increases (56). Michaels *et al.* also studied the interaction of radiosensitization and hypoxic cell toxicity *in vitro* and found that the toxic effects are at least additive and not independent (59).

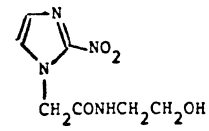
MISO plus drugs to improve efficacy

Added drug therapy has been attempted in many ways in order to reduce neurotoxicity without interfering with radiosensitization. Murine MISO toxicity measured by survival, weight gain, and neurological function was reduced by concomitant administration of pyridoxine (35).

Recalling the possible sensitizing mechanisms based on thiol depletion, added compounds which aid in this process might increase sensitizer efficacy. Pretreatment with buthionine sulfoximine inhibits the production of sulfhydryl glutathione (GSH) while pretreatment with compounds such as diethylmaleate (DEM) depletes existing GSH. Intracellular GSH levels can thus be significantly reduced so that radiation and MISO might be more effective (20, 85). The question of added toxicity resulting from the combined drugs must be assessed.

New nitro-based sensitizers

One approach to achieving improved sensitizer performance is to reduce neurotoxicity by reducing lipophilicity (15). N-(2-hydroxyethyl)-1-(2-nitro-1-imidazolyl)-acetamide (SR-2508) has been evaluated with this in mind (16). Its octanol/water partition coefficient P is only 0.046, leading to decreased neural uptake and reduced neurotoxicity in the mouse. At the same time SER values in mice are similar to those of MISO. These encouraging results have been confirmed by other workers (83). SR2508 has now completed Phase I trial. An earlier attempt along these lines was the use of the MISO metabolite desmethylmisonidaze (Ro-05-9963) which has a shorter biological half-life than MISO, and P = 0.1 leading to reduced uptake in the brain (99). In spite of these properties, the problem of peripheral neuropathy was not improved in clinical evaluations of desmethylmisonidazole.



SR-2508

Another approach to improved sensitizer performance has been to identify compounds more efficient than MISO, while hopefully not more toxic. The 2-nitroimidazole Ro 03-8799 seems to achieve this according to *in vitro* tests. Because the *in vitro* results showed that this compound was 5-10 times more efficient than MISO and because of the expected rapid elimination of the drug, it was selected for evaluation in the United Kingdom (63). However some reports seem to indicate that Ro-03-8779 is no better than MISO *in vivo* (100). As discussed in the section "Mechanisms", part of the problem may be concerned with tumor drug distribution, which may be pH-dependent because of the charge state of the compound. On the other hand, 2-nitrobenzimidazole as studied by Wright *et al.* (101) is charged but exhibited strong tumor sensitization.

In a similar attempt to reduce the neurotoxicity of misonidazole, Adams *et al.* have investigated 1-(2-nitro-1-imidazolyl)-3-(1-aziridinyl)-2-propanol (RSU-1069) (7). As this compound contains a basic side chain, it was hoped that this would prevent its penetration into neural tissues. This compound has a dual mode of action. It acts similarly to misonidazole as a radiosensitizer, but because it also contains an aziridine ring, it can also act as a monofunctional alkylating agent. This compound has shown very high radiosensitizing efficiency *in vitro* and *in vivo* (6). The dose-limiting toxicity of RSU-1069 now occurs in the gut, and work is underway to moderate this problem (8).

4-Bromomisonidazole was synthesized and tested recently (72). The drug was stable *in vivo*, and radiobiologically and pharmacologically similar to MISO except for high lipophilicity. Since it can be labelled with 82_{Br}, bromomisonidazole may be useful in imaging hypoxic tumor tissue.

The 5-nitroimidazole ornidazole (Ro-7-0207) was included in one of the original *in vitro* surveys which singled out MISO for its promise as a sensitizer (10). Reexamination of the data presented in this paper reveals that ornidazole was nearly as efficient a sensitizer as MISO. In a more recent paper (64), the *in vivo* effectiveness of ornidazole was measured using $LD_{50.6}$ in mice with hypoxia induced by near

asphyxiation. An SER value around 1.4 was achieved following single-dose irradiation. Since this compound, like 5-nitroimidazoles in general, is less electron affinic than MISO, it was felt it might produce less neurotoxicity while sensitizing hypoxic cells.

Several investigators have recognized the potential of nitrobenzimidazole compounds in terms of the expected high electron affinity and the great flexibility offered by the ring structure for subsequent molecular modification. In a survey of 17 2-substituted benzimidazoles, 2 were found to yield SER values around 2.0 *in vitro* (44), while Wright *et al.* found good sensitization in mouse tumors along with favorable normal tissue pharmacokinetics and no initial indications of neurotoxicity, using 50 mg/kg of injected 2-nitrobenzimidazole (101).

A number of hypoxic cell sensitizers have been found to produce unexpected results in terms of sensitization and cytotoxicity. For example, 4-nitroimidazoles appear to be a more efficient radiosensitizer than would be predicted from electron affinity (84). In addition, 5-phenoxysulphonyl-1-methyl-4-nitroimidazole (NSC 38087) is more cytotoxic to aerobic than to hypoxic cells (44).

Non-nitro sensitizers

Studies on the radiosensitizing properties of non-nitro compounds have been much less extensive than those on nitro compounds. Early work was with quinones such as indanedione (14), and indanetrione (ninhydrin) (37, 78). The investigation of these compounds was not pursued vigorously, but the information obtained from the observation of redox potentials and one electron reduction potentials of the quinones proved useful in the development of the more potent nitroimidazoles (65).

Though interest in this area has been sporadic, it seems to have revived recently because of the dose-limiting neurotoxic effects which at present seem to be inseparable from the radiosensitizing properties of the nitro compounds.

Infante et al. reported the activity of isoindole-4,7-dione mixtures. The one electron reduction potentials of the group of compounds were comparable to those of the nitroimidazole sensitizers, and may point to a new area of study (50).

Recently, attempts have been made to correlate the electron affinic properties of non-nitroimidazole derivatives with their radiosensitizing properties. In many cases the results have been disappointing. Several chemical groups (e.g., trifluoroacetyl, cyano), which would by Hammett correlations be predicted to confer radiosensitizing properties upon an imidazole ring, showed little or no activity (97). However, this study did show that furano- and imidazolo compounds substituted in the 2-position with a dicyanovinyl group had significant radiosensitizing activity. In addition, 2,4,5-tribromoimidazole also demonstrated potent activity, probably due to its ability to interfere with oxidative phosphorylation.

Clinical studies

By 1987 we can estimate that over 7,000 patients have been entered into over 100 trials of hypoxic cell radiosensitizers. Reviews covering some of this effort are available (34, 67, 68). Most of these studies have focused on MISO, and results have not produced enthusiasm. The reason is that neurotoxicity has imposed a limit on total MISO dosage, which in turn lowered the SER values to the point of producing no statistically significant gains even with fairly large patient populations. However, a clearer perspective on the eventual benefits expected from improved sensitizers in the near future should emerge from a review of the clinical trials starting with metronidazole, the various clinical strategies employed in order to improve response using MISO, and the current clinical use of improved sensitizers.

Metronidazole. Even though metronidazole has been generally abandoned because of its intrinsically poor sensitizing efficiency compared to MISO, the few clinical trials with metronidazole have suggested improvements in tumor response, if not in long-term survival. In one of the first clinical sensitizer studies, Urtasun *et al.* found statistically significant prolongation of survival when metronidazole was added to an unconventionally high-dose-per-fraction radiation delivery scheme (89). However, metronidazole plus high-dose radiation was not superior to the results of radiation alone delivered according to optimal fractionation schemes. The result was among the first of several trials which together led to the idea that sensitizers delivered in a suboptimal irradiation schedule might yield results equal to those from optimized schedules with radiation alone. The implications of this idea have been explored by Fowler (40). Recently another small trial of metronidazole and high-dose radiation for brain tumors has been reported (54). Although only 19 patients were treated, the increased median survival suggested improvement over the results expected historically from radiation alone delivered in conventional fractionation.

MISO. The first clinical experience with MISO was reported in 1976 (31, 41). By 1977, the United States Radiation Therapy Oncology Group (RTOG) began large-scale Phase I trials of MISO.

As reported by Phillips *et al.* (68), maximal concentrations of MISO in serum were reached between 1 and 4 hours following ingestion, followed by elimination characterized by a half-life of 14 hours. Tumor concentrations were 40–85% of those observed in serum. The Phase I trials revealed a dose-limiting toxicity in the form of peripheral sensory neuropathy, with paresthesias and numbness. In some cases the symptoms have persisted for several years. Phillips reviewed a spectrum of less limiting, more transient effects (68). Included are central nervous systems effects, ototoxicity, and nausea and vomiting. Overall experience with MISO has indicated that cumulative administered doses should not exceed 12 gm/m^2 given in 6 weeks, or 6 gm/m^2 in 1 week. On the other hand, a dose exceeding 2 gm/m^2 is expected to be required to achieve an SER of 1.5 (67). This SER value refers to single-dose treatment of systems whose response is dominated by hypoxic cell radioresistance, and would be diminished in conventional fractionated radiation therapy of less-than-totally hypoxic tumors given repeated low doses of radiation over protracted times during which reoxygenation might occur. Thus the effective expected SER for 2 gm/m^2 of MISO would be considerably less than 1.5. And 2 gm/m^2 could not be achieved for the large number of fractions in conventional radiation fraction schemes, so that novel radiation delivery schedules have been employed in

many Phase II and III trials of MISO. This makes it difficult to evalutate efficacy of sensitizers versus the best known conventional radiation treatment. A review of the various clinical trials reveals that most have employed MISO dosages closer to 1 gm/m^2 per treatment, and it is therefore not too surprising that results of Phase II and III trials have been disappointing.

Several approaches to reduce the neurotoxic effects of MISO have been attempted. Enzyme inducers such as phenytoin and phonobarbital have been shown to hasten the metabolism of MISO resulting in reduced half life (13, 60). Therefore, it seemed reasonable to expect that the peripheral neuropathy would be reduced by concomitant administration of those compounds with MISO. Although measured MISO levels post-irradiation have in fact been reduced by clinical application of phynytoin, peripheral neuropathy has not been alleviated (51). This casts doubt on the concept that there is a direct relationship between the accumulated MISO exposure and the resulting degree of neuropathy. On the other hand, the glucocorticoid dexamethasone was found fortuitously to reduce the severity of peripheral neuropathy, but the mechanism is not clear (51). Dexamethasone is, therefore, being used to increase the maximum tolerated dose of MISO in clinical trials (90).

Although the results so far with MISO have not been particularly encouraging, some interesting arguments have been raised in favor of the effort. Phillips points out that if the effective therapeutic gain factor is only 1.1 or 1.2, it will require careful long-term analysis of large, well-planned Phase III trials to detect small gains. If present, these small gains are nonetheless valuable (68) and pave the way for even better gains with more suitable new sensitizers. Fowler and Denekamp (39) have suggested that if MISO plus high-dose low-friction radiation can equal the results of the optimum radiation delivery scheme for a particular disease, then MISO plus high-dose radiation may be preferred in cases where the optimal scheme is unknown.

Other ideas have been advanced to increase the efficacy of MISO, or to broaden its application. These generally involve in combination with other drugs to increase sensitization (67) or the use of MISO as a cytotoxic agent in combination with chemotherapeutic agents such as 5-fluorouracil (82) or BCNU (90). MISO also has been found to chemosensitize cyclophosphamide, melphalan, and CCNU (18).

However, the direct approach to improving the clinical use of sensitizers has been to replace MISO superior compounds.

SR-2508 and Ro-03-8799: have passed phase I and II clinical trials and are currently being used in phase I and II clinical trials and are currently being used in phase III trials. From the preliminary data that are available (23), it appears that the cumulative dose of SR-2508 that can be administered is about 30 g/m^2. For misonidazole, the cumulative dose was in the range of 10–14 g/m^2. SR-2508 should therefore provide significant benefit, because of the higher doses that can be administered and also because of its favorable tumor plasms drug concentration ratio. The estimated sensistizer enhancement ratio with this drug is about 1.7 based on the measurement of tumor drug concentrations.

Ro-03-8799 has been shown (63) to have a maximum single dose of 1 g/m^2 with no serious observed toxicity. Multiple doses of 750 mg/m^2 can be safely administered. By measuring the tumor levels of Ro-03-8799 it has been estimated that the sensitizer enhancement ratio can reach as high as 1.6 in humans.

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14

HEAVY CHARGED PARTICLES IN CANCER TREATMENT

JOSEPH R. CASTRO

1. STATE OF THE ART

Effective radiation treatment is directed at maintaining structural and functional integrity of normal tissues while delivering tumoricidal radiation doses. Normal tissue is more likely to be preserved when: (a) the volume of irradiated normal tissues is reduced and (b) the biological effect is greater on tumor cells than normal cells. Heavy charged particle radiotherapy offers the potential of achieving this through a combination of advantageous dose delivery and greater biological effectiveness, thus offering an increased therapeutic ratio compared to low LET x-irradiation (23).

A heavy particle is defined as one with a mass greater than an electron. Both charged and neutral particles (neutrons) have been used on tumors. Neutrons have advantageous biological properties, but like x-rays, have energy deposition properties which often lead to irradiation of large amounts of normal tissues surrounding the tumor (11, 12, 16).

Among heavy charged particles, protons, helium and carbon ions appear to have the best dose localization properties; neon and silicon ions share some of the attributes of improved dose localization and in addition manifest an enhanced biological effect, evidenced by a diminished oxygen enhancement ratio, depressed enzymatic repair of radiation damage, diminished variation in radiosensitivity during various phases of the cell division cycle, greater than expected delay in cell division and decreased protective effects of neighboring cells in organized systems (2, 3, 14, 19, 20, 22).

In clinical studies at the University of California Lawrence Berkeley Laboratory (LBL), patients have received heavy charged particle irradiation as treatment of the following tumors (5, 9):

1. (1) Selected, advanced head and neck tumors
2. (2) Locally advanced carcinoma of the esophagus
3. (3) Locally advanced carcinoma of the prostate
4. (5) Non-small cell carcinoma of the pancreas
5. (13) Locally advanced carcinoma of the pancreas
6. (36) Uveal melanoma
7. (38) Chordoma, low grade chondrosarcoma and meningioma of the base of skull and juxtaspinal area
8. (41) Unresectable bone or soft tissue sarcoma
9. (52) Malignant glioma of the brain
10. (M) RBE studies (skin and subcutaneous metastases)

Over 800 patients have been irradiated with heavy charged particles, mostly with helium ions; about 250 patients have received treatment at least in part with heavier particles such as carbon, neon or silicon ions. At LBL, we utilize helium ions of about 232 mev/u, having a range in tissue of about 26 cm; this has proven clinically useful for dose localization therapy. The biological effects of helium appear similar to low LET irradiations and its clinical RBE values appear to be in the range of 1.2–1.4 (relative to 60 Cobalt).

Neon heavy charged particles are produced at LBL with energies of about 400–700 mev/u; the range in tissue reaches about 27 cm at the higher energy. Neon has some of the interesting dose localization attributes of protons and helium ions in its narrow lateral edge penumbra but has a fragmentation tail greater than helium or carbon. Its biological parameters approach those of neutrons with RBE values ranging from 2–3 relative to 60 Cobalt (5, 9, 21).

2. FUTURE DEVELOPMENT

The best results with heavy charged particles to date have been in those anatomical sites where the superb dose localization parameters of these ions can be advantageously utilized such as in tumors at the base of skull, juxtaspinal area, eye, selected head and neck sites or in soft tissue or bone. We must still continue to search for the optimal ion, treatment techniques and tumor sites for precision dose treatment. Thus we plan to continue our studies using the helium and carbon ion beams in collaboration with proton therapy studies underway in several laboratories in the U.S. and other countries.

We will also continue to seek other data of importance in the radiotherapy trial for optimization of therapy including:

1. Silicon and neon RBE data for various normal tissues.
2. Data of value in designing treatment schedules (cell cycle effects, OER data, SLD and PLD repair kinetics, LET and track structure studies).

Table 1. (Chordoma, chondrosarcoma, meningioma, neurilemmoma).

Pts	*Local control*	*Med surv.*	*Comp.*
49 (All)	34 (70%)	21 MOS	Brain nec 2
34**	27 (80%)	26 MOS	Sp cord inj 2 Cran nn 2 Brain nec 1

** previously untreated patients receiving definitive radiation doses

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3. Improved beam delivery and patient positioning techniques for better localized beam delivery and biologically more effective beams.
4. Analysis of the potential of other treatment technique (combined modality studies, predictive assays, hypoxic cell sensitizers or other radiosensitizers and heavy particles, modeling of heavy ion effects).
5. Evaluation of 2D or 3D scanned beams and integration of such data into improved clinical treatment planning.

3. CLINICAL EXPERIENCE

The attractive physical characteristics of heavy charged particle beams, particularly helium ions, have made possible treatment (11, 12, 16, 17) of certain tumors such as chordoma or chondrosarcoma close to the spinal cord wherein a higher tumor dose can be safely given with particles than with low LET x-irradiation (Table 1). To date, a total of 49 such patients received heavy charged particle irradiation for chordoma, chondrosarcoma, meningioma, or neurilemmoma of the base of skull or juxtaspinal area. Local control within the irradiated area was obtained in 35 of the 49 patients (70%). The median follow up is 21 months with a range from 3–90 months. Serious complications were seen in a small number of patients, with cranial nerve injury in 2, transverse myelitis in 1 and brain necrosis in 3 patients. We believe that there are additional tumors which will ultimately show benefit from treatment with heavy charged particles and plan to continue exploration of these sites, such as locally advanced soft tissue and bone sarcoma, para-aortic lymph node metastases, prostatic cancer and salivary gland or paranasal sinus tumors.

Advanced head and neck tumors

A small group of 42 patients with locally far advanced tumors of the head and neck area including the upper aerodigestive tract, paranasal sinuses, salivary gland, thyroid gland and neck have been irradiated all or in part with heavy charged particles. Toxicity has been within acceptable limits with generally moderate skin and mucosal reactions. In 7 patients treated with helium ions, 2 had local control and the mean survival was 12 months. With carbon ions, 1 of 4 patients had local control although he succumbed to a pharyngeal necrosis. Twenty-seven patients were treated with neon particles and 4 with silicon ions; 16/31 (50%) had local control in the radiated area with a mean survival of 12 months (range: 3–42 months). In view of the advanced nature of these lesions, the short survival is not surprising since many had metastases. Our experience with head and neck tumors needs to be augmented by further Phase I and II studies.

Carcinoma of the esophagus

Thirty-three (33) patients with advanced squamous carcinoma of the esophagus have received (8) at least 4000 equivalent-rads, 22 in the helium ion treated group and 11 in patients who received all or part of their treatment with neon ions. The helium ion irradiated patients had a local failure rate of 19/22 (82%) and a median survival of 9 months. For the 11 patients who received some or all of their treatment with neon particles, the local failure rate is 9/11 (82%) and the median survival is 11 months. We are considering an approach for combined chemotherapy and heavy particle irradiation for unresectable esophageal cancer.

Carcinoma of the prostate

We have begun a Phase III study for locally advanced carcinoma (T3-4) of the prostate based partly on the fact that the results in neutron and proton therapy (13) of these tumors suggest improved local control and survival. Our study tests whether the improved dose localization and high LET of neon heavy charged particles allow a higher and more effective dose to the prostate.

Carcinoma of the lung

A Phase I–II study of the use of neon heavy charged particles in treatment of advanced, unresectable non-small cancer of the lung has been carried out in patients with primary lung tumors.

Survival has ranged from 2–19 months; 5 patients have

Table 2. Uveal melanoma treated with helium radiotherapy 1977–1985.

Dose	*# Pts*	*Local failure*	*Mean follow up*	*Complications*
50 GyE	23	0	12 Mos	Rubeosis, glau: 2 pts
60 GyE	61	2	17 Mos	Neovasc glau: 10 pts
70 GyE	51	3	22 Mos	Neovasc glau: 2 pts
80 GyE	70	3	30 Mos	Neovasc glau: 19 pts
Totals	205	8 (4%)	22 Mos	33 pts

Enucleation: 16 pts (8%)
(complications) – 9
(local failure) – 5
(ring melanoma) – 1
(unk reason) – 1

Local failures: 8 pts (4%)
(enucleation – 5)
(rexrt – 3)

Distant metastases: 17 (8%)

1 GyE = Dose equivalent to 100 rads low LET irradiation

Table 3. Retention of useful vision 20/400 or better compared to tumor size and location of uveal melanoma.
Minimum follow up: 12 mos

	(Distance from fovea or optic n)		
Size	*< 3.0 mm*	*< 3.0 mm*	*Totals*
Small	5/6		5/6
Medium	23/39 (59%)	16/19 (84%)	39/58 (67%)
Large	26/38 (68%)	11/31 (35%)	37/69 (54%)
Extra large	8/14 (57%)	2/13 (15%)	10/27 (37%)
Totals	62/97 (64%)	29/73 (46%)	91/160 (57%)

local failure, 5 are questionable for local persistence and 2 remain stable at 7 and 19 months. These have been patients with relatively far advanced tumors so that a prospective randomized trial will be done to show whether neon heavy charged particles can offer an improvement over x-ray treatment of unresectable lung cancer.

Carcinoma of the pancreas

In over 150 patients treated for locally advanced carcinoma of the pancreas using heavy charged particles alone and combined with low LET x-irradiation (6, 7, 24), there is approximately a 15% incidence of local control and less than 5% of patients have a survival of greater than 2 years. The actuarial median survival is about 12 months. In a now closed Phase III trial (7) we compared helium charged particle radiotherapy + 5FU chemotherapy to low-LET split course radiotherapy + 5FU; 50 evaluable patients were entered in this study. There was only a slight trend to better survival and local control in the helium particle arm but this difference was not statistically significant. Because of the high rate of local failure and distant metastases, this study was terminated early in favor of Phase I–II trials with neon ions and more aggressive chemotherapy consisting of 5 Fluorouracil, Adriamycin and Mitomycin-C (FAM). Of patients receiving FAM plus neon heavy charged particle radiotherapy, about 20% have had serious GI toxicity with gastritis, ulcer or hemorrhage. It appears that new drug therapy active against pancreatic cancer will be needed to improve survival in this disease. Heavy charged particle therapy is useful in delivering a high local dose but probably should be combined with interstitial implant or intraoperative therapy for best local effect.

Uveal melanoma

The results in helium irradiation of uveal melanoma also show excellent results due to precise dose localization (5, 9, 10, 15). In over 200 treated patients, a tumor control rate of 95% with follow-up from 3 to 90 months (median: 28 months) has been achieved (Table 2). There has been a low level of complications. As with proton therapy for uveal melanoma (1, 18), there is also so far a low level (8%) of distant metastatic disease. However, we see about a 10–15% incidence of neovascularity in the eye, often leading to difficult-to-treat glaucoma. Other complications occur less often including perforated sclera, severe keratitis, painful or dry eye so that 10/205 patients required enucleation for complications, in addition to 5 patients for tumor progression. Overall, about 55% of patients have retained vision of 20/400 or better, more often in patients with small tumors and/or tumors more than 3 mm from the fovea or optic disc (Table 3).

Bone and soft tissue sarcoma

Thirty-six (36) patients with bone or soft tissue sarcoma have been treated with heavy charged particles or mixtures of low LET irradiation and particles during the years from 1978 to 1985, receiving doses ranging from a minimum of 50 GyE to a maximum of 78 GyE, with a mean of 65 GyE. The histology of these tumors included chondrosarcoma (11 pts), osteosarcoma (8 pts) and lesser numbers of patients with leiomyosarcoma, liposarcoma, rhabdomyosarcoma, neurofibrosarcoma or other sarcomata. Most patients had gross disease remaining post surgical debulking or were considered unresectable and had only biopsy. In patients with chondrosarcoma arising in the spine (post-partial resection), 8/10 patients have local control with a mean survival of 27 months. In the remaining 26 patients with other histologies, 12/26 (46%) are controlled within the irradiated area with a mean survival of 15 months. The control in the irradiated field in those completing 5000 rads or more was 19/36 (52%) with a mean survival of 18 months, range 2–75 months.

Serious complications were encountered in the CNS in 2 of the 36 patients. We expect to continue these studies to confirm the value of heavy charged particle radiotherapy in locally advanced bone or soft tissue sarcoma.

Table 4. Malignant glioma of the brain 1975–1984.

Histology	*# Pts*	*Status*	*Median survival*
Glioblastoma	18	Dead – 18 (3-s tumor) (15-local failure)	13 mos
Anaplastic Astrocytoma	14	Dead – 13 (13-local failure)	12.5 mos
Lower grade Astrocytoma	7	Alive – 4 at 13–56 mos Dead – 3 (3-local failure)	24 mos

Malignant glioma of brain

39 patients with malignant glioma of the brain have been irradiated through 1984 with heavy particles (4). Eighteen (18) patients had glioblastoma while 21 had anaplastic astrocytoma (14 pts) or lower grade tumors (7 pts). About half of these patients have received boost therapy with heavy charged particles after x-ray therapy to 4500–5000 rads. The other patients have received all of their treatment with neon ions. The median survival in the glioblastoma group is 11.4 months while in the anaplastic astrocytoma patients it is 7.1 months (Table 4). Most patients have died with tumor persistence.

We envision possible combination therapy with particles and chemotherapy in the future.

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15

INTRAOPERATIVE RADIOTHERAPY

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INTRODUCTION

Intraoperative radiotherapy (IOR) or "direct view irradiation" is a surgical radiotherapeutic team approach to unresectable or partially resectable malignant neoplasms. The technique permits the irradiation of a surgically exposed tumor or regional lymph nodes with an X-ray or particle beam during an operation. This direct visualization has several advantages. The technique permits accurate beam direction, precise limitation of the radiation effect to the tumor, and the ability to physically retract sensitive organs such as the skin, small intestines, and the lungs out of the treatment volume. This intraoperative boost dose can be preceded by or followed by conventional fractionated external beam irradiation. The net result is a higher radiation tumor dose without a concomitant increase in treatment morbidity and mortality.

Radiobiology

Preclinical animal studies conducted at several institutions in the United States provided the fundamental data on normal tissue tolerances to single high doses of intraoperative electrons. At Howard University Hospital, dogs were irradiated by Goldson (5) to the retroperitoneum, including the aorta and vena cava with single electron doses of 2000, 3000, and 4000 rad. After 16 months, the animals were sacrificed. Grossly, the aorta and vena cava appeared normal. Microscopically, there was some degree of intimal thickening of the vessels, but the overall integrity of the great vessels were maintained. In contrast, the small intestines tolerated intraoperative electrons poorly, with progressive ulcerations and ganagrenous bowel developing when single doses exceeded 2000 rad. Radiobiological studies carried out at the National Cancer Institute by Tepper *et al.* (15) demonstrated that dog aortas subjected to single doses of electron beam irradiation up to 5000 rad maintained structural integrity. Microscopically, there was subintimal and medial fibrosis in the aortic wall at doses above 3000 rad. The changes noted seemed to be dose-related, but there was no significant narrowing of the vessels.

Additional canine studies indicated the maximum tolerance doses were 1500 rad by the colon, 2000 rad by the kidney and bile duct, 2500 rad by the small intestine and 3000 rad by the bladder (4, 9–11, 13–15).

Technical aspects of intraoperative radiotherapy

Ideally, a dedicated intraoperative radiotherapy facility with an electron producing linear accelerator would be the preferred situation. At Howard, a supervoltage radiotherapy suite was converted into an operating facility (Figure 1). In hindsight, it now appears that the best arrangement is to have an operating room adjacent to the therapy suite with a lead door separating the two suites. This would allow for the accelerator to be utilized during the day for routine patient care and the simple transferring of the patient through the lead door for the short interlude required for intraoperative radiotherapy treatment. A third approach for initiating the intraoperative radiotherapy would be the transferring of an anesthesized patient from an operating room to the radiotherapy department and then transferring the patient back to the surgical suite for closure of the body cavity. Admittedly, this is not an attractive alternative, but this technique has been carried out successfully without complications at the National Cancer Institute, the Mayo Clinic and Massachusetts General Hospital. A fourth approach, currently being explored at Harvard is the installation of an orthovoltage X-ray machine into an existing operating room. The orthovoltage unit requires less radiation shielding and would be easily accommodated with renovations to an operating facility that was already outfitted for diagnostic radiology procedures.

Surgical equipment

Conventional surgical instrumentation would be utilized to carry out the needed resections or anastomosis predicated by the tumor site, histology and anatomical locations. Standard metal retractors would be used during the surgical procedure as well as the intraoperative phase of the procedure. No radiation activation of materials is produced with the energy of electrons currently employed for intraoperative radiotherapy.

Intraoperative radiotherapy equipment

An electron producing linear accelerator with energies of 6–20 MeV is currently the preferred modality for delivery of intraoperative radiotherapy treatments. Electron beams

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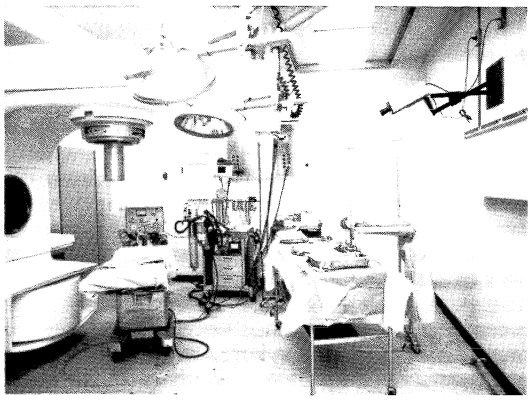

Figure 1. Special designed intraoperative radiotherapy suite at Howard University Hospital and Regional Cancer Center.

have the ideal characteristics for intraoperative radiotherapy. The sharp and rapid fall-off in depth dose protects the underlying tissues. The loss of skin sparing is no disadvantage for intraoperative radiotherapy because the skin is physically retracted out of the way of the electron beam. The bremsstrahlung (photon) contamination is less than five percent at all energies. The treatment times are short and are in the order of a few minutes.

Orthovoltage X-rays (40–200 kV) do not provide good dose homogeneity and penetrate far beyond the target volume and when treating body cavity organs, leaves a high differential bone absorption (Figure 2). Intraoperative radiotherapy using X-rays also requires long treatment times. However, with proper filtration to eliminate the low energy X-ray component, x-rays of 300 kV can be used for intraoperative radiotherapy. The advantages of orthovoltage intraoperative radiotherapy are the low cost of installation, easier service and less costly maintenance.

Specially designed intraoperative treatment collimators (or beam shapers) were fabricated for this unique treatment procedure. The electron collimator consists of an outer anodized or hard coated aluminum collimator and a telescoping inner lucite collimator, both 30 cm long and with $\frac{1}{4}$ inch thick walls. Both collimators overlap by 10 cm at 100 cm source to tumor distance (STD). The lucite collimator construction is such that it provides clear visualization of the tumor volume and confirms that the normal tissues have not drifted into the treatment field. The lucite collimator also acts as a secondary retraction device in that it provides a physical barrier between surrounding normal tissue and the treatment field. Thirdly, the telescoping effect of the collimator is an additional inherent safety factor. The inner

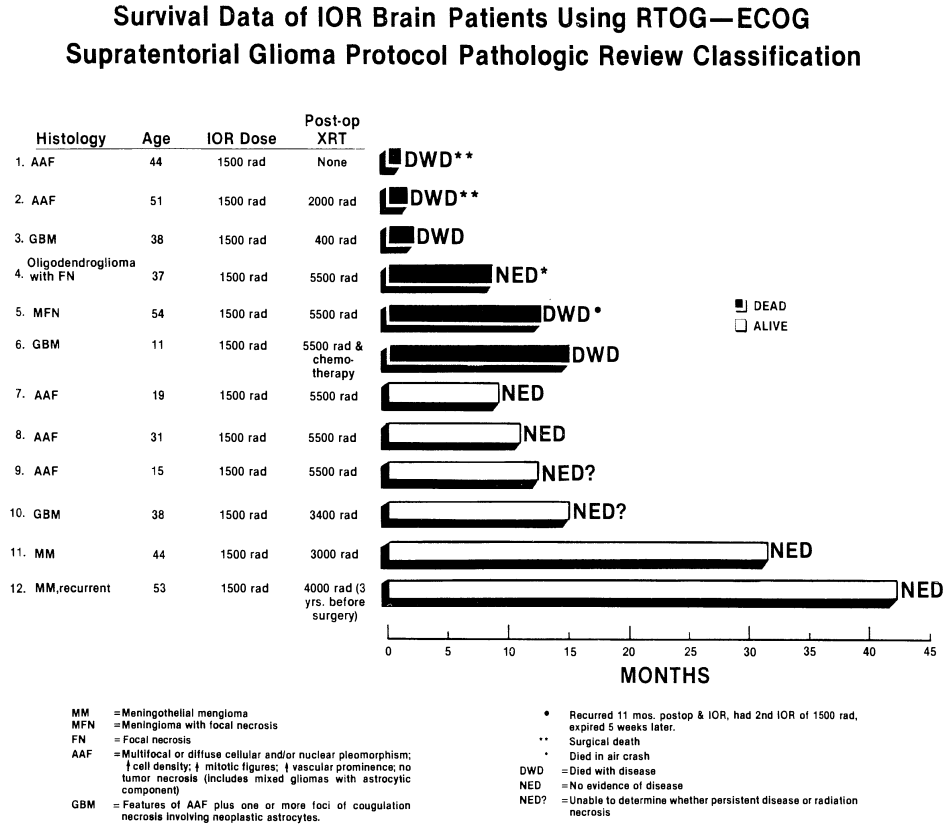

Figure 2. Survival data of IOR brain patients using RTOG – ECOG supratentorial glioma protocol pathologic review classification.

lucite portion of the collimator can telescope into the aluminum section for a distance of 20 cm before stopping. This characteristic prevents an undue static pressure against any vital structures that the collimator may rest upon during the intraoperative radiotherapy procedure. The treatment collimators come in various circular diameters as well as rectangular and square shapes. Certain collimators are designed with a bevelled edge to facilitate better insertion under the costal arch and against the pelvic side walls for certain clinical situations. The aluminum portion is gas sterilized. Both segments are numbered and stored in the cabinets in the IOR suite.

Standard anesthesia equipment and procedures are followed during surgery and intraoperative radiotherapy. However, in the short interval during which the dose is administered to the tumor, the anesthesia team as well as the rest of the surgical team must leave the room leaving the patient unattended. Therefore, additional anesthesia equipment needed is a multi-channel oscilloscope capable of monitoring remotely arterial blood pressure, respiration, electrocardiogram, and pulse rate. Beyond this, no extraordinary anesthesia equipment is required.

Procedure techniques

A surgical-radiotherapeutic team approach is the cornerstone to the ultimate favorable outcome of the procedure. Preoperative consultation between the team members provides for the best positioning of the patient on the surgical couch, the optimum site for placement and extent of the surgical incision. The surgeons, clear understanding of the capabilities of the intraoperative technique and the physical knowledge of the size and flexibility of the intraoperative cones will provide him with the fundamental data to offer the best exposure for the radiotherapist to perform the intraoperative technique.

In all anatomical locations, be they the cranium, thorax, abdomen, pelvis or soft tissues, maximal surgical resection of the tumor and its regional nodes should always be attempted. This provides the major reduction in tumor cell burden and increases the potential effect of the intraoperative radiotherapy as well as the postoperative conventional fractionated irradiation that follows. Along with the appropriate resections, and anastomoses, in many instances, additional dissection of the normal tissues in the immediate adjacent areas may be indicated so that they too may be removed from the intraoperative field, thereby minimizing late toxic effect treatment. Tissues that cannot be physically retracted out of the beam may be further protected by the use of sterilized lead shields or sheets which can be cut and fabricated in the operating room. Small bleeders may be controlled by the use of metal clips without the fear of them inducing any type of radiation scatter or shielding of micrometastases which may lie in their immediate vicinity. If at all possible, any anastomosis should be performed after the intraoperative procedure in order to reduce any unnecessary stress upon the anastomotic site as a result of the insertion of the intraoperative cone.

The intraoperative aspects of the procedure are conducted in the following manner. After surgical resection or exposure, the limits of the unresected primary or its tumor bed to regional nodes are determined and measured in all three dimensions. After determining the surface area of the region to be irradiated, the appropriate size and shape collimator is selected so as to encompass the tumor with a 1–2 cm margin of normal tissues. The selection of the appropriate electron beam energy is determined by the anterior-posterior thickness of the target volume. Technically, the electron energy is selected so that the 90 percent isodose line of that specific energy falls at least 1 cm deeper than the most posterior aspect of the target volume. Depending on the clinical presentation, either microscopic or gross disease, a single dose of electrons from 1000–3000 rad is selected. The appropriate treatment collimator is placed down over the target volume, care being made to retract normal tissues out of the way. Lap towels and metal retractors are also required to maintain the normal tissues in a safe position. The patient is then positioned under the collimator of the treatment gantry to which the aluminum portion of the treatment collimator has been attached. The surgical couch is then motorized or physically pumped up so as to dock or connect the lucite portion of the treatment collimator with the fixed aluminum portion. A scored mark has been etched on the lucite portion of the collimator. When this mark reaches the lower lip of the aluminum collimator, this clearly indicates that the source to tumor distance is 100 cm and is the indication to the physicist that the prescribed dose will be delivered without any distance factor corrections. Before leaving the treatment room, the anesthesiologist checks the patient's vital signs and the surgeon assesses the status of hemostasis. After confirmation of the patient's stability, all members of the team leave the IOR suite. The anesthesiologist monitors the patient's vital signs on the oscilloscope mounted inside the treatment control area and the intraoperative radiotherapy is initiated. The dose rate for most linear accelerators currently in use for this technique is approximately 500 rad per minute, resulting in treatment times usually lasting from 2–6 minutes depending on the single IOR dose prescribed. If during the delivery of the intraoperative dose any abnormalities are noted in the patient's vital signs, the accelerator can be turned off, allowing immediate entry of the surgical and anesthesia team into the room to assist the patient. Unlike interstitial implantation of radioisotopes, the patient is never radioactive.

Pancreatic cancer

Carcinoma of the pancreas represents the fourth leading cause of cancer death in the United States. Presently, the treatment of choice for a curative intent is surgery. Unfortunately the operative mortality for a curative resection is 20 to 25 percent, and the five-year survival for these patients is less than 10 percent. These patients must face a grave disease with little hope for a cure. As a result, most operations are done with a palliative intent.

Radiation therapy has been used in the past only for palliation, but, with the advent of high-energy photon and electron beam accelerators and of sophisticated interstitial implant techniques, radiotherapy has entered an era of curative intent in the treatment of pancreatic malignancies (2, 8, 9, 12). The total U.S. experience with intraoperative radiotherapy for the pancreas is currently greater than one hun-

dred and thirteen patients. Unfortunately, each center has taken a different approach to its management. The Massachusetts General Hospital series currently has the longest follow-up and the best median survival (7). In this series, patients judged unresectable at initial exploration receive low dose preoperative irradiation prior to a second exploration and only those who still have localized disease receive the intraoperative radiotherapy boost plus postoperative external beam irradiation. The intraoperative radiotherapy boost dose has been increased from 1750 rad to 2000 rad since evidence of local recurrence developed at the lower dose level and normal tissue tolerances were acceptable. In this series of twenty selected patients, sixteen were unresectable and the others were treated after a Whipple's operation (pancreatoduodenectomy). Median survival in the locally unresectable group as calculated from onset of treatment is $17\frac{1}{2}$ months. Although survival appears to be significantly better than that achieved with external beam irradiation with or without chemotherapy for unresectable disease, local failure still poses a significant problem with seven out of sixteen patients (44%) having persistent disease. Postoperative recovery was smoother and more rapid in those patients who received percutaneous biliary decompression and hyperalimentation to improve nitrogen balance prior to IOR. Post IOR gastric atony has been noted and is usually self-limiting after one or two weeks. Some patients also find benefit from exogenous pancreatic enzymes in the subsequent postoperative days.

Acute minor side effects of nausea, vomiting, and anorexia are usually observed within the first to fifth postoperative days. These symptoms are related to irradiation of the duodenum which cannot be completely isolated from the treatment portal in pancreatic head lesion treatment. Symptomatology was most pronounced in those patients with the poorest Karnofsky ratings and in those patients whose disease required larger treatment portals. On retrospective review of our Phase I toxicity study (6), those patients who fared best after intraoperative radiation were decompressed preoperatively.

At all doses and volumes, transient mild elevations in glucose and amylase were observed, particularly in patients who had a previous history of diabetes mellitus. No medical intervention was required for these patients.

Of the patients who expired and underwent autopsies, the histological findings of massive tumor necrosis with mild fibrosis reaction and islands of viable cancer were common. Therefore the IOR dose has been increased to 2500 rad to enhance tumor sterilization.

Gastric cancer

The incidence of gastric carcinoma has decreased markedly in the United States. Unfortunately, the five-year survival is still less than 10% and surgery continues to be the mainstay of treatment, but the results are discouraging.

Due to dose limiting normal structures, conventional external beam irradiation for gastric carcinoma is limited. A study done by Gunderson and Sosin reported a high local regional recurrence rate of 50–80% of patients who underwent reoperations and elective second look procedures (7).

With the limitations of external beam therapy and rate of local regional failures, the usefulness of intraoperative radiotherapy as a boost became evident.

The greatest experience with gastric carcinoma treated by intraoperative radiotherapy comes from Japan (1). In the Japanese series, the intraoperative radiotherapy is used primarily to treat residual microscopic disease in the tumor bed and all of the nodal sites around the celiac axis. Maximal surgical resection of the primary is attempted in all cases. A beveled treatment collimator is designed to fit under the costal arch and to cover the tumor bed as well as the celiac axis and to deliver a single intraoperative dose of from 2800–4000 rad. In order to evaluate the effectiveness of intraoperative radiotherapy, a sequential study was performed on the survival rate between patients treated by intraoperative irradiation plus surgery and those treated by surgery alone. Mainly, patients who were admitted to the Kyoto University Hospital on Tuesdays received an operation alone and those who were admitted on Fridays received surgery plus intraoperative radiotherapy. Except for a few cases, chemotherapy was not used in either group of patients. The total study population was 194 patients, who were stage I through IV with no patients having distant metastases. Of these, 84 received IOR in addition to surgery. Five year survival was comparable or better for the IOR group for stages I–III. Stage IV IOR patients had better survival rates compared to patients with surgery alone. Eighteen received surgery alone with no survivors after five years, while 27 patients received surgery plus IOR and 19.5% of this group survived five years.

Urinary bladder

Three Japanese centers have been using intraoperative radiotherapy plus fractionated external beam irradiation in the treatment of superficial bladder cancer (11). One hundred and sixteen patients have been treated thus far. Staging was based on bimanual examination under anesthesia, cystoscopy, excretory urogram, and pelvic angiography. The intraoperative radiotherapy was usually delivered through an open cystostomy generally using 4–6 MeV electrons delivering 2500–3000 rad by intraoperative radiotherapy. This was followed by postoperative irradiation to the whole urinary bladder to a dose of 3000–4000 rad in 15–20 days. The one, three and five year survival rates were 100%, 100% and 96.3% for T_1 cases and 100%, 87.2% and 61.6% for T_2 cases respectively. Heterotropic recurrences in the bladder were 5.3% within one year, 9.4% within two years and 19.3% within five years respectively. Normal bladder function was well preserved in all but five patients who underwent total cystectomy because of recurrences after radiotherapy and in one patient who underwent urinary diversion because of contracted bladder and progressive bilateral hydronephrosis.

Intracranial malignancies

In review of the literature, the use of IOR for intracranial malignancies can be traced to the work of Dyke and Davidoff who treated two patients with sarcoma of the brain at Columbia University Hospital with 3000 rad in 1934 and 1936 (3).

However, the major clinical experience in the United States using intraoperative radiotherapy for brain tumors has been limited to twelve patients treated at Howard University Hospital. The treatment protocol consisted of surgical resection or decompression followed by 1500 rad intraoperative radiotherapy. Postoperatively, the patients received fractionated external irradiation to the whole brain for a total of 5500 rad. Clinically, the inclusion of intraoperative radiotherapy with surgery plus conventional fractionated radiation has increased the dose potential to the tumor volume without a compensatory increase in morbidity or mortality from the combination of these procedures. Surgical decompression of the cranial vault, patient age, preoperative and postoperative Karnofsky status, and compliance with the treatment protocol appear to be variables influencing patient survival. Figure 2 summarizes patient demography and survival data in this pilot study.

IOR conclusions

IOR represents a surgical radiotherapeutic team approach to resectable and partially resectable malignant neoplasms of the various body cavities. It offers many patients a viable treatment option when complete surgical extirpation is impossible. Surgical cytoreduction, IOR plus or minus fractionated external beam irradiation and chemotherapy should increase local tumor control and survival with less morbidity and mortality.

Prolonged follow-ups and rebiopsies indicate that IOR can sterilize micrometastases in nodes and in malignant tumor beds. Complications, including: prolonged bleeding, delayed wound healing, increased infection rates, and organ necrosis secondary to IOR have been acceptably low.

Emerging clinical data warrant further clinical trials with intraoperative radiotherapy.

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16

OXYGEN MULTISTEP IMMUNOSTIMULATION AS A SIMPLE PROCESS AGAINST CANCER METASTASIS - A PROCESS TIMED TO THE METASTASIS PROMOTING LEUKOPENIC AND OXYGEN DEFICIENCY PHASES OF ESTABLISHED CANCER THERAPIES

MANFRED VON ARDENNE*

PROMOTION OF METASTASES BY THERAPY-INDUCED DECLINE OF CIRCULATING IMMUNE CELLS

Maximum potency of the host defense system is necessary in order to minimize the probability of dissemination of metastases. Since 1970 immunologic research has indicated more and more that nonspecific cellular defense mechanisms contribute most prominently to cancer control, and the number of immunocytes (leukocytes, T-lymphocytes etc.) per unit volume of blood is therefore of great importance. As shown in Figure 1, the concentration of immune cells, e.g., leukocytes, decreases fairly consistently as a consequence of the three most important tumor treatment regimens of today. This leukopenia weakens the host defense mechanisms just in the phase of cancer cell dissemination or, inversely, promotes metastasis.

A. Promotion of metastases by therapy-induced deterioration of the O_2 state

Although we have investigated the improvement of host defense by O_2-multistep processes since 1971, the impact of the individual oxygen state was widely recognized only recently.

Let the quantity of the oxygen uptake of the organism or of oxygen transport into host tissue be defined as O_2 state which can easily be calculated by the equation:

$$Q_{O_2} = \eta \times COP \times Hb_v,$$

where

η = utilization of the oxygen-binding capacity of blood, shortly termed as difference of oxygen saturation, that can routinely be determined from the arterial and venous pO_2 resting levels (3, 12);

COP = cardiac output = stroke volume (SV) × pulse rate f. Due to the reciprocity of SV and f, COP remains roughly unaffected by changes of η (3, 12);

Hb_v = Content of hemoglobin in blood, constant for the individual case.

Under normal conditions (at a given age and training state), the value of η describes roughly the relative magnitude of oxygen transport into tissue. Therefore, η may be considered a characteristic of the oxygen state and is used in this sense.

The fact that the dynamics of the O_2 state were considered only recently for attempts at optimizing tumor control may be due to three reasons:

1. In 1980 it became evident that peroxides are the main effectors of phagocytosing cells (20). The concentration of these peroxides depends on the actual oxygen supply of tissue, including the immune system.
2. In 1981 the routine determination of sufficiently representative values for the O_2 state (η) and the discovery of its dynamics were accomplished (12).
3. In 1981 we found that the O_2 state is severely impaired by conventional cancer therapies (7).

Typical examples of the deterioration of the oxygen state as a rapid response to cancer treatment by drugs, radiation or surgery are shown in Figure 2. The decrease of η therefore contributes to the further impairment of cancer control mechanisms in the phase of possible dissemination. The close correlation between the diminished value of η and the concomitant decrease of the number of circulating immune cells becomes evident as a critical factor resulting from established tumor therapies.

B. Development and rapid utilization of processes for decreasing probability of cancer dissemination as a major objective of present cancer research

More than 80% of all cancer deaths are caused by metastases as compared with only 10% by the primary tumor (24). The probability of dissemination depends strongly on the tumor stage and increases in proportion to the size of the primary tumor at the moment of first detection or treatment. Even in relatively early stages, when the first therapeutic measures are usually initiated, the probability of metastasis is already about 50 to 60% or higher for frequently occurring tumors such as breast and lung carcinomas or melanomas. The detection of metastases is often tantamount to the individual's sentence of death. These facts indicate that development and evaluation of methods for fighting against tumor spread should be a major objective of present cancer research. This is very serious and should initiate immediate activities, because in the past few decades there was no change in the relative number of cancer deaths despite billions of dollars invested worldwide in cancer research (statement by the Director of the National Cancer Institute.

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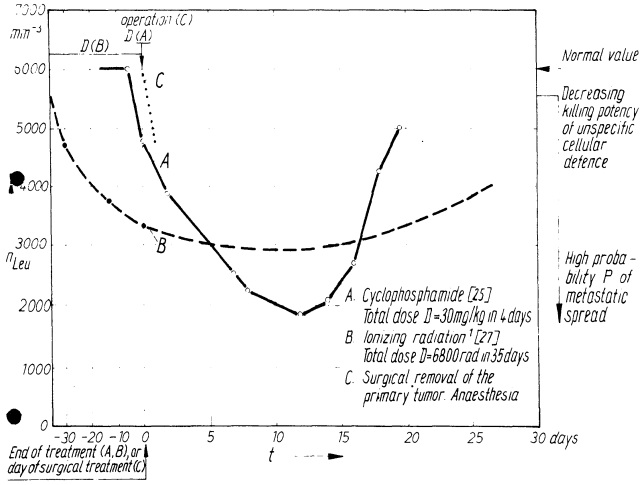

Figure 1. Examples showing decreasing numbers of leukocytes n_{Leu} (leukopenia) after drug (A), radiation (B) and surgical treatment (C), respectively. – Data in part from [25] [27].
[1]Volume of the primary tumor $\approx 800\,cm^3$

Bethesda, MD, 1980). In his profound criticism of present cancer management, Krokowski (24) pointed out that in the past 20–25 years all attempts to improve the cure rate substantially failed. Prevention of metastatic spread, which should be initiated before and immediately after first treatment of the primary tumor, may be the most opportune course for leading cancer treatment to new horizons. For the above-mentioned reasons, we have been dealing with the development and optimization of a particular process for reducing the probability of tumor spread (1–6; 9–11; 14–17).

C. The primary events of metastatic spread

It has been shown in most cases that metastatic spread begins at the moment of the first (diagnostic or therapeutic) manipulations of the primary tumor (24). According to Figure 3, about 1–2 $\times$ 10^4 cancer cells are disseminated into the body tissue by such manipulations (19, 22, 24, 28) although this represents only a small fraction of the number of cancer cells in the primary tumor at that time. For bronchial or mammary carcinomas the tumor specific "spreading factors" are in the order of 10^{-4} to 10^{-6} for the tumor sizes indicated in Figure 3. The problem is to stimulate the lowered cellular host defense in order to kill all the cell clones developing from the 2 $\times$ 10^4 scattered cancer cells. The prophylactic process should possess a large reserve power for killing cells (Figure 3, N scale) and should be initiated no later than one month after the moment of metastatic spread since there are relatively short replication times of cancer cells in developing metastases.

D. Concept of the O_2-multistep immunostimulation

In order to decrease metastatic spread temporary elevation

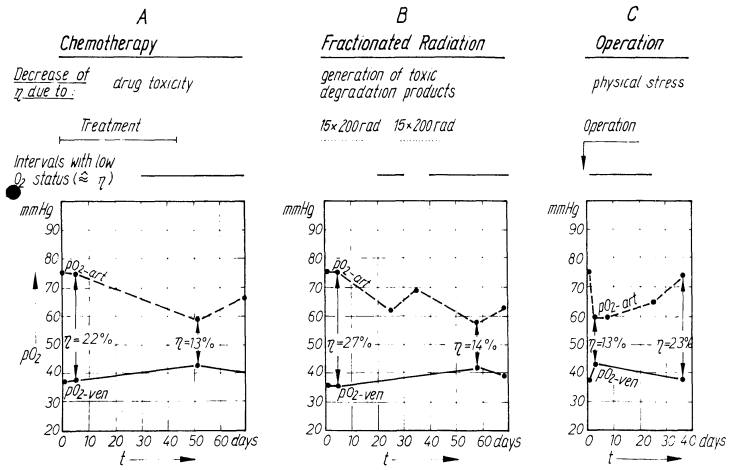

Figure 2. Remarkable deterioration of the O_2 status (i.e. decline of η as the main factor of oxygen transport to tissue) after tumor therapy by drugs (A), fractionated radiation (B), or surgery (C), – Figures for orientation.

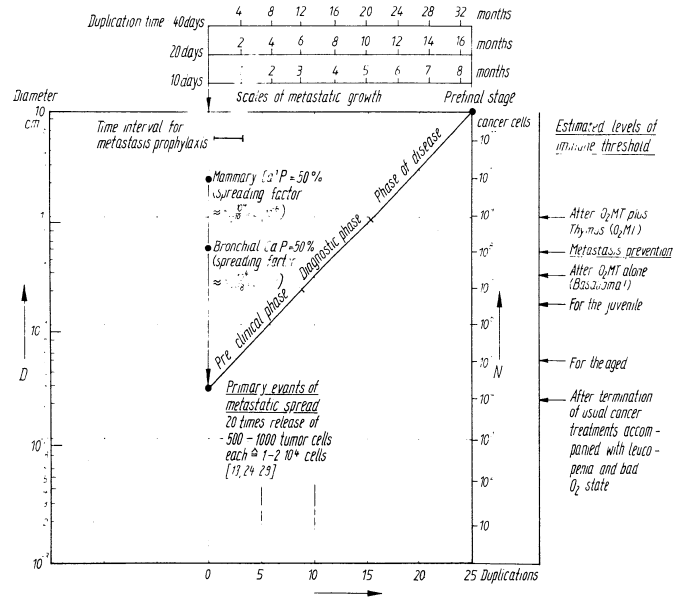

Figure 3. Primary events of metastatic spread ($N \approx 1 \div 2 \cdot 10^4$ tumor cells) in comparison to the estimated levels of immune threshold. P = probability of metastatic spread.
[1][21]

of the number of circulating immune cells has to be combined synchronously with continued increase of oxygen transport into host tissues by intensive variants of the O_2-multistep therapy (3). The subtly timed concerted action of stimulated immune cell proliferation and improved oxygen supply is termed "O_2-multistep immunostimulation" (4) and should be applied immediately after the first treatment of the primary tumor, i.e., in the hazardous phase of dissemination triggered by diagnostic or therapeutic interventions. The interrelations between the two basic steps of this procedure and their synergistic effects are compiled in Table 1. The oxygen transport into tissue represents the limiting factor of (chemical) energy supply. It is therefore not surprising that continued improvement of the O_2 state intensifies remarkably the many-sided energy-dependent mechanisms of cellular tumor defense (see column 3 in Table 1).

E. Synchronized elevation of the number of circulating immune cells by stimulation of their generation

Progress has been made in the field of immunostimulation by thymus extracts. The whole extract could be separated into its immunosuppressive and immunostimulating peptide fractions; the latter is now available in purified form as 1 ml (6.5 mg) ampoules (ISTP thymus preparation by Dr. Jaeger, manufactured by the company Dr. Kurt Mulli, Nachf., Otto-Hahn-Str. 2, D-7844 Neuenburg, FRG). Figure 4

Table 1. Synergistic effects of O_2-multistep immunostimulation.

Step	*Phenomenon*	*Effects**	*References and remarks*
1. Chemical stimulation of cellular defense by BA 1–4 and/or ISTP peptide mixture from thymus extract	Temporary elevation of the number of leukocytes and lymphocytes	1. Significantly enhanced proliferation of immune cells over several days or even weeks	[14, 15, 16]
2. Increase of oxygen transport into all parts of the body by intensive variants of the O_2-multistep therapy	Temporary (3–4 days) elevation of O_2 transport up to 250% of the average measured at 70 yrs of age	2.1 Stimulation of cell migration by improved energy supply	[7]
	Synergistic triple effect:	2.2 Stimulation of chemotaxis	Richter (1980)
		2.3 Enhanced peroxide formation in phagocytosing cells	[20] Peroxides as main contributing factors to phygocytosis

* Energy- and oxygen-dependent processes

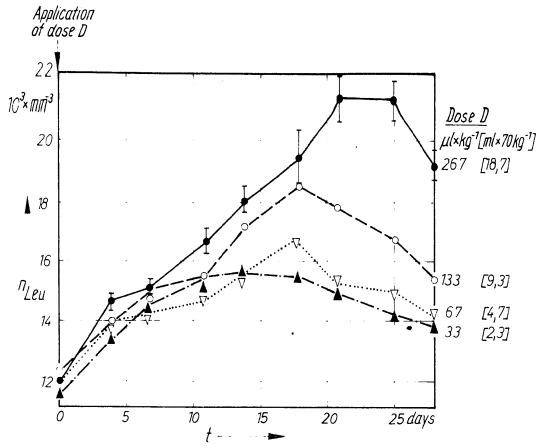

Figure 4. Increase of number of leukocytes (n_{Leu}) in the peripheral blood after stimulation of the body defense mechanisms by single application of different doses of thymus extract.
Results obtained on male Wistar rats (mean body weight 150 g, 5 animals per group). Purified thymus extract ISTP (without immunosuppressive fractions) kindly provided by Dr. K Mulli KG, D-7844 Neuenburg. Intramuscular application of 40 (●), 20 (○), 10 (▽) and 5 μl (▲) per 150 g body weight at Day 0.
Unpublished results by W. Krüger and P.G. Reitnauer, 1983.

shows increasing leukocyte counts in the peripheral blood of Wistar rats during a three-week period after single application of various well-tolerated doses of this preparation. In this system a corresponding amount of the whole thymus extract proved to be almost fully as active as the ISTP fraction (not shown here). Hence, that preparation can be used as well, if the purified ISTP fraction should not be available. The old immunomodulators such as BCG and levamisol (23) no longer are competitive. The usefulness of other substances such as lysolecithin, preparations from living cells, lithium carbonate etc. has not yet been evaluated definitely.

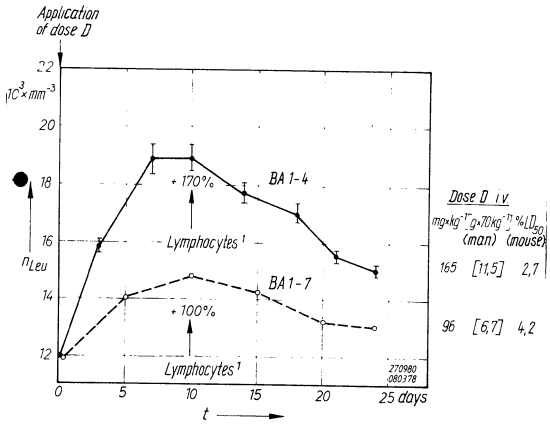

Figure 5. Increase of number of leukocytes n_{Leu} in the peripheral blood after stimulation of the body defense mechanisms by single application of the immunomodulators BA 1–4 (2-cyanoethyl-urea) and BA1–7. Results obtained on Wistar rats by P.G. Reitnauer, 1978/80
Raise in number of lymphocytes as indicated in case of BA1–4, the increase of thrombocytes was about 29%.

2-Cyanoethyl urea (BA 1–4) was tested in the same manner as ISTP (14, 16) (Figure 5). This substance exerts its maximum effect 7 days after application, whereas the ISTP effect does not culminate before day 16. On these grounds it seems probable that both preparations stimulate different areas of the cellular immune system, and there is hope that a combiantion of them will act synergistically.

Animal experiments (Figure 6) indicate that drug-induced leukopenia can be compensated for, or at least, attenuated by modulators of this type. Unfortunately, BA 1–4[1] is not yet commercially available.

F. Synchronized re-elevation of decreased O_2 state

In the course of studies on the O_2-multistep therapy we found a trigger mechanism of the microcirculation in the cellular vessel walls that controls the bloodflow at the venous ends of all capillaries in the organism, synchronously and equidirectionally dependent on the oxygen (energetic) state (8). On passing a certain oxygen threshold the mechanism increases or decreases capillary bloodflow. Its integral effect is reflected in changes of the arterial and venous pO_2 resting levels and is, therefore, measurable. This unexpected trigger effect is due to a feedback mechanism of bloodflow at the venous end of capillaries (3, 8) and continues until strong external influences on the O_2 (energetic) state cause a shift. The discovery of this "switching" mechanism and of its response to variants of the O_2-multistep therapy facilitates the continued re-elevation of oxygen transport into tissues (expressed as η) after impairment by stressful events such as cancer therapy (3). The 36 h-multistep therapy process turned out to be the most practicable treatment, as described in the following section. The increase of η attainable by this method is most pronounced for lowered initial values by stress, preferably induced by stress, preferably in old age. It should be mentioned that in cases of physical weakness, circulatory lability and bedrest, values of η are generally in the order of only 12–15% as compared to the normal 20–25% (12).

A typical example showing improved oxygen transport by means of the 36 h-multistep therapy process after distress induced by cancer therapy is given in Figure 7. When HOT*-UVI[2] treatment is added as adjuvant measure to the regular 36 h-process, the gain in η is even greater for several days. In the example shown, η is increased from 13 to 48%, i.e., 3.7 fold!

For HOT*-UVI treatment (26) about 70 ml of venous blood is withdrawn from the patient, mixed with citrate solution (12:1 v/v), uv-irradiated in a quartz cuvette for some minutes, and re-infused intravenously. For this entirely riskless procedure, which needs no bubble oxygenator, a special apparatus is commercially available (Manufacturer: VEK Präcitronic, DDR-8016 Dresden, GDR). The main parts are a quartz lamp emitting short-wave uv light and a quartz cuvette of short path length.

[1] Presently this compound is synthesized on a small scale in our Institute and will be manufactured later by VEB Arzneimittelwerk Dresden.

[2] HOT* = Hematogenic Oxygenation Therapy (the asterisk indicates a special variant of it); UVI = Ultraviolet Irradiation (of blood).

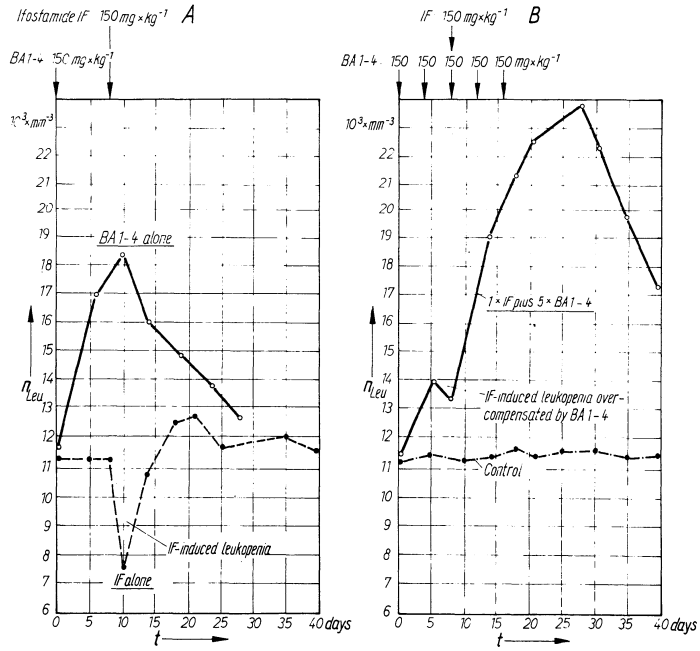

Figure 6. Number of leukocytes (n_{Leu}) as function of time t after a single dose of 150 mg/kg BA1–4 of Hosfamide, respectively (A), and the effect of combined treatment (B). – Control: no treatment. – Results obtained on Wistar rats (8 animals per group).

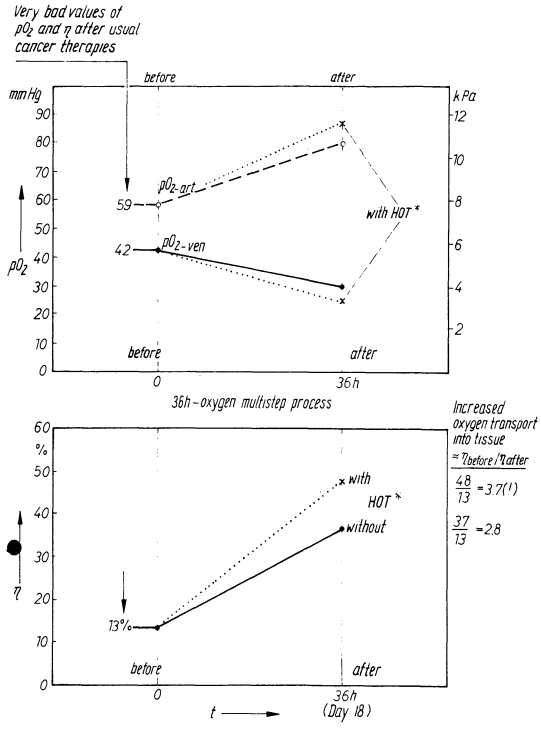

Figure 7. Example showing the marked increase (factor 3.7!) of oxygen transport to tissue (approximately represented by η), which is impaired by distressing effects of common cancer therapies, by means of the 36 h-oxygen multistep therapy process with or without UVI-HOT* treatment.

The effect of uv irradiation of the patient's own blood, both on pO_2 and η is shown in Figure 8 (18). However, the positive effect of the additional HOT*UVI treatment on pO_2 and η generally lasts for only a few days as compared to the long-term effect of the O_2-multistep process. However, to overcome the problem of metastatic spread every measure which improves the O_2 state is welcome even if only for a few days. Further research must determine whether the adjuvant HOT*-UVI method is essential for preventing metastasis or whether the 36 h-multistep therapy process alone will succeed. Presently, it seems opportune not to omit the HOT*-UVI step. In conclusion the O_2-multistep measures discussed above are capable of increasing oxygen transport into host tissues, particularly into the body's defense system, up to three-fold the degree detectable after conventional tumor therapies.

G. Treatment schedule of the O_2-multistep immunostimulation

The treatment schedule for the intensive variant of the O_2-multistep immunostimulation is presented in Figure 9. The single steps are coordinated so that the maximum effect is reached around day 18. Several simplified variants have been derived which are summarized with their indications and efficiency ranges (Table 2). To combat metastases, it is recommended to employ the standard variant (No. 2), because it offers certain "stand-by power." When this variant has proven effective, one can pass carefully to simpler and less powerful variants.

As for the optimum starting time of this process, it must be considered that metastasizing cancer cells may arise si-

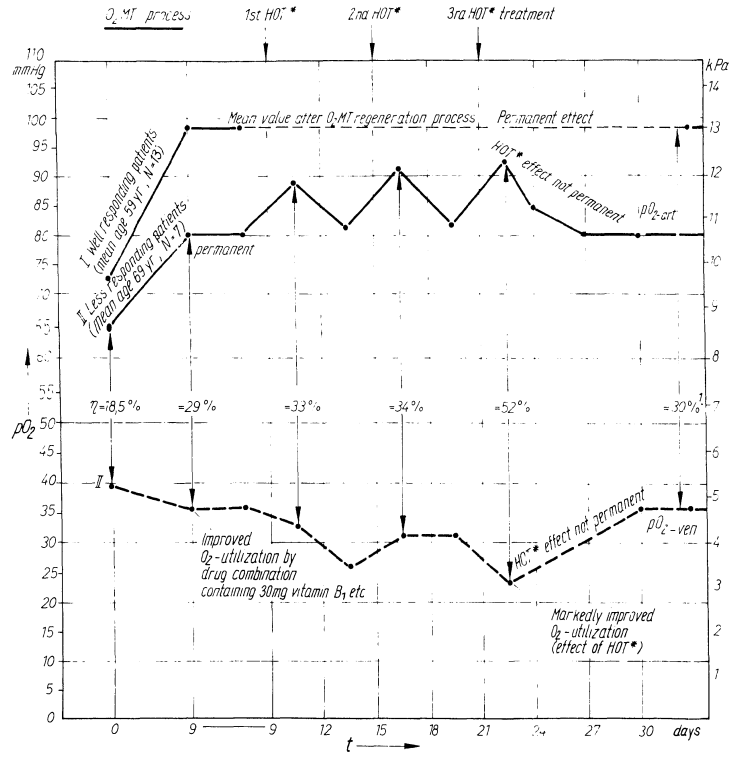

Figure 8. Examples showing the increase of arterial pO_2 and decrease of venous pO_2 by the O_2 multistep regeneration process combined with three times HOT* treatment.
η = Degree of utilization of oxygen-binding capacity of blood.
[1]On the fairly exact assumption that there is no essential decrease of cardiac output.

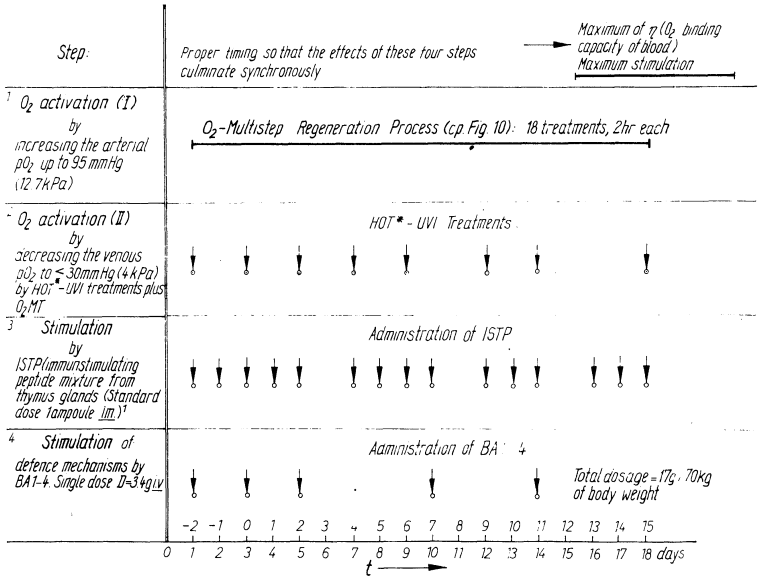

Figure 9. Schedule of O_2-multistep prophylaxis to cancer metastases and recurrencies by combining the O_2-multistep regeneration process + HOT*-UVI treatment with stimulation of body defense mechanisms by ISTP thymus extract + BA1–4 (2-cyano-ethyl urea)[2]. When linked with the Cancer Multistep Therapy (CMT) this process is followed by hyperthermia (41°C – 120 min) combined with synchronous hyperglycemia (5–8 times the normal) on Day 18. For details of CMT see [1, 13].
[1] 1 ampoule containing 1 ml = 6.5 mg of immunostimulating peptide mixture from thymus extracts (Manufacturer Dr. Kurt Mulli, K.G., D 7844 Neuenburg/FRG). Total dosage: 15 ampoules. – Combination with Step 4: Stimulation by BA1–4 (for details see [4]).
[2] At present prepared by our Institute; prospective manufacturer: VEB Arzneimittelwerk Dresden/GDR

Table 2. Variants of O_2-multistep immunostimulation, their indications and efficiency ranges.

No	*Variant*	*Combination of steps as indicated in Fig. 9*	*Efficiency range expressed as number of cancer cells killed*[2]	*Indication*	*Treatments per annum*	*Remarks*
1	Intensive variant	1, 2, 3, 4 (+fasting)	8×10^9 $>2 \times 10^{11}$	Therapy (also for palliative treatment)	1–2 1	Cure on life with cancer[2] (for clinically controlled therapy see [3])
2	Standard variant[2]	1, 2, 3, or 1, 2, 4	1×10^9	Prevention of metastases (unexhausted reserves)		Treatment within 10 days after any conventional cancer therapy
3	Standard variant, simplified	1, 3 or 1, 4	$\approx 5 \times 10^8$	General cancer prevention (juvenile years)	1	
4.	Non-invasive variant	1 with oral applications of stimulants	$\approx 2 \times 10^8$	General cancer prevention	1	Orally applicable stimulants under investigation
5	O_2-multistep therapy alone	1 as 36 h-process	5×10^7	Prevention of metastases? (no reserve)	1	Treatment within 3 days after any conventional cancer therapy

[1] These numbers are assessed from experimental observations
[2] Also as constituent of the Cancer Multistep Therapy

multaneously with the original tumor. In order to prevent metastases of this type, i.e., when the tumor cell emboli are still small, the process should be initiated as soon as possible after diagnosis of the primary tumor. Spreading cell aggregates like these are indeed thought to be destroyed by the body's unstimulated natural immune system, but they can attach to distant sites when the defense is severely weakened due to conventional therapeutic measures. These considerations indicate that even in case of early dissemination the preventive process started immediately after treatment of the original tumor may also be purposeful. When linked with the Cancer Multistep Therapy (CMT) this process is followed by hyperthermia (41°C – 120 min) combined with synchronous hyperglycemia (5–8 times the normal) on day 18. For details see (1, 13).

Each variant of the O_2-multistep immunostimulation includes the 36 h-oxygen multistep process (Step 1 in Figure 9) divided into 18 treatments of 2 hours each. All essential details of the program II of the 36 h-oxygen multistep process using Alupent for increasing cardiac output are given in Figure 10 (3). In brief, for a good response to this process consisting of three special measures, it is of great importance that the prescribed flow rate of 4 l/min O_2 is fully available to the lungs. This is met by using a special breathing mask made of flexible polyethylene (Figure 11). Thus, oxygen is delivered without loss, even if the patient breathes through nose and mouth.

Investigation and design of the simplest possible variant system for general prevention of cancer is a future project. It does not seem that by practicing such a method once a year, the incidence of cancer will be reduced drastically. For optimizing treatments orally applicable stimulants or combined preparations might play an important role. Further studies must clarify whether the 15 min-O_2-multistep quick process, which is suitable also for outpatients, can be applied in the same way as the above-mentioned extended 36 h-process. A method for general cancer prevention should be extraordinarily simple; this is, however, a future challenge.

H. Efficiency range of the O_2-multistep immunostimulation

In devising prophylactic methods for metastases two values should be known:

1. The total number of cancer cells spread during metastasis. This figure results from the number of cancer cells initially released multiplied both by the number of cell duplications until the onset of the immunostimulating process and a safety factor.
2. The efficiency range of the different variants of O_2-immunostimulation. The total number N of cancer cells to be killed by metastasis prophylaxis can be calculated according to the equation

$$N = N_{prim} \times 2^{ND},$$

where

N_{prim} - the number of initially released cancer cells, i.e., 2×10^4 (see: (24), and Figure 3)
ND = Number of duplications until onset of the prophylactic treatment.

As is known, duplication times of metastases are generally shorter than those of primary tumors and are in the order of 5–30 days, depending on type and localization and the original tumor (24). Therefore, any prophylactic process

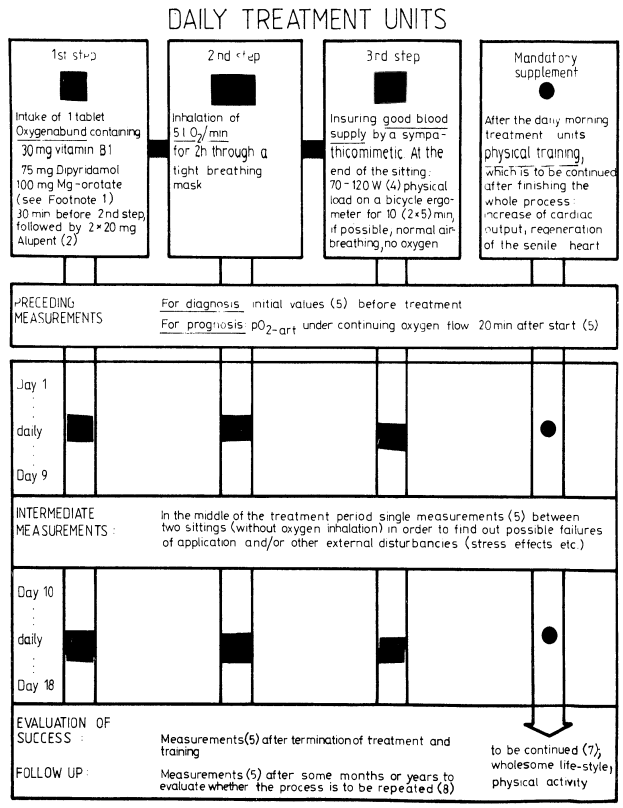

Figure 10. Leaflet for doctors and patients, which explains the protocol of the 36 h-O_2 multistep therapy exemplified by the "18 days cure".
(1) Add 1 g Vitamin C if desired
(2) Monitoring oxygen flow
(3) For hypotensive patients: drugs (e.g. Novodral retard or Mephentemin) should be given for increasing blood pressure amplitude
(4) Measurement of arterial oxygen partial pressure (pO_2-art) in capillary blood from the "arterialized" (hyperemic) ear-lobe after 10 min rest about at the same time of day by using a special device, e.g., MO 10 Universal pO_2. Meter manufactured by VEK Präcitronic, 8019 Dresden/GDR
(5) Measurement of venous oxygen partial pressure (pO_2-ven) in blood from the untied vena cubitalis/9/
(6) pO_2-art $\geqq$ 125 mm Hg indicates good response
(7) Daily intake of Oxygenabund 30 min before starting exercise to decrease pO_2-ven
(8) Decreased values of η reflect stressful events such as cytostatic treatment, irradiation, operations etc.

should be initiated as soon as possible after termination of usual tumor treatments. On the assumption that:
- the duplication time is (unfavorably) short, i.e., 5 days, and;
- the prophylactic process is performed 20 days (= 4 duplication times) after initial metastatic spread, the number of cancer cells to be destroyed is approximately 3×10^5. This number is multiplied by a safety factor of 10^2.

Then, it must be checked which variant of the O_2-multistep immunostimulation is capable of killing tumor cell numbers of this magnitude. The effective range can be determined from the disappearance of tumor cell aggregates in humans. Table 2 indicates the efficiency of different variants of the O_2-multistep immunostimulation assessed from such observations. After performance of the standard variant (No. 2 in Table 2), disappearance of mammary tumor nodes 1 cm in diameter, i.e., about 10^9 tumor cells, could be seen repeatedly. By application of O_2-multistep therapy alone, i.e., without immunostimulants and HOT*-UVI treatment but by elevation of η up to 52%, a basalioma consisting of about 5×10^7 cells disappeared as shown in Figure 12. From these measurements (note the values of η!) the correlation between oxygenation state and immune state becomes obvious.

The graph in Figure 13 demonstrates the relation between the efficiency ranges of variants of the O_2-multistep immunostimulation and the different growth kinetics of tumors. At the right hand side of this graph, the number of tumor cells which have to be killed by effective metastasis prophylaxis as well as by a general preventive measure applied once a year are additionally indicated. It shows clearly that in terms of the number of destroyable cancer cells the variants of the O_2-multistep immunostimulation present an increased effectiveness in the order of some powers of ten. Whereas large parts of the host's defense system are daily bound up in removing decaying normal

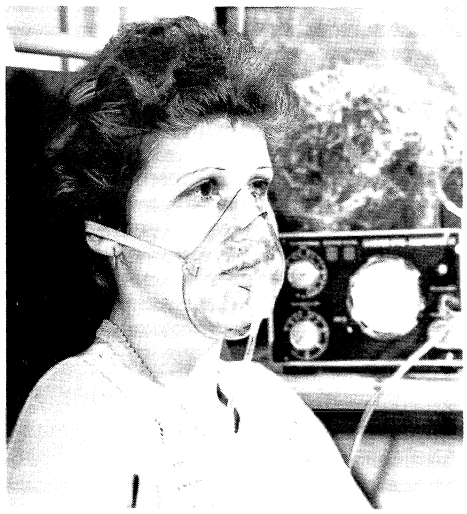

Figure 11. The new oxygen applicator made from polyethylene, manufactured by SM-Gesellschaft für Kur- und Sauerstoff-Mehrschritt-Therapie, D-8942 Ottobeuren, or G. Weinmann, D-2000 Hamburg 54, FRG. – Advantages: oxygen application through nose and mouth; reading with glasses possible; comfortable enough for use at sleep; low oxygen loss; disposable material.

other "daily necessities", the additional defense capacity gained by the methods described can primarily serve to attack cancer cells.

The case reported in Figure 14 rests on the assumption that the standard variant of the O_2-multistep immunostimulation[3] even enters the therapeutic region.

Most recently, a very simplified variant of the Oxygen Multistep Immunostimulation, which is also applicable to outpatients, has been developed and tested successfully. The protocol is given in Figure 15. One of the first results obtained by using this method shows lessening of precancerous states (Figure 16). This finding indicates additionally that the treatment variant in the upper part of Figure 15 might be effective as *common cancer prophylaxis.*

Rapid introduction into practice

On the 7th Cancer Congress of the German Democratic Republic we suggested long term studies in the prevention of cancer metastasis (9).

Definite data will not be available before 10 years for several reasons such as latency of metastases, evaluation of year survival, lacking research capacity in hospitals etc. Meanwhile an alarmingly high number of cancer patients die daily from their metastases.

Clinical oncologists should already be encouraged to recommend established facilities for O_2-multistep immunostimulation to their patients who should apply to these institutions for appropriate treatment as soon as possible after termination of conventional cancer therapy.

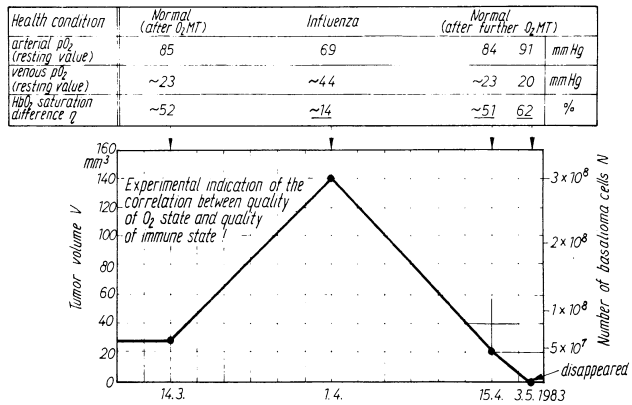

Figure 12. Dependency of the steady state between cell proliferation and cell killing by body defense mechanisms from the oxygen state of organism (mainly determined by η) in case of a basalioma on a 76-yr-old male patient – Destruction of at least 5×10^7 basalioma cells only by O_2 multistep therapy without UVI-HOT*

[3] As a consequence of further numerous clinical observations (pain relief, retardation or cessation of tumor growth, survival with increased quality of life despite cancer), a physicians' community for utilization of O_2-multistep immunostimulation was founded under the leadership of Dr. SH Wolf of Bad Wildungen/W. Germany. This group held two symposia in June of 1983 and 1984.

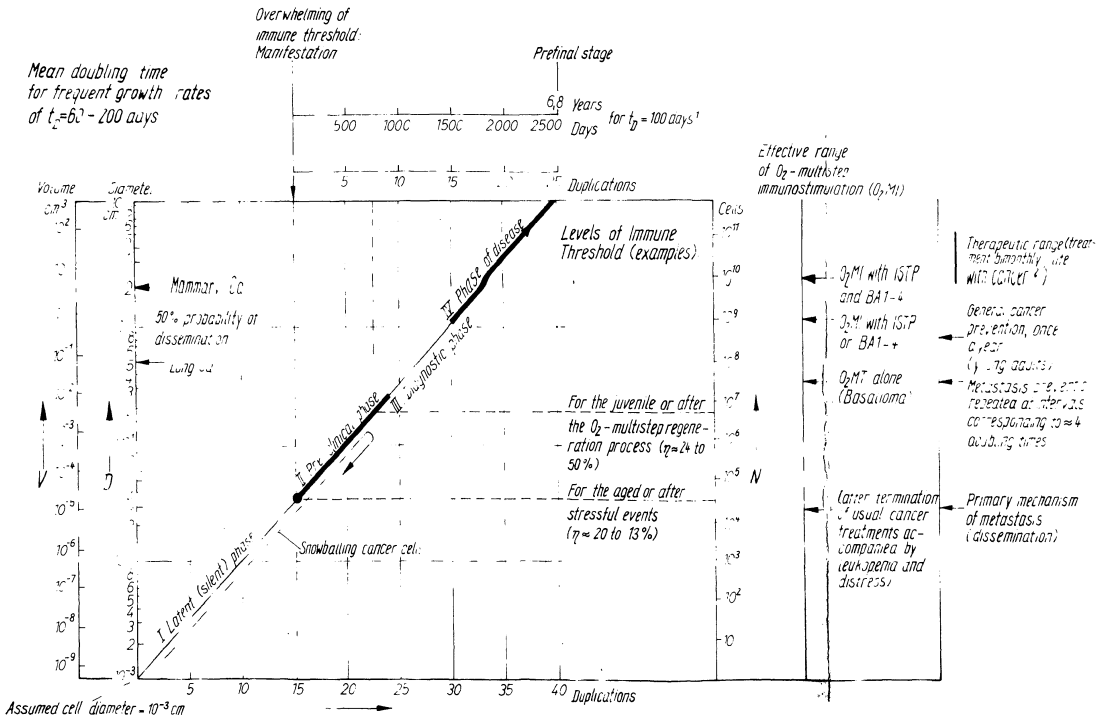

Figure 13. Effective range of variants of O_2-multistep immunostimulation in relation to the different growth dynamics of tumors
[1] Actually, cancer cell doubling time t_D is not constant but increases with increasing tumor diameters (D ≧ 1 cm) [24]

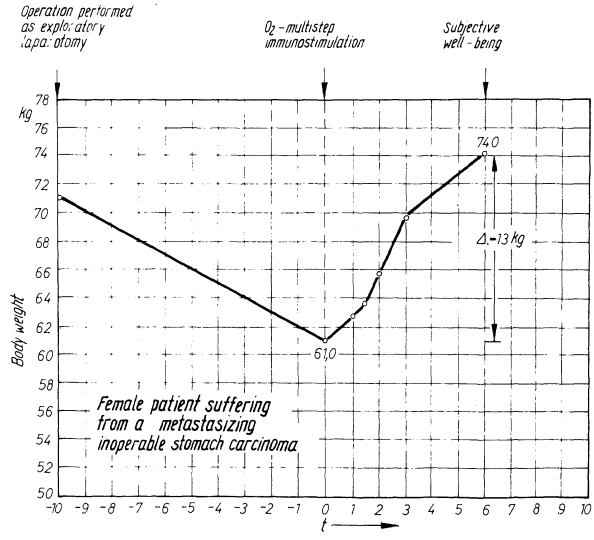

Figure 14. Body weight changes and course of the disease of a 73-yr-old female inoperable cancer patient (Code No IMS 01 WM) before and after O_2-multistep immunostimulation.

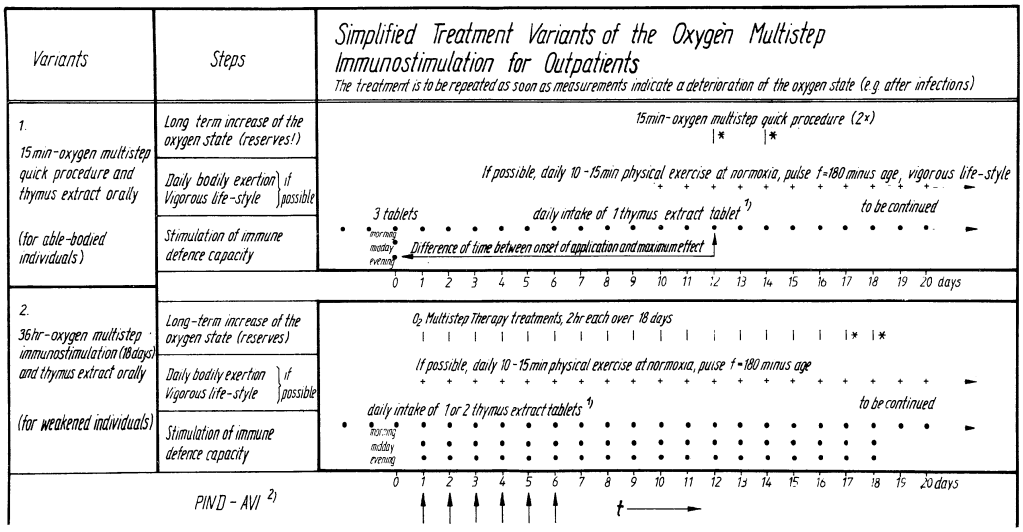

Figure 15. Treatment protocols of the simplified 15 min and 36 hr variants of the oxygen multistep immunostimulation.
After preliminary results this multistep procedure seems to be effective in the following ways: diminishes the general susceptibility to disease, reduces the severity of diseases and crises in old age, improves quality of life and performance, and may prevent early stages of primary cancers and metastasis (assessed effective range: killing of up to 2×10^8 malignant cells). For serious cases of this type the blood glucose level should temporarily be increased up to 5×10^{-3} g/ml for 6 hr after the treatments marked by an asterisk.[3]
[1] Containing 240 mg dry extract from calf thymus. Manufacturer: Dr. Kurt Mulli, Otto-Hahn-Str. 2, D-7844 Neuenburg, FRG.
[2] For special cases, the immunomodulator PIND-AVI, prepared in the Institut für Medizinische Mikrobiologie, Infektions- und Seuchenmedizin (Head: Prof. Dr. A Mayr) der Universität, D-8000 München, FRG., can be applied additionally as follows: 2 mg (1 vial) daily *im.* on the days 1–6 for both variants. (Synergistic effect expected due to other effector mechanisms)
[3] The selective overacidification of the malignant foci released by this measure accelerates the chemotactic attraction of leukocytes in this area.

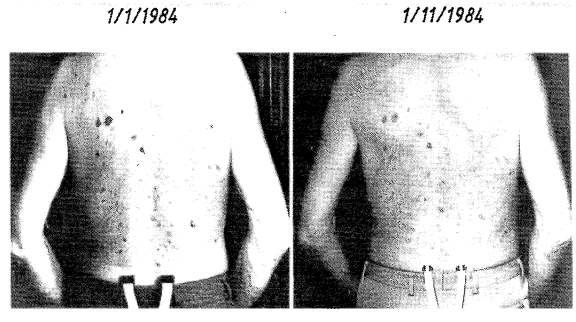

Figure 16. Lessening of dangerous precancerous dorsal skin lesions[1] *within 10 months* by daily oral intake of thymus extract (one coated tablet[2]) and by maintenance of a good oxygen state ($\eta \approx 40\%$, Q_{O_2} at rest ≈ 0.4 l/min) through 15 min-oxygen multistep quick procedures. Daily brushing of the affected sites for 20 sec. – 73-yr-old male patient
[1] This finding (flattening and depigmentation of the dysplasias) indicates that the very simplified variant of the O_2 multistep immunostimulation might possibly become a common cancer prophylaxis
[2] Thymus "Mulli" (one tablet contains 240 mg dry extract from calf thymus). Manufacturer: Dr. K. Mulli, D-7844 Neuenburg, FRG

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17

CANCER MULTISTEP THERAPY: PRINCIPLES AND STATE OF THE ART

MANFRED VON ARDENNE[1]

DEVELOPMENT OF ONCOLOGIC HYPERTHERMIA

Attempts to destroy primary tumors by local hyperthermia are not recent (4, 5, 10). In continuation of these efforts, the following developments have been pursued.

A. Extreme whole body hyperthermia

By placing cancer patients in a dual-chamber hot water bath, body temperatures of 43°C could be maintained for 60 min, if the head were cooled with water of 10°C in a separate compartment. With this treatment initial tumor remissions occurred (9, 31), and some inoperable tumors became operable after the treatment.

B. Local and regional two-step hyperthermia

With this protocol the circulatory complications sometimes occurring in older patients could be decreased. The whole body was heated first to a tolerable temperature of 41–41.5°C; then the temperature was increased locally 42.5–43°C only in the tissue bearing the primary tumor or in areas suspected of carrying metastases (15).

C. Local hyperthermic perfusion

A perfusion device which encompassed a rotary oxygenator, roller pump and heat exchanger was devised to provide partial extreme hyperthermia (6). Heated blood with chemotherapeutic agents was thus available for regional perfusion. This method was improved and extended by Cavaliere and colleagues (3, 8) and others (11).

D. Synergism of hyperthermia and hyperglycemia

Aerobic tumor glycolysis is stimulated by a 5- to 8-fold increase in the systemic blood glucose level for several hours; this causes the pH of the cancerous tissue to drop about 1 unit below normal (38) (Figure 1). The time course is shown in Figure 2 (49). The drop in pH leads to a selective increased heat sensitivity of the cancer cells and tissues in the order of several degrees C (Figure 3) (32). Thermal sensitization of cancer tissue is important for deep seated cancerous tissues that can hardly be heated to the temperature of 42.5°C needed for destruction of the cancer cells. However, the synergism between hyperthermia and hyperglycemia (49) received little attention for more than 10 years.

1. Proof for the acidification of micrometastases

Using a glass microelectrode we found that micrometastases of only about 1 nm^3 (10^6 tumor cells) were also appreciably lower in pH (52) (Figure 4). The study demonstrated that metastases are susceptible to the combined treatment of hyperthermia and hyperglycemia. However, metastases with few cells, where the pH is not readily lowered, can be destroyed by the method of oxygen multistep immunostimulation (30).

2. Circulatory support by oxygen multistep measures at hyperthermia with high core temperatures: *1969–1983*

The incorporation of oxygen multistep measures into the entire treatment program proved to be invaluable for the stabilization of the cardiovascular system during long lasting hyperthermia at body temperatures up to 41.5°C (19, 27, 34).

3. Radiological variant of the Cancer Multistep Therapy. Hyperthermia plus hyperglycemia and local irradiation at reduced doses.

The importance of the combination of local irradiation and hyperthermia was emphasized by us (19, 33, 48). These publications have often been overlooked, although the value of hyperthermia as part of clinical cancer therapy was recognized through this combination (2, 13, 14). The main effect, namely to lower the total radiation dose, for example to 1200 rad, is due to the so-called lysosomal cytolytic chain reaction (2, 16, 19). The powerful activation of lysosomal enzymes at pH values <6.5 produced by hyperglycemia results in selective increase of the radiation effects in the cancer tissue.

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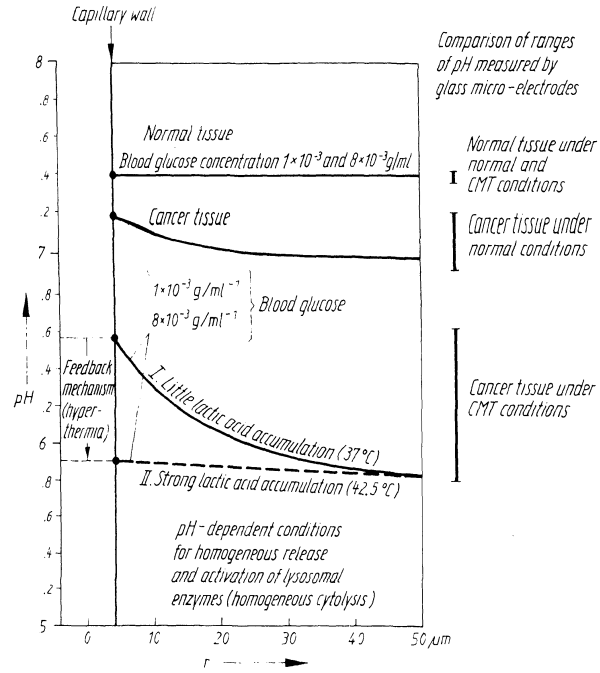

Figure 1. The pH value as function of the distance r from the capillary axis in the venous capillary region under normal and CMT conditions, calculated for the steady-state and tumor volumes exceeding 100 mm^3. (From A. and M. von Ardenne, 1977 [38]) Result: Selective pH difference in tissues of about 1.4 and in capillaries of about 0.8-1.4 units.-Multiply utilizable source of selectivity during the CMT.

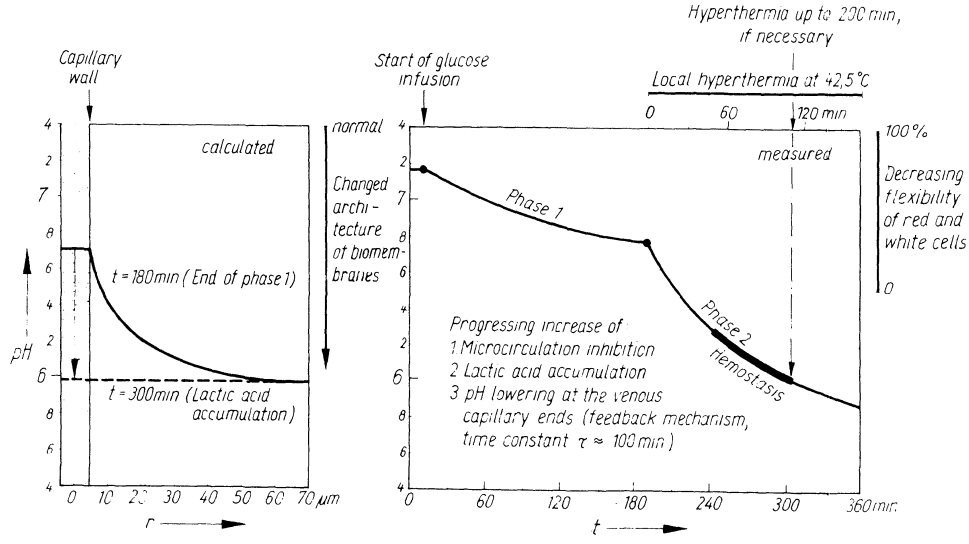

Figure 2. Calculated pH value in cancer tissue as function of the distance r from the venous end of a capillary at a systemic blood glucose concentration of $C_G = 5 \times 10^3$ g/ml at the times t = 180 and 300 min (A) as compared to the pH measured *in vivo* (glass micro electrode) near a tumor capillary wall after glucose infusion and local hyperthermia at 42.5°C (B). Wistar rat bearing a $g_{\ddot{u}}$ DS carcinosarcoma. Complete tumor remission and cure. Expt. from 1969 [38, 41]

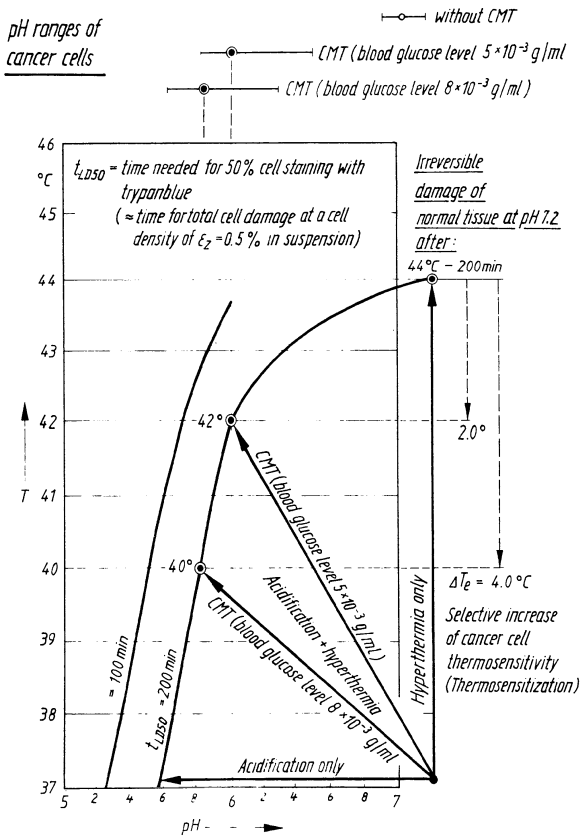

Figure 3. Synergism of hyperthermia - hyperglycemia. *In vitro* measurements showing the interrelations between hyperthermia temperature T, cancer tissue pH and exposure time $t \approx t_{LD50}$ for total damage of cancer cells. Ehrlich ascites cells (0.5%) in lactalbumin-yeast extract medium, glucose concentration 10^{-2} mg/ml, $pO_2 = 2$mmHg, pH values adjusted by lactic acid. Expts. of 1968 [32]

4. Hyperthermia plus hyperglycemia plus adjuvant measures.

Our research initiated to optimize the "Cancer Multistep Therapy" (CMT) represents a continuing *development*, which finally resulted in the CMT concept (22, 24). It must be emphasized that any development is necessarily linked with certain changes. Progress can only be achieved from the permanent interplay of trial and error. Modifications of our concepts were concerned exclusively with the improvement of the hyperthermia technique as well as the dosage and timing of adjuvant steps, whereas the backbone of the CMT concept, namely the combination of *hyperthermia plus hyperglycemia* has been unchanged since 1965. *Adjuvant steps* are not only measures for additional killing of tumor cells such as *radiotherapy* (at reduced doses), *chemotherapy*

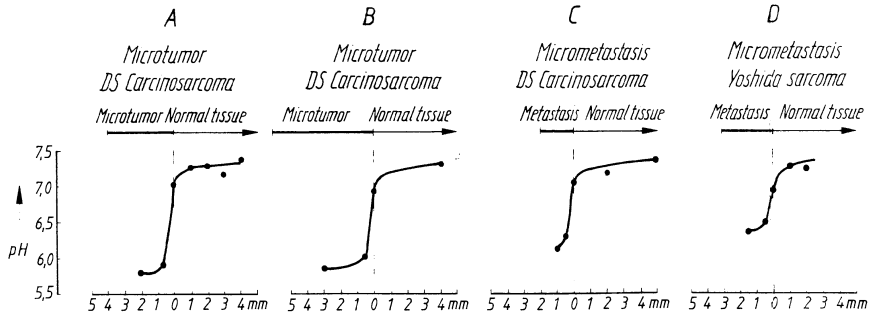

Figure 4. The pH profiles inside and outside optimally acidified microtumors and micrometastases of different size and type. *In vivo* measurements made 1969 [52] using pH glass microelectrodes having sensitive tips of about 80 μm. Duration of hyperglycemia: t = 200 min at a blood glucose level of 4×10^{-3} g/ml.

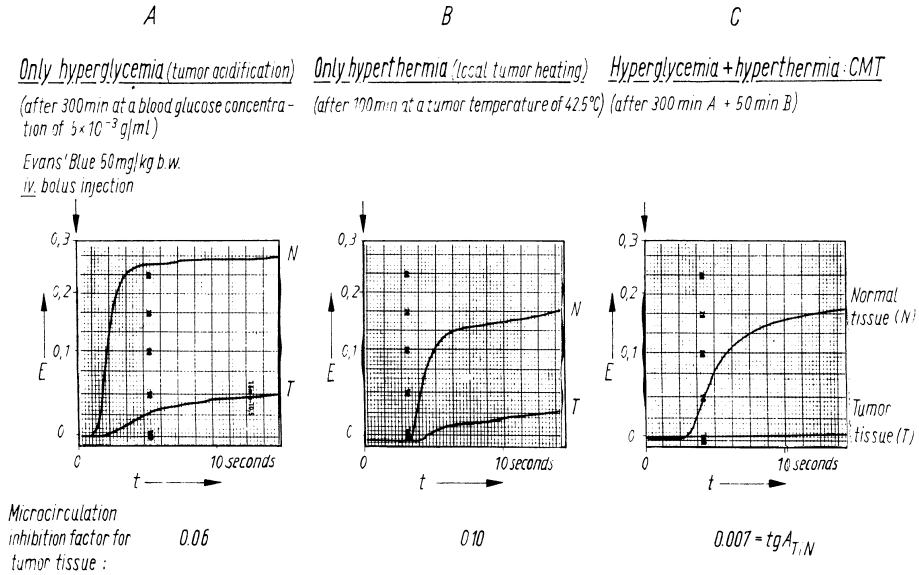

Figure 5. Selective inhibition of blood microcirculation in tumor tissue by hyperglycemia (A), hyperthermia (B)[1] and the combination of both (C)[2]. Simultaneous photoelectronic recording of the intravasal dye influx into normal (N) and tumor tissue (T) measured as tissue extinction after a single iv. bolus injection (during 0.3 seconds) of Evans' Blue. – DS carcinosarcoma-bearing Wistar rats weighing 200–250 g, tumor size ≈ 7 g

[1] This result reveals that even hyperthermia effects *selective* blood microcirculation inhibition, since the microcirculation in normal tissue remains almost unaffected.

[2] Same result 24 h later!

(for triggering the lysosomal cytolytic chain reaction) and the *oxygen multistep immunostimulation* (to strengthen the body defenses), but also include measures directed against the unavoidable side effects of hyperthermia, drugs and radiation (25, 27). The introduction of such supporting measures marks an important stage which should be emphasized: the oxygen multistep procedures generally support any kind of cancer therapy. They can contribute to an increased quality of life, stabilization of circulation and to designing time saving therapy regimens.

5. Selective inhibition or interruption of blood microcirculation in tumor tissues

Studies of the selective impairment of blood microcirculation in tumor tissues by 42.5°C-hyperthermia and optimized lowering of tumor pH (20), resulted in discovery, design and clinical application of a novel therapeutic principle for fighting cancer (39, 42, 44): the selective and irreversible occlusion and destruction of the tumor vasculature by the combined measures of the CMT. The synergism of hyperthermia and hyperglycemia is also effective for inhibition of microcirculation and selective triggering of hemostasis (44) as illustrated in Figure 5.

6. The CMT Selectotherm procedure for homogeneous two-step regional hyperthermia: *1975–1984*

Progress both for clinical hyperthermia in general and particularly for CMT was achieved in 1975 by the invention of the *CMT Selectotherm method* (M. and Th. von Ardenne, 21). By using specific applicators this radiofrequency heating procedure allows a surprisingly uniform heat deposition even in deep-seated tumors. This technique was improved recently (40, 50, 53, 54).

The Dresden studies of development and design of oncologic hyperthermia represent also stages of the elaboration of the Cancer Multistep Therapy. About 250 experimental and theoretical papers from our Institute are the scientific basis of the CMT concept of today.

7. Treatment protocol for cancer multistep therapy

Type, dosage and timing of the major and adjuvant steps of the CMT are given in Table 1 and Figure 6. The entries in the table refer to the three standard variants of this concept, as explained in the following sections:

A: Variant with ionizing radiation
B: Variant with anticancer drugs
C: Variant with "releasing attack" (without radiation and drugs).

a. Oxygen multistep immunostimulation and conditioning

Oxygen multistep immunostimulation (30) is an integral part of the recent CMT concept since it serves simultaneously as a conditioning measure for the patient (29), for killing tumor cells escaping the major CMT steps and for fighting metastasis after diagnostic or therapeutic manipulations of the primary tumor (30).

This procedure effects a long-lasting improvement in the oxygen state of patients and a significant increase of the

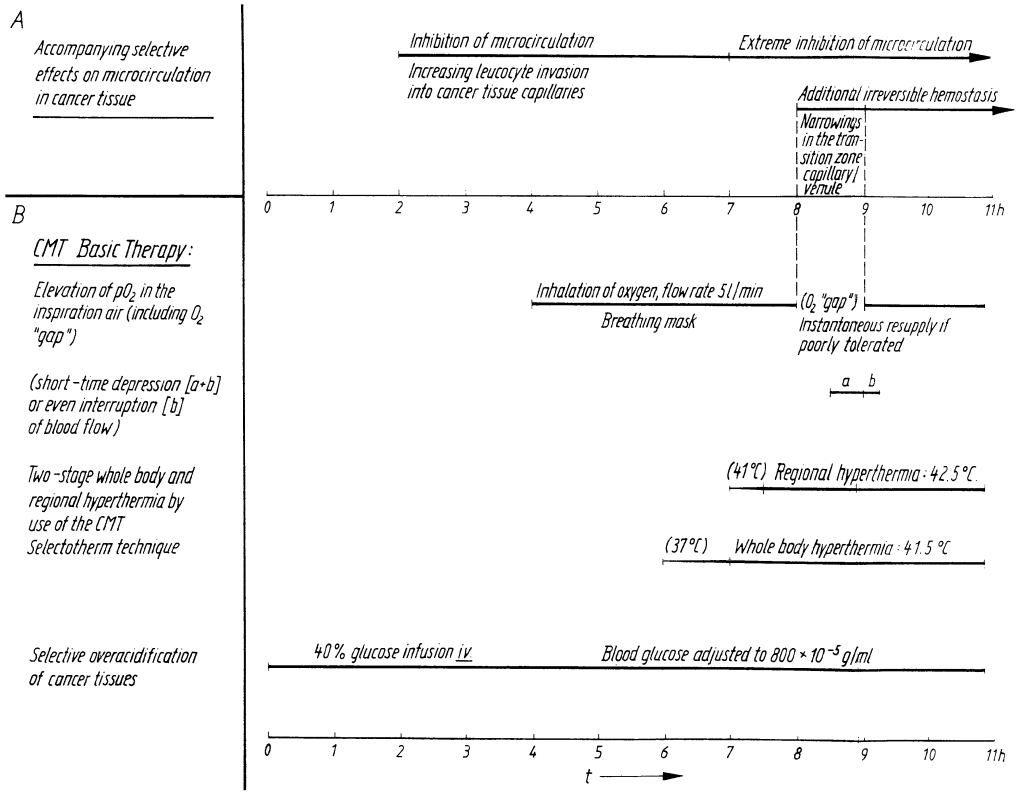

Figure 6. Selective microcirculatory effects in cancer tissues (A) and their relations to the time dependency of compulsory measures within the schedule of the CMT basic therapy (B).

unspecific cellular defenses (27, 30). It consists of a powerful stimulation of the cellular defense (production of immune cells) combined with an increased oxygen transport, as great as possible, into all tissues of the body. Animal experiments had shown that unspecific immunostimulation by 2-cyanoethyl urea (our code name BA 1-4), thymus extracts, or other immunomodulators persists for more than 20 days and is very effective and well tolerated. The elevation of oxygen transport to activate immune cells is achieved by combining the standard 36 hr-oxygen multistep process (27) with re-infusion of the patient's own blood after UV irradiation, repeated for several days (51). The oxygen multistep immunostimulation protocol is given in Table 1; this treatment covers not only the prophylactic range but extends over a therapeutic region (killing capacity up to 10^9 malignant cells). The measures for improving the oxygen status are of particular importance for the CMT variants using radiation or chemotherapy, because irradiation and drugs can critically impair the oxygen status of the organism (25, 30). The treatment protocol is timed so that its effect occurs one day before and one day after the major therapy.

b. Ionizing radiation and cytotoxic drugs for triggering cytolysis

To improve the therapeutic efficiency of the major therapy (hyperthermia + hyperglycemia) the addition of a "releasing attack" may be useful. If a primary tumor or a major metastasis is to be attacked locally, ionizing radiation can be recommended. To affect the different tumor cell fractions in the sensitive phase of the cell cycle, a low total dose of about 1200 rad should be divided into two equal doses within an interval of 12 hours (23). When drugs are preferred for the "releasing attack" as a systemic measure, it is recommended to employ a drug for which an antidote is known (for example, the pair Methotrexate and Leucovorin). In such a case, the drug is given first as an extremely high-dose which becomes evenly distributed in the whole body. A relatively high amount of the drug will be entrapped in the tumor after about 5 hours, because at that time the tumor vasculature is progressingly occluded by the other measures of the CMT. The antidote, when given at this point will hardly penetrate this barrier and not reduce the desired drug effect in the target; on the other hand, the antidote can display fully its compensating effect in all healthy tissues of the organism (28).

c. The increase of the blood glucose concentration to stimulate aerobic glycolysis in cancer cells

According to our own clinical experience and that made by our clinical collaborators, it is possible to increase the blood glucose concentration up to 8 mg/ml for many hours without risk, if the application is made under a "protective

Table 1. Treatment Scheme according to the Cancer Multistep Therapy Concept 1977/84
Deadline: Oct. 31, 1984

Characteristics Effects: Segregation of cancer tissues from circulation by selective occlusion of their vessels. Triggering of lysosomal cytolysis. Tissue destruction. Regression and cicatrization with low circulatory burden by toxic degradation products. Fighting metastasis.

Methods: Two-step hyperthermia (whole body at 41°C, regional in a body section of 42.5°C). Cardiovascular relief by the Oxygen Multistep Therapy. Utilization of the extremely pH-sensitive interactions between red cells, white cells and platelets. Utilization of the $pO_{2\text{-ven}}$-controlled switching mechanism of microcirculation for selective blood flow interruption. Oxygen Multistep Immunostimulation.

Fractionation: Repeatable treatment for primary tumors and metastases as well in intervals of about 8 days.

		Therapeutic measure (Step)					
Treatment phase	*Treatment element*	*No.*[1]	*Measure*	*Dosage (per 75 kg. b.w.)*	*Day of application*	*Timing*	*References*[2] *(Remarks)*
1.	1.1 Lysosomal labilization	1.1.1	[Retinol palmitate, orally	[D = Σ 30 × 16^6IE]	−7 to 7	3 equal doses, daily	No. 62, 71, 92, 96, 203. (optionally]
	1.2	1.2.1	Stimulation of unspecific immune defense, permanent improvement of the O_2 state (value of η) by		−2 to 15	see paper No. 308, Fig. 17	No. 163, 194, 264, 266 new (No. 79, 92, 96, 108, 116, 119)
			1) 2 h oxygen multistep therapy	Orziprenalin (Alupent) 20 mg orally	−2 to 15	before each sitting	
	Oxygen Multistep Immunostimulation		daily for 18 days	Oxygenabund, 5 lO_2/min	−2 to 15	one sitting daily	No. 298, 308
Conditioning			2) 7 × HOT* treatment	Standard HOT* (optionally)	−2 to 15	on Day 0, 2, 4, 6/8, 11, 15	
			3) 15 i.m. injections (or 15 × 3 tablets[5]) if thymus peptide ISTP (Mulli)	1 ampoule (2 ml, 13 mg), each	0 to 15	on Day −2, −1, 0, 1, 2, 4, 5, 6, 7/9, 10, 11, 13, 14, 15	total 15 ampoules or 45 tabl.[5]
				Dose = 3.4 g 2-cyanoethyl urea, each	−2 to 15	on Day −2, 0, 2, 7/11	total 17 g
			[4) 5 i.v. injections of BA 1–4]		−2 to 11		
2. Releasing attack: Triggering of cytolysis and cytolytic chain reaction in acidified tumor tissues	2.1 Localized measure	2.1.1	Ionizing radiation for local treatment of primary tumor and major metastases (cells damaged in their sensitive cell cycle phase initiate cytolysis)				No. 65, 69, 77, 88, 92, 96, 148, 151, 154, 171, 201
				D = 600 R	7	13 hr (on the eve) and 1 hr	
				D = 600 R	8	before main therapy	No. 203
	2.2 Systemic measure	2.2.1	[Cancerotoxic drugs for systemic treatment of primary tumor and metastases (Prospective application of CMT Selectines)]	Depending on the drug used (high dosage for "bolus" application)	8 7	At onset of main therapy (cancerotoxics) or on the eve (cancerostatics); antidote if possible, at t = 3 h	No. 174, 183a, 201, 306 No. 151, 169, 173, 174, 176, 180]
3.	3.1	3.1.1	Glucose (40% solution containing 16 IE soluble insulin per 500 ml)[4] (Selective long-term stimulation of aerobic glycolysis of cancer cells)	$Dt_A^{-1} \approx 1.9$ g/min	8 Day of main therapy	from t = 0–10.50 h	No. 23, 31, 59, 76, 85, 87, 112, 116, 117, 204, 211
				(Blood glucose concentration adjusted and monitored at		from t = 5.30–7.30 h blood glucose level	No. 64 (Insulin) No. 155, 112, 174

	Selective and irreversible vascular occlusion in cancer tissue	3.1.2 WBH	Whole body hyperthermia (WBH)	Regional hyperthermia: Section 1 (primary tumor); fractionation, if necessary	800 mg/100 ml). T_{core}: 41.5°(41°)C Additional heating of a body section to T_H 42.5°–43.0°C (Two-step hyperthermia using the CMT Selectotherm equipment; double-applicator systems)	8	at 800 mg/100 ml Heating up 6.00–7.00 h 60 min Head cooling, cardiovascular support; myocardial protection, if necessary (e.g., by Strodival special) Heating up 7.00–7.30 h 30 min Constant phase 7.30–10.50 h 200 min (aim)	3.1.2 No. 164, 13, 18, 76, 113, 159, 183a 3.1.3 No. 189, 196, 197, 198, 213, 223, 233, 238, 308 Whole body hyperthermia: scanning around the body. Regional hyperthermia: reduced scanned areas, fine adjustment by water mattress
Main therapy		3.1.3 RH		Section 2 (metastatic region)				
	(Final stage: confluent large-area necrosis)	3.1.4	[Short-term blood flow reduction or interruption localized or systemic		Intermittent blood flow interruption (inflatable cuff)	8	Section 1:t = 9.00–9.15 h Section 2:t = 12.00–12.15 h	No. 225, 226, 275, 277 (General reduction of blood flow effects temperature increase in deep-seated tumors!)
					[Reduction of systolic blood pressure to about 80 mm Hg[3]	8	Section 1:t = 8.30–9.15 h Section 2:t = 12.00–12.15 h]	
	3.2	3.2.1	Mineral balance (K^+)		D ≈ 10 mval K^+ per 100 g infunded glucose	8	from t = 0–10.50 h	
	Adjuvant measures	3.2.2	Increase of pO_2 in breathing air		5 l O_2/min (≈ 400 mm Hg) (ideal value $pO_{2\text{-art}}$ ≈ 120 mm Hg)	8	from t = 4.00–8.00 h; to be continued if patient's general condition deteriorates	
		3.2.3	Infusions of vitamins B_1 and C		D = 100 mg B, and 1 g C	8	at t_0 and 10.50 h	
4. *Rehabilitation* (repeated application if necessary)	4.1 Oxygen Multistep Therapy	4.1.1	Repetition of Oxygen Multistep Therapy, if the value of η, measured e.g. on Day 22, is too low		see Tables in the respective book (column of References)	10 to 18	see Tables in the respective book	No. 307, program II; M.v. Ardenne, Oxygen Multistep Therapy 4th edition, Thieme, Stuttgart 1987

[1] The numbers of first-rank measures (essential) are underlined, other numbers indicate optimal measures.
[2] The numbers indicated in this column refer to the (numbered) papers of the book by M. von Ardenne, "Theoretische und experimentelle Grundlagen der Krebs-Mehrschritt-Therapie", 2nd edition, VEB Verlag Volk und Gesundheit, Berlin 1970/71, and to papers published thereafter (further informations or reprints on request).
[3] Or, at least, prevention of any RR increase.
[4] By means of a catheter inserted into the vena cava superior via vena subclavia.
[5] Daily oral intake of 3 coated tablets thymus extract "Mulli".

shield" of oxygen (oxygen multistep therapy) and the mineral balance is kept within normal bounds by substituting electrolytes (K^+) (12). The effective stimulation of aerobic glycolysis and the powerful, selective acidification of cancer cells by an increased blood glucose level depends largely on the intact blood-brain (17) and the blood-nerve barrier (18). These barriers prevent the nervous tissues which have a limited aerobic glycolytic capacity against hyperglycemia. Still another favorable circumstance is responsible for the stimulation of tumor cell glycolysis *in vivo*. The overwhelming fraction of the malignant cells in a tumor are poorly supplied (35, 37) *in vivo* and their glycolytic capacity is not exhausted at normal blood glucose levels. Consequently, an excessive glucose offer results in a remarkably enhanced lactic acid production.

The hyperthermic step is not initiated before about 6 hours after the start of the glucose infusion. At this point, steady-stage conditions have developed and inhibition of the microcirculation has progressed in the tumor. The latter has another beneficial effect, as the tumor cooling by blood convection ceases and the tumor temperature increases above the level of the surrounding host tissue. However, this leads to a significant temperature gain only in tumors larger than about 1.5 cm in diameter (54).

d. Two-step regional hyperthermia at 42.5°C

An example showing the temperature course in the host tissue surrounding a tumor (T_H) and in the center of the body (T_C), when the first generation CMT Selectotherm device was used, is given in Figure 7 A as compared to the ideal temperature course to be achieved (7 B). Although the theoretical temperatures could not fully be attained in this case, the treatment was successful. The reason for this was probably that due to the remarkably reduced cooling of blood by convection as is envisaged in the CMT, the tumor temperature T_T (not measurable then) was actually higher than the (measured) temperature T_H in the adjacent tissue. Furthermore the temperature-decreasing effect of heat conduction is, as a rule, strongly reduced in bronchial carcinomas. When the core temperature is elevated from 37°C to 41.5°C, the HbO_2 binding curve is shifted to the right (cp. Figure 126 in (27). This can lead to a collapse, generally in the elderly after long-term hyperthermia. However, the onset of such complications is effectively counteracted by the application of oxygen, by which the arterial pO_2 is usually increased to 100 mm Hg (13.3 kPa) as indicated in Table 1.

Oxygen application can be interrupted during regional hyperthermia ("oxygen gap" in Figure 6) until the first early signs of incompatibility occur. The rationale of this transitory measure is to reduce the pO_2 at the venous ends of the tumor capillaries, which in return produces narrowing in the transition zone near the venules (26) and favors hemostasis.

In order to achieve tumor regression by means of the CMT, it is of primary importance *to bring two physical parameters to certain values in the tumor tissue:*

first - the pH value in the intercapillary space of the tumor and at the venous ends of its capillaries to 6.2 and 6.6, respectively;

second - the intratumoral temperature to $\geqslant 42.5$°C.

Both physical figures are measurable and they can be monitored in the individual case, so one can check whether or not the desired values are achieved or even exceeded.

According to clinical experience, the major obstacle of practical realization is that a temperature of 42.5°C or above must be attained even in such cancerous tissues which are not readily achievable by the external energy source. Overwhelming this difficulty for a given tumor is mainly a physical and technological problem. For cancers, near the surface, local hyperthermic radiofrequency devices often proved to be sufficient (2, 13, 14). Therefore, the CMT protocol according to Table 1 is also practicable with moderate expenditure for the treatment of superficial tumors. This statement, however, does not yet apply to deep-seated tumors. For this, we developed a novel RF technique for two-step regional hyperthermia which, as compared to other known devices, improves the energy deposition even in deep-seated malignant lesions, as detailed below. In contrast to usual arrays for *local* hyperthermia, the Selectotherm procedure succeeds in attaining the necessary temperature in a circumscribed *region* of the body, due to the scanning motion of the applicator system. Thus, it becomes possible to attack metastases of unknown localization. In this context, the stepwise heating of different body sections as is indicated in Table 1 should be mentioned. By using such a *fractionated* heating pattern one could even envisage a whole-body protocol for the treatment of disseminated cancer. We consider the possibility to fight metastases by means of fractionated regional hyperthermia to be a prominent difference and significant progress, compared to most methods known in hyperthermic oncology.

8. Selective, irreversible arrest of cancer tissue microcirculation in cancer tissue as an extremely pH-sensitive and therapeutically utilizable biomechanism

As shown by Raman laser and X-ray diffraction studies, biomembranes undergo a remarkable alteration of their architecture, when the pH changes from 7.4 to 6.5 (24). These structural rearrangements occur in fractions of a second. This notice stimulated research on microcirculation of the blood for therapeutic utilization within the framework of the CMT concept.* The outcome of these studies is outlined in Figure 8 and summarizes the processes that proceed in the transition zone between capillaries and venules at low pH values and that may irreversibly stop the microcirculation. We learned that the basic mechanism of the pH-induced rearrangement of biomembranes has at least *four rheological consequences* (24).

The pH drop effects a drastic decrease in flexibility of both *erythrocytes* and *leukocytes*. Stiffened red cells crowd in the capillaries, red and white cells come into close interaction, and white cells adhere at the endothelium of the venules. These processes increase the probability of generation of microthrombi. Finally, the inner vessel walls become sticky, and non-enzyme-mediated fibrinogen deposits at the endothelium can be detected (43, 45, 46, 47). These multiple effects of the basic mechanism outlined above result in a

* In this early phase we obtained numerous kind suggestions from AL Copley, L Dintenfass, H-G Lasch, G Schmid-Schönbein, H Schmid-Schönbein and O Westphal.

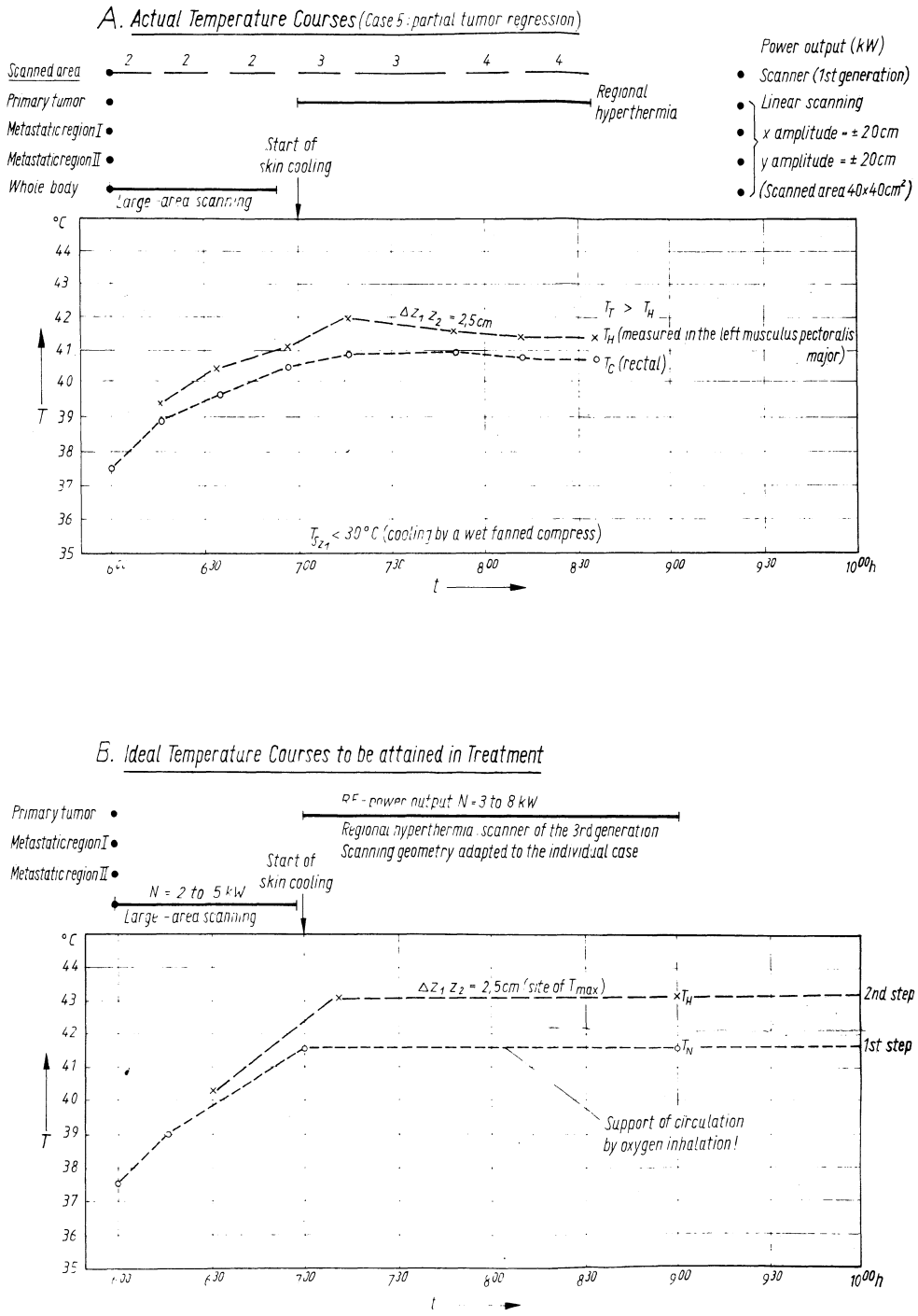

Figure 7. CMT Selectotherm regimen and tissue temperatures as function of the time t after onset of the two-step hyperthermia according to the CMT program. (R = 8.5cm, $\Delta Z_0 Z_1$ = 10 cm) Symbols: T_H = temperature of the (peritumoral) host tissue, T_T = tumor temperature, T_C = core temperature, T_S = skin temperature

A. Actual temperature courses, case No. 5, code R.E.BKC-03:47yr-old female patient with a left-sided inoperable bronchial carcinoma, date of treatment: Feb. 14, 1980, follow-up Sep. 8, 1980, partial regression

B. Ideal temperature courses to be attained in treatment by using the double applicator system, optimized power output and temperature control (heat insulation, thermostatized water mattress).

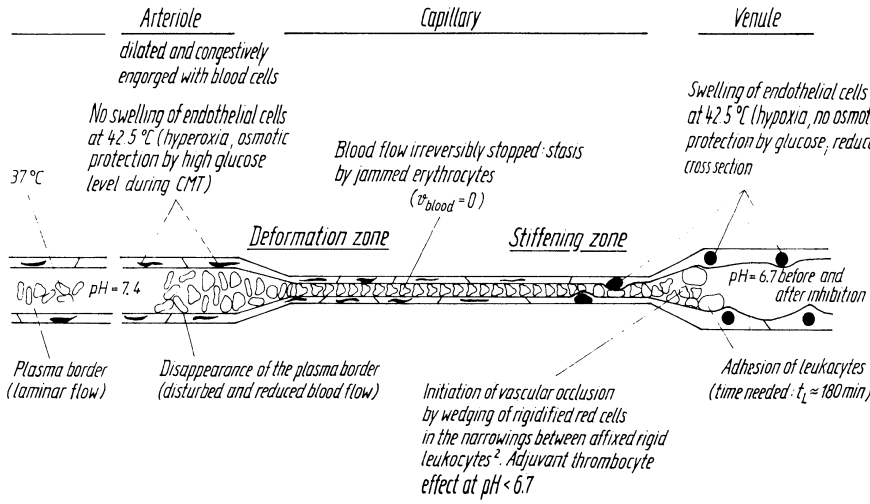

Figure 8. Suggestions on the mechanism of vascular occlusion in the capillary-postcapillary bed (hemostasis, microthrombosis) by combining optimized tumor tissue acidification (pH $\approx$ 6.0) with regional hyperthermia at 42.5°C (CMT Selectotherm technique), possibly combined with short-term blood pressure reduction[1]. CMT concept 1977/84. Primary effect: Structural rearrangement of biomembranes (rigidification of red and white cells at pH decrease from 7.4 to $<$ 6.7 in the transition zone of the venous capillary ends.

[1]Short-term blood flow inhibition, e.g., by local or systemic reduction of the propulsing blood pressure, promotes the onset of vascular obstruction (reduced shear decreases blood fluidity).

[2]Rich in lysosomes, discharge and activation of lysosomal enzymes at low pH: additional contribution to blood cell stiffening. Further: accumulation of platelets and fibrin deposits in the proximate parts of the venules.

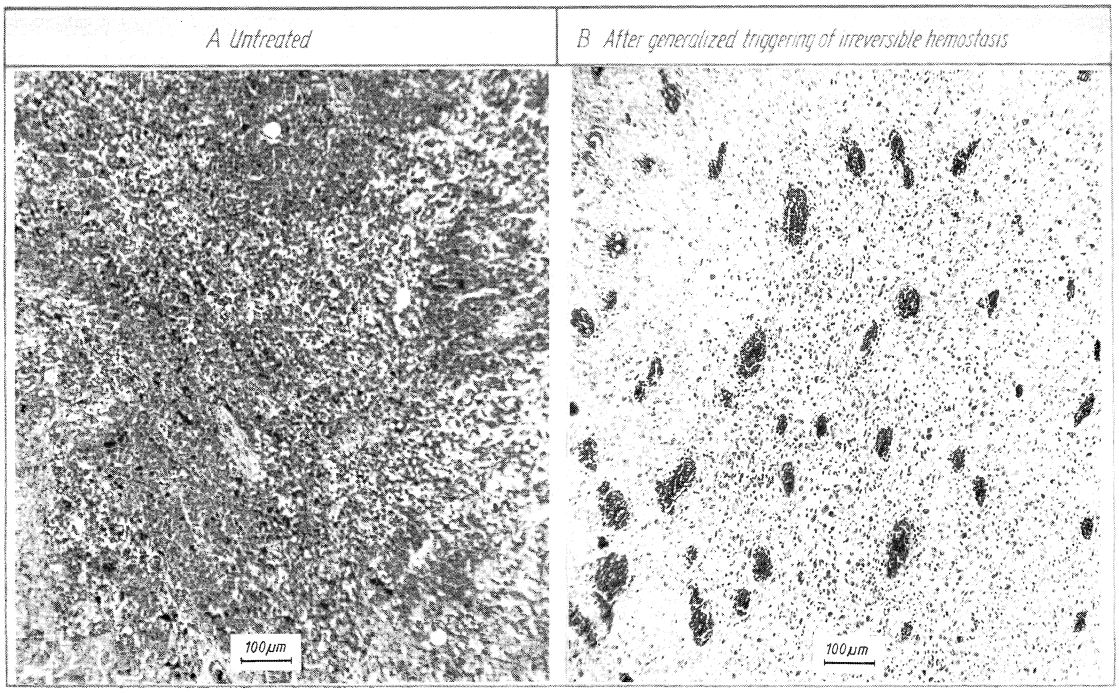

Figure 9. Histological sections of a rat DS carcinosarcoma before and after selective segregation of the tumor tissue from the circulation by triggering irreversible hemostasis by means of hyperacidification and local hyperthermia at 42.5°C. The blood vessels are dilated and occluded (B). Trichrom staining after Goldner. Green filter.

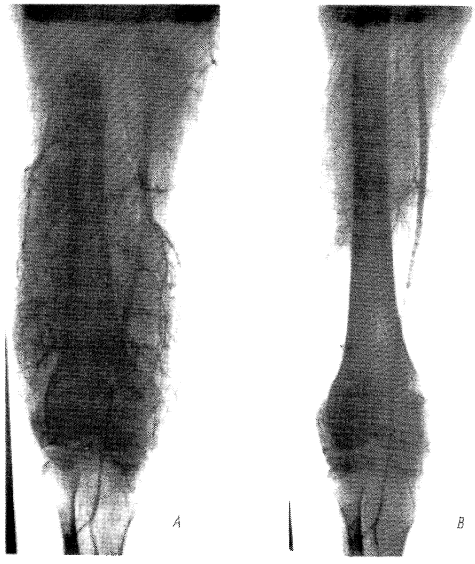

Figure 10. Arteriography of a clear cell sarcoma above the right knee before (A) and three weeks after treatment acco.ding to the 1977 CMT concept using a simplified procedure. The well vascularized tumor (left) was no more demonstrable after treatment (right), the healthy tissue remained unaffected.CMT pilot treatment M. von Ardenne, Clinic R. Barke, Medical Academy, Dresden.

remarkable pH sensitivity of blood microcirculation; it is almost the same mechanism that is responsible for shock and heart infarction. Moreover, the selective triggering of hemostasis is favored by endothelial cell swelling elicited by hyperthermia at 42.5°C and oxygen deficiency ("oxygen gap" in the treatment protocol of Figure 6). The measurements previously shown in Figure 5 revealed that not only optimized tumor acidification but also hyperthermia itself reduces the blood microcirculation in tumor tissues to one tenth of the normal. The consequence is that by combining hyperglycemia with hyperthermia according to the CMT protocol, the blood microcirculation in a tumor is reduced to 1% or less, which corresponds virtually to cessation of bloodflow (42). However, bloodflow inhibition fails, when at normal leukocyte and platelet counts the hyperthermic phase is reduced from 100–120 min to only 25 min.

The irreversible vascular occlusion in the tumor effects its starvation and decay as well as its segregation from circulation. Consequently, toxic tumor degradation products cannot overrun the bloodstream and provoke critical intoxication; moreover, the danger of internal bleeding from the damaged tumor is reduced. Histological sections of a rat tumor showing generalized and irreversible hemostasis after treatment are given in Figure 9 (55).

In contrast to all other weapons used against cancer to now, the method of selective bloodflow inhibition in tumors differs greatly in that the primary targets are no longer tumor cells with their largely variable properties but blood cells having a relatively small biologic variability. Thus, the selective occlusion of tumor vessels might lead to a concept of broad applicability in therapy, because the irreversible halt in a supply of substrate results in an accelerated starvation of solid tumors.

Figure 11. Disappearance of a lymph node metastasis as demonstrated by lymphography after local hyperthermia (A: before, B: 3 weeks after CMT treatment). The same case as in Figure 10.

In clinical CMT trials, we were able to demonstrate for the first time that human cancers disappear by selective occlusion of tumor tissue capillaries, i.e. by tumor starvation.* In Figure 10 the X-ray arteriograms of a 2 kg clear cell sarcoma above the knee are compared. The tumor regressed completely within three weeks after CMT treatment. A pelvic metastasis disappeared as well in the same patient (Figure 11). After a follow-up time of more than 5 years no signs of recurrency or metastasis could be detected so that this patient can be considered to be cured after CMT treatment. Further results of clinical trials, in which selective

* Accomplished in close cooperation with Prof. Dr. R. Barke, director of the Radiologic Clinic of the Medical Academy Dresden.

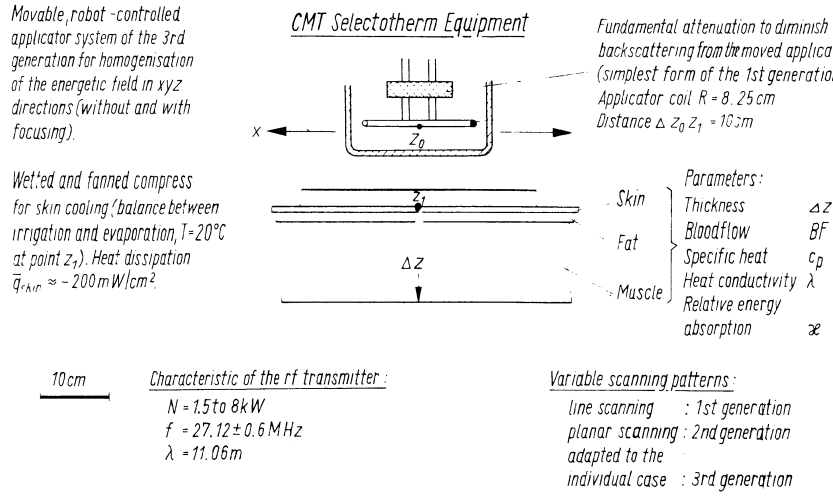

Figure 12. Selective regional CMT scanning hyperthermia according to M. and Th von Ardenne. Schematic of the one-system variant representing an acceptable compromise on the chosen geometric parameters ($R\Delta Z_0 Z_1$ etc.). Additional skin cooling, core temperature $T^c \approx 41.5°C$ (Minimum 41.0°C). - The three-layered model tissue (skin-fat-muscle) and its parameters.

destruction of smaller tumor vessels could be detected clearly, were reported by Barke *et al.* (1). From other treatments showing only partial remissions and from tumor temperature measurements made during the phase of regional heating, there emerged the necessity for increasing the heat effects in the depth of the tumor.

9. State of the art and perspectives of the CMT Selectotherm technique for two-step regional hyperthermia

It is the aim of the CMT Selectotherm procedure to produce temperatures of 42.5–43.0°C in the host tissue of a body region suspected of harboring cancer. In the circumscribed

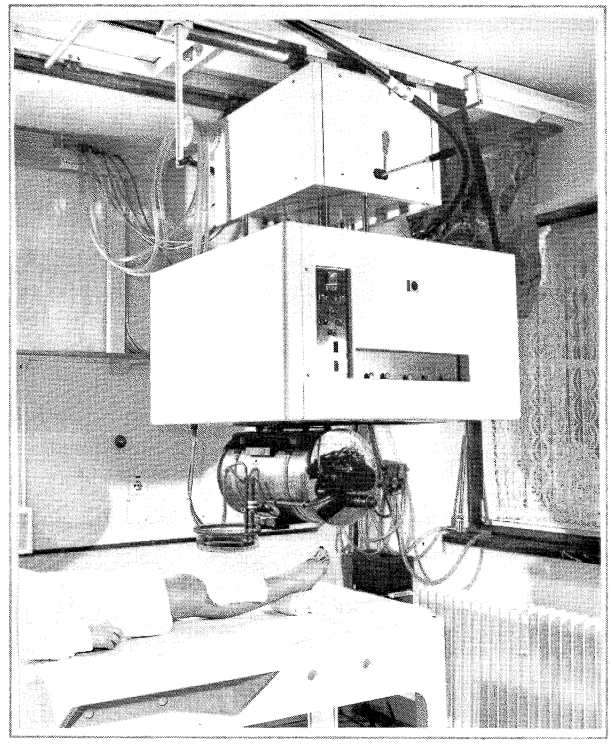

Figure 13. Applicator system (1st generation) of the 8KW CMT Selectotherm device used for clinical trials in the Forschungsinstitut Manfred von Ardenne from 1979 to 1984. The driving unit for the x-ray scanning motion is installed in the box above the applicator.

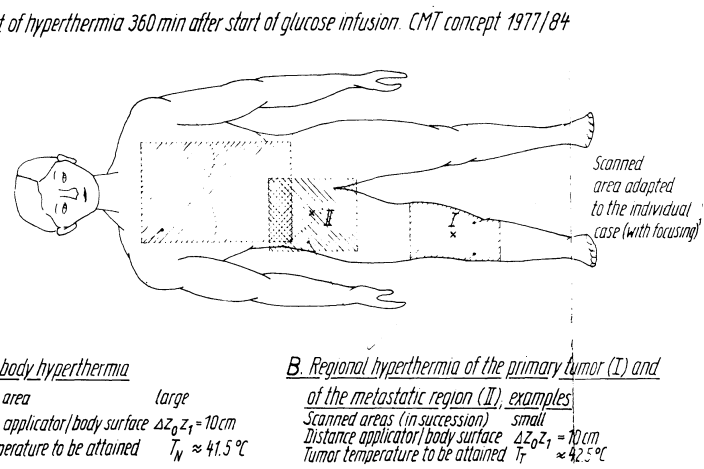

Figure 14. Scanned areas of different size and shape, generated by the two-step whole body/regional hyperthermia using the CMT Selectotherm equipment. Fine adjustment of T_N by appropriate choice of the power output N of the transmitter and of the initial warming-up period as well as by means of a thermostated water mattress below the patient.
[1]For example, motion of the dual system applicator on a cylinder envelope, the axis of which goes through the center of the tumor.

section to be treated the temperature must not exceed 44°C, which would lead to burns in normal tissue at treatment periods of some hours, and must not fall below 42.5°C, which is necessary for tumor damage. When one realizes that there is a therapeutically utilizable margin of only T = 1.5°C and that the regions to be treated extend often to the center of the body (12 cm of depth), the difficulties involved in the solution of this task become obvious. Any effective hyperthermia technique has, therefore, to guarantee

- a sufficient *homogeneous* temperature distribution in the target area and
- a sufficient *depths of penetration.*

The practical realization of these demands proves to be very difficult and has by no means been met optimally and comprehensively by any of the known hyperthermic techniques. We are trying to approach this goal by using a particular variant of short-wave diathermy. Our CMT Selectotherm technique is characterized by

- preferential use of inductive short-wave diathermy (induction of eddy currents in the biological object),
- permanent, optimized movement of the applicator over and around the patient's body (scanning),
- specific design of the applicator and combination of inductive and capacitive variants of short-wave diathermy, if applicable.

By using inductive short-wave hyperthermia, the subcutaneous fatty tissue is thermally relieved (7). Reflection phenomena at tissue borders and the depth-dependency of energy absorption, both occurring increasingly at higher frequencies, can be avoided and reduced, respectively. The permanent movement of the applicator over the body (scanning principle) allows a remarkable uniformity of the temperature distribution, which is very inhomogeneous by nature due to the heating principle applied (RF diathermy).

The simplest variant of the applicator system of the first generation is described in Figure 12. A photograph of this system used from 1979–1984 for clinical CMT trials is reproduced in Figure 13. By means of this previous array the results shown in Figures 10 and 11 and those reported by Barke *et al.* (1) have been obtained. The device serves for heating the whole body to the desired core temperature and also for additional temperature elevation in the body region to be treated (cf. Figure 7). For attaining a core temperature of T = 41.5°C (at least 41.0°C), a large scanning area is chosen first, whereas thereafter for the additional temperature elevation for regional treatment the scanning area is reduced. Figure 14 exemplifies proportions and positions of the scanned areas adapted to the individual case.

In most cases the lesions are located more than 1.5 cm below the surface. In these instances, *skin cooling* is provided if necessary, by which the temperature maximum is shifted to about $\Delta Z \approx 2.5$ cm the body's surface (Figure 15). Then, a somewhat increased energy deposition can be achieved in deeper-seated tissue layers, when the power output of the Selectotherm transmitter is enhanced.

In order to get the highest possible *penetration depth* at the site to be treated, *the core temperature must be elevated as far as it is tolerated by the patient*, before in the regional hyperthermic phase the target temperature can be further increased up to 42.5°C. For the treatment of deep-seated tumors, we aim at a core temperature of 41.5°C during this regional heating period, whereby the probability of any possible complications is drastically reduced by breathing oxygen (5 l/min) to improve the substrate supply in the brain. For cancers near the surface, a core temperature of 40.5–41.0°C is generally sufficient.

In early 1984, basic physical studies guided us to a newly designed applicator system (28). As compared to the two-dimensional scanning applicator hitherto used, the new system can move three-dimensionally which promised a more extended penetration depth and an improved adaptation of the energy-supply to the individual case. Figure 16 shows a photo of the new device, its robot control having a great number of degrees of freedom for spatial motion. The applicator runs on polygonal spatial tracks, the parameters of which can be adjusted at choice between up to 30 different fixed points. During movement, the main axis of the applica-

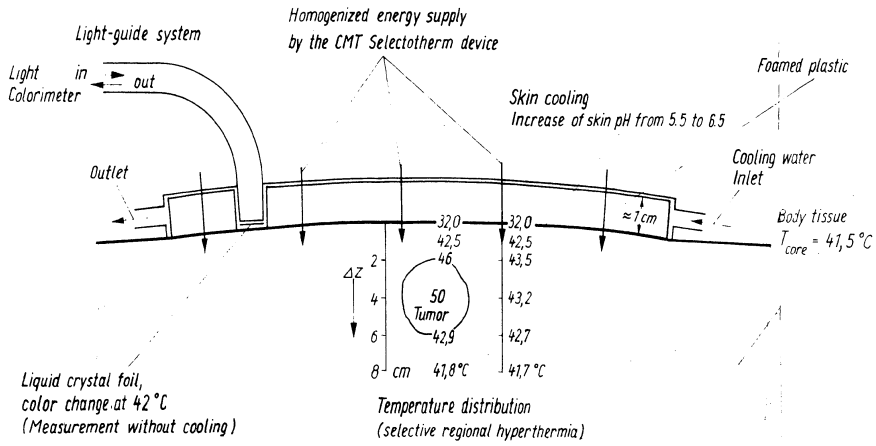

Figure 15. Exemplified temperature distribution by using the CMT-Selectotherm procedure. Pain as indicator for $T_{max} \approx 43.5°C$ (normal tissue). Tumor microcirculation inhibited by the CMT, i.e., blood convection cooling almost abolished in tumor tissue.

tor can be continuously inclined. Due to its extraordinary mobility the device is easily adaptable to varying working conditions. For example, deep seated targets can be heated continuously from *different directions* (crossfire technique, Figure 17), by which the effect in depth is improved.

Such studies in development of a new applicator system with improved spatial mobility became necessary because with the old simple magnetic dipole applicator, we had been confronted with a detrimental effect. We propose the name "body contour effect" for this important phenomenon. When this applicator moves on planar scanning tracks exceeding the body's boundaries, disturbances arise from the interplay of currents induced in the body with the geometry and conductivity of the sections involved (Figure 18). "Hot spots" (burns) can occur and limit prematurely the penetration depth of the CMT Selectotherm technique. Attempts to obviate the body contour effect and, if possible, to extend the penetration depth resulted in an improved double-system applicator (Figure 19) and a so-called butterfly applicator derived from the former. The most prominent advantage of the new system is that by chosing antiparallel current circuits in the 8-shaped applicator the maximum power density produced in the body is concentrated around the axis of the system.

The temperature course derived from measurements on pigs with the new CMT Selectotherm technique is shown in Figure 20. Temperature profiles (specific absorption rates) measured in an agar phantom simulating conductivity and dimensions of the body and with two types of applicators but the same circular scanning pattern are shown in Figures 21 and 22. The comparison of these results illustrates the progress made by the development of the double-system butterfly applicator with respect to the increased effect in depth and the attenuation of the body contour effect as well.

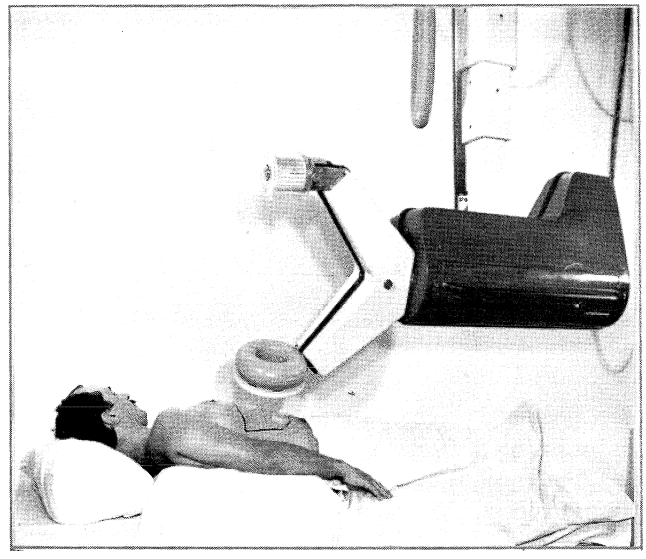

Figure 16. Applicator system (3rd generation) with freely adjustable, robot-controlled spatial scanning motion.

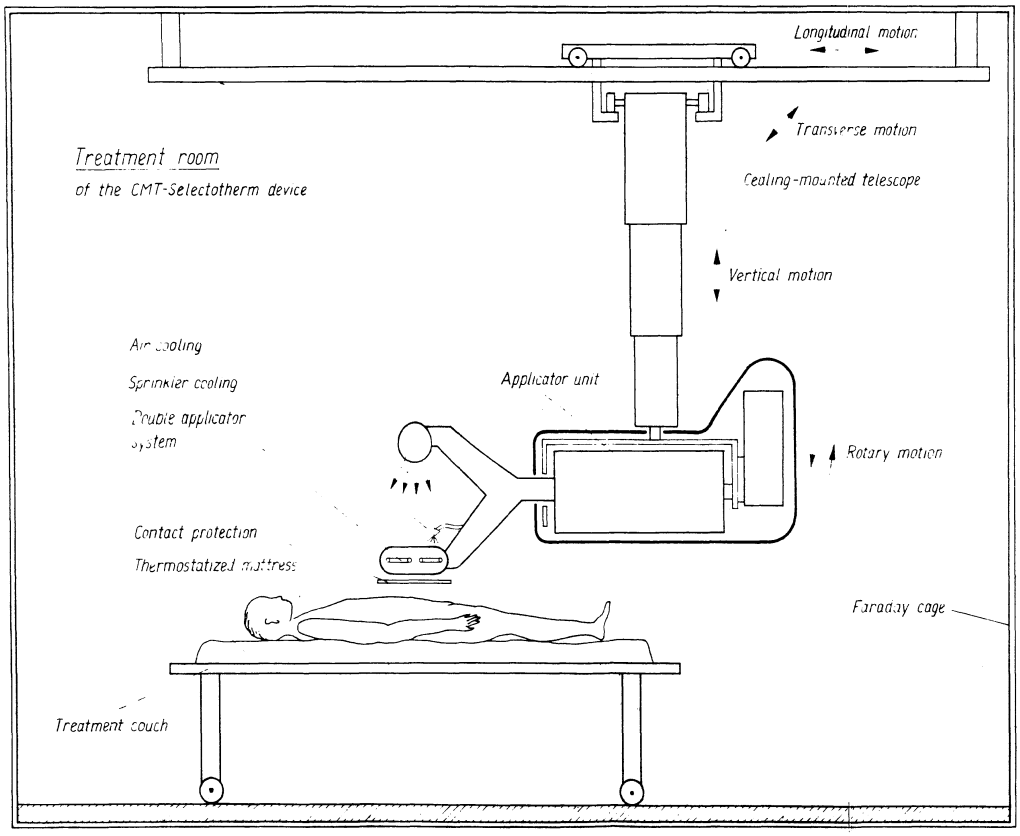

Figure 17. Design of the applicator system of the third generation with robot-controlled, freely adjustable spatial scanning patterns.

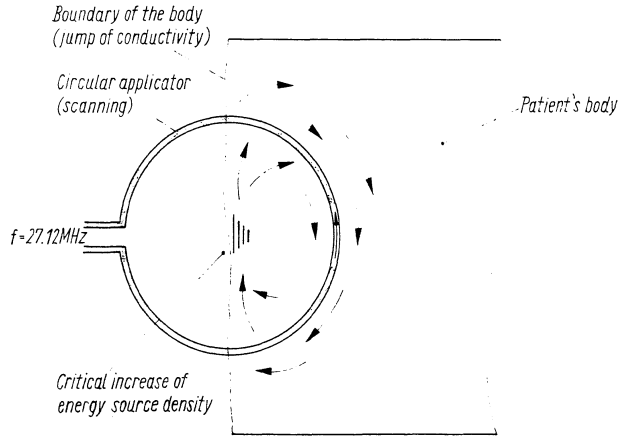

Figure 18. Overheating of border tissues due to the body contour effect. Possible momentary energy density distribution during scanning.

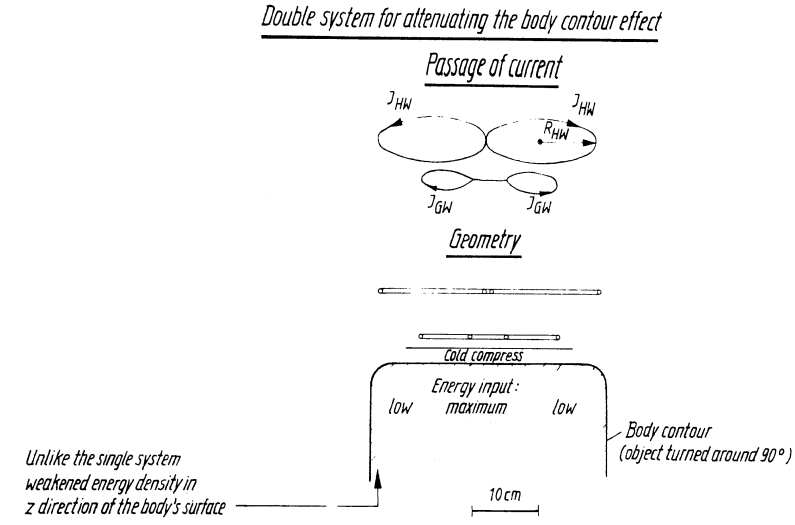

Figure 19. Passage of current and geometry of the Selectotherm double applicator system[1] characterized by attenuation of overshooting energy densities at body contours in z direction and by field straining.

[1] Patent pending (M.von Ardenne, G. Böhme, M. Zimmermann and H.-J. Götting)

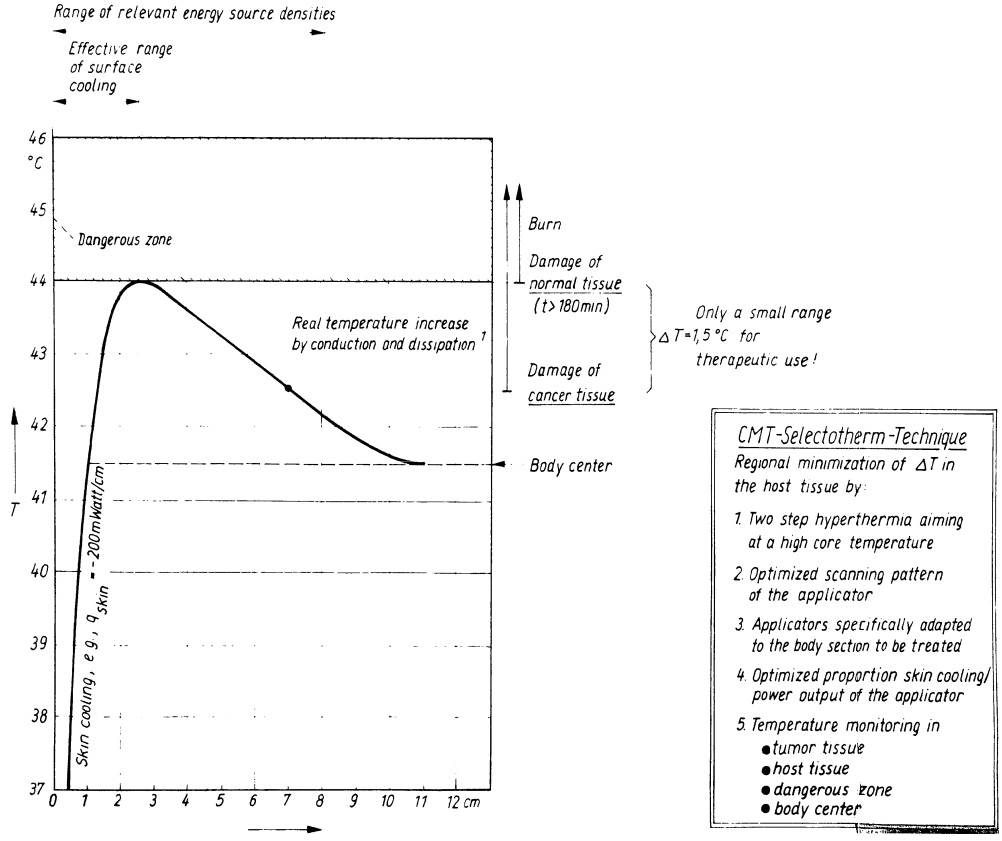

Figure 20. Schematic representation of the principle mode of action of the CMT Selectotherm technique by means of a didactic model for homogeneous and non-vascularized tissues.
[1]The actual course depends on the anatomy of the particular body region. Cooling effects: conduction, evaporation (e.g., in the lungs), blood convection. Local heat regulation. Optimized CMT regimen based on the flexibility of the Selectotherm process.

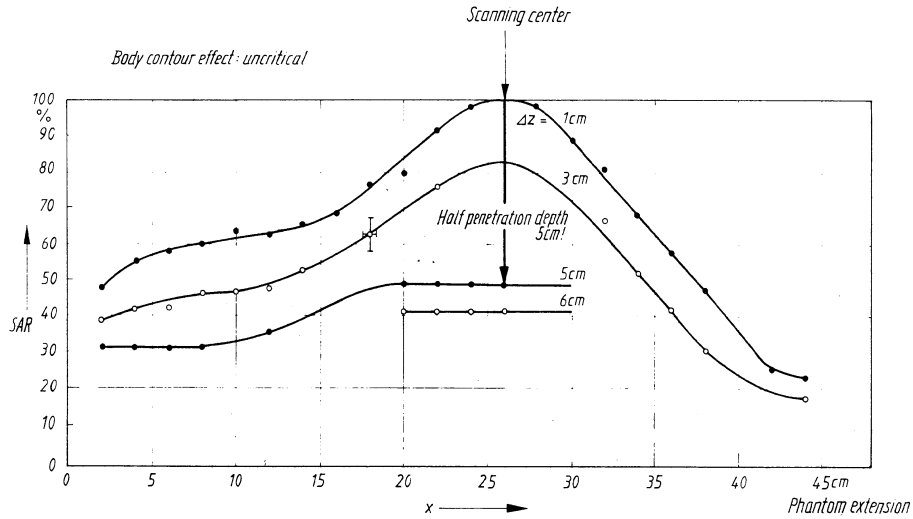

Figure 21. Specific absorption rate (SAR) in a homogeneous agar phantom (45 × 52 × 9 cm; conductivity $\kappa \approx 1\Omega^{-1}m^{-1}$) at circular scanning ($R_0$ = 10 cm) with a circular coil (diameter R = 8.25 cm). Distance between applicator and phantom $\Delta Z_0 Z_1$ = 11 cm. Measurements by M. Zimmermann.

10. Tissue temperature measuring within the CMT Selectotherm technique

For attaining a therapeutic effect it is necessary to reach and to maintain a temperature T_H of about 42.5°C in the peritumoral host tissue: First measuring point. Further, in order to avoid burns, the temperature must not exceed 42.5°C at any site of the body for a longer period of time. To meet this demand the temperature must be determined at sites where "hot spots" can occur, i.e. in tissue layers located 1.5–2.5 cm below the skin, which depends on the intensity of skin cooling, or at critical internal or external tissue borders: Second measuring point. The *actual temperature* (or temperature distribution) *in the tumor* is another very informative parameter: Third measuring point. Due to the selectively reduced convection cooling, this temperature often exceed T_H. The very important core temperature can be determined accurately in body cavities either by RF-immune techniques (56) or by classical means, provided that in this case the RF field is switched off for a short period of time: Fourth measuring point.

The temperature measurements 1–3 should be perform-

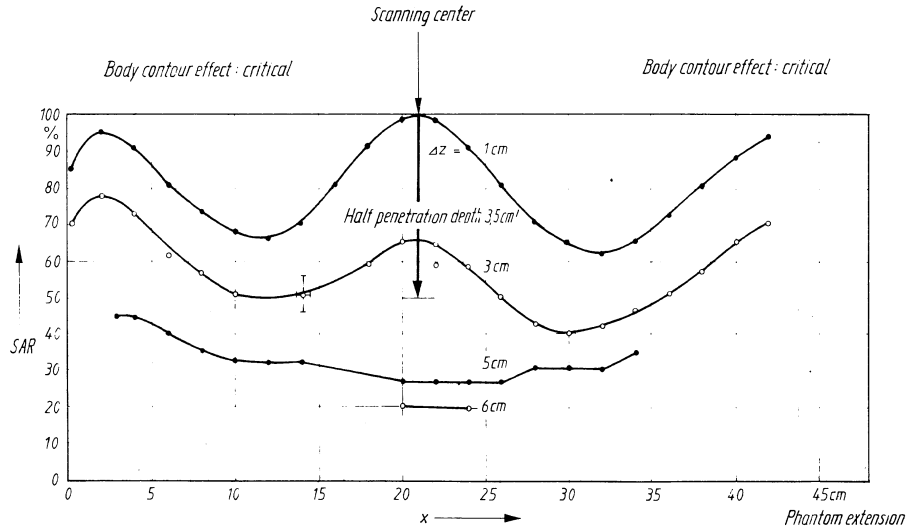

Figure 22. Specific absorption rate (SAR) in a homogeneous agar phantom (45 × 52 × 9 cm; conductivity $\kappa \approx 1\ \Omega^{-1}m^{-1}$) at circular scanning ($R_0$ = 10 cm) with the butterfly applicator (double system of two inclined coils). Distance between applicator and phantom $\Delta Z_0 Z_1$ = 11 cm. — Gain in half penetration depth by this applicator: $\frac{5.0}{3.5}$ = 1.43 (cp. Fig. 21). Measurements by M. Zimmermann.

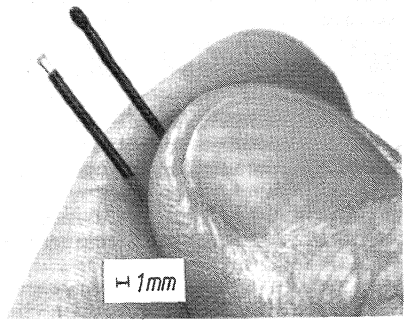

Figure 23. Sensor tip containing a spezial luminophore and connected with a light guide. Open (left) and encapsulated ready for use (right). (Quartz en Silice Co., France).

able under the influence of the RF field; the probes should be as least invasive as possible and be placed in deeper tissue layers. A convenient *fluoroptic technique* described by Wickersheim and Alves (56) was further adapted (Figure 23). At the tip of a thin light guide (800 μm in diameter) there is the sensor proper containing a *luminophore on the basis of rare earths* (La, Eu), the spectral emission of which is temperature-dependent. The sensor is excited by UV_A light fed into the fiber optic, and the emitted fluorescence is guided back the same way to the detector unit. Its set-up (optical separation of the two spectral lines of 513 and 625 nm and their processing) is schematically shown in Figure 24. The absolutely smooth TEFZEL surface of the sensor can be cleaned and sterilized easily and permits friction-free gliding in the tissue. Due to these properties, its accuracy to ± 0.1°C and the easily performable switching from one sensor to the other, this thermometric device turns out an indispensable aid for clinical CMT Selectotherm trials.

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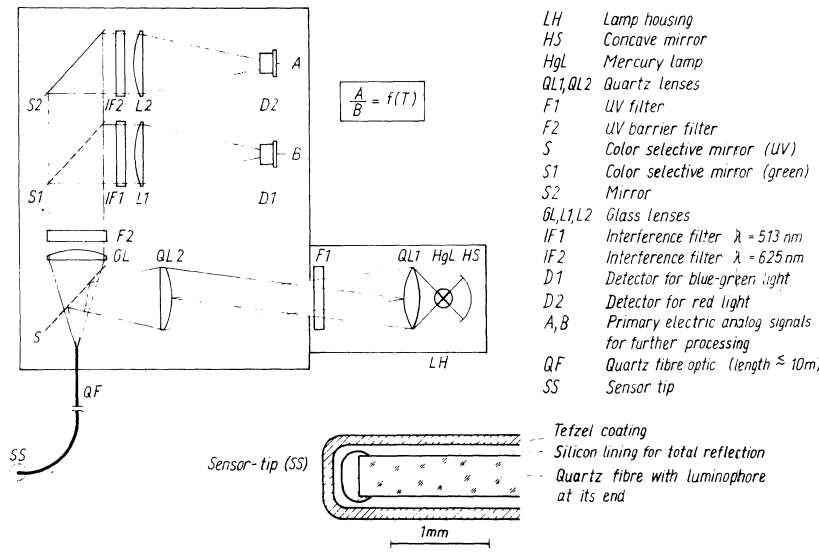

Figure 24. Schematic representation of the optical part of the temperature measuring unit "Lumitherm" (up to four sensors).

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18

PHOTOSENSITIZERS AS DIAGNOSTIC AND THERAPEUTIC TOOLS IN ONCOLOGY

ATUL C. MEHTA, JOSEPH A. GOLISH and MUZAFFAR AHMAD

Photosensitizers are chemicals that can be activated *in situ* by penetrating light of a specific wavelength, leading either to fluorescence of a different wavelength; tissue necrosis or both. Most malignant and some premalignant tissues retain these photochemically active substances in higher concentrations and for longer durations than surrounding normal tissues. The exceptions are regenerating or embryonic tissue, inflammatory processes and organs rich in reticuloendothelial cells, such as liver, kidney, spleen, skin and, to some extent, muscles. Photosensitizers, which are relatively nontoxic and safe for humans and animals, are currently being studied for diagnostic and therapeutic applications.

Photosensitizers have been known to us for almost a century. In 1900, Rabb (37) reported the lethal effects of a combination of acridin orange dye and ordinary light on *Paramecium*. In 1903, von Tappenier reported the first therapeutic use of photosensitizers; he used eosin and white light to treat skin tumors (43). Later, a variety of photosensitizers was reported having either *in vitro* or *in vivo* photodynamic activity, including barberine sulphate, fluorescein, eosin, tetracycline and variety of porphyrins. To date, however, there is no known ideal nontoxic photosensitizing substance which is selectively taken up by malignant tissue and is activated by a wavelength not absorbed by normal tissue.

Of all the available photosensitizers, porphyrins have received the most attention. The chapter is a brief review of the current literature on porphyrins as photosensitizers for diagnostic and therapeutic uses.

Policard (35) was the first to notice photodynamic property of endogenous porphyrins in animals and in a man dying from infection by hemolytic bacteria. In 1942, Auler (4) was the first to experimentally produce red fluorescence in implanted rat tumors following systemic injection of hematoporphyrin. During the early 1960s, Lipson (26, 27, 29) reported that a porphyrin derivative had a greater affinity toward malignant tissue than hematoporphyrin and used it to diagnose tumors of various organ systems in humans. In 1966, Lipson (28) also described the first therapeutic use of this porphyrin derivative in a patient with recurrent breast carcinoma.

After extensive animal experiments using a variety of porphyrins, three were found to have photosensitizing properties *in vivo*; tetraphenylporphine sulfonate (TPPS), hematoporphyrin derivative (Hpd), and dihematoporphyrine ether (DHE). Because of its poor serum clearance rate and related neurotoxicity, TPPS has remained of little clinical significance.

Hematoporphyrin derivative (Hpd) and dihematoporphyrin ether (DHE)

Hpd is prepared by acetylating hematoporphyrin (nonmetallic porphyrin derived from hemoglobin) with a glacial acetic acid and sulfuric acid mixture followed by hydrolysis and recrystallization under near-neutral or basic pH. This method was first described by Swartz (41) in 1960. Hpd thus produced still contains a variety of porphyrins without photosensitizing properties, such as hydroxyethylvinyldeutroporphyrin (HVD), protoporphyrin, and the parent compound, hematoporphyrin, constituting almost 50% of the total volume (17). Many other minor porphyrins with questionable photosensitizing activity have also been identified in varying concentrations. Thus, although a very active photosensitizer, Hpd harbors many impurities.

In 1981, using gel exclusion chromatography, Dougherty (17) separated Hpd into various porphyrin fractions depending on their molecular weight and size. He was able to demonstrate the presence of high molecular weight (up to 60,000), orange-brown porphyrin aggregates constituting almost 45% of total Hpd. With subsequent animal experiments, this material was found to be responsible for the photosensitizing action of Hpd. With extensive chemical analysis, this substance has been tentatively identified as Bis-1-[3(1-hydroxyethyl) deutroporphyrin-8-yl] ethyl ether. This substance is generally known as dihematoporphyrin ether (DHE) (17) (Figure 1).

The major benefit of this substance is markedly reduced skin photosensitivity for an equivalent tumoricidal dose, at least in animals, compared with Hpd.

Distribution

After systemic administration, Hpd and DHE are distributed throughout the body. In six hours, most normal tissues clear porphyrins but tumor tissue retains them for several days. Lung tissue retains these chemicals for 12 hours and kidney, liver, bone marrow, and splenic tissue retain them for 24 hours in higher concentrations than tumor tissue; however, this ratio reverses within 48 hours (15). Skin retains these chemicals, at least in minute amounts for 30 days or longer. Some reduction in their concentration in tumor tissue with time is mainly due to tumor cell proliferation (19). Inflammed, but noncancerous tissue, may take longer than normal tissue to clear Hpd or DHE, but not as long as malignant tissue. Within the tumor itself, level of porphyrins

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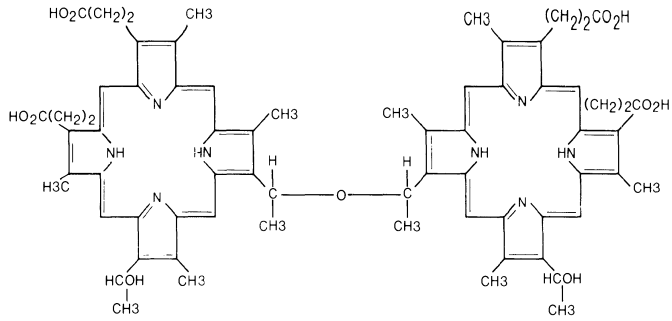

Figure 1. Chemical structure of dihematoporphyrin ether.

is at least five times higher in macrophages, mast cells, and fibroblasts of vascular stroma than in the actual tumor cells. It is still unclear whether or not endothelial cells of tumor vessels retain this material (14). One study has shown that fluorescence from adenocarcinomas is usually greater than that from sarcomas (18). It has also been reported that, in general, spontaneous tumors in animals show more fluorescence than transplanted tumors, although it is possible that the degree of fluorescence may not be proportional to the concentration of metabolites responsible for cytocidal effects (13). Within normal tissue, Hpd and DHE is mainly retained by reticuloendothelial cells and only minimally by the actual organ cells. For example, hepatocytes in the liver retain little if any of the drug, while Kupffer cells retain the most.

Tissue uptake

It is thought that once Hpd is injected intravenously, some of its active component, DHE, is bound to serum proteins, some gets phagocytosed by vascular stroma, and the remainder escapes into the interstitial fluid. Larger aggregates of DHE with high molecular weight are thought to get trapped within the interstitium of the tumor for a prolonged period. DHE, which is highly lipophilic, binds with the cellular membrane and slowly diffuses into the cytoplasm. Within the cells, DHE gets disaggregated and releases porphyrins, which rapidly bind with hydrophobic loci. Fluorescence microscopy has demonstrated this deposition to occur around the plasma membrane and within intracellular vesicles around the perinuclear area whereas no fluorescence is noted within the nucleus (24). Based on electron microscopy, Hpd seems to be deposited in significant amounts in mitochondria as well (8, 33).

MECHANISM OF ACTION

Production of fluorescence

One of the unique properties of Hpd and DHE is the production of salmon-red fluorescence when they are exposed to light in the spectrum between ultraviolet and red light (360–609 nm). The maximum excitation is by violet light of 405 nm. In presence of phosphate buffer, fluorescence is produced at two characteristic peaks of 618 nm and 676 nm. *In vivo*, these peaks are at 632 nm and 697 nm (Figure 2). DHE, an active ingredient of Hpd, has a strong tendency to self-associate and form large aggregates in aqueous solution. In this form, it does not fluoresce. It is likely that, once taken up by tissue in this form, DHE gets disaggregated and that emits fluorescence under proper conditions. How these excited chemicals actually produce fluorescence by shifting their energy state is a matter for nuclear physicist and beyond the scope of this chapter (3).

Cytocidal effects

The cytocidal effect of Hpd and DHE is mainly due to the production of singlet oxygen once they are excited by a light of wavelength near 620 nm. In this process, the energy is transferred from the excited singlet state to the triplet state and then to oxygen to produce singlet oxygen (32). Singlet oxygen irreversibly oxydizes essential cellular components, producing tissue necrosis (Figure 3). Hence oxygen is usually required for Hpd and DHE to produce cytocidal effects. Oxygen species other than singlet oxygen, as well as oxydation products subsequently formed, are also suspected to participate in killing cells (13). Rapid and progressive cell death actually starts one to ten hours after the treatment with light of specific wavelength. The first changes occur around the microvasculature, partly because changes in the vascular stroma reduce the blood supply. Eventually, there is rupture of the blood vessels, extravasation, and tumor death. Intracellular changes such as crosslinking of membrane proteins, inhibition of transport across the cell membrane, inactivation of enzymes, enzyme loss, mitochondrial dysfunction, membrane lysis, and impaired protein synthesis have been reported to contribute to cell death (13).

It is this property of photosensitizers that is currently being studied in the palliative as well as curative treatment of malignancies in various organ systems. This form of therapy is usually referred to as photodynamic therapy (PDT).

Dosage and administration

The recommended human dosage of DHE is 2 mg/kg of body weight given as a slow intravenous bolus over several

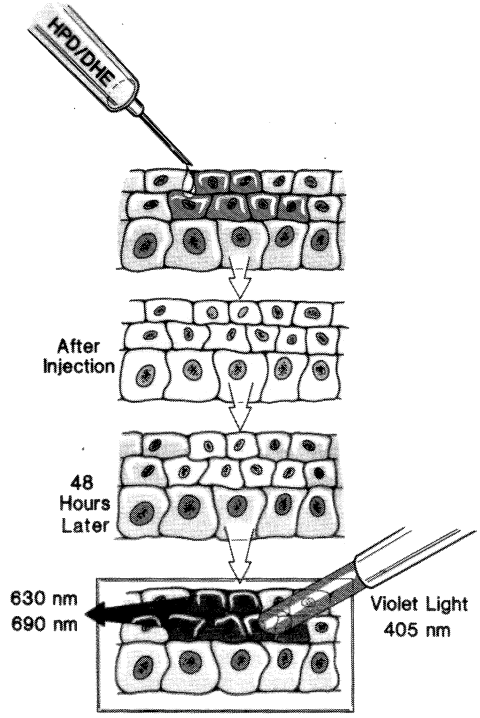

Figure 2. Schematic diagram of fluorescence production using photosensitizers.

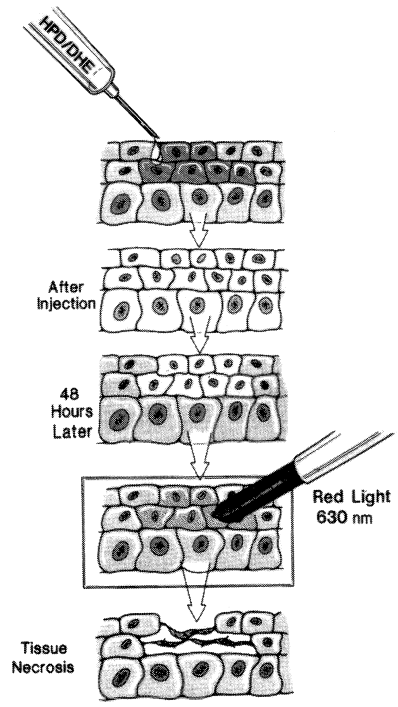

Figure 3. Schematic diagram of cytocidal effects of photosensitizers.

minutes through preexisting, normal saline I.V. drip tubing, taking precautions to avoid extravasation. It has been well documented in animal studies that only half the amount of DHE as Hpd is required to produce similar drug levels in the tissue since DHE is the only part of Hpd that is retained by the tissue (31). We would like to caution readers that both Hpd and DHE are still undergoing trials in the United States and have not been approved for clinical use in humans.

TOXICOLOGY

Dougherty (17) has studied the toxicity of these photosensitizers extensively using albino-Swiss mice. Keeping these rodents in the dark after intraperitoneal injection, he found LD^{50} for Hpd to be 275 mg/kg at 24 hours. At 14 days, LD^{50} was 230 mg/kg for male and 180 mg/kg for female mice. Repeated dosage of 50 mg/kg/day for five days and 25 mg/kg/day for ten days caused no deaths. Deaths were attributed to liver and kidney necrosis. Damage to spleen and thymus tissue was also noted, but bone marrow was unaffected. Obviously, toxicity was more pronounced when animals were exposed to light. LD^{50} for Hpd was 7.5 mg/kg for 24 hours as well as for 14 days in shaved animals after five hours' exposure to infrared filtered xenon light. Signs of hypoxia were evident before death. The cause of death was considered to be shock syndrome. Shaving was essential to produce these lethal effects. Milligrams per milligram, DHE was found to be twice as toxic as Hpd.

Side effects

Skin photosensitivity is the only known side effect of photosensitizers. Because skin retains these chemicals in enough quantities to produce marked photosensitivity, usually for 30 days and as long as 80 days, patients must avoid exposure to sunlight, cover exposed areas of the body, and use sunscreens for at least four weeks. Limited areas of skin can then be exposed to sun and checked for any reaction before the patient can return to regular outdoor activities. Extravasation of Hpd followed by undue exposure to sunlight has caused extreme photosensitivity, sloughing, and ulceration of the skin. Precautions should be taken to avoid extravasation. Photosensitivity is not cumulative.

DIAGNOSTIC AND THERAPEUTIC INSTRUMENTATION

The role of photosensitizers in oncology is two-fold, diagnostic and therapeutic. Both these applications need light of a specific wavelength and a system to deliver light to the target organ.

The fluorescence of Hpd and DHE at 630 and 690 nm when exposed to ultraviolet to violet light is used to detect superficial tumors or carcinomas *in situ* that usually cannot be detected by the naked eye or endoscopic examination. Since ultraviolet light is carcinogenic, violet light with a wavelength of more than 400 nm is used for these purposes.

Cortes (12) uses mercury arc lamp to obtain violet light which is then transmitted through the illuminating guide of a double-lumen fiberoptic scope alternating with white light. While Profio (36) and Kato (22) use a krypton ion laser to produce violet light at 405 nm, which is transmitted via a 400 μm thin, flexible quartz filament. Forty-eight hours after intravenous injection of Hpd or DHE, target areas are exposed to this violet light and screened for fluorescence.

The fluorescence is difficult to detect because small tumors retain very small amounts of photosensitizer and because optical losses through the fiberoptic scopes commonly used for this purpose are high. Some form of wavelength detector device is necessary. Cortese (12) uses a device that converts these fluorescent light signals into audio signals and follows them to localize the malignant areas. For the same purpose, Profio (36) uses a phosphor screen image intensifier that converts the light signals to green fluorescence and amplifies them 30,000 times, whereas Kato (22) employs ultrasensitive TV cameras to increase the fluorescence gain. After the tumors are localized, multiple biopsies are performed to confirm the diagnosis.

The cytocidal property of Hpd and DHE is now being studied in palliative as well as curative treatment of malignant neoplasms in various organs. A tunable, argon-pumped rhodamine B dye laser is used to produce red light at 630 nm wavelength which excites these porphyrins to produce the cytocidal effects. This light is transmitted through the flexible, 400 μm, thin quartz filament and delivered to the target organ. Fiber with a flat tip is generally used for superficial treatment where the angle of laser beam divergence can be varied from 22–53°. Different lenses placed at the tip can modify the spot size as needed. Cylindrical diffusers of various lengths with full or lateral exposure can be capped at the tip for interstitial (intralesion) application. For treatment inside the hollow organs, such as the urinary bladder, a spherical fiber tip is preferred. In the past, filtered xenon arc lamp light with a wavelength of 600–700 nm was used for this similar purpose. Even though wavelengths shorter than 630 nm are better absorbed by these chemicals, this wavelength penetrates tissue more deeply and hence is chosen for this purpose (14). From three to 72 hours after intravenous administration of Hpd or DHE, the target organ is exposed to the red light and 60–240 Joules/cm^2 of tumor surface area of light energy is delivered either in interstitial or intraluminal (superficial) fashion. Tissue destruction usually occurs within 0.5–1 cm from the surface or from the point of fiber insertion. Larger lesions are best treated by combining superficial and interstitial therapy or by inserting multiple fibers. Tissue necrosis is produced by the chemical reaction that starts within one to ten hours and may take several hours to complete. If the results are unsatisfactory, the entire procedure can be repeated.

RESULTS

Tumor localization

Occult or early malignancy of internal structures, such as the endobronchial tree or gastrointestinal tract, is difficult to diagnose because they produce no signs, symptoms, or typical endoscopic findings. The diagnostic usefulness of photosensitizers is being investigated for such cases. Kato (20) tested photosensitizers in a group of patients already known to have bronchogenic carcinoma of all different cell types. False negative results occurred in three of 36 patients in whom lesions were either submucosal or covered by blood or necrotic tissue. Weak fluorescence was detected in areas with severe atypical metaplasia in three other patients. Aida *et al.* (1, 2) demonstrated similar effects in patients with malignancies involving the gastrointestinal tract. In 1982, Benson (7) also documented the effectiveness of Hpd in localizing dysplastic and neoplastic transitional cell carcinoma of the bladder.

In a Mayo Clinic study of 11 patients with true occult lung carcinoma, photosensitizers were instrumental in locating the tumor site in eight patients. In two of the remaining three in whom no fluorescence was detected, the lesions were eventually found beyond the range of fiberoptic bronchoscope, and thus there was actually only one false negative result (21).

These studies clearly demonstrate the potential of photosensitizers in early detection of neoplasms in various organs. However, false positive and false negative results do occur, and histological examination of biopsies obtained from the fluorescent areas must be confirmed in every case.

THERAPEUTIC APPLICATIONS

Dermatology

PDT has been used to treat a variety of primary and secondary skin cancers. In his small study, Tokadu (43) found PDT to be quite effective in treating multifocal carcinoma *in situ*, Bowen's disease, and squamous cell carcinoma. Even pigmented lesions such as melanomas, which are thought to absorb laser light poorly, responded well. Dougherty (16) proved the effectiveness of PDT in the management of metastatic skin lesions from breast cancer, despite prior radiation and without affecting surrounding normal tissue. Effectiveness varied with the size of the lesion, dose of photosensitizers, amount of light energy delivered and prior radiation therapy. PDT has also been demonstrated to be useful in treating Kaposi's sarcoma as well as mycosis fungoides. The reported complications are pain after treatment for larger skin lesions from eschar formation and increased photosensitization in patients with prior treatment with a combination of doxorubicin and radiation therapy (14).

Pulmonary medicine

PDT has proved useful for the potential cure of unresectable early-stage (invasion limited to bronchial mucosa) lung cancers, in reducing the extent of lung resection to preserve lung function in patients with early stage and stage 1 lung cancers and in palliation of unresectable airway obstructions. In Hayata's study, a subgroup of eight patients with early stage, inoperable, squamous cell carcinoma all had complete remission during 13 to 64 months follow-up (19, 23). One patient was alive at 64 months. In a similar study by Cortese, (11, 10) eight of 19 patients had complete response during

eight to 57 months of follow-up using a maximum of two PDTs. Complications noted were sunburn (3), minor hemoptysis (4), and temporary airway obstruction from post-treatment bronchial edema (4).

Hayata's five other cases with early-stage disease and three patients with stage 1 squamous cell carcinoma underwent lung resection after PDT treatment. Histological examination showed a complete response in two and > 60% response in three patients in the first group and > 60% response in all three in the second group. All patients were disease-free after seven to 41 months of follow-up. Five other patients with stage 1 disease were treated with PDT alone, and macroscopic examination showed complete response in tow and > 60% in the remaining patients (19). These studies strongly emphasized the therapeutic value of PDT in the cases with early-stage lung cancer. However, PDT is not yet recommended as a sole curative therapy because of limited period of follow-up, technical difficulties in delivering sufficient laser light to all target areas, and inadequate data on actual amount of light energy being delivered to the treatment site.

PDT helped four of Kato's (20) five patients with bronchogenic carcinoma who had unresectable disease because of local extent allowing them to undergo resectional lung surgery. In five of the other six patients it helped to reduce the extent of resection from pneumonectomy to lobectomy. Disease recurred in two patients at 11 and 20 months, respectively. Five patients died with metastatic disease. The longest disease-free survival was 20 months.

PDT may be able to play an important role in the palliation of partial or total airway obstruction caused by malignant processes. In his study of 72 patients with unresectable endobronchial lesions from nonsmall cell bronchogenic carcinomas, metastatic colon, thyroid, melanoma, and breast carcinomas, adenocystic carcinoma, carcinoid, and malignant fibrous histiocytoma, Balchum (5) could establish total patency of the airway with no macroscopic evidence of residual tumor in all. A maximum of two treatments were required. Only one patient had partial response. In all patients "clean-up" bronchoscopy to remove necrotic tumor debris was performed 48–72 hours after PDT treatment. Parallel improvement in symptoms, pulmonary function studies, and radiographic findings occurred. Infection and dyspnea from retention of secretions were the immediate minor complications requiring use of antibiotics and additional bronchoscopy, respectively. The long-term complication was hemoptysis four to eight weeks after treatment, probably from slow sloughing of the involved vessels. He concluded that in selected patients, PDT is an effective, reliable, and relatively safe adjuvant palliative therapy to relieve airway obstruction from malignant processes.

It was an overall observation from all the studies that because of unpredictable extent and variable size and shape of these lesions, a higher-than-usually-recommended light dose was delivered to treat lung cancer patients.

Urology

The majority of bladder tumors are superficial and multifocal on presentation. Despite aggressive treatment, > 50% of the patients have recurrences within two years. The potential of PDT is being studied in the management of such neoplasms. Schumaker (39) showed complete remission in five patients with *in situ* cancer of bladder during one year of follow-up. Ohi (34) demonstrated complete remission for six to 18 months in eight of 11 patients with stage T1 and T2 *de novo* tumors as well as recurrent bladder tumors, following PDT treatment. In Benson's (6) study, all 15 patients with recurrent and resistant *in situ* bladder tumors, initial complete remission was observed, but in 38 months follow-up, eight had recurrences at either the treated site or at remote sites. In one other patient with recurrent hematuria, palliative treatment with PDT was successful. To approach the frequently encountered multicentricity of the bladder tumor, a light-bulb shaped fiber tip was used to deliver superficial whole-bladder therapy in eight patients. *In situ* tumors disappeared completely and T1 and Ta lesions responded partially. Frequency, urgency, and temporary shrinkage of bladder size were side effects of this form of therapy.

Gastroenterology

PDT may play a role in gastroenterology similar to that in pulmonary medicine, i.e., to radiate early-stage cancers or to palliate extensive lesions. Aida (1) used PDT to treat four patients with early-stage carcinoma of the esophagus. Complete endoscopic response for the period of 14 and 24 months was found in two patients. Partial response was seen in the remaining two who underwent resection following PDT treatment. In five of his patients with extensive carcinoma of esophagus, only partial response was noted. In ten patients with early gastric carcinoma, there was complete response in five, significant response in four, and partial response in one (2). In eight patients with advanced gastric carcinoma, only partial response was observed. McCaughan (30) used PDT to treat seven patients with dysphagia from subtotal occlusion of esophagus by primary neoplasm and produced palliation in all. One patient was free of symptoms for nine months. The complications substernal chest pain and tracheoesophageal fistula have been reported (14).

Otolaryngology

Wile (45) used PDT to treat 21 patients with unresectable and recurrent head and neck carcinomas arising from various sites. Six patients had complete response, while 11 had partial response at four weeks. One patient with cancer of the tongue survived 18 months disease-free before dying from a vascular event. Another patient with similar malignancy was alive at 12 months without evidence of recurrence. However, the remaining patients did demonstrate residual tumor and early recurrences. Taketa (42) treated six patients with head and neck malignancies involving the larynx, oropharyngeal mucosa, and tongue. The size of the tumor mass was reduced in all, but deeper areas of the tumor site remained positive for neoplastic cells.

Gynecology

Soma (40) used PDT to treat a variety of *de novo* and recurrent neoplasms involving female genitalia, including primary and secondary lesions of the vagina, cancer of vulva, and intraepithelial carcinoma of the uterus. Patients belonged to *in situ*, 1 and 1b stages of the disease. Internal lesions were treated using a modified colonoscope. A majority of patients required two to three procedures and when necessary adjuvant chemotherapy or radiation therapy was used. Out of 13, nine showed complete remission, and one patient with primary vaginal carcinoma showed no evidence of recurrence for at least two years of follow-up. Three other patients demonstrated significant response. There were no complications. Rettenmaier (38) treated six patients with recurrent vaginal, cervical, and endometrial tumors; two patients showed complete response.

CONCLUSIONS

Photosensitizers possess great diagnostic and therapeutic potential. Investigation in recent years has made their application in oncology almost a reality. Photosensitizers have been shown to be safe, noninvasive and effective for diagnosing and radiating early-stage neoplasms and palliating extensive lesions, even after conventional surgical, radiation, or chemotherapy. PDT may possibly cure in situ lesions without surgery and reduce extent of surgery for invasive yet resectable lesions.

Establishing uniform dosimetry, devising ways of reducing skin photosensitization and increasing the depth of cytocidal effects require further study. Their synergism with hyperthermia, Nd-Yag laser photoresection, and brachytherapy are the areas of future endeavor.

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19

CHRONOBIOLOGY, RADIOBIOLOGY AND STEPS TOWARD THE TIMING OF CANCER RADIOTHERAPY

FRANCINE HALBERG, JULIA HALBERG, ERNA HALBERG and FRANZ HALBERG

1. STATUS QUO

Radiotherapists and most radiobiologists approach the organism and its responsiveness, e.g., its radiosensitivity, as a more or less constant entity. Radiotherapy is hence scheduled during 'regular' working hours on weekdays only, as a function of practicality. In the past few decades, however, a quantitative science, chronobiology, has developed. This science shows that the host harboring a malignancy and some malignancies themselves are dynamic entities. Human and other hosts exhibit recurrent changes. These can be assessed by inferential statistical means. Thus, a variety of bioperiodicities is uncovered. Their frequencies range from 1 cycle in less than 20 hours (ultradians), over one in about 24 hours (circadians) to one cycle in more than 28 hours (infradians). Infradians include changes with a frequency of 1 cycle in about a week (circaseptans) or in about a year (circannuals), as well as about 30-day cycles (circatrigintans). These latter cycles are found in menstrually cycling women (but also years before the first and decades after the last menstruation); furthermore, circatrigintans are also found in men (66). Documentation of the pertinence to therapy of chronobiologic schedules for obtaining optimal results is forthcoming, primarily with respect to circadians. Accordingly, concern in radiotherapy and radiobiology for timing according to the principles of chronobiology seems to be justified. This concern is here examined in a historical perspective of this field and primarily with a display of original circadian data. These data are illustrative rather than inclusive. This chapter complements several others on related topics in this volume concerned with prevention (109) as well as treatment (21, 110).

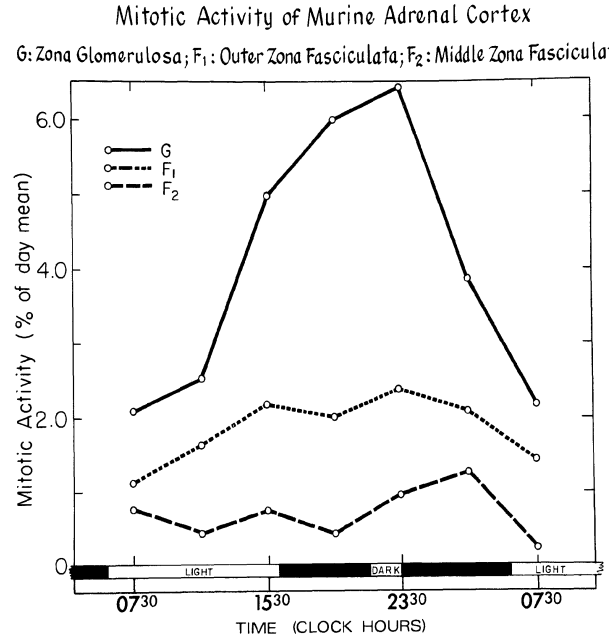

Figure 1. Cell division in different parts of the same tissue undergoes changes of a different extent, here depicted for viewing by the naked eye. Such a so-called macroscopic approach suffices for qualitative impressions but not for a quantitative comparison of characteristics. © Halberg et al., 1959.

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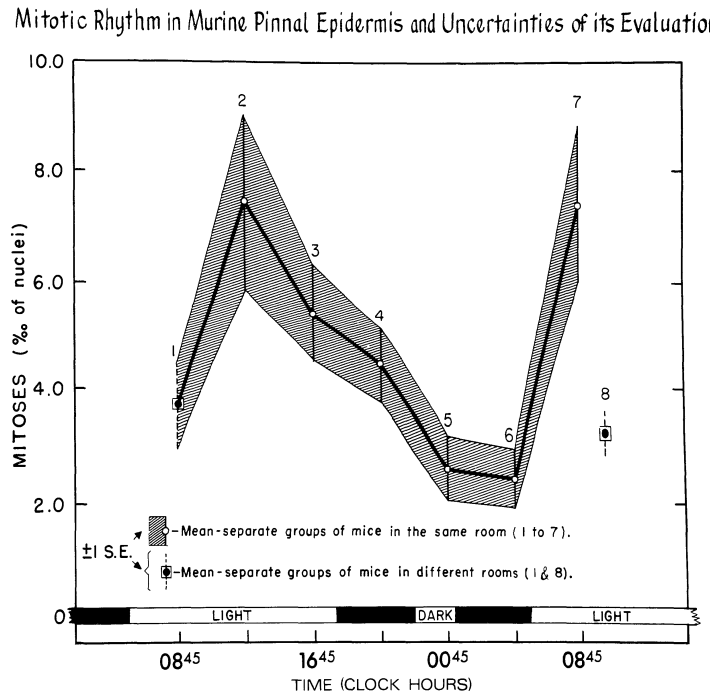


Figure 2. Effect on mitotic rate in ear pinnal epidermis of repeated sampling from (7 separate groups of mice kept in) the same room. Group 8 kept in a different room has a different rate, as compared to the concomitantly studied rate of animals kept in a room previously entered for sampling on 6 prior occasions. © Halberg, 1959.

2. UNQUALIFIED TIME-OF-DAY EFFECTS RATHER THAN RANDOM VARIABILITY

Kellicott (117) and Karsten (116) described a 'daily periodicity' of cell division in plants. A change along the 24-hour scale in the mitotic activity of a mammalian tissue was reported by Fortuyn-van Leiden, who studied nuclear division in the cat (37). In follow-up work, changes along the 24- hr scale have been documented in numerous cellular and metabolic variables at different levels of organization (42, 48, 54, 57, 141, 154, 155, 159). Bioperiodicity can in fact be demonstrated in any mitotically active tissue from properly standardized apparently healthy organisms, including human beings (46, 48, 105, 168, 169, 177). The extent of change within a day varies, e.g., in different cells of the same tissue, Figure 1, or in different tissues, Figures 1–3. A peak in the mitotic index rhythm may be up to 4 or even 20 times the trough value. The timing of mitotic bioperiodicities in different tissues of the same organ, e.g., the pancreas of the mouse, can also vary (69, 72). The approximate minimal and maximal values and the times of their occurrence are predictable within limits from one experiment to the next, even under slightly different (if standardized) conditions. The overall mean may be different in 2 sets of conditions being compared, but the times of the minimum and maximum can be similar. Figure 3 shows the extent of reproducibility in timing of the change in mitoses of growing mouse liver, studied with and without the use of colchicine. Mitotic peaks in this case are similarly timed (69).

The implication that cells *in vivo* divide asynchronously, underlying current radiotherapeutic practice, has repeatedly been shown to be unjustified, Figures 1 to 4 (101). Bioperiodicity is reported for the effect of radiation upon the intact organism as a whole and on the basis of mitotic studies on intact untreated organisms, including unicells (63); bioperiodicity is reported even at the level of a single cell, such as *Gonyaulax polyedra* (97), and is also a characteristic of microorganisms (63, 157).

Radiobiologic data are often reported as a percentage of control, where the control is the value at the zero timepoint. It is not valid to compare an experimental curve with an assumed straight-line control, which reflects only one point on a circadian curve (14, 15, 16; cf. also 80). The approximately sinusoidal curve resulting when mitoses in some tissues are plotted as a function of time resembles that used to demonstrate *in vivo* the classical radiation-induced mitotic delay and subsequent recovery (160).

3. CHRONOCYTOKINETICS: THE CIRCADIAN CELL CYCLE

Circadian mitotic changes have been reviewed and documented as ubiquitous and amenable to gradual (not abrupt) schedule shifts (46, 48, 69, 141, 169, 177). Some of these bioperiodicities have also been explored in relation to related biochemical phenomena, bottom of Figure 4, and underlying mechanisms: cellular, adrenocortical, pituitary and other (46, 52, 54, 57, 69, 70, 94). These observations were made at about the same time as the proposition (110) and development (53, 80) of circadian-time-invariant cell kinetics.

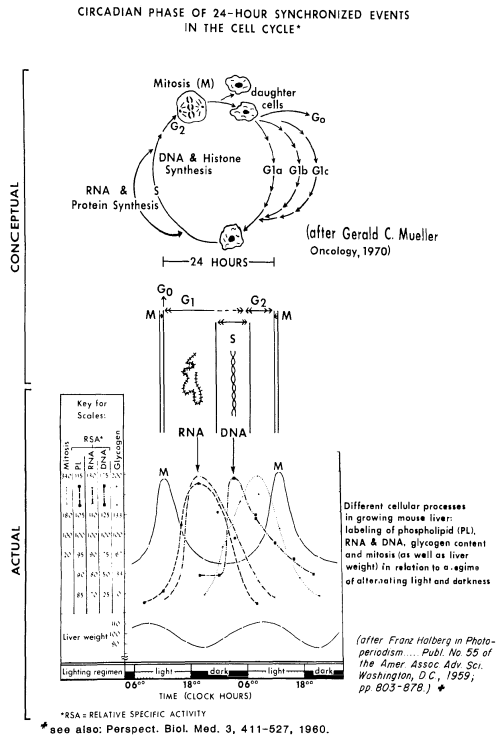


Figure 3. In the absence of any treatment (dashed line), cell division in growing mouse liver undergoes changes from less than 1 mitosis (in 100 oil-immersion fields) to, on the average, more than 10 mitoses. When colchicine is given 4 hours before each sampling, the circadian change occurs around the higher mean value, yet the timing of the bioperiodicity is similar to that seen in the absence of any treatment. (When colchicine is allowed to act for longer spans, the timing of the mitotic rhythm may be distorted.) © Halberg, 1959.

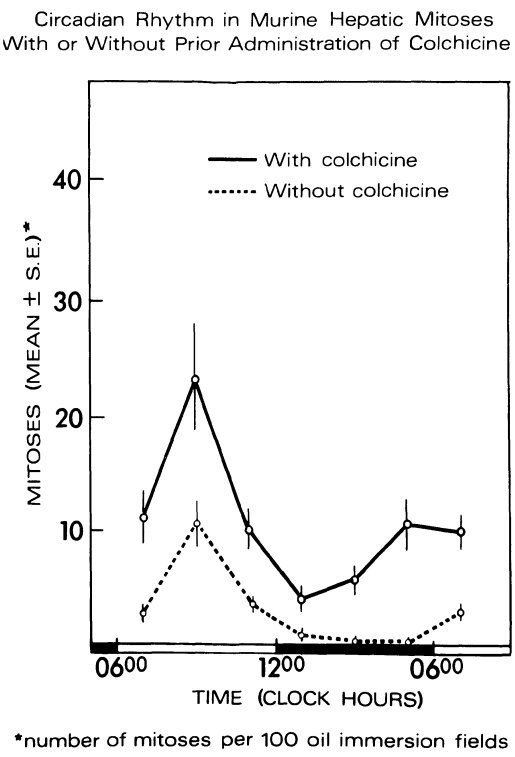


Figure 4. Concept of a chronobiologically not specified generalized cell cycle (top) vs. a circadian cell cycle (bottom). The latter shows a sequence of metabolic events in relation to cell division, specified for species (mouse), age (of about 5 weeks) and circadian stage. The 'activation', first, of phospholipid and RNA labeling is followed with a lag by the circadian increase in the labeling of DNA. This macroscopic view does not allow the resolution of any differences between (a membrane phenomenon,) phospholipid labeling and (a cytoplasmic phenomenon,) RNA labeling. A lead in phase of phospholipid in relation to RNA labeling is resolved by cosinor analysis (not shown). © Halberg *et al.*, 1958.

Before the end of the 50s, a combination of classical histology, radioactive tracer methods and wet chemistry had led to the documentation of a circadian cell cycle in rodent liver (bottom of Figure 4). Mitosis was followed, in this order, by the labeling of phospholipid, RNA and DNA, then by glycogen deposition and eventually by the next wave of mitosis. It should be emphasized, of course, that the circadian cell cycle in growing liver includes, for the case of DNA synthesis and mitosis, the contribution only of a (relatively small) dividing subpopulation, whereas the timing of the other metabolic variables represents a much larger non-dividing subpopulation in the same organ. Nonetheless, a first temporal if global map was obtained with the results at the bottom of Figure 4, for an immature growing mammalian organ. This map represents a tangible since time-specified alternative to the conventional time-unspecified homeostatic line of thought shown in the upper part of Figure 4. A sequential 'activation' of metabolic processes, demonstrated in the 50s (69), served to test the site of action of agents such as somatotropic hormone (72).

The degree of generality of this sequence of circadian cellular events may be suspected from the similarity of the internal timing of certain metabolic processes and mitosis in several different models of life. This similarity can be seen for RNA vs. DNA labeling vs. mitosis in immature growing mouse liver, in a unicell (27, 28, 31), and with respect to DNA vs. mitosis in hamster cheek pouch epithelium (112, 113, 114) and in human bone marrow (127). Analyses (by the cosinor method) reveal a difference of a similar order of magnitude and direction in timing between indices of DNA and mitosis in human beings, mice, hamsters and a unicell.

In tetrahymena cells, Scherbaum and Zeuthen (167) achieved a relatively high degree of synchronization of DNA replication and of mitosis by heat shocks spaced one cell generation apart. Work in the growing liver of a mammal has the disadvantage that DNA synthesis occurs in a much smaller fraction of cells than in the case of tetrahymena. The merit of mammalian hepatic work lies in the fact that a host of systemic and local variables, including DNA metabolism and mitoses, exhibit, without shocks, a light-dark-synchronized or a free-running bioperiodicity that is already mapped (58). The circadian frequency and/or acrophase (defined in Figure 5) synchronization (69, 73) for different processes in the same organ (labeling of phospholipid, RNA and DNA as well as mitosis in growing mouse liver) is altered in continuous light (58). Studies under presumably constant conditions reveal changes in the rela-

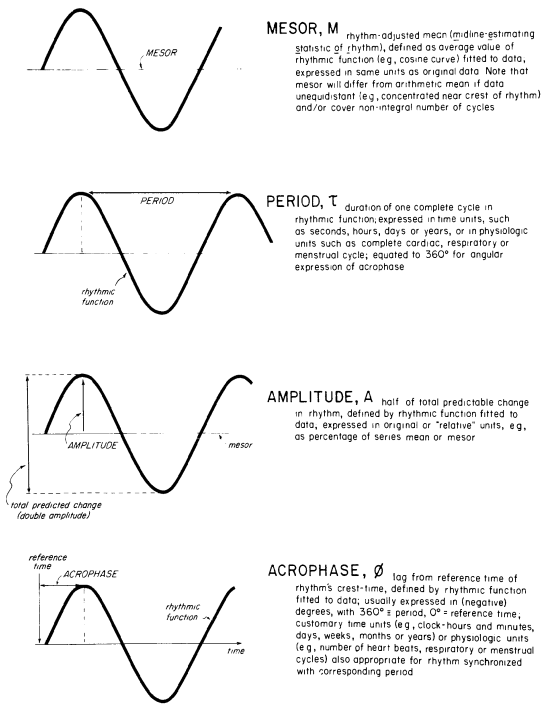


Figure 5. Some characteristics of a rhythm. © Halberg, 1969.

tions of loosely coupled rhythmic webs. The manipulation of several synchronizers (62, 91) can serve attempts to separate tightly coupled circadian webs, for a critical analysis, *inter alia*, of problems of chronocytokinetics, and may be complemented by a (chronobiologically designed) timed or periodic administration of growth-inhibiting and -promoting agents (72, 125).

Circadian metabolic and mitotic bioperiodicities also stand out clearly in certain regenerative processes, e.g., when the proportion of cells participating in division is substantially enlarged by a procedure such as partial hepatectomy (69) and in diseases such as certain cancers (72). Circadian variation is often found in labeling and mitotic studies (2, 13, 16, 19, 30, 39, 40, 43, 69, 107, 111, 112, 113, 114, 121, 138, 139, 142, 143, 149, 150, 151, 152, 171, 174, 182, 190, 191). Bioperiodicity in cell division is the more important when it influences widely used indices of kinetics and responses to agents such as pituitary growth hormone (72, 125) and anticancer drugs (62, 72, 99, 173, 174).

Among several other approaches tending towards chronocytokinetics (74), Guiguet *et al.* (44) proposed a mathematical model of cellular events allowing for the circadian variations in any or all of the stages of an unidentified cell cycle. This was done by inclusion of sinusoidal variations in the probabilities of transition from any compartment of the cell cycle to the next.

The role that further knowledge in classical cytokinetics may play in improving chemotherapeutic schedules has been questioned (189). The acrophase, ϕ, of DNA synthesis in the bone marrow of rats (72, 173) occurs around an administration time corresponding to high murine tolerance of some so-called 'S-phase specific' myelosuppressive oncostatic drugs. Such results underline the need for combining chronocytokinetics with chronopharmacokinetics in the study of cell damage as a result of oncostatic agents, to clarify the controversy stemming from a time-unspecified cytokinetic approach in the management of cancer therapy.

4. EARLY RESULTS ON TIMING DRUGS

By 1814, a French physician, Virey, had suggested that timing is important for the administration of drugs. In 1931, Ågren, Wilander and Jorpes provided pertinent experimental data. To study insulin-induced convulsions in the mouse, these authors gave insulin at 6 different times of day to groups of about 50 mice. In one experiment, the insulin dose was fixed (at 0.0075 units/20 gm body weight). All animals had been deprived of food in the evening preceding the start of testing. The large differences in response to insulin found by these authors were clearly not feeding phenomena. The authors deserve credit for demonstrating more generally, for the case of murine liver glycogen as well, that feeding is not the 'cause' of rhythms, a comment still made with respect to rhythms today. Even if they described an effect of 'time of day', without further qualification, and did not analyze their data by inferential statistical parameter estimation, Ågren et al. initiated the experimental study of time-dependent drug responses. Our analysis (by the so-called cosinor method) (62) of their published data reveals that the no-rhythm assumption can be rejected at the 2% level.

A dramatic time-dependence for another drug effect was reported by Carlsson and Serin in 1950. These authors described the effect of an overdose of nikethamide, an analeptic drug, by the endpoint of lethality; they reported large differences for the effect of the same doses given at different times. With inferential statistical tools (the cosinor method), applied to the data of Carlsson and Serin, the statistical significance of the findings can again be documented ($P < 0.03$) (17).

Such laboratory observations were paralleled by complementary reports of time-dependent effects in illustrative clinical cases by Menzel (128, 129, 130, 132, 133, 134, 135). Menzel and his school (136, 137) documented different drug effects at different times of day; they deserve credit further for building a machine for patterned drug administration and for introducing temperature monitoring and curve-fitting for the determination of drug effects. Menzel even fitted the harmonics of a 24-hour period to his data. Thus, clinical as well as basic data for interpreting and quantifying the effect of what we now call circadian variation were available by the 1940s and 1950s. Two sets of critical ingredients were missing.

One set was purely conceptual and biologic. This was the realization first that one dealt, in an open system with endogenous and exogenous components (3, 48), i.e., one dealt neither with an unidentified immovable fixed cosmic pattern, nor with equally unqualified 'endogenous' clocks; discussions of the alternatives 'exogenous' vs. 'endogenous' as either/or propositions, became a sterile matter, once the adrenal mechanisms and eventually their interrelations, on the one hand with the pituitary, hypothalamus and pineal and, on the other hand, with a circadian cell cycle, were resolved. It could be shown that the adrenal cortex is particularly involved in the amplitude of the periodicity, as such, of certain mammalian variables (46, 75, 94, 115)) and in the timing of certain events or stages within a given period. As one bioperiodicity after the other was described, it became obvious that recurrent changes are a prominent feature of the dynamics of all life, rather than merely an expression of specific biologic mechanisms. Reference to

clocks and calendars was no substitute for pertinent data on mechanisms.

The other set of missing ingredients was primarily methodologic, namely a combination of biologic and statistical designs for the study of characteristics such as the period, the overall timing of the recurrent change (apart from any instantaneous phase) and the overall amplitude as well as waveform of a bioperiodicity in inferential statistical terms.

5. ADRENAL CORTICAL CYCLE, SYNCHRONIZATION, PHASE-SHIFT, FREE-RUN AND HEREDITY

By 1940, Hamar reported on the obliteration of a within-day change in glucose resorption following adrenalectomy. In the early 1950s, several lines of evidence in Minnesota led to the recognition of the adrenal gland as a critical mechanism underlying many physiologic about-24-hour rhythms, such as those in serum corticosteroids, blood eosinophils and hepatic phospholipid labeling (46, 69, 75, 92, 94).

Moreover, it was found that the adrenal cortical cycle was preparatory for daily activity, rather than a mere response to activity (46). By the use of marker bioperiodicity, namely core temperature, to be discussed below, it could be shown that under certain conditions, such as blinding, each mouse showed its own period, which was close to 24 hours, yet differed statistically significantly from precisely 24.0 hours. Such an about-24-h periodicity, with a period slightly shorter than 24 h, reported by the 50s (47, 48) was replicated and further extended in scope in the 60s (103). Very similar average periods have been reported for mice kept in continuous darkness, in a review in the 70s (144).

Free-running similar to that established in the experimental laboratory was soon documented for human beings as well, not only for a few weeks of social isolation, during which transients are likely to occur, but during several months of isolation in caves (53, 86, 181). Moreover, free-running bioperiodicities were also found in relation to the biologic 'week' and the 'year' (66, 78, 185, 196). The week is a hereditary feature even of flies (106), springtails (126) and a unicell (179). In the eighties, on the basis of studies in twins reared apart, it has been shown for several circadian parameters of human heart rate that we are dealing with characteristics that are in part inherited (57, 96), as may be the circadians of Neurospora crassa (32, 33, 34, 35)), and the ultradians as well as circadians of *Drosophila melanogaster* (118, 158).

Biologically, it also had to be demonstrated that the timing of circadian mitotic and many other bioperiodicities can be phase-shifted, e.g., by manipulating external lighting and/or feeding schedules, the synchronizers (59, 61, 69, 92, 93) or drugs (56). It had been known earlier that changes in a few variables, such as motor activity, are amenable to shifts in time location, but these were regarded as exceptions. On the basis of a study covering only the first few days after a change in lighting regimen, it had been reported by an outstanding scholar (7, 8, 9, 10, 11) that mammalian mitotic bioperiodicity cannot be phase-shifted. This mistaken assumption prevailed with respect to many variables. This assumption (that most physiologic variables cannot be changed in their time location) stemmed from the circumstances that shifts in the location along the 24-h scale of mammalian bioperiodicities are not abrupt phenomena, but occur gradually. The rate of adjustment often does not exceed 1 hour/day in mammals (59, 61, 69). Rates of adjustment reveal asymmetries in shift-time, noted when the same variable is assessed as a function of delaying vs. advancing schedule shifts. The adjustment rate may also differ among certain variables of the same organism, shifted in the same direction; the shift of certain mitotic within-day changes can be particularly slow (61). The adjustment may take weeks, e.g., for pinnal mitoses (61) or months when oppositely timed synchronizers are involved (92).

It also had to be demonstrated that circadian mitotic and other rhythms persist after the removal of the eyes in mice, with free-running periods that have no known environmental counterpart (69), as do other variables after blinding (47, 48, 55, 93). These experimental facts laid to rest the suggestion that one dealt merely with effects of time of day (not further defined). The same facts also led to the standardization of external conditions in chronobiologic studies that were previously ignored not only by those who believed in a purely 'cosmic' origin of rhythms (not further qualified), but also by many who preferred to write about 'endogenicity' but again without further qualification or documentation of any underlying physiologic mechanisms in specified anatomical locations.

Incidentally, the documentation of the degree of generality of the phase-shifting of circadian rhythms at different organization levels (59) led to the institution of light switches in most experimental (rodent) laboratories. Once it was shown that recurrent changes in serum corticosterone and blood sugar in hepatic phospholipid, RNA, DNA and mitosis of ear pinna as well as susceptibility-resistance cycles to ethanol or noise, could all be shifted by manipulating the lighting regimen, it was impossible to ignore a standardization of the lighting regimen in studies on rodents. The equivalent standardization of conditions for observation has yet to be achieved in most human studies (64).

6. FROM 'STRESS RESPONSES' TO CHRONOBIOLOGIC DESIGNS AND ANALYSES

Mostly ignored, up to the early 50s, were available means for estimating the likelihood of whether one dealt with random variability or rather with a statistically validatable recurrent phenomenon. The relative contributions of external and internal components of bioperiodicity could not be assessed without the use of methods to estimate the uncertainty in differentiating bioperiodicity from randomness. The biologic approach, e.g., by the 'remove and replace' method (46) and the inferential assessment of results, both were indispensable. Estimates were needed of the uncertainty of the characteristics derived from curve-fitting and ways to compare one or several rhythm characteristics in two or more time series. The need for reference standards was acute. Such standards were needed for dynamic as well as static parameters of bioperiodicity and for single values. These standards, called chronodesms (57, 74) were eventually built for illustrative purposes on the basis of data specified in terms of clock-hour, calendar date, living routine and the risk of developing certain diseases, for groups of subjects

studied under specified conditions with serially dependent sampling (57, 74). This was warranted once some problems of sampling effects were overcome by the so-called serially-independent design.

The use of separate groups of comparable experimental animals at different test-times was a procedure dictated early by the fact that in assessing the endpoint death, one had to use separate groups of animals at different test times. By the same token, when some potentially handicapping or injuring endpoint, such as a convulsion, was the criterion, it seemed logical again to use different groups. In the case of a seemingly innocuous procedure, serially independent sampling as to individuals was also useful, since it served as a control in work aiming to assess the effect of repeated sampling of blood. Even when no more was done to an animal than picking it up repeatedly for a measurement, e.g., of rectal temperature, the effect of doing this could not be assessed without serially independent controls. At the outset, the latter were needed generally, in order to demonstrate a bioperiodicity.

A design limiting interference by handling to a single occasion, in combination with Student's t-test, was a way to rule out the still-common misconception that the effect of repeated sampling is a determinant of the within-day changes rather than merely a modifier. Prominent recurrent changes in the number of mitoses or in that of circulating eosinophil blood cells were rigorously established by the use of serially independent sampling designs (12, 90). In the absence of such designs, changes in mitotic count, blood eosinophils and many other physiologic variables had been dismissed as trivial phenomena, i.e., as epiphenomena, matters of second-order or, as was the (still prevailing) custom of the time, as 'stress responses' (23).

It was, and still is tempting to say that the body's motor activity, the meals and a host of other physical or emotional stimuli impinging upon an organism represent the stress of daily life (143). The reference to stress as a synonym for life is noncontributory since it is redundant. Moreover, the inference that changes within the physiologic range are trivial is tragic. It is not commonly realized that this attitude draws a curtain of ignorance over the very range in which function usually occurs. The task on hand is the use, when possible, of the 'remove and replace' approach, in the study of external as well as of internal factors. The critical role played by the adrenal cortex in the maintenance of the circadian bioperiodicity of circulating eosinophil cells, pinnal mitoses and hepatic phospholipid labelling was documented by this method (69, 75, 94).

With respect to meals, one can render them equidistant (75) or study the timing of a single meal (57), or investigate the effect of inanition (20, 66). Long-term social isolation can also be studied as a reference standard, to remove social effects (53). Whether one wishes to assess, by elimination, the effect of one or the other, external or internal factor, in relation to 'ordinary' synchronized bioperiodicities or free-running ones as a reference standard, at first one must ascertain the effect of what one does to obtain repeated measurements.

The availability of genetically comparable inbred rodents allowed one to use separate yet comparable groups of individuals at different test times (90). The construction of special environments with control at least of light, temperature and humidity allowed the carrying out of such tests under similar environmental conditions (92). It was thus possible to obtain, at 2 different times, a single sample from (2 groups of) separate yet comparable animals. The results obtained were studied by procedures such as Student's t-test. Thus, one could examine at least the statistical significance of the phenomenon under study. With such a design, the large changes in circulating blood eosinophil count were described as a statistically as well as biologically significant and spontaneously cyclic phenomenon (90, 92). It was then no longer possible to attribute a bioperiodic response to the stimuli associated with sampling. The stimuli which had to be applied in order to obtain a single sample carried no information regarding a 24-hour or other cycle. This is not to say, however, that the single stimuli had no effect. The response to a placebo (71) and to handling (46), can be assessed by sensitive chronobiologic approaches; if one does so, one also finds that those responses vary in a predictable since bioperiodic fashion at different circadian times.

A comparison of serially dependent and independent information is desirable and feasible in the absence of any known cyclic (e.g., 24-hour) information. Variables such as motor activity or core temperature that can be monitored with minimal or no interference are particularly suited for this purpose. Time series on core temperature, obtained the hard way by manual measurement (47, 48) or more elegantly by telemetry (83), provided indications for the time of sampling on biochemical and/or other variables of primary interest. First, however, the internal time relations had to be mapped under specified conditions (e.g., alternating light and darkness and continuous darkness) (48, 58). With such work in hand, the serially dependently measured variable core temperature served as a marker rhythm for the period of a change in susceptibility to ethanol (50).

It became clear that the demonstration of spontaneous within-day differences, dramatic though these may be, does not yield a reliable measure of a rhythm's timing and extent of change. Procedures such as periodograms or least-squares rhythmometry had to be made available before the period and other characteristics of a bioperiodicity became amenable to statistical estimation, eventually with their uncertainties. Some of the missing ingredients for an inferential statistical design and for analyses yielding parameters have long been available. The least-squares method had been published by Gauss in 1809. Schuster had provided periodograms by 1898. The systematic use of such methods and other in biomedicine, however, had to wait until the 50s (47, 49, 84, 91).

As isolation studies were begun, it also became obvious that the classical periodograms and autocorrelograms (49), Fourier analyses and power spectra (6) dependent upon equispaced data were not satisfactory. Methods were needed to deal with unequally-spaced data. Whenever a subject being observed and the observer both had to participate actively in a test, a choice had to be made. Sleep had to be interrupted if the subject was willing to be awakened and if comparable observers were willing to take turns in staying up for testing during rest spans. Alternatively, one had to be content with a data gap equivalent to the sleep span. One could then try to make up for the nightly data loss by the collection of dense data during waking. There were added reasons for seeking methods for the analysis of unequidistant data. Some observers were not available at all times. Some of the individuals being observed were unavailable at certain times. The waveforms of some of the bioperiodicities to be analyzed required dense sampling at certain times in a cycle, e.g., around ovulation, yet sparse samples sufficed at other times.

Against this background, multiple regression techniques by least-squares were introduced for rhythmometry (66) and soon complemented by a combination of linear-nonlinear least-squares analyses (73). It became apparent that even sparse data covering short spans of one or two lengths of a period to be examined, could be analyzed once it was recognized that bioperiodicities with certain periods, e.g., of about 24 hours or of about 1 year, are ubiquitous. Such evidence had become available by the late 60s (54). It was then a relatively simple matter to introduce the family of cosinor and related methods of data analysis (66, 77, 89). These complemented the use of analyses of variance, notably of data expressed as percentage of series mean, in the case of marked differences in operating level among individual series (92).

These methods involved the least-squares fit of a fundamental cosine curve with a period near that which was anticipated to characterize the data and of harmonics, if the waveform rendered this desirable. The statistical significance of the phenomenon thus quantified was checked by a conventional F-test, and by the use of

other inferential statistical methods described elsewhere (5, 22, 77, 89, 140). With computer tools available, a rhythm was then defined as a recurrent change once the predictability of its characteristics, including preferably the waveform, was estimated by inferential statistical methods.

7. FROM (MACROSCOPIC) BIOPERIODICITY TO (MICROSCOPIC) RHYTHM

Inspection by the naked eye of original data such as those in Figures 1–4 constitutes a so-called macroscopic approach and is indispensable to visualize a bioperiodicity. Classical statistical tests support the statistical significance of the changes in these figures. A probability (p) value indicates that the results are not likely a consequence of random sampling error. This approach is useful but incomplete. One need not be satisfied with the mere demonstration of a 'time effect'. Instead, in raising the question as to whether the results would have been obtained by chance, the 'no-rhythm' (null) hypothesis may be tested for a given period anticipated to characterize the data. In so doing, one also keeps in mind the need to obtain estimates of rhythm characteristics and of their uncertainties, a task described as parameter estimation.

Indeed, the macroscopic approach is best complemented by a so-called microscopic approach. The latter provides point-and-interval estimates of rhythm characteristics, thereby also allowing for a further statistical testing of results. The adjective 'microscopic' is used by analogy, e.g., to light microscopy in the study of spatial structure. The macroscopic approach to time series is as important as is the inspection by the naked eye of a tumor by the surgeon. To carry this analogy further, a microscope in conjunction with a set of histologic methods and experience is usually needed to ascertain whether the tumor is benign or malignant. By the same token, prior information on rhythms substitutes for the histologist's 'experience' in a temporal microscopic approach. The latter approach is needed for the objective resolution of quantitative characteristics in biologic time series. For this purpose, one fits mathematical models to time series and estimates the parameters of these fitted models, while testing for statistical significance.

In the microscopic context of this paper, as noted above, a rhythm is a component of biologic time series, i.e., a recurrent phenomenon formulated algorithmically and demonstrated as being periodic by inferential statistical means. A circadian rhythm may be 'isolated' by testing the null hypothesis of the zero-amplitude ($A = 0$) of a 24-hour cosine function fitted to the data. This fit is done by least squares (41). If the null hypothesis of no rhythm ($A = 0$) is rejected at or below the 5% level of statistical significance, a rhythm is described, provided that some criteria of regression diagnostic tests are met (5). Concomitantly certain parameters are determined as illustrated and defined in more detail in Figure 5. These rhythm parameters or characteristics are given with their 95% confidence intervals, measures of the uncertainty in their estimation (not shown).

Rhythm characteristics include the midline-estimating statistics of rhythm, the MESOR, a rhythm-adjusted mean; the period, τ, or its reciprocal, the frequency, f; the amplitude, A, a measure of the extent of predictable change; and the acrophase, ϕ, a measure of the overall timing, namely the lag (time from a defined reference time-point) of the crest in the 24-h cosine model best approximating the data; and the waveform, quantified by the (A, ϕ) of harmonics of the fundamental cosine curve fitted (if not by signal averaging).

When the period is likely to be synchronized with some environmental, e.g., 24-h, schedule and one wishes to check on whether there is synchronization or desynchronization, the chronobiologic serial section is a method of choice (66). This method is applicable to time series covering cycles of the component of interest. The period is anticipated on the basis of prior or separate information. A data section of fixed length, called an interval, is next defined for this kind of analysis. Thereafter, that interval is displaced throughout the time series, in increments, also chosen *a priori* by the analyst. At the outset, the interval may be equal to the period fitted and the increment to one-sixth of the interval or the average time span between consecutive observations. A single cosine with a constant period is fitted to the data in each interval. Thus, rhythm parameter estimates are obtained for each interval as a function of time. These parameters can be displayed in parallel with the original data.

If the data intervals chosen for analysis overlap, the resulting analyses are not statistically independent, but useful for a macroscopic check of the microscopic results. Analyses of this kind, based on data in overlapping intervals, have been called 'pergressive' (66, 124, 184) while analyses of data in non-overlapping intervals can be called 'fractionated' ones (66, 73, 77). The interval may be manipulated, e.g. shortened to better detect any patterns as a function of time (in acrophase and/or other characteristic) and/or lengthened, if this is needed to achieve, if possible, statistical significance, i.e., to obtain a $P < 0.05$ in a zero-amplitude test (77) say in 50% or more of the intervals. Thereby, the degree of consistency or stability (biostationarity) of the parameter estimates is examined (73).

This method is in keeping with an approach introduced as a 'moving' amplitude and phase by Stumpf (184) and is comparable, in results, to complex demodulation (5, 123). The display of moving rhythm parameters as a function of time is reminiscent of the smoothness resulting from a moving average, primarily when utilizing a pergressive approach. This procedure provides valuable information in the presence of non-stationarities, e.g., when rhythm parameters are slowly varying with time or when, at a given instant, the synchronizer schedule and/or the organism's time structure are altered, e.g., during drug therapy.

Figure 6 shows the different acrophase behavior of 3 urinary variables in the same patient, the displays in the 3 rows being parts of 3 serial sections. Other parts of those sections which are not directly pertinent to timing are not shown. The ordinates extend from midnight, equated to 0°, over noon, equated to $-180°$, to the following midnight, equated to $-360°$ (or 0°). The first two rows show a rather stable acrophase for the circadian rhythms in urinary potassium excretion and urine volume, before and after radiotherapy and also during radiotherapy. As indicated at the bottom of Fig. 6, aspirin was added for a while to the treatment initiated earlier. The interval used for analysis is of 240 hours, the increment and the period fitted are both of 24 hours. The abscissa extends from December 19, 1968, to January 14, 1969. It can be seen from the third row of Figure 6 that the acrophases slant upward. These acrophases occur later and later, as time proceeds. For the case of the circadian rhythm in urinary sodium excretion, radiotherapy is associated with a delaying acrophase drift. The 95% confidence intervals of each acrophase are also shown, as dots above and below the line of acrophases. There is a clearly phase-drifting (if not a free-running) rhythm in sodium excretion, until the time when aspirin treatment is also given (while radiotherapy continues). Aspirin administration leads to an apparent synchronization of the rhythm's acro-

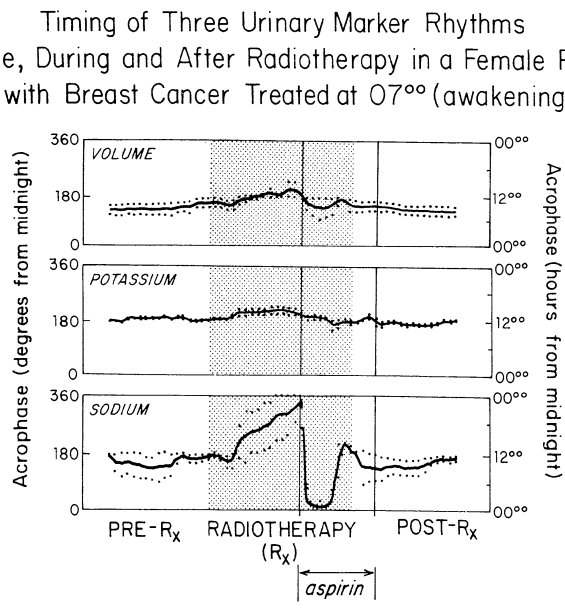


Figure 6. Chronobiologic serial sections reveal the effect of chronoradiotherapy of a patient on a diurnal activity-nocturnal rest routine. Treatment is associated with an increase in dispersion of the acrophases of all circadian rhythms and with a phase-drift of the circadian acrophase of urinary sodium. The latter drift is apparently synchronized by aspirin treatment. © Halberg et al., 1974.

phase with the 24-hour routine. For a few days the acrophase is located near midnight, rather than near noon. Eventually, an acrophase located near noon, in its habitual time relation, is seen, in keeping with the acrophase, before radiotherapy. These chronobiologic serial sections of urinary data in Figure 6 and elsewhere (18) show that some circadian rhythms (which are quite prominent in health; 57) persist in a case of cancer, before and after mastectomy. The sections also show that at least the circadian rhythm in urinary sodium excretion can be radically altered during radiotherapy. During radiotherapy, an increase in the confidence interval of the acrophase for the circadian rhythm in urinary potassium excretion is also seen in Figure 6. These intervals can be seen during radiotherapy, yet are barely discerned before or after radiotherapy.

When only short and sparse time series are available (rather than longitudinal data such as those underlying Figure 6), single and population-mean cosinor methods may be applicable (73, 77, 89). These procedures again involve an analytical method and a graphical presentation of results. The rhythm parameters are obtained by the fit with the least squares method, e.g., of a 24-h cosine curve, to data such as those as in the left half of Figure 7. A polar plot, shown on the right of Figure 7, conveniently and usefully presents the results of the statistical analysis. In this presentation, the amplitude and acrophase are represented as a directed line. The length of that line (vector) indicates the amplitude of the rhythm. The orientation of the vector, e.g., its direction with respect to the circular scale, indicates the acrophase of the rhythm. The circular scale covers one period or 360°. A 95% confidence region for the amplitude-acrophase pair is shown by an error ellipse around the tip of the vector. An ellipse not overlapping the center of the circle indicates that the amplitude is different from zero and thus a rhythm with a period near that of the fitted cosine is statistically significant (73, 77, 89, 100, 140).

While developed for the analysis of short and sparse time series, the single cosinor procedure is applicable to any one biologic time series anticipated to be characterized by a rhythm with at least an approximately known period. Figure 7 lists, in a table at the bottom right, estimates of the MESOR, i.e., the rhythm-adjusted mean percent regression/week, with the corresponding error. The double circadian amplitude is an estimate of the total predictable change/week, accounted for by the circadian rhythm. The circadian acrophase is at $-354°$, i.e., 6° from the tumor temperature peak. The latter time is equated to 0° or 360°. The best treatment time acrophase is within 24 minutes of the tumor temperature peak. With treatment given at this time, the most favorable tumor regression can be anticipated.

Figure 7 shows how the single cosinor method, along with the point estimates of the MESOR, amplitude and acrophase, also provides estimates of dispersion, such as the confidence intervals for the rhythm parameters (A, ϕ). Provided the residuals are independent random normal deviates with mean zero and unknown variance, the F-statistic (72, 77) can be used, as already noted, to derive an elliptical confidence region for (A, ϕ). The uncertainty of the ϕ can be derived from tangents drawn to the ellipse, as also shown in Figure 7. This F-statistic can also be used to test the null hypothesis of a zero (e.g., circadian) amplitude. If this hypothesis is rejected at an acceptable probability level, e.g., at or below 5%, it can be assumed that the fluctuation in the data is not random, but rather cyclic with the anticipated period or with a period near the one fitted. Indeed, it can be seen further in Figure 7 that the 95% confidence region does not overlap the center of the graph, called the pole. A statistical validation of the bioperiodicity is thus provided; we are now speaking of a rhythm (57, 66, 68, 89).

Sometimes, a single cosinor can be computed on data derived from different individuals or groups of individuals, each sampled at a different time or, in the case of the data summarized in Figure 7,

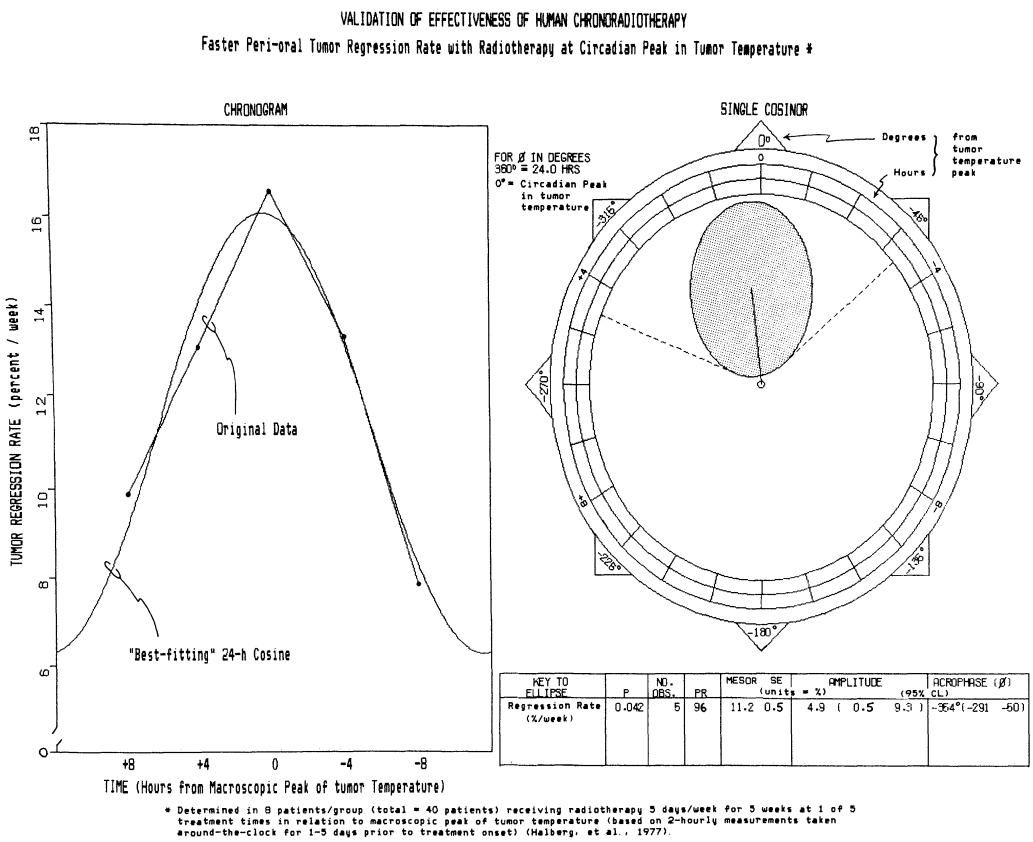


Figure 7. Illustration of the single cosinor method of data analysis. Original data are regression rates (in per cent per week) plotted as a function of the timing of radiotherapy in relation to peak tumor temperature. In the left half, treatment at the temperature peak or 4 or 8 hours before the peak, or 4 or 8 hours after the peak is described by 0, +4, +8, −4 and −8, respectively. The 24-hour cosine curve best fitting these results is also shown on the left. Summary of the same data in a polar display is shown on the right (see text). © Halberg, 1977.

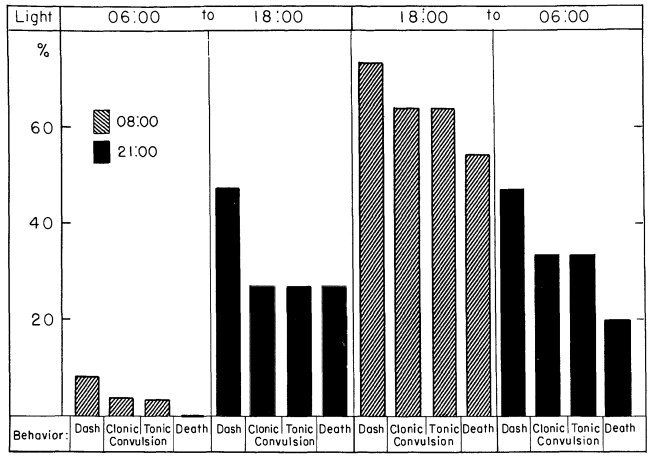


Figure 8. Response to noise at 2 different circadian times of separate groups of comparable mice susceptible to audiogenic convulsions. Results on mice maintained in light from 06^{00} to 18^{00}, alternating with darkness from 18^{00} to 06^{00}, are shown in the left half of the graph. The right half of the graph shows an increase in overall susceptibility and a persisting, yet differently timed, bioperiodicity, following a reversal of the time location of the lighting regimen. © Halberg *et al.*, 1958.

each treated at a different time. Similar analyses of data on patients receiving chemotherapy at different times in relation to a prospectively determined marker rhythm are discussed elsewhere in this volume (21, 22).

8. CHRONORADIOBIOLOGY

By the mid-50s, it was known that in mice susceptible to audiogenic convulsions, the application of a physical stimulus, namely a certain level of noise, had dramatically different outcomes as a function of when, along the 24-hour scale, the exposure to noise took place (60). At one time, exposure to noise had nearly no effect: it led to few convulsions and no death, Figure 8. At another time, a sizable percentage of comparable mice exposed to the same noise level responded with convulsions, many of them lethal (59. 60, 75).

It was also known by then that the circadian adrenal cycle could be synchronized by the lighting regimen and was amenable to phase-shifts by changes in time location of a regimen of 12 hours of light alternating with 12 hours of darkness (46, 90, 92, 95). It was interesting, as already noted, to ask whether the effects of a change in the lighting regimen, described as the dominant synchronizer of circadian rhythms, may not be restricted to a few functions such as motor activity and to anticipate that a shift in a susceptibility rhythm may also well be achieved, if a sufficient number of days was allowed to elapse following the inversion of the lighting regimen. Accordingly, the possibility of phase-shifting the circadian response to a physical factor such as noise in audiogenically-susceptible mice was indeed tested and the amenability to phase-shifting demonstrated, Figure 8 (76).

At about the same time, work pertinent to radiation and biologic rhythms was triggered by the endeavor of shielding (by timing) the mammalian host against the toxicity of radiation. In the 50s, with the knowledge that the rhythmic response to noise was both dramatic in extent and amenable to a change in time location by the manipulation of lighting, Webb Haymaker, then with the Armed Forces Institute of Pathology in Washington, DC, called one of us in behalf of the National Aeronautics and Space Administration (NASA) to ask whether we could find the best time to send an astronaut through the then-newly discovered Van Allen radiation belt. Thus the stage was set not only whether a mammal may be more resistant to whole-body X-irradiation at one time along the 24-h scale than at another, but also to ask whether such a susceptibility rhythm, if it existed, was amenable to phase-shifting. This question was answered in the affirmative in 1958 and reported orally at symposia sponsored by the American Association for the Advancement of Science (69) and in a discussion of the LD50 at the Cold Spring Harbor Symposium on Biologic Clocks (49). This work, carried out in the fifties following Haymaker's request, is shown in Figure 9.

With Figure 9, we turn from the time-dependence of the response to the stimulus noise to the response to another physical stimulus: irradiation. Figure 9 summarizes a study carried out under standardized conditions on 2 groups, each of 210 singly-housed mice, standardized for several weeks on 2 different lighting regimens. These regimens differed by 12 hours in terms of the time location of the daily 12-h light spans. Subgroups of 10 from a total of 30 mice from each room, at each of 6 circadian times, were then exposed to 400, 450 or 500 roentgens of total body x-irradiation, respectively. In this test, it was shown that the response to whole-body irradiation varies drastically along the 24-hour scale. From mortality at each dose, at each test-time, for mice on each regimen, a dose was computed which killed 50% of the

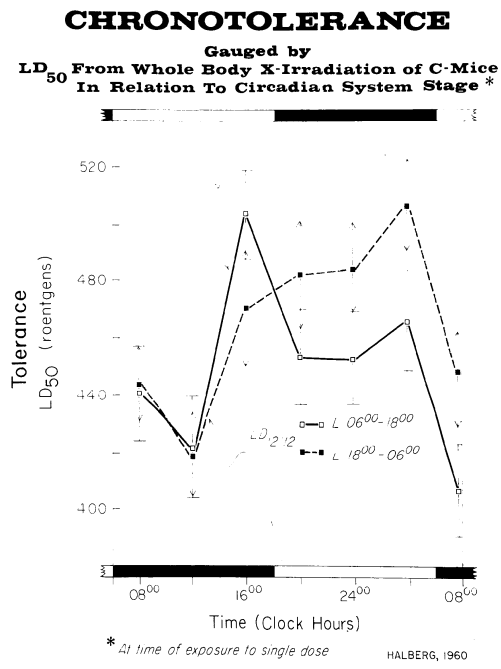


Figure 9. Chronoradiotolerance. See text. © Halberg, 1960a.

animals within 30 days (a so-called LD50/30); it varied by nearly 100 roentgens as a function of exposure time along the 24-hr scale. What was critical with respect to the time of exposure was not the clock-hour. It was shown in this same first experiment that the time location of the rhythm in

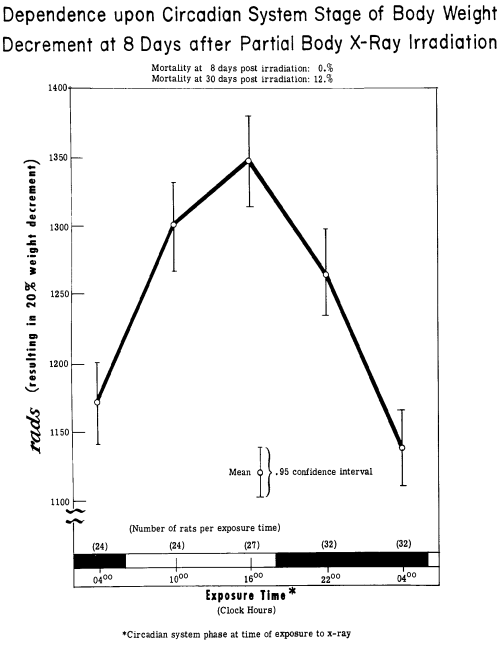
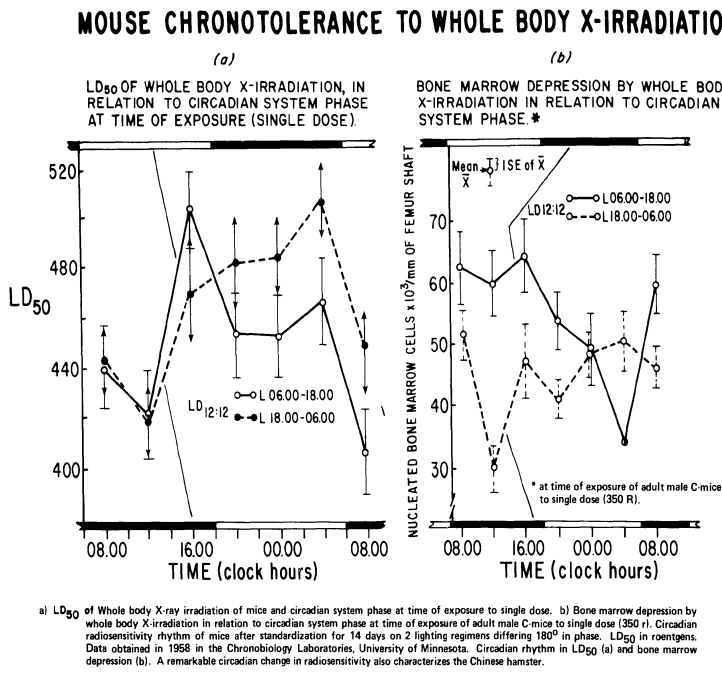


Figure 10. Circadian change in susceptibility of rats to partial body x-ray irradiation (38).



a) LD_{50} of Whole body X-ray irradiation of mice and circadian system phase at time of exposure to single dose. b) Bone marrow depression by whole body X-irradiation in relation to circadian system phase at time of exposure of adult male C-mice to single dose (350 r). Circadian radiosensitivity rhythm of mice after standardization for 14 days on 2 lighting regimens differing 180° in phase. LD_{50} in roentgens. Data obtained in 1958 in the Chronobiology Laboratories, University of Minnesota. Circadian rhythm in LD_{50} (a) and bone marrow depression (b). A remarkable circadian change in radiosensitivity also characterizes the Chinese hamster.

Figure 11. Change in susceptibility of the murine bone marrow to whole-body x-irradiation, gauged by the number of nucleated cells in femur shaft on right of figure (102). Results are aligned with the chronoradiotolerance of the entire organism, shown on the left of the figure (and also in Figure 9). © Halberg, 1960a.

radiotolerance was shifted by manipulating the lighting regimen. The highest dose of radiation (highest tolerance) was found during the second half of the light (rest) span, as can be seen from Figure 9 (49).

Circadian radiation sensitivity-resistance cycles were also documented for body weight loss in rats after upper hemibody irradiation ((38), Figure 10). For doses compatible with a 30-day survival of 88% of the animals, the dose required to obtain 20% weight loss in the animals at 8 days after irradiation varied as a function of circadian system stage at exposure time. The time of highest resistance of the animals against radiation-induced weight loss occurred during the second half of the light span (LD12: 12, $L06^{00}$–18^{00}), corresponding to the time of lowest mortality from the exposure to x-irradiation of mice and to that of rats exposed to whole-body irradiation by the same investigators and others. Comparably timed cycles of susceptibility and resistance to radiation thus were found for total and partial body irradiation and for the endpoints death and weight loss.

These results were extended by others, although not as a rule by the same approach (102, 120, 145, 146, 147, 192, 194). Ueno (192) and Vacek and Rotkovska (194) studied endogenous spleen colony-forming units at 10 days after irradiation. They observed a higher number of colonies in the spleens of animals irradiated at a time of greatest radioresistance, as gauged by the 30-day survival rate. These authors suggested that a higher number of hematopoietic stem cells might underlie differences in the mortality of mice irradiated at different circadian system stages. Pizzarello and Witkofski (146) measured the mitotic activity and incorporation of [^{3}H] thymidine into mouse bone marrow. DNA synthesis and mitotic rate were highest during the daily dark span (LD12: 12), coinciding with the time of highest radiosensitivity in our original studies and in earlier follow-up studies by these investigators. Two studies (161, 183) reported no circadian rhythm in mouse or rat radiotolerance; the analysis of the Rugh series by Pizzarello, however, reported a

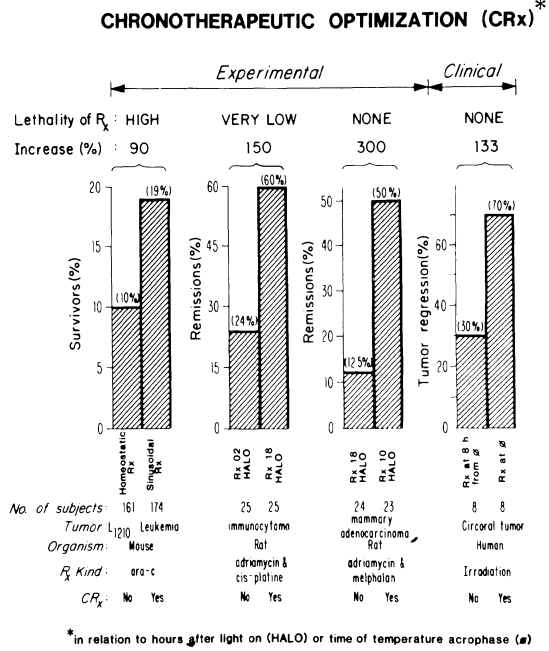


Figure 12. Chronotherapeutic optimization of cancer treatment in experimental animals (first 6 columns), and in clinical radiotherapy research (last 2 columns). In each pair, the column on the right shows the gain from timing, with the column on the left in each pair serving as reference standard. The last two columns describe the study already presented in Figure 7, to illustrate a cosinor method. This study is summarized further in Figure 15 and Table 2. © Halberg, 1977.

Table 1. Site of lesion in timed-treatment-groups investigated.

		Treatment group				
Site of lesion	*At peak (M)**	*4 h before M*	*4 h after M*	*8 h before M*	*8 h after M*	*Control (as usual)*
Tongue	1	4	3	3	3	6
Buccal mucosa	4	1	–	2	1	3
Tonsil	1	1	1	2	–	3
Alveolus	2	–	1	–	–	–
Maxillary antrum	–	2	–	–	1	–
Floor of mouth	–	–	1	–	1	–
Pharynx	–	–	–	1	1	–
Lip	–	1	1	–	–	–
Palate	–	–	–	–	1	–

*Macrophase.

circadian rhythmicity. The Straube study tested irradiation effects at only two times in the cycle. This approach does not necessarily assess the absence of a circadian rhythm, since, in the presence of a rhythm, samples at 2 timepoints may be taken from midline crossings rather than from a peak and a trough. A circadian change in radiotolerance has also been reported for intestinal crypt cell survival (4, 108).

Haus *et al.* (102) studied the number of nucleated bone marrow cells per mm femur shaft after exposure to 350 R whole-body irradiation at different circadian times. The reduction in the number of nucleated bone marrow cells in animals exposed at different circadian system phases was neither equal nor randomly varying. A circadian stage-dependence of this effect was seen in animals on a regular LD12: 12 lighting regimen and was changed in time location for animals on an inverted lighting regimen, Figure 11. The bone marrow depression rhythm was nearly in antiphase for mice on regular and reversed lighting regimens. In both groups, the largest number of nucleated bone marrow cells was found following irradiation during the second half of the light (rest) span (102). Again this time corresponds to the time of highest radioresistance for the same mice in the original study (49). Thus, it appears that the time of highest tolerance to radiation occurs late in the daily rest span in the species of animals studied, when the mitotic rate in bone marrow is lowest. Radiation-induced lethality and toxicity are sequels of cell lethality. It is concluded that radiation sensitivity is circadian stage-dependent.

9. MORE ON THE IMPORTANCE OF TIMING

Apart from the store of papers in experimental chronoradiobiology, the promise of timing human radiotherapy can also be considered in the light of the benefits demonstrated for the timing of other types of therapy, again mostly in the laboratory. Cases in point are the chemotherapy and immunotherapy of cancer and even therapy by placebo quite broadly (71, 188). In the laboratory, the cure rate of cancer is improved by the timing of ara-C in the treatment of an L1210 mouse leukemia, as shown in the first 2 columns of Figure 12. This gain, however, is achieved at the price of substantial toxicity (72, 99, 173). With much less toxicity, in the case of a rat plasmacytoma, cures are achieved by the timing of the interval between doxorubicin and cisplatin,

Table 2. Summary of study by Deka *et al.*

		Patients investigated					
Treatment (Rx) group		*A*	*B*	*C*	*D*	*E*	*F*
Rx time (hrs)*		0	−4	+4	−8	+8	unspecified 'as usual'
N patients		8	8	8	8	8	10
N lost to follow-up		1	1	1	2	2	1
Month of loss from follow-up		12	12	18	1&12	3&18	3
% disease-free at 2-year follow-up (N disease-free:N with recurrence) Evaluation time**							
3		7:1	4:4	4:4	4:4	4:4	5:5
6		7:1	4:4	3:5	4:4	2:6	4:6
12		6:2	3:5	3:5	3:5	2:6	4:6
24	a	5:3	2:6	2:6	2:6	1:5	3:7
	b	5:2	2:5	2:5	2:4	1:5	3:6
% disease-free	a	62.5	25	25	25	12.5	30
at 24 months	b	71.4	28.6	28.6	33.3	16.7	33.3

*Hours from tumor temperature peak.
**Months from end of treatment course. Losses from follow-up a) regarded as failures or b) excluded from study.

cures that are further improved by circadian timing, whereas otherwise cures are not obtained with these agents in the particular plasmacytoma investigated. The results are summarized in columns 3 and 4 of Figure 12 (65). With no deaths attributable to treatment, the number of remissions of a rat breast cancer is nearly tripled by the right timing of chemotherapy with doxorubicin and melphalan, as seen in the fifth and sixth columns in Figure 12 (82, 122). The background data are thus available and the procedures are developed to carry such results to the clinic (21, 110).

In the clinic, as yet only in a single study on a limited number of subjects, Table 1, the timing of radiotherapy is associated with the doubling of a two-year disease-free survival of patients with perioral cancers, Figures 7 and 15 (on the right), and in Table 2. These data are further examined below to check their validity and the promise, more generally, of marker rhythms (25, 110). The additional work supports inferences from earlier analyses (24, 73).

10. CLINICAL CHRONORADIOTHERAPY

In the original around-the-clock study of the effect of whole-body irradiation in mice (49), Figure 9, rectal temperature profiles were also measured (not shown). The temperature profile just before the start of irradiation served as a gauge of the state of the organism at the time of exposure, as a so-called marker rhythm for treatment timing. A marker rhythm is sought on a variable which can be readily measured and should reflect the timing of a predictable change in susceptibility, even if the variable itself is not causally related to the phenomenon investigated. The rectal temperature of the host had already served as a marker rhythm to gauge the free-running rhythm in continuous darkness of the murine rhythm in susceptibility to ethanol (50). Tumor temperature, in turn, was used in an attempt to assess rhythmic changes in tumor sensitivity. The bioperiodicity of temperature substituted for the more specific rhythm in tumor mitoses. As yet, repeated measurements, as a function of time, of mitoses *in vivo* perturb the host, are difficult and costly. Hence, the stage of a circadian change in tumor temperature was used as an unspecific marker rhythm for the tumor's sensitivity to treatment. The aim was to increase the effectiveness of radiation by selectively timing treatment according to this circadian marker rhythm.

The initial work was planned in 1969 as part of the International Biologic Program. A U.S. team (of which one author [Franz Halberg] was the head and another [Francine Halberg] a member) was assembled by the U.S. National Research Council. This team sought chronobiologic applications in joint research by Indian and U.S. scientists. We presented the dramatic changes in radiosensitivity, shown in Figure 9, to Indian radiotherapists. We agreed with the then-head of radiotherapy of the All-India Institute for Medical Research in New Delhi on the testing of any merits of timed radiotherapy on patients with large cancers associated with the chewing of tobacco, betel nut and lime. The response to therapy could be readily assessed at least semiquantitatively on these large perioral tumors. The task of the senior author (then in high school) was to demonstate the feasibility, even at an early age, of systematic oral temperature and other self-measurements. Subsequently, temperature over the tumor was measured with an oral thermometer (as a marker for the chronoradiotherapy of oral cancers) (rather than by patient self-measurement) by the staff of B.D. Gupta (with whom the original plans were made while he was at the radiotherapy department in New Delhi).

Two studies were carried out with external beam radiation by B.D. Gupta at the Post-Graduate Institute for Medical Education and Research (Chandigarh, India), one with

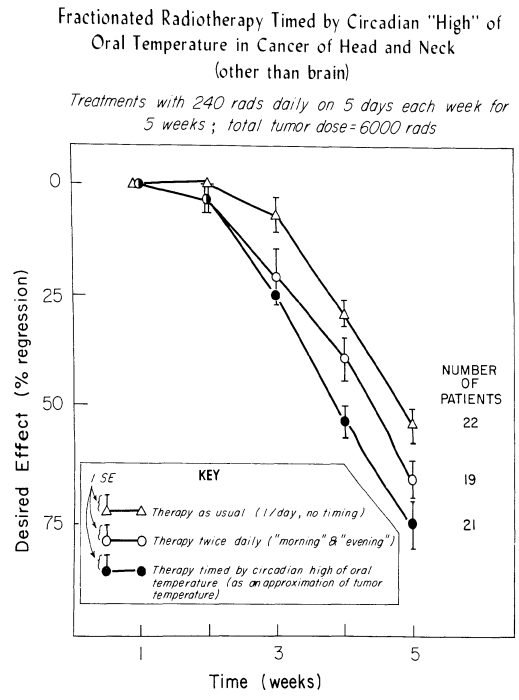


Figure 13.. Study by Dutta and Gupta on clinical chronoradiotherapy. (See text.)

T.K. Dutta, Figures 13 and 14, and another with A.C. Deka (24), Figures 7 and 15. Prospectively, these studies were macroscopic in that a plot of tumor temperatures was examined by the naked eye for the time of the highest point in the pattern of the temperature series: the peak (or macrophase) (110).

Groups each consisting of 8 patients were scheduled for 5 weeks of radiotherapy, 5 days out of 7/week. The breakdown of patients assigned at random to the treatment groups is shown in Table 1. Timepoints of treatment in relation to the tumor temperature are at the peak, i.e., at 0 hours from the peak, at 4 hours after the peak (−4) or at 4 hours before the peak (+4) or at 8 hours after (−8) or at 8 hours before (+8) the peak. Data from a ('control') group treated with no regard for timing ('as usual') are also available. The graph on the left of Figure 7 shows the different average regression rates/week, as a function of specified treatment time. The graph on the left of Figure 15 shows data on the best and worst tumor regression rate during treatment, again as a function of treatment time.

The data on tumor regression are retrospectively analyzed by microscopic chronobiologic methods. The acrophase, i.e., the time lag from a defined reference time point (the acrophase reference) to the highest point of the 24-h cosine function best approximating all results is shown on the right of Figure 7. Regression is optimal with treatment near the time of peak tumor temperature (62).

Table 2 shows remission rates of the various groups of subjects who had received timed (or 'as usual') treatment and who were available during an admittedly all-too-short follow-up for only 2 years. With the results on groups A to E, Table 2 also provides the corresponding rates for patients

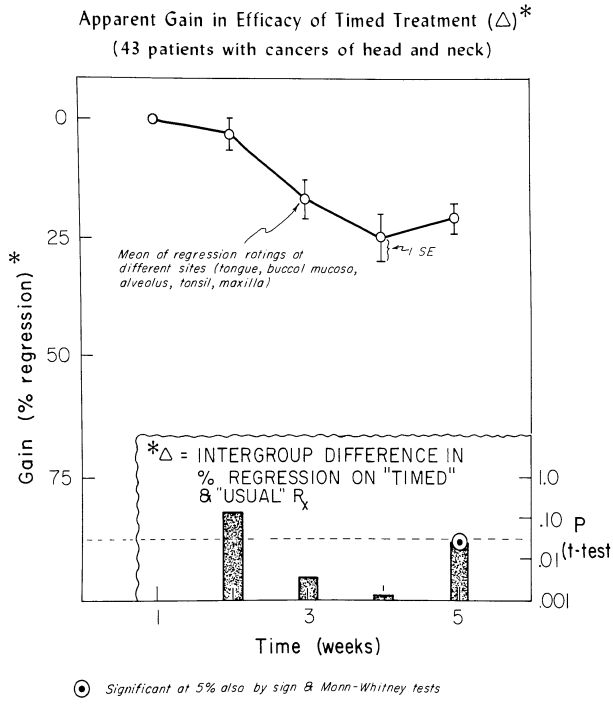


Figure 14. Summary of study in Figure 13. (See text.)

treated concomitantly 'as usual' (in the last column, F). Patients lost to follow-up are regarded as therapeutic failures in one summary. The table also shows results excluding those patients from consideration. These latter results are given in boldface, for the 2-year follow-up only. Results at the 2-year follow-up are also displayed in Figure 15. These data visualize the strikingly different result of treatment at the peak of tumor temperature. The figure also shows that the results as a whole are clearly nonsinusoidal. Accordingly, when the data on the remissions at 24 months are assigned to the six treatment times, in relation to peak tumor temperature time as 0°, a single cosinor based on the fit of a 24-hour cosine curve does not resolve any rhythm. In the light of a test of peak values in physiopathologic time series (166), ratios between patients free of disease at 2 years and the number of patients with recurrence, reveal a statistically significant advantage of chronoradiotherapy at peak tumor temperature.

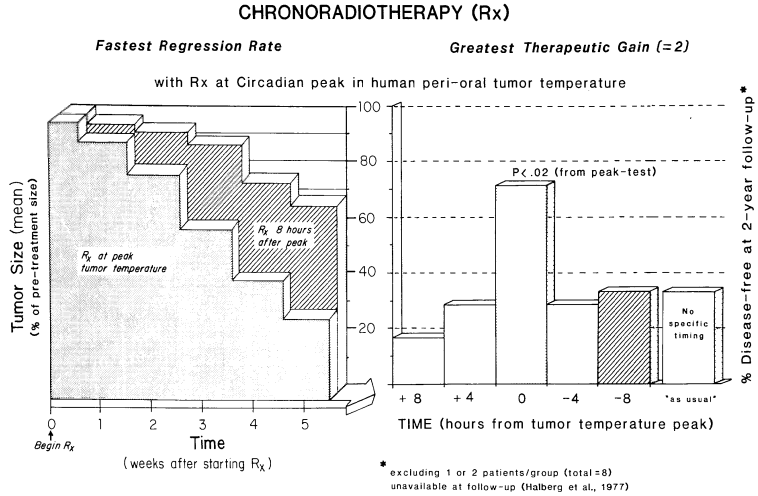


Figure 15. Summary of study by Deka and Gupta. (See text.)

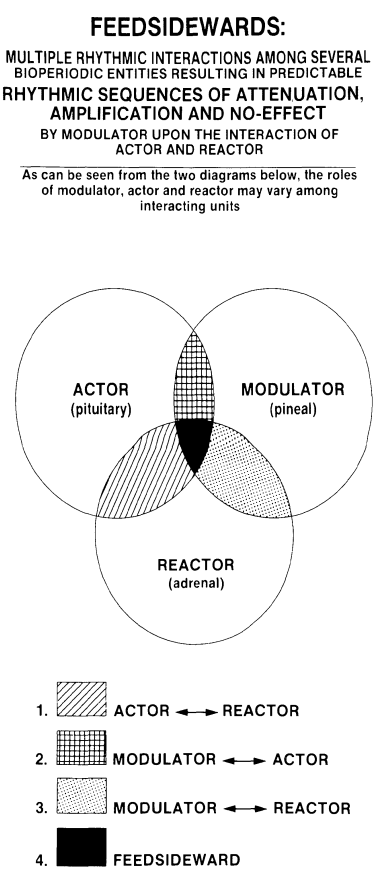


Figure 16. Chronomodulation by feed-sidewards. (See text.) © Halberg *et al.*, 1985.

11. A- TO D- AND ALPHA- TO DELTA-RHYTHMS

The clinical results are not surprising. We have seen that circadian rhythms are particularly prominent in cell division and several other cellular processes, under ordinary conditions in the field or laboratory (46, 48). Spontaneous circadian rhythms in cell division are observed in the absence of stimuli other than those in a unicell's standardized environment (31) or in modern human beings' daily routine (36, 168). We have seen a variety of response rhythms as well, which are sufficiently critical to tip the scale between death and survival. In ordering these variations, we can describe those found *in vivo* by roman letters and those studied *ex vivo* by Greek letters. We speak of series, unless the assumption of zero-amplitude (no-rhythm) has been rejected. If so, the series become rhythms.

Spontaneous (circadian or other) variations (occurring in the absence of intentional stimulation other than a daily routine), such as those in mitoses or in hormone concentrations of different tissues, e.g., in adrenal and serum corticosterone (56, 66) and in pituitary ACTH content (193), all constitute first-order data series. These first-order series may be described, if they represent data obtained *in vivo*, as a-series, or if they are data from *ex vivo* work, as alpha-series. Either a- or alpha-series become a- or alpha-rhythms once the no-rhythm assumption is rejected.

The (*in vitro*) alpha-rhythm is of immediate interest to those treating cancer. A separation of cells for *time-dependent* treatment *ex vivo*, prior to their reinfusion, should be explored. Moreover, the alpha-marker rhythms can determine the best treatment time, before treatment actually begins, by the use of test procedures that involve large doses of drugs not applicable *in vivo*. Any *ex vivo* treatment should take the different orders of *ex vivo* rhythms into account.

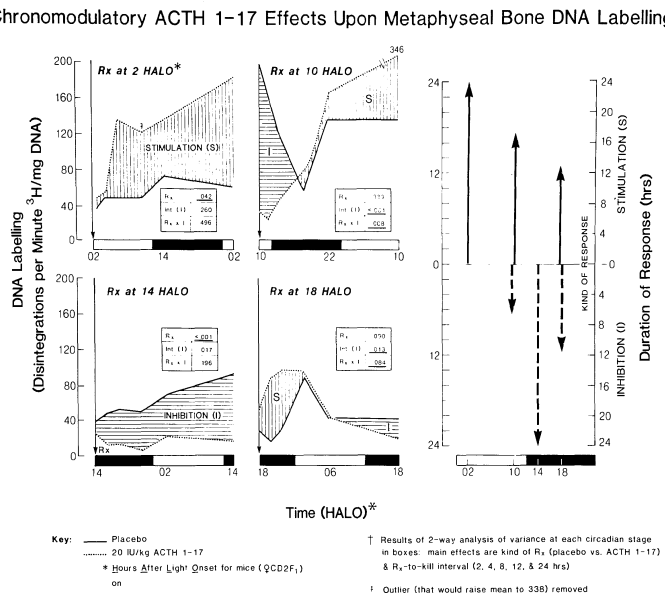


Figure 17. The same dose of the same molecule can stimulate (top left) or inhibit (bottom left) DNA synthesis, as a function only of timing (196). Thus, chronobiology adds a new dimension, yet to be utilized in the clinic.

Apart from spontaneous rhythms, the varied responses of an organism to a number of agents, including radiation as well as drugs, are also eminently rhythmic. a-rhythms are modified into b-rhythms by unusual exogenous stimulation such as the injection of a placebo or ACTH (51, 68, 71, 72, 175, 176). The changing responses along a 24-h scale of human serum cortisol or of murine adrenal corticosterone (e.g., to ACTH 1–17) (82) can be denoted as second-order or b-series and, once their statistical significance is validated, as b-rhythms (88). The rhythmic corticosterone response to ACTH (a second-order or b-rhythm), which modifies the spontaneous change in corticosterone (an a-rhythm) is modified, in turn, by aqueous pineal homogenate or melatonin into a third-order or c-rhythm. This pineal modulation of pituitary-adrenal interactions constitutes a so-called feed-sideward, leading to chronomodulation, namely to a sequence of amplification, no effect or attenuation of the ACTH 1–17 (57, 162, 164).

Over the past several decades, *in vitro* and *in vivo* work has also shown that, among other variables, the adrenal response to exogenous ACTH varies dramatically not only as a function of the stage of circadian and circannual rhythms (100), but also with other, e.g., about-7-day (circaseptan) rhythms (57, 70, 87). Moreover, when an a-, b- or c-rhythm, e.g., a circadian one, is being manipulated, there arise series of changes with the next lower frequency of the system, e.g., circaseptan (about-7-day) frequencies (88, 165).

For instance, the circadian system synchronized by a 24-h cyclic lighting regimen, may be shifted at intervals of different length for different groups of comparable unicells (179) or flies (106). A so-called frequency response, showing that even an isolated unicell can count to 7, in an endpoint as basic as growth, is the result (179). The same circaseptan response may be achieved in the springtail by a manipulation of environmental temperature at different intervals (126). Such manipulations of circadians at different intervals along the scale of circaseptans reveal a family of circadian-circaseptan d-series *in vivo* and delta-series *in vitro*. These latter result not only from a varying extent but also from the diverse timing of repeated circadian manipulation along the scale of circaseptans and of other rhythms with even lower frequency.

The mechanisms underlying such circaseptans have yet to be elucidated. The adrenal cortex is essential for the maintenance of some rhythms (69, 75, 94). The pituitary and hypothalamus interact with it (69); the suprachiasmatic nuclei represent an integrator of amplitude and timing, yet properly analyzed rhythms persist in their absence (81). The pineal is dispensable for the maintenance of adrenal corticosterone and pituitary ACTH rhythms (187), yet it clearly and directly modulates the adrenal *ex vivo* (162, 163, 164, 165). Moreover, the pineal displays circaseptans even *in vitro* (57). Circaseptans further characterize the effects of aqueous pineal homogenate upon the ACTH 1–17-stimulated production of corticosterone *ex vivo* (165). Pineal-pituitary-adrenal intermodulations, already resolved *in vitro*, so-called feedsidewards, Figure 16 (88), will have to be quantified *in vivo* in relation to radiation effects, in order to optimize the benefits to be derived from radiation itself and from sensitizing therapy, given repeatedly (daily) with a circaseptan (5 days out of 7) pattern.

At this writing, we can only speculate as to the role of radiation in the scheme of rhythms and vice versa. At first, one may assume that radiation brings about a beta-rhythm, i.e., a response rhythm in different structures affected by it. If radiation also stimulates one or several links in the pineal-hypothalamic-pituitary-adrenal network, it will trigger an endocrine beta-rhythm which, in turn, may be modulated by the effect of substances released in response to radiation at the cellular level and vice versa. Moreover, when a course of irradiation is given for 5 days out of 7, fourth-order or d-rhythms are likely to arise in the response to entire courses of radiation (88).

As we learn more about the interactions among suprachiasmatic, pituitary, pineal and adrenal factors, which constitute a documented feed-sideward mechanism, Figure 16 (88), we may become able to investigate the effect of radiation as such and of radiosensitizers in a systematic chronobiologic (rather than homeostatic) approach. This may be desirable, unless we do not care to distinguish between a 24-hour stimulation or a 24-hour inhibition of DNA-synthesis. Figure 17 (196) shows such responses to the administration of the same dose of the same molecule to comparable groups of animals. By the same token, the timing of radiation should indeed be scrutinized for an enhancement of desired and a reduction of undesired effects.

12. OUTLOOK

In the light of this available evidence, clinical follow-up research, further scrutinizing the merits of timing, can be advocated. As a minimum, the clock-hour and calendar date of each treatment should be recorded and the course of radiation kept consistent throughout. An analysis of desired and undesired effects (with such a minimal investment in a computer age) should be telling. Preferably, in addition, work with marker rhythms should also be done on an appropriate scale. It may, however, require some rescheduling of the already-costly operation of a radiotherapy facility and/or a socially-costly rescheduling of the patient's routine. In considering the around-the-clock operation of a radiation facility, at least research at the outset, the circumstance can be cited that shift-work is certainly the rule rather than the exception in hospitals. The alternative is to modify the subject's sleep-wakefulness routine. This change is accepted by any shift-worker, a disruption of relations with family and/or friends notwithstanding. Conflict will be encountered only if the patient's work and the routine for a conveniently scheduled treatment diverge.

Other solutions may also be sought. The optimal treatment time may be preset to a convenient clock-hour. In the rodent, the optimal tolerance of the host for at least one oncostatic drug may be set to any desired treatment time (71). ACTH 1–17, given 24 h prior to treatment, will present, to any time, a tolerance of mice to doxorubicin equivalent to that encountered at the circadian time of best tolerance (71). Such presetting of the best state has been achieved for only one chemotherapeutic agent, only in a laboratory rodent. The pertinent evidence has been reviewed in detail elsewhere (71). This precedent of presetting host tolerance will have to be complemented by an as-yet not documented similar presetting of highest tumor radiosensitivity to the desired treatment time. These are but avenues for future research in clinical chronoradiotherapy.

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ADDENDUM

A new as-yet experimental strategy has entered most recently into the chronobiologic approach to cancer prevention as

well as treatment. Several lines of evidence, obtained independently by different groups of investigators, converge to support the proposition that exclusive reliance upon the best circadian timing or even the best circadian, circaseptan and circannual timing may not suffice for a full optimization of cancer chronotherapy. These new results in no way detract from the basic demonstrations of a circadian rhythm in 1) the usual toxicologic criterion, the dose that kills 50% of mice exposed to whole-body irradiation (Halberg, 1960) and 2) a gain of 2 recorded by the criterion of the 2-year (superficially) disease-free survival for the chronoradiotherapy of certain advanced human perioral cancers (Deka *et al.*, 1976; Halberg *et al.*, 1977).

We have also obtained most encouraging results in the case of clinical chemotherapy following the demonstration of a circadian rhythm in the therapeutic index of doxorubicin in rodents (Halberg *et al.*, 1973). With a combination of doxorubicin and cisplatin, we reported time-dependent responses in LOU rats inoculated with a plasmacytoma, when the tactic for chronotherapy was simply to test each agent separately around the clock on separate groups of rodents, thus to seek a time of best tolerance for each and then to see whether, by treatment with both drugs at their particular optimal tolerance times, the efficacy of a combination treatment could be improved. Indeed, this was the case (Halberg and Delbarre, 1979).

In clinical follow-up work started on the basis of this finding, toxicity was reduced when the same two drugs were given with a certain timing 12 hours apart, as an interval that had proved advantageous in the studies on rats. A first stage of this clinical study by Hrushesky et al. (1981) involved the alternation, in consecutive months, of the two-drug combination from clock-hours deemed to be best to times believed to be worst. Adult patients with advanced-stage solid (ovarian or bladder) malignancies (diurnally waking: 07^{00}–22^{00}), eating mostly at 08^{00}, 12^{00} and 17^{00}, were treated monthly with 60 mg/m^2 of doxorubicin followed 12 hours later by cisplatin. Patients were randomized to receive chemotherapy beginning either 1 h before the habitual awakening (at 06^{00}: schedule A) or 11 hours after habitual awakening (at 18^{00}: schedule B), and then crossed over to the other schedule with a continuing alternation of schedule throughout the 9 months of treatment. As compared to treatment with the worst timing, the best treatment timing was associated with less toxicity, assessed on monthly bases (Hrushesky *et al.*, 1981). More specifically, in a month after treatment was given at the time deemed to be best, but not with treatment at the time deemed to be worst, the total white and platelet counts and other indices of myelotoxicity had on the average returned to or exceeded their MESOR preceding the given treatment course. The first clinical study stage (A+B) was followed by a two-armed approach in which the drugs were given to separate groups of patients consistently either at the times deemed to be best (Schedule A only) or at those deemed worst (Schedule B only) with the endeavor to assess the optimal treatment timing by marker rhythms at least retrospectively (see a Hermida and Halberg 101 (101b); b Cornélissen and Halberg 21, this volume).

The original approach with alternating treatment timing was not strictly comparable with the following two-armed approach, since the two stages were set up in succession rather than concomitantly. (The group of patients on the A + B treatment had entered that study before the two-armed approach was started on new patients.) With this qualification, however, there was a noteworthy finding. These patients with advanced ovarian and bladder malignancies, who received, 12 hours apart, a combination treatment of doxorubicin and cisplatin, at the best times on one day and at the worst time a month later, and so on (schedule A + B in alternation) did better not only (as anticipated) than those who, in the subsequent study, consistently received treatment at the worst time (B), but also did better than those receiving treatment consistently at the best time (A). The patients on the alternating A + B treatment schedule also did much better than historical controls (Hrushesky, 1987).

The foregoing results deserve further comment in several ways. A clinical two-timepoint design, whether designed as alternating A and B or as A or B, is not recommended for chronotherapeutic work when no prior evidence with the drug(s) on the given species examined is available. A 5- or 6-armed approach is preferred and has proved its value with an ACTH analogue (Halberg *et al.*, 1982) and further in chronotherapy with cyclosporine administered by pumps (Cavallini *et al.*, 1986; Halberg 1987). The 5-timepoint approach was successful in chronoradiotherapy (Deka, 1976; Halberg *et al.*, 1977). Apart from the foregoing, the relative merit of the A+B schedule as compared to the A-only schedule suggests that factors other than the best timing must be considered and will concern the major desired effect of treatment: the killing of the last cancer cell.

Thus, the stage is set to seek for reasons that may account for the as-yet lacking therapeutic benefit from clinical cancer

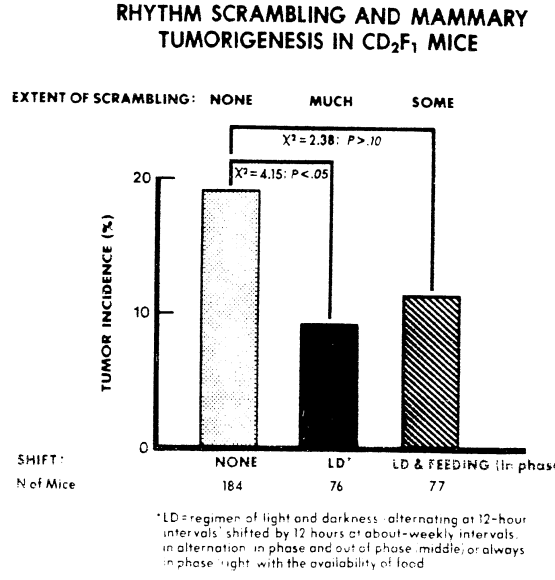

Figure 1. Statistically significant reduction in tumor incidence by strategic rhythm scrambling by a shift of LD alone (middle), which results in food availability in light or dark span, in alternation, i.e., food availability (the feed-starve rhythm) is alternately in phase and out of phase with the lighting schedule. By contrast, a concomitant shift of both LD and feeding (right) keeps the schedule of lighting in phase with that in food availability (i.e., with the feed-starve rhythm), but nonetheless brings about some rhythm scrambling. For the analysis of data on core temperature as a potential marker rhythm, see Figures 2 and 3 and Table 1.

Table 1. Amplitude ratios of 12-h and 24-h components of model* fitted to telemetered core temperatures of female $CD2F_1$ mice investigated on three synchronizer-shift schedules**

Group; meal-schedules	*Thermal circasemidian–circadian amplitude*											
	↓ *shift*						↓ *shift*					
AL free	0.23	0.34	0.44	0.30	0.09	0.33	0.17	0.31	0.30	0.17	0.22	0.29
F fixed	1.22	0.95	0.27	0.34	1.17	1.48	1.19	1.61	1.94	0.38	0.21	0.47
S shifted	2.29	0.66	0.57	0.28	0.31	0.36	1.59	1.54	1.07	0.31	0.10	0.43

*A model of combined 24-hour and 12-hour cosine curves was fitted as cosinor (Halberg, 1969; Bingham et al., 1982), to data covering consecutive 24-hour spans from each of three groups investigated.

**Weekly shifts in time location of a regimen of light and darkness alternating every 12 hours, while one group was on ad libitum feeding (AL), another group was kept on a fixed (F) meal-time (so that for one week, food availability and darkness were in phase and for the next out of phase, etc.), while, for a third group, meal-time was shifted concomitantly with lighting, so that feeding and lighting were always in phase (S). Note that ratios are consistently below unity (actually below 0.35) in animals subjected to a manipulation only of lighting, whereas with a manipulation in-phase of both lighting and meals (S), the ratios are above unity on four occasions out of 13 and with antiphasic manipulation (F), they are above unity six times out of 13. If core temperature is a marker rhythm for defense by natural killer cells or phagocytosis, the redistribution, on at least some days, of such defense may mop up cells that are about to become cancerous at times other than those when defenses are at their usual circadian peak.

chronochemotherapy, notwithstanding one's ability to administer more of a chemotherapeutic drug (achieved by the lesser toxicity due to timing). Hints concerning factors that may contribute to, if not account for, the foregoing outcomes came from two different experimental studies, done independently. One of these represented the conclusion of life span studies covering nearly two decades (Nelson and Halberg, cited by Luce, 1970), published recently (Halberg *et al.*, 1986; Nelson and Halberg, 1986); the other was an abstract of work carried out presumably without any information on the Minnesotan life span studies and their outcome (Carlebach and Ashkenazi, 1987).

In Minnesota, a substantial reduction in breast tumor incidence has been achieved by what may be called a targeted scrambling of rhythms. The finding was made in female CD2F1 mice kept in light (L) alternating with darkness (D) at 12-hour intervals (LD12: 12) (Halberg *et al.*, 1986). Some of these CD2F1 mice, feeding freely, were subjected to weekly 12-hr shifts of the LD12: 12 schedule beginning at either 7, 20 or 52 weeks of age and continuing until death. Other mice were meal-fed, eating about 25% less than the freely-feeding mice, and, from 7 weeks of age until death, experienced weekly 12-hr shifts of the LD12: 12 schedule alone (with mealtime fixed) or shifts of both the LD12: 12 schedule and mealtime (Nelson and Halberg, 1986). In the mice with fixed mealtime exposed to weekly lighting regimen shifts, breast tumor incidence was reduced from 19.6 to 9.2% ($P < .05$), Figure 1 (Halberg *et al.*, 1986).

We had previously demonstrated an ordered restructuring of circadian rhythms in rodents involving drastic changes of amplitude and of internal timing by shifts of lighting and feeding schedules (Nelson *et al.*, 1975). Such a tactical scrambling of rhythms comes to mind in viewing the new results in Figure 1. To explore the state of the circadian system in these more recent studies, core temperature had been telemetered from some of the CD2F1 mice, as a potential marker rhythm. A macroscopic view of the temperature data (Nelson and Halberg, 1986) already suggests differences in thermal responses of these animals to the different shift schedules. In the case of meal-fed animals, an increased prominence from time to time of an about 12-hour rhythm can be seen in Table 1. As can be seen further from the ratios of 12- and 24-hour cosine components in this table, there was a recurrent change from a prominent circadian rhythm, described by ratios much smaller than unity, into a transiently circasemidian one, quantified by ratios approximating or exceeding unity.

The changes in circadian amplitude can also be seen from Figure 2, a chronobiologic serial section, summarizing data from 6 female CD2F1 mice on a fixed meal-feeding schedule and subjected to weekly shifts of the lighting schedule (i.e., antiphase changes in lighting and feeding schedules). In the third row of this figure, the amplitude represents the distance between the lower curve (for the midline-estimating statistic of rhythm, the MESOR, M) and the upper curve. Changes in circadian amplitude are apparent while the phase of the circadian component in the fourth row of the figure is rather stably anchored by its synchronizer, the time of food availability (shown by hatching diagonally from the upper left to lower right). Darkness is shown in this acrophase section at the bottom of Figure 2 by hatching diagonally from the lower left to the upper right. it is apparent in the chronogram at the top of the figure that at the end of the span between the first and second shifts, indicated by vertical dashed lines and, on the abscissa by the Roman numerals I and II, the range covered by the temperatures is much wider than it is immediately after the shift. This increase in temperature range occurs after a shift when a daily span of darkness and that of food availability are out of phase, i.e., with the meal in early light. The thermal range is reduced as soon as there is a shift back so that the dark span of the lighting regimen and the span of food availability are again in phase, i.e., with the meal in early darkness.

What increases during the last 2 days of the week following the first shift, however, is not just the range, but the predictable extent of the circasemidian rhythm, its double amplitude, as can be seen in the second row of Table 1. Accordingly, the circadian amplitude decreases when the rhythm becomes circasemidian (Table 1) or at least more irregular to the naked eye, e.g., toward the end of the week following the first shift when food is available during early light. For 2 days after the second shift, the circadian amplitude remains smaller than the circasemidian one, only to increase later in the week following the synphasic shift, i.e.,

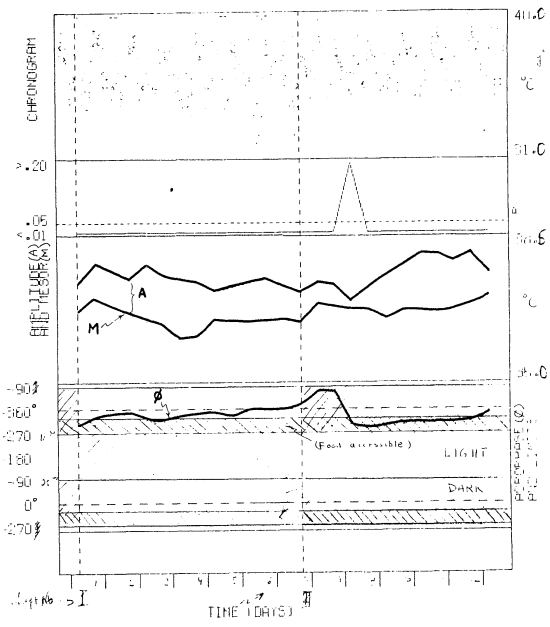

Figure 2. Chronobiologic serial section of core temperature telemetered from a group of 6 female CD2F$_1$ mice subjected to repeated 180°-shifts of a schedule of light (L) and darkness (D) alternating at 12-hour intervals (LD12:12). Only two shifts are shown here. Data series from June 3, at 18^{00}, to June 16, 1981, 18^{30}, from each of 6 animals were transformed into hourly averages and pooled into a single series. Data intervals of 24 hours were then fitted as a pool with a 24-hour cosine curve by the cosinor method (Halberg F., Carandente F., Cornélissen G, Katinas GS: Glossary of chronobiology. Chronobiologia 4, Suppl. 1, 1977, 189pp.). These intervals were then displayed with increments of 12 hours through this pool. The original pooled data are shown on top, with each hourly average intraperitoneal temperature from a single animal corresponding to a dot. The consecutive points in the several rows below the top row connected by lines are the results of the 24-h cosine curve fit to consecutive overlapping 24-hour intervals (the overlap, as noted, being 12 hours). The P-values from a zero-amplitude test are shown in the second row; the MESOR (M) is shown as the bottom curve in the third row, with a standard deviation of M shown as the distance between the M and a dot below it. The amplitude (A) is given as the distance between the two curves in the third row, the standard error of A as the distance between the upper curve of the third row and the dot above the A curve. The curve in the last section, for acrophases, describes the timing of the high values in the rhythm along a vertical scale from $-270°$ (6 p.m.) over 0° ≡ midnight, $-90°$ (6 a.m.), $-180°$ (noon), $-270°$ (6 p.m.) to the next $-360°$ (or 0°), i.e., midnight, and again $-90°$ (6 a.m.). Diagonal hatching from lower left to upper right indicates times of darkness; hatching from upper left to lower right shows times of food availability.

the shift when mealtime and darkness are moved into phase along the 24-hour scale.

For comparison, Figure 3 shows another chronobiologic serial section representing data from a pool of 6 female CD2F1 mice subjected to consistently synphasic changes in lighting and meal schedule. There are drastic changes in amplitude (with the amplitude estimate again being lowest when the data plot shows two peaks per 24 hours). Figure 3 shows further that the thermal acrophase follows, with a lag of two or three days, the changed time of the availability of food. Both the amplitude and phase behaviour differ between the sets of animals in Figures 2 and 3. They are presented in detail for a consideration in the context of the results in Table 1 and in the even broader and critical context of an attempt to interpret the mechanism of the reduction in breast tumor incidence, shown in Figure 1. Clearly, the differences in cancer incidence of the two scrambled groups are relatively small, even if only the animals on the antiphasic, probably more scrambled, regimen are statistically significantly lower in breast cancer incidence than meal-fed controls, consuming a diet similarly restricted in total calories. At this time, one can only say that Table 1 and Figures 2 and 3 show two different thermal marker rhythm features associated with schedule shifts. Figure 2 shows that the circadian thermal acrophase is reasonably stable near the span of food availability on schedules that involve changes along the 24-hour scale of the time relation between the two competing synchronizers, the lighting and feeding cycles. In Figure 3, by contrast, there is a pronounced weekly shift of the circadian acrophase of body core temperature following every synchronizer shift. To the extent to which the data pools for two weeks in Figures 2 and 3 represent long-term behavior of the larger groups of animals investigated, they may be interpreted to indicate two rather different acrophase behaviors of the circadian system. When the shifts occur always synphasically for the two synchronizers, the acrophase is moved greatly and thus changes weekly in its time location, whereas in the case of shifts into antiphase one week and back into phase in the next week of the two environmental cycles, the acrophase moves by less than 180°. The critical question, of course, in this context, is whether defenses against cancer may behave in a way similar to temperature, and if indeed they did, whether the role of a circasemidian period emphasized in Table 1 may be the critical feature to be considered as a marker for a distribution of defense by schedule-shifts.

It is pertinent in this context that blood cell counts and the actual activities of natural killer cells and phagocytes are eminently circadian rhythmic phenomena under usual conditions in experimental animals (Fernandes *et al.*, 1981) and human beings (Williams *et al.*, 1981); Halberg, Sánchez and Fernandez, 1983; Gatti *et al.*, 1985, 1987). This bioperiodicity means that for part of the day, immunosurveillance will be at its peak. By contrast, during another part of the day, at the trough of immunosurveillance, the reduced defense may not suffice. For optimal resistance at times of the usual trough, an occasional redistribution of defenses is required in such a way that, every so often, most if not all of the 24-hour span is covered with immunosurveillance. This can be achieved, among other possibilities, by a circadian-to-circasemidian change.

Unless this is done, the remarkable circadian rhythmicity of our humoral, cellular and neuroendocrine defense features, may constitute a two-edged sword. Even if treatment is properly timed to meet most of the cancer at the right time (when it is most susceptible), some cancer cells or cells about to become cancerous may be out of phase with the rest. There are usually stragglers and/or early risers among cells that become cancerous. These may escape the concerted major attack by natural defences and/or by radiotherapy,

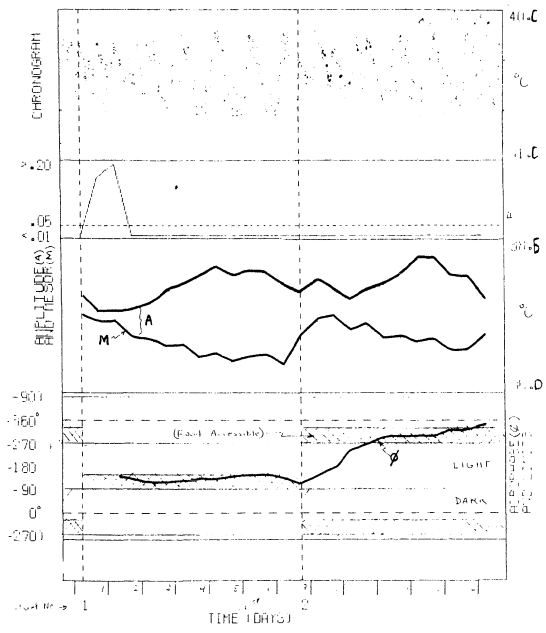

Figure 3. Chronobiologic thermal serial section of data from a group of 6 female $CD2F_1$ mice subjected to repeated 180°-shifts of LD12: 12 schedule, while the time location of food availability was fixed for the same about-4-hour span per day (namely for a time span compatible with a food consumption that was about 25% lower than that of controls feeding ad libitum but was not different from that of similarly meal-fed mice not subjected to shifts of the lighting regimen). For other details, see legend to Figure 2.

chemotherapy or immunotherapy. If, then, some of these therapeutic agents and/or the host defenses and/or the budding cancer cells are redistributed by strategic scrambling, the treatment may be much more effective.

This line of thought is further supported by independent investigations by Carlebach and Ashkenazi (1987), who report that temporal disorder may indeed be beneficial in the context of therapy. The foregoing conclusion, that in certain pathological conditions, temporal disorder may be of advantage to the organism, is properly qualified. Carlebach and Ashkenazi examined the presence of a rhythmic, endogenous 'defense system' against cancer cells [on] 24 groups of C57BL/6J male mice . . . inoculated with EL4 ascites [on an] LD14:10 regimen [and on] BALB/C male mice . . . inoculated with [a] plasmacytoma' and report on 'a . . . circasemidian rhythm in the susceptibility of the mice to the lethal effect of the inoculated cells. In order to examine if distortion of the host's temporal order would widen the time-range effectiveness of the "defense system", the two mice strains were inoculated with the respective cell lines and divided into two groups. One group was kept in a LD14: 10 regimen. The second group was exposed to continuous, randomly changing photoperiodic signals. The results showed that the latter group exhibited a distorted pattern of locomotor activity and high levels of life expectancy which differed significantly from those of the synchronized groups. Furthermore, recovery (low percentage) occurred only among the desynchronized animals. These observations suggest that, when temporal order prevails, host's susceptibility to, or/and defense against, cancer cells exhibit circasemidian rhythmicity. The distortion of host's temporal order increased the defense output. Thus, it seems that in certain pathological conditions, temporal disorder may be of advantage to the organism.'

One may add to the 'certain pathological conditions' a 'certain scrambling'. Evidence obtained in our laboratory shows that not any 'distortion of host temporal order increased the defense output.' It may be emphasized that scrambling may not be best done casually, but should preferably be done strategically.

Data obtained in our laboratory by Wu *et al.* (in preparation) concerning the interaction of schedule shifts with an immunomodulatory antibiotic, the third-generation cephalosporine, cefodizime, document this point. As a function of the conditions prevailing at the start of scrambling and/or other factors, a manipulation of the LD12: 12 regimen can either render the antibiotic effective in mobilizing defense or conversely shorten survival time.

In the search for the optimal circadian (Cavallini *et al.*, 1986) or circaseptan (Liu *et al.*, 1986) time for the administration of cyclosporine and other potentially harmful agents (Halberg, 1962, 1969; Reinberg and Halberg, 1971; Reinberg and Smolensky, 1983), it was already realized that the wrong timing can be detrimental (Halberg, 1962). Hence the use of marker rhythms has been strongly recommended (Halberg *et al.*, 1977; Hermida *et al.*, 1986a and b). In the case of scrambling as well, reliance upon marker rhythms seems to be indispensable and actually has been used in work thus far by Halberg *et al.* (1986), Carlebach and Ashkenazi (1987), Bingham *et al.* (1987) and Wu *et al.* (1987). With pertinent, readily monitored marker rhythms available, one will have to wed what can be regarded as complementary than competing strategies. Unquestionably, at certain times the body is more resistant to potentially harmful treatments, including radiation (Halberg, 1960; Haus *et al.*, 1973). This fact should be exploited. Accordingly, one should administer an agent such as radiotherapy at the time of highest resistance by host structures susceptible to harm from the treatment. A marker rhythm can then convey information on the best timing for minimal toxicity. What is even more important, certainly the critical consideration when a tumor rhythm can be demonstrated, is the need to identify the time of the tumor's highest sensitivity to radiation. This approach has doubled the two-year survival time in the case of certain accessible perioral cancers.

There is, however, a feature to the war against cancer, which applies to all wars. As Gen. Carl von Clausewitz (1832–37) already recognized in the Napoleonic era (when wars were fought primarily by armies): 'The war with armies is also to some extent guided by the *laws of probability* [emphasis mine] rather than only by the rules of logic.' Indeed, like those concerned about wars on battlefields, the strategists exploiting chronobiology must recognize that certain biologic rhythms obey a statistical rather than purely deterministic causality. Clausewitz also wrote that 'an infinity of unforeseen circumstances make plans fall short of the mark' and adds that 'everything is simple in war, but the simplest thing is difficult.'

By analogy, in the war against cancer, the latter point can be construed to indicate the need for controls on every aspect of a problem in experiments and the need for multiple

marker rhythms in treating a given patient. The clinical equivalent controls are indeed the multiple marker rhythms for body defense. The choice of these marker rhythms is a tactical matter. Different tactics may be taken into account in the overall strategy. For instance, body defense may relate to a number of variables that under usual conditions are more or less rhythmic, ranging from natural killer cells and phagocytes to the different granulocytic and monocytic responses. The more of these marker rhythms one monitors, the more likely one will find the best timing for their mobilization and appropriate distribution, just as multiple marker rhythms may be used for avoiding the toxicity of a drug, e.g., to the bone marrow. Whenever one can afford it, one may use, in addition to a leukocyte count, counts also of one or the other kind of cell (granulocyte, erythrocyte or platelet). Again, for reducing toxicity in another organ such as the heart or kidney, one may single out the appropriate variables, or several of them for each cardiotoxicity or renotoxicity, if one can afford it. But just as one cannot deploy troops everywhere everytime, one cannot rely on too many marker rhythms all the time, and the task ahead of us still lies in finding those that are most cost-effective. These different marker rhythm tactics have to become part of an attack on cancer at the times of best mobilization of defense, best desired effect and least toxicity.

The strategy to adopt, in the light of all available evidence, aims at the last cell kill, taking into account that, on the one hand, a majority of cells in the host and the cancer may be synchronized along the 24-hour and other scales. Accordingly, one should direct the timing of a major attack on cancer cells whenever these are most sensitive. On the other hand, one must not forget about guerrillas; we already mentioned stragglers and early risers. There is a need for defense against them as well. Unless the last cell kill is achieved, the process of cancer will not be stopped; the cancer cell that should preferably be a prey to the organism's defenses or, when these do not suffice, a prey to the treatment directed against it, once treatment fails, eventually becomes a predator that kills the organism. It is sheer common sense to spread some of the defenses in such a way as to take care of cancerous early birds and stragglers. This may be done by the shifting of light-dark and meal schedules in the experimental laboratory, in order to manipulate, to one's advantage, the rhythms in both host and cancer, or by stystematically changing the administration times of treatments with some interspersed scrambling in such a way that any treatment becomes more effective on fighting both the main force of the cancer and the guerrillas. Already, Jinyi Wu in our laboratory has shown that an immunomodulatory drug becomes effective (when it would not be otherwise), in the case of lighting regimen shifts implemented every second day. In this ongoing study by Wu et al. in our laboratory (in preparation), a transplantable LOU rat plasmacytoma is being treated. In the CD2F1 study, we are dealing with spontaneous mammary carcinogenesis, the prevention of the development of half of these spontaneous cancers. The strict control of food intake in the CD2F1 study is noteworthy. The Figure 1 results may qualify with their implications as the basis for a new strategic approach.

It is emphasized, however, that neither a deterministic fixed optimal timing nor a purely random treatment should be advocated. As we have also seen in our laboratory (again in studies on a transplantable LOU plasmacytoma), scrambling can be ineffective or even harmful (Wu *et al.*, in preparation). A statistical cauality (Halberg, 1946) will have to be kept in mind with a number of tactical features characterized by it, whether one's treatment may be radiation aimed primarily at the cancer cell or a mobilization of the organism's defenses achieved concurrently, or an even broader chronobiologic strategy of the attack against cancer.

Only with such qualification may one explore the extent to which these considerations also apply to radiotherapy, a promising matter for investigation. To start with, the noisy situation in the clinic notwithstanding, one may compare, retrospectively and evenually prospectively (if retrospective studies warrant it), the effects of similar radiation schedules on comparable patient groups when, for two groups, the circadian timing is fixed and regarded, at least on a population basis, as either optimal or undesirable on the one hand, and on the other hand, for a third group, the circadian timing of schedules has been changed from treatment to treatment, inadvertently if not intentionally.

An anecdote about an actual experience by a member of the family of some authors of this paper, who was to receive radiotherapy after a mastectomy, is pertinent here. A radiotherapist who went on a vacation told the patient that the treatment was best delayed until he returned. For all too long, such timing by convenience rather than pertinence has governed many treatment schemes including those of radiotherapy. Much work remains to be done on the alternatives here alluded to. In view of the technology involved in marker rhythmometry, this work cannot be done on the back burner. Oncology stands on the threshold of a revolution in prevention as well as diagnosis and treatment, based on the combination of several emerging technologies with an understanding of their effect of rhythms. We already have miniaturized portable personal long-term ambulatory monitors of biologic variables that undergo large-scale, spontaneously recurrent and reactive changes and that await testing for their use as marker rhythms. We also already have data base systems to acquire and analyze the volumes of data obtained by personal monitoring, and statistical procedures to model the biologic rhythms in the data, and from them to devise several strategies for optimal dosage time pertinence for specific individuals. By both allowing for treatment at optimal times and mixing a strategic scrambling that takes rhythms into account rather than proceeding randomly, one may plan a successful strategy, not only in the war against cancer, but more broadly in the body's defense against disease.

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20

ENDOCRINE THERAPY OF BREAST CANCER

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INTRODUCTION

Breast cancer is the most common malignancy affecting women in the western world. It is estimated that one in fifteen women will develop this disease during her lifetime. Approximately 110,000 new cases are diagnosed each year in the United States alone. If we assume that 30,000 will be cured by the initial surgery, 80,000 will develop metastasis at some point after mastectomy. Of these approximately 27,000 have hormone-responsive disease. Since the average survival from the time of mastectomy in these patients is 10 years (53), the prevalence of hormone-dependent breast cancer which has already relapsed or will recur sometime later is in excess of a quarter million in the United States alone. These figures underscore the magnitude of the impact that hormone-responsive breast cancer has on our society. It is the purpose of this manuscript to review our knowledge on the endocrine factors affecting tumor growth and, in particular, its applicability to the development of treatment strategies.

The hormone responsiveness of human breast cancer was first discovered by Beatson in 1896 who reported the success of ovariectomy in inducing tumor regression in some premenopausal patients with advanced disease (8). It is now established that approximately one-third of human breast carcinomas are hormone-responsive and regress following a variety of endocrine manipulations (69). It has been recently recognized that hormone-dependent breast cancer has different characteristics when compared to its hormone-independent counterpart. It tends to be histologically more differentiated (21), has a slower growth rate as evidenced by a usually low labelling index (94) and, more importantly, is associated with a better prognosis and longer overall survival (36). It is important to identify *a priori* those patients harboring hormone responsive tumors in order to appropriately select the therapy of both advanced and early disease. The introduction of estrogen and later progesterone receptor measurements in tumor biopsy specimens has allowed a reasonable good separation between hormone-dependent and independent breast carcinomas (59). It is now well known that when both receptors are present the likelihood of response of advanced disease to endocrine therapy is around 80%. In contrast, when they are absent, the success rate of hormonal manipulations is at best 10%. More recently, receptor status has been found to be important also in selecting appropriate therapy for early breast cancer. Two randomized prospective studies of adjuvant therapy involving women with stage II disease have revealed that only receptor positive patients benefit from the addition of the antiestrogen tamoxifen to combination chemotherapy (20, 28). Finally, receptor status has been found to have an important prognostic value. Patients whose primary tumors are positive for estrogen and progesterone receptors have been found to have an overall longer survival irrespectively of the treatments used (13, 36).

Despite the fact that over 80 years have elapsed since the initial discovery of the hormone dependency of human breast cancer, the individual roles played by the various hormones in affecting mitogenesis remain to be fully elucidated. Although recent evidence suggests that estrogens may play a predominant role, other steroid and peptide hormones may also have a significant effect on tumor growth.

ESTROGENS

Strong evidence in favor of a role of estrogens in supporting breast cancer growth is provided by the well established success of ovariectomy in inducing tumor regression in some premenopausal patients (64). Since the ovaries are the main source of estrogens in menstruating women, it is felt that castration induces its palliative effect through suppression of estrogen secretion. After an initial response to ovariectomy, surgical adrenalectomy and hypophysectomy performed at the time of relapse have been found to induce further remissions in 50% and 90% of patients respectively (41, 76). This additional palliation is thought to be due to a further decline in circulating estrogens obtained with these ablative procedures. The adrenal glands in fact are a major source of Δ_4-androstenedione which is then peripherally converted to estrone, the predominant circulating estrogen in women after the menopause. Consequently, significant estrogen depletion can be achieved in postmenopausal patients by removing the adrenal glands or by suppressing their trophic hormone ACTH via pituitary ablation. It was difficult, however, to be certain about the individual effect played by estrogens on breast cancer growth on the basis of the palliative effects obtained with these ablative procedures. Surgical ovariectomy, adrenalectomy, and hypophysectomy in fact produce additional perturbations in the hormonal milieu of the host besides lowering the circulating level of estrogens of potential clinical relevance, for instance, may be the suppression of androgens and, in the case of hypophysectomy, the suppression of the lactogenic hormones, growth hormone and prolactin. Furthermore, since es-

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trogens stimulate prolactin secretion from the pituitary (73), it has always been difficult to ascertain whether they act directly at the tumor level or indirectly by affecting prolactin secretion. Early evidence, to be reviewed in a later section of this chapter, suggested that indeed this latter mechanism might be the predominant one by which estrogens influence breast cancer growth.

In the last 15 years, however, several lines of evidence have suggested that estrogens directly stimulate tumor growth and are probably the predominant hormones involved in the mitogenesis of hormone-responsive human breast cancer. In this regard a major advance in breast cancer research was the discovery that a significant proportion of tumors, like normal estrogen target tissues, contain a binding protein in their cytosols, called estrogen receptor, which is able to bind estradiol with high affinity and specificity (58). The discovery of the estrogen receptors has not only been clinically useful, as discussed above, but has also provided support for a direct effect of estrogen on breast cancer growth. It would be, however, incorrect to equate estrogen-dependency of breast cancer with the presence of estrogen receptors in the tumor cytosol. It is, in fact, well known that at least 35% of estrogen receptor positive tumors are not hormone-responsive at all (59). It is conceivable that in the process of dedifferentiation some tumors may have retained the ability of the normal breast tissue to bind estradiol but may be unable to carry on subsequent steps of estrogen action. In support of this concept is the observation that hormone-dependent tumors can be better identified by the additional presence of progesterone receptors, the synthesis of which depends on an intact estrogen action machinery (27). However, to further illustrate the complexity of this matter, at least 20% of tumors that are both estrogen and progesterone receptor positive are hormone-independent (10). Thus, it appears that in some tumors estrogens are able to exert their action as far as stimulating progesterone receptor synthesis but not as far as initiating mitogenesis. In support of this concept are the results of our experiments performed in hypophysectomized rats bearing the hormone-responsive 7,12-dimethylbenz-(a)anthracene ((DMBA)-induced mammary tumor (6). Under these circumstances, we were able to demonstrate that estradiol administration was fully capable of stimulating progesterone receptor synthesis but had no discernible effect on tumor growth.

Perhaps the most compelling evidence in favor of a direct effect of estrogens on human mammary cancer growth has been provided by the potent antitumor activity of antiestrogen therapy. Numerous reports have not described the efficacy and safety of the antiestrogen tamoxifen in the treatment of postmenopausal women with advanced breast cancer (38, 50, 62). The mechanism of tumor regression induced by tamoxifen appears to be through inhibition of estrogen action at the estrogen receptor level in the tumor (30). In the attempt to exclude possible indirect effects of tamoxifen we have performed detailed hormonal measurements in women on chronic treatment with this drug. We have observed that in postmenopausal patients chronic tamoxifen administration did not alter the circulating levels of growth hormone, prolactin, estrone, estradiol, and estriol and only slightly suppressed serum gonadotropins (50, 51).

Further evidence in favor of the direct antitumor action of tamoxifen is provided by our finding that antiestrogen therapy induced significant palliation in a group of patients with metastatic breast cancer who had previously undergone surgical hypophysectomy (44). In these women, pituitary hormones were undetectable after provocative stimuli but estrogens were present in their serum although at low levels. These observations suggest that even small amounts of estrogens are able to stimulate the growth of some human breast cancers probably through a direct mechanism not requiring the presence of the pituitary gland. These findings are in agreement with *in vitro* studies by Lippman *et al.* (39, 40) who demonstrated a direct stimulatory effect of estrogens on the growth of the MCF-7 human breast cancer cell line grown in tissue culture.

The role of antiestrogen therapy in the treatment of advanced breast cancer in premenopausal patients is not at present fully established. It appears that tamoxifen has a definite antitumor effect in this group of patients with a response rate similar to that obtained with oophorectomy (47, 77). It is of concern, however, that antiestrogen therapy does not suppress menses in most women, thus suggesting that estrogen action is not completely blocked. This is probably due to the fact that tamoxifen in premenopausal women markedly increases estrogen production by the ovaries (47). The resulting high serum estrogen levels may, in turn, at least partially offset the antiestrogenic and possibly antitumor action of tamoxifen. This latter possibility is suggested by the observation that ovariectomy has induced remissions after relapse and, even more important, failure to antiestrogen therapy (31, 47, 77). Thus, at present, it appears that castration is still a necessary procedure to achieve optimal palliation of hormone-responsive breast cancer in premenopausal women. Recently, analogs of GnRH have received considerable attention as possible substitutes of surgical ovariectomy. These compounds, in fact, given chronically in a continuous fashion, have been found to produce a paradoxic inhibition of gonadotropin secretion and, consequently, gonadal steroidogenesis (35). The antitumor effect of such compounds has been found to be similar to that of surgical ovariectomy.

Further evidence in favor of a major role played by estrogens in supporting breast cancer mitogenesis has been provided by the recent introduction in the therapeutic armamentarium of the aromatase inhibitor, aminoglutethimide. This drug has been found to exert its antitumor action through inhibition of estrogen formation from the androgen precursors in postmenopausal women (85). Incidentally, it should be noted that the aromatase activity of the ovary is resistant to the action of aminoglutethimide and thus this drug is not effective in the treatment of breast cancer in premenopausal patients (86). In contrast, in postmenopausal women, aminoglutethimide has been found to be highly effective with an antitumor action similar to that of surgical adrenalectomy (87), and hypophysectomy (23). Since tamoxifen and aminoglutethimide work through similar mechanisms, the former by inhibiting estrogen action, the latter estrogen biosynthesis, it might be expected that patients should not respond when crossed from one treatment to the other. On the contrary, it has been shown that response to aminoglutethimide occurs in 50% of patients who had initially responded to tamoxifen and then relapsed and in 25% of patients who had actually failed to respond

to antiestrogen therapy (84). It has also been found that when aminoglutethimide is successfully used first, response to subsequent therapy with tamoxifen occurs only in 21% of patients (84). Because of this observation and the fewer side effects associated with antiestrogen therapy, it is preferable to use tamoxifen first followed by aminoglutethimide at the time of relapse. The reason why the sequential use of tamoxifen and aminoglutethimide is more effective than the reverse sequence is at present not known.

Although a predominant role of estrogens in affecting the growth of human breast cancer is now well established, the exact mode by which estrogens influence tumor mitogenesis is largely unknown. As mentioned above, the mechanism by which estrogens stimulate progesterone receptor synthesis is probably different from that employed to promote tumor growth at least in a significant fraction of patients. This statement is based on the observation that at least 20% of tumors positive for both estrogen and progesterone receptors fail to respond to endocrine therapy. On the other hand, approximately 30% of tumors positive for estrogen receptors but negative for progesterone receptors regress after hormonal manipulation (10, 59). In elegant experiments conducted in human breast cancer *in vitro*, Chalbos *et al.* (12) were able to demonstrate that physiologic doses of estradiol directly stimulate the proliferation of cancer cells after having stimulated the synthesis of 60,000 dalton proteins released into the medium. This finding supports the hypothesis that cancer growth may be autostimulated by estrogen-regulated proteins released by the cancer cells. In recent experiments performed in the experimental N-nitrosomethyl-urea-induced rat mammary tumor grown in culture in soft agar, we have tested whether the polyamines (putrescine, spermidine, spermine) may mediate the effect of estrogens on tumor growth. Our data indicate that, in this system, the integrity of the polyamine pathway is essential for full expression of the mitogenic effect of estradiol (54). While necessary, however, polyamines are not sufficient mediators of estrogen action (54). Preliminary data suggest that, in our experimental mammary tumor *in vitro*, estradiol may stimulate tumor growth through an autocrine mechanism which closely interacts with the polyamine pathway (Manni *et al.*, unpublished observations).

Finally, estrogens appear to have an unexplained paradoxical effect on breast cancer growth depending on their dose. Whereas in physiological amounts, as discussed above, estrogens stimulate mammary cancer growth, at pharmacological doses, they induce tumor regression both in rats (49) and women (33). Administration of high-dose estrogens is a well established hormonal therapy of metastatic breast cancer in postmenopausal patients (33). In two recent randomized clinical trials, it has been found to possess the same antitumor effect as inhibition of estrogen action with the antiestrogen tamoxifen (9, 29). The mechanism of antitumor activity of large doses of estrogens remains unknown. Various possibilities include suppression of pituitary function (101), inhibition of peripheral action of prolactin (34) and "down-regulation" of the estrogen receptors (43, 46).

In conclusion, although estrogens clearly play a predominant role in the growth of hormone-responsive human breast cancer, the exact mechanism(s) by which they affect mitogenesis at the molecular level is largely unknown. A better understanding of the role played by estrogens in the process of tumor cell division may allow us to treat breast cancer more effectively. For instance, it may enable us to optimally affect cell kinetics to enhance tumor responsiveness to cytotoxic chemotherapy. At present, treatment regimens aimed at "synchronizing" tumor cell growth are largely devised on an empirical basis.

PROGESTINS

Progestational agents such as megesterol, norethisterone acetate and medroxyprogesterone acetate (MPA) have been known since the 50's to induce remissions in 10 to 30% of women with advanced breast cancer (5, 22, 92, 93, 95). The mechanism of the antitumor action of progestins is largely unknown. Some evidence suggests that estrogens may be required for the optimal antitumor effect of progestins. It has been shown, in fact, that postmenopausal patients whose endogenous estrogen levels are sufficient to cornify the vaginal mucosa have a higher remission rate with progesterone therapy than patients with an atrophic vaginal smear (29% vs. 6%, $p < 0.03$) (92, 93). In addition, a synergistic effect between estrogens and progestins in inducing tumor regression has been suggested (17). Since estrogens have been found to stimulate progesterone receptor synthesis in human breast cancer (27), it is possible that at least one of the mechanisms of action of progestins may be mediated through the progesterone receptor itself at the tumor level. Since the antiestrogen tamoxifen has also been found to stimulate progesterone receptor synthesis in human breast cancer (27), one might anticipate an enhanced antitumor effect with antiestrogen and progestational therapy combined. In a recent limited study, however, where the therapeutic effect of tamoxifen (30 mg/day) was compared to that of tamoxifen combined with MPA (100 mg/day, orally), this hypothesis was not substantiated (61). It was indeed shown that the response rate in women treated with tamoxifen alone was higher than in women treated with the combined therapy (45 vs. 26%) although the difference was not statistically significant. Currently, it appears more profitable to use progestins sequentially after antiestrogen therapy rather than in combination. In a recent clinical trial, it has indeed been shown that after an initial response to tamoxifen, 31% of patients obtained further remissions to megesterol acetate (160 mg/day) while an additional 33% achieved stabilization of disease (82).

At present, large clinical trials, where the ability of the progesterone receptors to predict response to progestational therapy is rigorously tested, are not available. In a recent study, Clavel *et al.* (14) were unable to increase the proportion of patients responsive to progestin therapy through selection based on progesterone receptor presence in the tumor. It should be stressed, however, that the number of patients in this study was too small to draw a definitive conclusion.

Progestins, however, have been found to bind not only to the progesterone receptors but also to the androgen and glucocorticoid receptors in human breast cancer (96), thus, opening the possibility of alternative models of action at the tumor level. In addition to a direct effect, progestins may exert an indirect effect on the tumor through inhibition of

pituitary function since they have been found to suppress gonadotropins (81), ACTH (55), and growth hormone (89). Finally, it has been suggested that at least in some patients, progestins may be converted to estrogens (83).

Considerable emphasis has recently been placed on a superior antitumor effect of massive doses of MPA (> 500 mg/day) primarily by Pannuti and co-workers. Using this treatment modality in several clinical trials, these investigators reported response rates of about 45% and average durations of response ranging between 6 and 13 months (66, 67, 68). The superiority of massive doses of MPA over conventional doses (< 500 mg/day) has been challenged by DeLena *et al.* (18) who observed a remission rate of only 28% with a median duration of 6 months when giving MPA (1000–1500 mg/day) to women who were heavily pretreated with chemotherapy and endocrine therapy. These authors feel that the striking results reported by others with high-dose MPA could at least in part be explained by selection of patients who had received very little prior treatment in the form of chemotherapy and endocrine therapy. A recent prospective study, however, has provided some support for a superior antitumor effect of high doses of MPA in comparison with conventional amounts.

ANDROGENS

Androgen therapy has been known to be successful in the treatment of advanced breast cancer for almost 40 years (2, 63). The most commonly used androgens in clinical practice include testosterone propionate (2, 88), calusterone (11) and fluoxymesterone (45). Overall androgens have been considered somewhat less effective than estrogens in inducing tumor regressions with an overall remission rate of 21% reported in a large series of 580 patients (16).

The mechanism by which androgens induce breast cancer regression is unknown but several hypotheses can be considered. Androgens may exert their antitumor action by conversion to estrogens, since both adipose tissue and breast tumors can aromatize various steroid precursors to active estrogens (1, 57). This mechanism, however, cannot account for the antitumor effect of fluoxymesterone, a commonly used androgen which is not aromatized. Some evidence obtained in experimental rat mammary tumors suggests that androgens may diminish the sensitivity of the tumor to prolactin. This hypothesis is based on the observation that pharmacologic androgen administration reduces prolactin receptor content of the tumor (15) and that its tumoristatic effect can be reversed by high doses of prolactin (78).

In premenopausal patients, androgens may also work through suppression of gonadotropin secretion and consequent cessation of ovarian function. This possibility is supported by the observation that the majority of premenopausal women developed amenorrhea while taking pharmacologic doses of androgens (32).

Particularly puzzling is the recent observation that androgens may affect breast cancer mitogenesis through the estrogen receptor. In experiments conducted in the MCF-7 breast cancer cell line in culture, Zava *et al.* (100) observed that pharmacologic concentrations (10^{-6} M) of dihydrotestosterone (DHT) were able to translocate the estrogen receptors to the nucleus and induce a specific product of estrogen action, namely progesterone receptors. This concentration of DHT had been previously shown by Lippman *et al.* (39, 40) using the same system, to markedly stimulate cancer cell mitosis as assessed by incorporation of ^{3}H-Thymidine into DNA. It is difficult, however, to know the clinical relevance of these observations since high-dose androgens *in vivo* are known to induce tumor regression. In one study, however, where the effect of various doses of androgens on breast tumor regression in rats were tested, Heise *et al.* (25) observed that very high concentrations of androgens were less effective than lower concentrations and in some instances, they appeared to stimulate tumor growth. Thus, it appears that although pharmacological doses of androgens usually induce regression through a different mechanism, they may be able, in some cases, to activate tumor growth through binding to the estrogen receptors.

Finally, androgens could work directly on the tumor through the androgen receptor which has been described in human breast cancer (99). Of interest in this regard are our results with fluoxymesterone (10 mg orally twice a day) in 19 women who had been previously treated with tamoxifen and hypophysectomy (48). We observed that 7 obtained further remission from androgen therapy for an average period of 10 months. These results suggest that at least one of the mechanisms by which androgens induce breast cancer regression is not an antiestrogenic effect or an indirect action mediated through the pituitary, but perhaps a direct effect at the tumor level.

Recently, androgen therapy with fluoxymesterone was evaluated in combination with tamoxifen. In a randomized clinical trial, Tormey *et al.* (98) observed that the combination treatment was superior to tamoxifen alone in terms of response rate (38% vs. 15%, $p = 0.016$) and time to treatment failure (180 vs. 64 days, $p = 0.01$). These data suggest that the two treatments affect different subpopulations of tumor cells. This concept is further strengthened by our trial of androgen therapy used sequentially after tamoxifen (48). We observed a remission rate of 38% with fluoxymesterone in a group of 37 patients who had been previously treated with tamoxifen. Furthermore, therapy was equally effective in patients who had responded and in those who had failed to benefit from previous treatment with antiestrogens.

CORTICOSTEROIDS

Corticosteroids together with insulin and other hormones play an essential role in the growth and differentiation of the rodent mammary gland (97). The influence of these hormones on the growth of human breast tissue, including breast cancer, is less well understood. Administration of pharmacologic doses of glucocorticoids is well known to induce remissions in some women with advanced breast cancer. In a recent comprehensive review of the literature, Henderson and Canellos (26) reported that of a total of 589 patients treated with these drugs, an average of 23% responded with a response rate ranging from 0 to 43% in different trials. The short duration of response, usually averaging 3 months (70, 91) and the high incidence of serious side effects markedly limit, in general, the use of corticosteroid therapy in this disease since alternative treatments are available which are less toxic and more effective. There

are, however, selected circumstances where glucocorticoid treatment can be very valuable. One of them is hypercalcemia usually associated with widespread skeletal metastasis. When used in combination with hydration and forced diuresis with either furosemide or ethacrinic acid, corticosteroid therapy can effectively lower serum calcium (56). Obviously, the definitive management of hypercalcemia rests upon effective tumor control with specific therapy. When this is achieved corticosteroids can be reduced and discontinued, avoiding the undesirable side effects associated with long-term treatment. Another circumstance where corticosteroids are extremely helpful is in the presence of cerebral metastasis (37). In this case, their beneficial effect can be attributed to the reduction of the edema surrounding the metastasis which accounts in part for the symptoms of increased pressure and the presence of neurologic deficits. Here, the glucocorticoids with their potent anti-inflammatory activity are more effecacious than those which have a significant sodium retaining potential. The agent of choice is dexamethasone, which is usually given at the initial dose of 10 mg intravenously followed by 4 mg every 6 hr. The treatment is then tapered off over a few weeks period at which time the more lasting effect of whole brain irradiation has already started taking place.

Glucocorticoids, particularly prednisone, are also frequently used in association with cytotoxic drugs. It is, however, not clear if prednisone is an essential component of combination chemotherapy. While some studies have shown that corticosteroids add little to the combination regimen (79), others have shown a distinct advantage in including prednisone (7, 80).

Finally, prednisone has been used in the setting of adjuvant therapy of breast cancer. Notable in this regard is the report by Meakin *et al.* (60) who used prednisone as an adjunct to prophylactic ovarian irradiation. These authors observed a significant improvement in disease-free survival and overall survival at 10 years in premenopausal women, age 45 to 50 yrs., treated with ovarian irradiation and prednisone when compared to the group treated with ovarian irradiation alone or to the control group. Traditionally, the antitumor action of glucocorticoids in breast cancer has been attributed to the suppression of adrenal estrogen precursor secretion through inhibition of the hypothalamic-pituitary-adrenal axis. Recently, however, Osborne *et al.* (65) have shown that at least in human breast cancer cells in culture, glucocorticoids can directly inhibit tumor growth. The possibility of a direct effect at the tumor level is supported by the observation that glucocorticoid receptors have been detected in over 50% of human breast tumor samples (3). In a study including a limited number of patients, Allegra *et al.* (4) found that glucocorticoid receptors were of limited value in predicting response to endocrine therapy. Perhaps the most significant finding of their study was that glucocorticoid receptor positivity did not alter the low response rate to endocrine therapy observed in estrogen receptor negative tumors. Up-to-date, nobody has correlated glucocorticoid receptor status with response to corticosteroid therapy specifically.

PITUITARY HORMONES

Removal of the pituitary gland has long been known to be a highly effective therapy of metastatic breast cancer (74). A major mechanism of the antitumor effect of hypophysectomy is thought to be suppression of pituitary ACTH which results in decreased secretion of estrogen precursors by the adrenal gland and ultimately lowering of circulating estrogen levels. It has been suspected, however, that an additional mode of action of pituitary ablation may be suppression of the lactogenic hormones, growth hormone and prolactin, which have been shown to play a predominant role in the growth of experimental mammary tumors (52). Support for this latter mechanism of action is provided by the fact that in at least two clinical trials, one of which is prospective and randomized, hypophysectomy has been shown to be superior to adrenalectomy in its antitumor effect (24, 75).

A major area of controversy in the literature has been the relative role of estrogen and prolactin in supporting the growth of hormone-responsive breast cancer. Since estrogens are well known to stimulate prolactin secretion (73), it is possible that the mitogenic effect of estrogens on the tumor may be mediated through the pituitary gland. Early data in the literature both in women (74, 75) and in rats (90) seemed to support this conclusion. It was indeed observed that administration of physiological doses of estradiol were able to reactivate to tumor growth after ovariectomy but not after hypophysectomy. In contrast, when prolactin was administered to rats following pituitary ablation, tumor growth was promptly restored (73). The concept of prolactin dependency of human breast cancer was, however, seriously put in doubt when antiprolactin drugs, such as lergotrile mesylate and bromocriptine, were introduced in clinical trials in the treatment of metastatic breast cancer. It was, in fact, disappointing to observe that, despite adequate suppression of serum prolactin, these drugs had only a marginal palliative effect (19, 71). However, a final negative conclusion about the role of lactogenic hormones in human breast cancer cannot yet be drawn. In fact, in addition to prolactin, human growth hormone is also lactogenic and, thus, it may be necessary to suppress both growth hormone and prolactin secretion to obtain optimal palliation in men.

As discussed above in detail, the introduction of antiestrogen therapy with tamoxifen has indicated that estrogens are the predominant hormones stimulating the growth of human breast cancer probably through a direct effect at the tumor level. In the attempt to establish whether, besides estrogen, a pituitary factor also plays an individual role in supporting human breast cancer growth, we performed surgical hypophysectomies in patients who had been previously treated with tamoxifen and had either relapsed or failed (48). In a total of 61 patients we observed that 39% obtained an objective remission with a median response approaching one year. Although response to hypophysectomy was higher (60%) in patients who had initialy responded to tamoxifen, it is noteworthy that 6 of 22 patients who had failed to respond to antiestrogens, obtained a remission from removal of the pituitary gland. These data suggested that a "pituitary factor", perhaps growth hormone and prolactin, play an individual role in the growth of some human breast cancers. Support for this hypothesis was also provided by the observation that specific prolactin receptors are present in a significant number of human breast tumors (72).

Recent data, however, on the sequential use of aminoglutethimide after tamoxifen cast some doubts on this con-

cept. Under these circumstances, suppression of estrogen biosynthesis with this aromatase inhibitor yielded a response rate which was very similar to that reported above with hypophysectomy (84, 85). This observation raises the possibility that the antitumor effect of hypophysectomy after tamoxifen may be mediated through suppression of estrogens rather than lactogenic hormones.

In conclusion, at present, definitive evidence in favor of an individual role of growth hormone and prolactin in supporting human breast cancer growth *in vivo* is lacking. The pituitary gland appears to play its major role through stimulation of adrenal estrogen precursors via ACTH.

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21

SELECTED ASPECTS OF BIOLOGICAL CANCER THERAPY

ALBERT LANDSBERGER

INTRODUCTION

Biological cancer therapy involves a pronounced understanding of the tumor and host relationship. In certain neoplastic diseases other methods of chemotherapy since the early '50th and radiotherapy were successful as in acute leukemias of the young, in others they failed as in many solid tumors,* in a third group they were ineffective. For many types of tumors new approaches are needed and biological cancer therapy has to be seen as one of these avenues.

Experience has demonstrated that, just like in chemotherapy, better therapeutic results can be expected from a combination of two or more biologies because of the differentiated effects of the individual substances. But here, exactly, lies also the weak point of biotherapy: while we do have sufficient results with single preparations, there are almost no studies or animal experiments devoted to polybiotherapy, that is, with combinations of several biologics for the same tumor group. The aim of biotherapy should be to change a permissive tumor situation to a nonpermissive one.

Therapy with viscum album-extracts

These have been used in cancer therapy since 1920. Positive clinical experiences in tumor patients were confirmed through animal experiments. Total extracts have a direct cytostatic effect and an indirect effect through stimulation of the cellular immune system. Because of their great sensitivity to heat and chemicals, the delay rates differ depending on the process of extraction used (80, 83). Helixor (R), an aqueous cold-extraction product effects a delay already at a 0.01 mg/ml culture medium (50, 82) (see Table 1). Alkaloids at a concentration of 2.3 mg/ml block the reproduction of L/1210 leukemia cells 100% (46).

Lectins at a concentration of 0.4 mg/ml agglutinize Ehrlich-Ascites tumor cells (EAT-cells), sarcoma Ia and Soy/16-leukemia cells. Because of their specificity for galactose groups they also agglutinize erythrocytes, but the agglutination of malignant cells is stronger and can be achieved at lower concentrations (71). Viscotoxins, first characterized as peptides of lower molecular weight in 1948 exhibit a necrotizing and cardio-toxic effect. Samuelsson (78, 79) isolated a polypeptide mixture of the same name but different composition, which also seems to inhibit cell partition (56); its ID/50 for human KB-cells is 0.01 mg/ml. viscotoxins have an alpha-helix-structure (87); because of their affinity for nucleic bases they do not attach stochasically but selectively in certain gene regions, thus blocking their activity (87).

An isolation product of basic proteins (VP 16) inhibits the partition of HeLa-cells beginning at a threshold dosis of 0.0001 ug/ml of culture medium; its ID/50 is 12 decimal powers lower than that for Endoxan. In addition to all these, viscumic acid, a polysaccharide, also seems to exhibit an antibiotic effect (72). ID/50 is the dosis which inhibits cell growth by 50%.

In a clinical test, 63 patients with liver metastases caused by various primary tumors were treated with Helixor (Viscum album). The survival rate of treated patients was 8.1 months, 1 yr. survival 40.3% as compared with 2.5 months and 6.6% of a control group.

Thymus extracts and therapy (Zoch)

The thymus plays a central role in the production and differentation of precursor cells for the immuno competent T-lymphocytes. As support cells, suppressor cells, cytotoxic cells, emory cells, they are responsible for humoral, especially for cell-specific immunity. About 95% of all T/o-cells are destroyed by phagocytosis and only 5% of the precursor cells are formed into immuno-competent T-cells which, in cooperation with B-lymphocytes and macrophages defend the body against viruses, fungi, bacteria and neoplasms.

Table 1 lists the various enzymes derived from the thymus glands generally of young calves. The first group catalyses the reaction stages for the formation and repair of DNA, which provides the genetic information for the biosynthesis of protein molecules necessary for metabolism. The enzymes of the second group are responsible for purine metabolism which, in turn, is of importance for the synthesis of nucleic acids. Genetic defects in ADA can lead to a combined destruction of the B- and T-cell systems; the absence of PNP can lead to isolated effects on the T-cell system. In the third group, the relatively high SOD-activity found in young calves is indicative of the active macrophages which destroy 95% of the T/o-cells; similarly, protein-carboxy-O-methyltransferase is responsible for leukocyte-chemotaxis.

The specific proteins listed in Table 2 are not enzymes, but have regulative functions for the formation of nucleic acids. The histamine receptor proteins are localized at the mem-

* which are the most common neoplasms

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Table 1. Enzymes in juvenile calf thymus.

Ribonucleotidereductase
Terminal deoxynucleotidyl-transferase (TdT)
DNA-kinase; hypoxanthine-DNA-glycosylase
Histone-acetyl-transferase
Ribonuclease HI; deoxyribonuclease
5′-Nucleotidase
Adenosine deaminase (ADA)
Purinenucleoside phosphorylase (PNP)
Methylthioadenosine phosphorylase (MTA-P)
Endopeptidase (Protease)
Exopeptidase
-D-Galactosyltransferase
Neuroaminidase
Glucose-6-phosphate dehydrogenase
Superoxidedismutase (SOD)
Glutathione reductase
Protein-carboxy-O-methyltransferase
Phosphatase
Transasminase

Table 2. Special proteins in juvenile calf thymus.

Histamine receptor protein H_1	50,000 D
H_2	40,000 D
DNA binding protein	27,000 D
DNA–RNA helix destabilizing protein	20,000 D
DNA synthesis regulating factors	10,000 D

branes of the thymocytes. Histamine is a catalyst for various physiological reactions of the membrane receptor system.

Table 3 lists the most important peptides and proteins which have been isolated to date from calf-thymus-glands.

Treatment reports: 26 patients with mamma carcinoma were treated with thymus extract; 18 of the treated patients (70%) showed a markedly reduced CEA (Carcino-Embryonic-Antigen Test), accompanied by improved subjective conditions (73). A group of patients with advanced intestinal tract cancer was treated with thymus extract; within 3 years only 15% showed metastases, as compared to 45% of the untreated group, of which 4 also died. In a group having metastasizing colon cancer, supplementary viscum album therapy led to a doubling of the statistical survival time (28). Supplementary therapy with thymus extracts proved especially successful in tumor patients having a reduced defense system. Resistocell (R).

Table 3. Isolated peptides and proteins with biological activities from juvenile calf thymus*.

Daltons (approx.)	*Name*	*Researcher(s)*
307–612	Glutathione	Folkers
600	TP-5	G. Goldstein
1,000	Thymulin (FTS) Zn	Bach, Dardenne
2,000	$Thymosin_7$	A. Goldstein
3,100	$Thymosin_1$	A. Goldstein
3,400	Thymic humoral factor (THF)	Trainin
4,400	$Thymosin_4$	Horecker
5,300	$Thymosin_4$	A. Goldstein
5,600	Thymopoietin II	G. Goldstein
6,000	Thymone B	Folkers
6,000	Thymone C	Folkers
7,000	Thymone A	Folke
10,000	TSGF	Soder
15,000–16,000	HTH	Comsa, Zeppezauer
17,000	LSH_h	Luckey
80,000	LSH_r	Luckey
57,000–68,000	Hypocal	Mizutani
120,000	Protein	

* Adapted after different authors.

This preparation is a mixture of fetal mesenchyme (lyophilized), syn. fetal connective tissue from sheep fetuses obtained during the last gestation quarter, of heparin and a polyglucose. From the investigations performed during 1968 it became apparant that this combination produced effects which could not be achieved with the individual components, namely, the stimulation of the immune system and tissue regeneration. Experience has shown that this preparation is effective in tumor therapy as a supplement to radiation and/or chemotherapy; it can also be used when internal therapy is not or no longer effective. We also consider using it postoperatively, when the presence of metastases cannot be demonstrated, but when we find a high percentage of micrometastases. In this case, toxic chemotherapy has not produced any positive results.

In a test for lymphocyte-transformation of spleen lymphocytes following the intraperitoneal application of a mixture of Resistocell 1 and 11 in 10 female C57B16 mice, the integration of H-thymidin in test and control groups was investigated. Results are tabulated in Table 4 and shown graphically in Figure 1.

Several animal tests have shown that following immunostimulation with fetal, soluble and structural antigens (Resistocell), tumor growth in rats was clearly delayed. Histological tests showed an increase in lymphocytes in the spleens of the animals. The tests also showed, that with

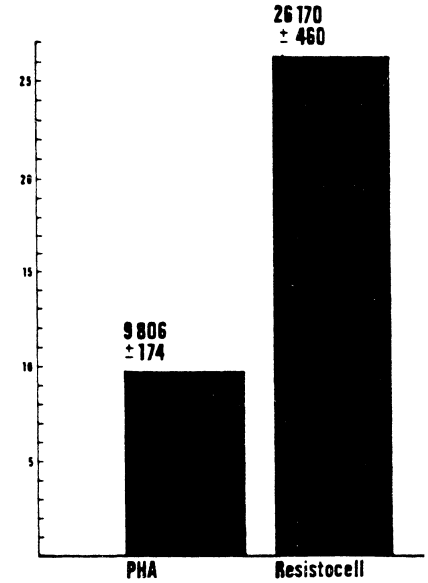

Figure 1. Lymphocyte-transformation test using U-thymidin/resistocell-PHA.

Table 4. Lymphocyte-transformation in c57 B16-mice following i.p.-resistocell-injection.

		Without PHA cpm	*with 10y PHA cpm*
Control (n = 10)	physiological cell-solution 7.5 ml/kg	161 ± 22	9806 ± 174
Test group	100 mg l +6.0 ml 11/kg	205 ± 9	26170 ± 460

respect to lymphocyte stimulation, Resistocell was clearly superior to Phythemagglutin (PHA).

Recent investigations have shown that interferon therapy can be successful in virus diseases (herpes), and for certain cancer types (leukemias). It was reported that Resistocell induces the formation of interferon.

Treatment with lyophilized, fetal tissues (Resistocell, bone marrow) has also led to a reduction in radiation-induced tumors in animal models, as shown by a decrease in tumor incidence or reduced growth rates. Seven months-old albino rats were subjected to whole body irradiation with 600 cGy. Spontaneous tumors appear generally in 10–20% of the animals at age 24 months, in all organs. The results are compiled in Table 5. Table 5: Percent tumor-incidence following 600 cGy whole body irradiation of the rat, as a formation of supplemental immunostimulation with fetal, xenogenic, lyophilized connective tissue or bone marrow cells, determined 3-1/2, 4-1/2 and 6 months following irradiation.

In another animal experiment, cytostatically produced side effects could be reduced with supplemental Resistocell treatment. The following cytostatica were used:
Adriamycin : 2.5 mg per kg body weight and week
Endoxan : 30.0 mg per kg body weight and week
Mitomyxan S: 0.5 mg per kg body weight and week
Supplemental treatment with Resistocell, 50 mg per kg body weight and week. The results are summarized in Tables 6 and 7.

The histological investigations of the spleen have shown that in animals treated only with cytostatica, a distinct damage to the lymphatic tissue could be observed. The observation confirms earlier reports of such damages caused by antineoblastic chemotherapy. Addition of Resistocell reduced or completely prevented such damage. One case of

Table 5. Percent tumor incidence determined $3\frac{1}{2}$, $4\frac{1}{2}$, and 6 months after total body irradiation (600 cGy), in the rat, independent of additional immunostimulation with fetal xenogenic, lyophilized connective tissue and bone marrow cells.

Experimental group	*Tumor incidence (%) after total body irradiation*		
	$3\frac{1}{2}$ Months	*$4\frac{1}{2}$ Months*	*6 Months*
Unirradiated & Untreated control group	0	0	0
(Total body irradiation (600 cGy), (TBI)) Untreated control group	55	55	55
TBI, fetal connective tissue, 8 days before and 4 days after TBI	10**	15**	25*
TBI, fetal bone marrow, 8 days before TBI	25 n.s.	25 n.s.	25 n.s.
TBI, fetal bone marrow, 8 days before and 4 days after TBI	15**	15**	15**
TBI, fetal bone marrow, 4 days before and 100 days after TBI	30 n.s.	45 n.s.	45 n.s.
TBI, fetal connective tissue and fetal bone marrow 8 days before TBI	35 n.s.	35 n.s.	50 n.s.
TBI, fetal connective tissue and fetal bone marrow 8 days before and 4 days after TBI	30 n.s.	35 n.s.	50 n.s.

Significant analysis; n.s. = nonsignificant, $p > 0.10$, * = significant $p < 0.05$ ** = significant $p < 0.01$; chi-square test.

Table 6. Histological investigation of the spleen*.

Test group	*Number of animals*	*Total area of lymphocyte walls, including germ centers per 2.72 mm² spleen tissues mean values in mm² × 10^{-3}*	*Standard deviation from mean value*
I control, untreated	12	244.8	±24.7
II control treated with fetal connective tissue	12	369.8	±16.5
III Adriamycin treated	12	140.8	±15.2
IV Adriamycin treated fetal connective tissue	12	259.5	±14.9
V Mitomycin treated	12	162.9	±15.2
VI Mitomycin treated, plus connective tissue	12	272.3	±15.0
VII Endoxan treated	12	33.4	±6.9
VIII Endoxan treated, plus fetal connective tissue	12	66.2	±9.7

*Evaluation of the area of the lymphocyte walls, including the germ cells in the white pulpa. The groups treated additionally with Resistocell show, with $p < 0.01$ (two-factorial variance analysis) a significant increase of the total lymphocyte wall area including germ cells as compared with control groups.

Table 7. Histological investigations of liver, heart, and blood smear.

Test group	*Number of animals*	*Number of centro lobular liver cell nuclei per visual field enlarged 1000 times*		*Percentage of granulocytes in blood smear*		*Area of interstitial heart muscle edema in μm^2 per 0.21 mm^2 heart muscle area*	
		mean value:	*standard deviation*	*mean value:*	*standard deviation*	*mean value:*	*standard deviation*
I control group untreated	12	33.25	±0.8	9.5	±1.5	–	
II control, treated with Resistocell	12	32.6	±0.5	13.5	±1.2	–	
III Adriamycin treated	12	23.5	±2.3	12.5	±1.4	5825.6	±1017.9
IV Adriamycin treated with Resistocell	12	28.4	±0.4	29.6	±2.3	1824.7	±343.1
V Mitomycin treated	12	24.5	±0.5	9.9	±0.9	–	
VI Mitomycin treated with Resistocell	12	26.7	±0.4	21.2	±2.4	–	
VII Endoxan treated	12	22.5	±0.6	25.6	±3.1	–	
VIII Endoxan treated with Resistocell	12	26.3	±0.5	32.4	±3.1	–	

Liver: During chemotherapy, the groups treated with Resistocell showed with $P < 0.01$ significantly reduced damage as compared to the control groups

Heart: Supplemental treatment with Resistocell reduced significantly the area of the interstitial heart muscle edema induced by Adriamycin.

Granulocytes: Additional treatment with fetal connective tissue showed with $P < 0.01$ a significant increase in the fraction of granulocytes in the blood smear.

granulocytopenia could be distinctly improved; the same is true of liver and heart. It is possible, that Resistocell can even prevent Adriamycin myocardosis.

Evaluation of the area of the lymphocyte walls, including the germ cells in the white pulpa. The groups treated additionally with Resistocell show, with $p<0.01$ (two-factorial variance analysis) a significant increase of the total lymphocyte wall area including germ cells as compared with control groups.

Clinical study

An established tumor-therapy scheme (CMFVP/Rad in the SKIT-Process) could be improved by adding Resistocell. The median survival rate of patients with advanced mammary carcinoma could be increased from 22.2 to 29.3 months.

A better result of the immuno-modulated group came from the positive late result. The first year of observation found no difference between the groups (Figure 2), possibly because the body's own immune system would act as a stabilizing therapeuticum only after the clinical remission of the disease; also, the supplementary therapy did not start before the 4th and 10th months. The increase in survival based on additive immunomodulation resulted for all types of regressions, for loco-regional relapses as well as for osseous and visceral metastases. Locoregional relapses improved from a medium survival rate of 30 to 43 months, the osseous type from 23 to 27, and the visceral type from 12 to 19 months.

Grouped by age, the patients also show a uniform increase in median survival rate following additional immunomodulation. Figures 3 and 4 show the tendency towards the late effect of immuno-modulation. In younger patients the survival rate improves from 24 to 43 months, in older ones only from 22 to 25. A comparison of the 1 year survival rate, gave no significant differences, but it became significant for a 5-year rate. (CMFVP/Rad = chemotherapy/radiotherapy).

Comparison of the survival curves of patients with ad-

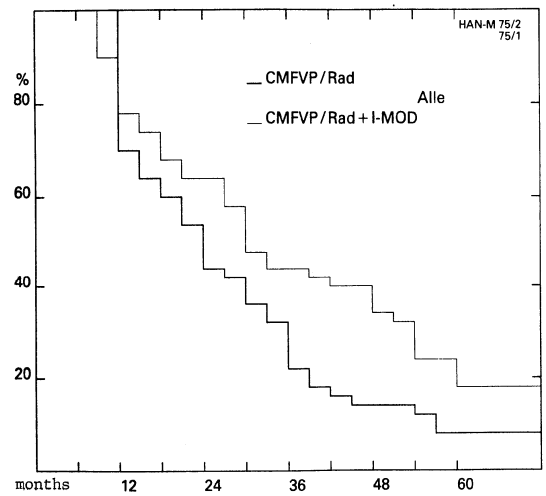

Figure 2. Comparison of the survival curves of patients with advanced mamma carcinoma, treated only with the CMFVP/Rad basic scheme (Study HAN-M 75/2) and those who had an additional immunomodulation (Study HAN-M 76/1). The 46 patients of the basic therapy did significantly worse as far as life expectancy than the 57 women receiving a Resistocell supplement ($p = 0.05$). The difference appears in the life-table only after the 24th month.

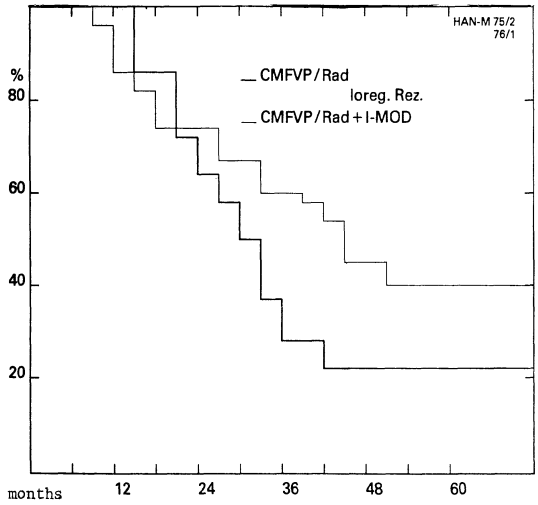

Figure 3. Comparison of the survival curves for the locoregional regression in the studies HAN-M 75/2 (CMFP/Rad and HAN-M 76/1-A (+I-MOD) for 15 or 28 patients did not show a statistically significant difference in life expectancy (p = 0.24). The apparent tendency for a better result in the patient group "immunomodulation" cannot be justified statistically because of the relatively low number of patients. The better prognosis for Resistocell-patients also becomes obvious only after the 24th month.

vanced mamma carcinoma, treated only with the CMFVP/Rad basic scheme (Study HAN-M 76/1). The 46 patients of the basic therapy did significantly worse as far as life expectancy than the 57 women receiving a Resistocell supplement (p = 0.05). The difference appears in the life-table only after the 24th month.

Comparision of the survival curves for the locoregional regression in the studies HAN-M 75/c(CMFP/Rad) and

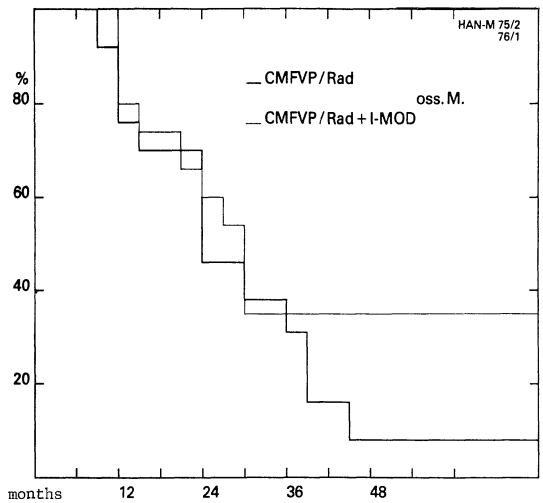

Figure 4. Statistical comparison for the osseous metastasis type in the studies HAN-M 75/2 (CMFVP/Rad) and HAN-M 76/1-A (+1-MOD) shows no difference for 13 or 15 patients in life expectancy (p = 0.41). The plateau of the immunomodulation-curve after the 30th month is due to a few "patients at risk".

HAN-M 76/1A (+ I-MOD) for 15 or 28 patients did not show a statistically significant difference in life expectancy (p = 0.24). The apparent tendency for a better result in the patient group "immuno-modulation" cannot be justified statistically because of the relatively low number of patients. The better prognosis for Resistocell-patients also becomes obvious only after the 24th month.

Statistical comparison for the osseous metastasis type in the suties HAN-M 75/2 (CMFVP/Rad) and HAN-M 76/1-A (+ I-Mo.), shows no difference for 13 or 15 patients in life expectancy (p = 0.04). The plateau of the immuno modulation-curve after the 30th month is due to a few "patients at risk".

Rise of NK-cellactivity by resistocell (RES)

Natural killer cells (NK) have the ability to destroy tumor cells and virus infected cells without prior immunization. NK-cells become stimulated by interferon and interleukin I and II. We investigated the capability of NK-cells with a test of cytotoxicity, because the production of interferon as well as interleukin develops through fetal mesenchyme (combination therapy resistocell). During this process, radioactive chromium is released and quantitatively measured using previously marked cells.

Material and methods

Human peripheral Blood Leukocytes (HPBL) were isolated by centrifugal elutriation, washed three times with Hanks-balanced-salt-solution (HBSS) and adjusted at a concentration of 10^6 cells per ml culture medium (RPMI supplemented with 10% human AB-Serum and 1% Penicillin/Streptomycin).

Resistocell was added at a concentration of 10% thinning dilution to the cells. The stock solution was prepared as described in the users' guide.

After overnight incubation the cells were washed and diluted into a 96 well plate (100 μl per well). A 4 h Cr release assay was performed. As a tumor cell line the K 562 cells were used.

The cells grow in suspension cultures in our laboratory; the cultures are free of mycoplasmia.

Cytotoxicity assay

The tumor cell lines were labelled with 100 μCi ^{51}Cr for 2 h at 37°C. They were then washed three times with RPMI with 10% FBS. For testing the cytolytic activity effector target ratios were adjusted to 10:1, 5:1, 1:1, 1:5 and the cells were incubated for 4 or 18 h.

Baseline spontaneous Cr release was determined by incubating targets without effectors. Total chromium release was measured in wells to which Triton X-100 had been added. After harvesting, radioactivity was calculated by the following formula.

$$\% \text{ specific lysis} = 100 \times \frac{\text{Test release} - \text{low control}}{\text{high control} - \text{low control}}$$

Polyerga

This extract was developed by Kuhlmey (57) during 30 years of research, based on the observation that the cancer cell partition rate correlates with the glycolysis level. Low glycolysis can lead to a partial dissolving of the cancer cells because of lack of nutrients. A nontoxic lowering of glycolysis can be achieved with Polyerga.

A study involving stomach-cancer patients of stages T3 and T4 gave the following results for postoperative therapy with Polyerga, evaluated on a 5-year survival rate:
Group 1: surgery and treatment + Polyerga: 38%
Group 2: surgery + treatment: 12%
It was observed that if Polyerga-group-patients survived the first year, they generally reached the 5-year limit. In the control groups, a continuous dying-off could be observed after the first year.

Enzymetherapy (66)

A total of 5 mammographies can double the mamma carcinoma risk in women – a reason good enough for us to use Wombencyme during mastopathia fibrosa when there is no urgent reason to suspect palpation. A study of 550 patients treated for about 4 weeks with Wombencyme supplemented with vitamin E in the form of E-Wulsin has shown that about 80% do no longer require dangerous radiation mammography; equal results could be obtained by thermography or with ultrasound.

A second, and no less important indication for enzyme therapy was provided by the prophylaxis of the lymphoedema. Following ablatio mammae, 49 patients were treated with Wombencyme over a two-year period. No lymphoedema appeared during those two years in the Wombencyme-group, while the control group of 33 patients exhibited 7 cases. This result is the more important if we consider that enzyme treatment has obtained an essential place in the remission phase following cytostatic treatment to reduce the relapse risk.

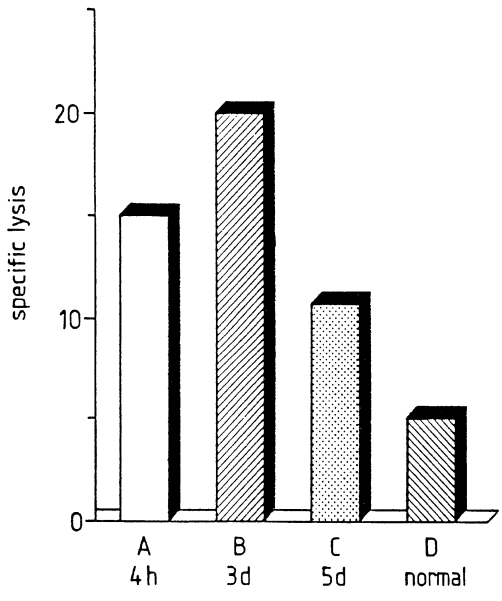

Figure 5. A. Peripheral blood cells were stimulated overnight with resistocell. Concentration: one ampulla of 20 ml medium + cells (10° pro ml) following NK-assay, 4 hours, against K 562 cells. E:T, 10:1.
B. As above (A), the stimulation was continued over a 3-day period.
C. Stimulation continued over a 5-day period.
D. Spontaneous NK activity in the blood.

The basic idea underlying enzyme treatment is to eliminate long-term chemotherapy. If chemotherapy is indicated, depending on the type of tumor and the state of the disease, then we apply a very high dosis, up to the dosis maxima tolerabitis, measured in terms of bone marrow toxicity, where white blood cells can be reduced to less than 200/mm/3. After this radical, short-term chemotherapy, we advanced enzyme treatment and other measures to stimulate the body's own immune system into action and to secure therapeutically induced remission.

Another indication of enzyme application is found in intratumor injection, involving a very high dosis. At a hit quota of 50% tumors can be brought into colliquative necrosis. This is possible, of course, only, if the tumor can be reached with a needle.

Local enzyme therapy using Wobe-Mugos starts on a larger scale in carcinomatous cavernous effusions where it is possible to achieve almost total remission for pleural carcinomatosis. Similar results were obtained against ascite formation generally originating from an ovarial carcinoma.

That our method of treatment is superior to "standard" treatments, using radiation followed by chemotherapy, can be seen from the following study: Between 1970 and 1974 we treated 58 male patients with stages III and IV teratomatous tumors of the testes; 13 are still alive (1983); of the 25 who died, 5 had brain metastases and 3 secondary tumors. But with Ifosfamid and cisplatinum now available, we can expect, generally, full remission following radiation, chemotherapy and enzyme treatment.

It is the goal of enzyme therapy to stabilize remission, not to exert a primary influence on extended masses of tumors. This requires the application of extremely high doses of proteolytic enzymes. Remissions can be induced in about 70% of all cancer cases, but in only a few cases we were able to stabilize a remission or to effect a cure. Even this is possible only through long-term biological methods, applied very carefully. We still have no control at all over gastrointestinal adenocarcinoma.

A study concerning the effect of very high doses of vitamin A on bronchial carcinoma has demonstrated that vitamin A must be given to the point where the patient will have vitamin A poisoning. This method of treatment is not without danger. Of the group of patients without vitamin A only 20% survived the first year, but 61% of the vitamin A group; 12 years later, there are no survivors from the group that underwent irradiation only, while 8 of 45 of the radiation/vitamin A group are still alive, resulting in a cure-rate of about 20%. This is the more amazing if we consider that all patients had been beyond surgical treatment and, statistically, had a survival time of only 6-7 months.

Summary

Biological extracts and methods are regarded only as sup-

plementary to the established tumor therapy methods (surgery, radiation, chemo- and hormone-therapy). Generally, they belong to the large group of immunestimulating substances; in part with regenerative effects. Like in chemotherapy, polybiotherapy is more effective than a singular substance. It is important, that the quality of life of tumor patients can be greatly improved through biological therapy.

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22

IMMUNOTHERAPY

RICHARD A. MALMGREN

Although immunotherapy of cancer seemed, when it was first conceived (46), the ideal form of treatment, relying as it does on the host's own defense mechanisms, it was not until successful control of cancer in the autologous host had been demonstrated that enthusiasm seemed justified. The first true demonstration of the existence of tumor immunity was observed in mice by Gross in 1943 (65). This was followed by evidence that chemically induced animal tumors were antigenic in the host (52) and that the antigenicity was individually tumor specific (136). In virus induced tumors in rodents the antigenicity was found to be virus specific (67, 152). Evidence for the existence of tumor antigens in humans can only be determined by *in vitro* techniques and there have been numerous reports in the last 20 years describing such antigens (71, 122, 172, 165, 111, 22).

Evidence was already at hand that transplantation immunity in animals was due to cellular rather than humoral factors (135) although the possibility that a combination of the two was necessary was not determined. At about the same time it was shown that carcinogenic agents interfere with the immune system in mice (113) and recent studies (42) have demonstrated that the toxic effect is on the T helper cells since it could be prevented by the lymphokine IL-2. A similar toxicity must have affected the B cells for antibody formation was also depressed.

The theory of immunosurveillance was proposed (27) which postulated that neoplastic cells are developing continuously but are recognized by the presence of tumor specific antigens as foreign and are destroyed by the immune system. A defect or interference with the normal function of the immune system would thus result in cancer as a disease entity. For the oncogenic virus, a defective immune system may only mean introduction of the virus into the host before the normal immune system has become functional. In the case of the mouse this is a short time after birth. Immune surveillance has been an attractive hypothesis and has had a strong influence on the field of tumor immunology and encouraged a search for methods of immunotherapy. It also has suggested an association between tumor development and a functional immune system. Attempts to correlate the phylogenetic appearance of neoplasms with development of an immune system have been only partially successful. Although the cellular immune system seems to first appear in lower vertebrates where tumors also are seen (60), tumors do occur in invertebrates (40). Some form of immunity does seem to be present in the invertebrates, however, as evidenced by the rejection of foreign tissue grafts by annelids (38, 39). If one considers phagocytosis a form of cellular immunity then it is present in protozoan as well (39).

An alternative immunologic theory for the progressive growth of a neoplasm is that the tumor specific antigens are weak or of a low concentration so the immune system does not react to them until it is too late to successfully destroy the mass of tumor cells.

Which of these theories is correct of course influences the direction taken to effect successful immunotherapy. If a weak or low concentration of tumor antigens is at fault, increasing the quantity or antigenicity by active immunization would be appropriate. On the other hand, if a defective immunosurveillance mechanism exists, ways to augment, stimulate, or modify it would be the method of choice. At present there is evidence supportive of both theories. Studies using *in vitro* techniques of the various cellular components involved in immunity and *in vivo* delayed hypersensitivity tests of animals and patients with cancer have demonstrated that there is indeed a depression of their immune state (69, 161) and this correlates in a general way with the prognosis (47). That is, incompetence of cellular immunity is associated with a poor prognosis. Although attempts to favorably influence survival of cancer patients using autologous tumor immunization have not been entirely encouraging (9), when combined with nonspecific immune modulators the results have been better than with either alone (24, 121).

Throughout this consideration of immunotherapy the assumption is made that data obtained from animal tumor systems are transferable to humans. A number of authors working in the field of cancer immunology using animal models have cautioned against this assumption, and their thoughts have been summarized and included in Hewitt's report on the relevance of animal models to human tumor immunology (75). An attempt to judge each animal study in this context is beyond the scope of this review. However, attention will be called to those studies where differences between animal and human results have been observed.

IMMUNE SYSTEM STIMULATORS

Microbial agents

As is true throughout the history of medicine, much that is known has been discovered empirically and later rationally explained. This is well illustrated in the development of immunotherapy. The use of such terms as agent, factor, nonspecific, etc. are instituted when empiricism is involved. So, as the agents that are being studied for their immunotherapeutic effect are considered here, the groupings are

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perhaps arbitrary for their mechanisms of action may prove to be overlapping.

Perhaps the most extensively tested method for stimulation of the immune system has been the use of various microbial agents or fractions prepared from them. This approach began with the well known usefulness of Freund's adjuvant as an immune stimulant technique. The active ingredient is the tubercle bacillus. When this organism, *Bacillus Calmette-Guerin* (BCG), was administered to animals it was found that the development of both viral and carcinogen induced tumors could be prevented and existing tumors destroyed (127). Direct injection of BCG into the tumor seemed to have a particular beneficial effect, even causing metastatic lesions to disappear (192). Treatment of human cancer with this agent has given encouraging but not universally successful results. As with the animal tumors it would appear that treating human cancer patients by intralesional administration of BCG gives better results than systemic administration by scarification. Most human immunotherapy studies using BCG involve treatment combined with surgery, chemotherapy or autologous cancer vaccine. In the treatment of lung cancer, BCG given intrapleurally in stage I non-oat cell carcinoma resulted in a longer survival rate in the treated group (ll, 116, 187). Similar results were obtained by intralesional injections (79). Systemic treatment with BCG in stage I and II cases did not seem to influence the survival rate (133, 134).

BCG treatment of patients with melanoma where the skin location of the metastases made intralesional treatment easier demonstrated improvement in the duration of remission and survival rate (123, 184). Similar treatment when combined with chemotherapy failed to be effective in stage I and II melanoma patients (159).

Acute myelogenous leukemia patients have experienced a significantly longer period of remission and survival time when treated with BCG plus allogenic cancer cells (193). However, in another study, the use of irradiated allogenic cancer cells alone was equally effective (183).

The other microbial agent that has been extensively tested is *Corynebacterium parvum*. The mode of action of this organism seems to be similar to BCG, and in experimental animals it has been shown to be as effective as BCG (101, 185). *C. parvum* has the advantage of being administered as a killed organism but its toxic side effects are a disadvantage. In clinical trials the results to date seem similar to those with BCG, including a preliminary report of 40 percent regression of metastatic lung lesions when given intravenously (83). In studies of the cellular components of the immune system involved in the modulating effect of *C. parvum* therapy it was found that there was an increase in natural killer cells and monocyte antibody dependent cellular cytotoxicity in patients with myelogenous leukemia treated with *C. parvum* (78).

An improved prognosis was seen in melanomas over 3 mm thick (8) but this was not the case when the *C. parvum* was administered systemically (intramuscularly) (37).

Another bacterial agent, OK432, a heat-killed lyophilized powder prepared from *Streptococcus*, was found to have an immunostimulatory effect resulting in a prolonged survival of patients with a number of different types of cancer (171).

The beneficial effect demonstrated on a number of neoplasms in animals and humans treated with whole microorganism suggest the possibility that improved results with fewer toxic side effects might be achieved with a purified preparation of the active principal of these organisms. Extracts of BCG, *C. parvum* and a number of microorganisms have been tested for their cancer therapeutic effect. Encouraging results have been obtained with a number of these preparations (138, 29, 12). Methanol extract residue (MER) of BCG, a nonviable particulate material that may have the attributes of BCG without the toxicity, is an attractive alternative. It has been found to have antitumor activity in animal systems (180), and some of the studies in humans have been encouraging (103). It was without effect, however, when given intradermally (125, 177), and in one study it interfered with the immunotherapeutic effect of neuraminidase treated myelogenous leukemia cells (14).

It is hoped that a more purified substance, muramyl dipeptide (MDP) from BCG, may prove even more useful and when combined with trehalose diesters (TDM), another microbacterium product that has been effective in the treatment of animal tumors (117). TDM in combination with endotoxin from other microorganisms such as *Salmonella* have effected cures of 90 percent in experimental animals (93).

A purified glycoprotein extracted from *Klebsiella pneumonia* serotype 2 called c-1821 has the unique feature among such agents of being nontoxic and effective by the oral route. It has been shown to enhance the immune response in animals and delayed cutaneous hypersensitivity in cancer patients. *In vitro*, increased levels of lymphocyte c AMP and c GMP have been demonstrated (104). What its cancer therapeutic effect may be remains to be seen.

The mechanism of action of the microbial agents seems to be through macrophage (74) and natural killer cell activation. There is also evidence that antibody dependent cytotoxicity mediated cells are involved (78, 96). Peptides produced by the gastrointestine have been shown to influence the immune system of mice in a way inhibitory to the effect of a number of exogenous activating agents (100). Inhibition was also observed when diethylstilbestrol was used in conjunction with the modulating agent Propionibacterium acnes (1).

SYNTHETIC ACTIVATING AGENTS

One of the earliest attempts to use immunotherapy in humans was the treatment of skin cancers with the delayed hypersensitivity sensitizing agent DNCB (178). Other agents that effect a similar response have more recently been evaluated and shown to be effective even in such tumors as mycosis fungoides (76).

A growing number of synthetic compounds have been prepared that have a stimulating effect on one or more cellular components of the immune system. A few of those most extensively studied will be considered here.

Levamisol, an antihelminthic, was found to augment Brucella vaccine in mice. *In vitro* it increased macrophage and polymorphonuclear leucocyte phagocytosis, leucocyte migration inhibition, lymphocyte nucleic acid and protein synthesis and lymphocyte cytotoxicity effect. In cancer patients it did not seem to increase the delayed hypersensitivity response (30). It has had little effect on animal tumors when

used alone, but in combination with chemotherapy (155, 160) or radiation (3) it resulted in retardation of tumor growth and increase in survival time. In humans with operable bronchogenic squamous cell carcinoma when an adequate dose of levamisol was given, therapeutic results were achieved (109). It has been tested on a variety of other human neoplasms with some beneficial effect noted for breast cancer and lymphocytic and nonlymphocytic leukemia (17, 31, 132). Another study showed no improvement in breast cancer (130, 154) and melanoma (163).

Azimexon has been shown to have beneficial effects on animal tumors (18), and it also provides protection against the toxic effects of cyclophosphamide and X-radiation (62) making it a particularly attractive agent for combined immuno- and chemotherapy. The effect seems to be achieved by stimulating the bone marrow cells and increasing the numbers of circulating T cells. Restoration of immune function in anergic cancer patients has been shown (34). More clinical trials are anticipated.

Pyran copolymer (MVE) has been reported to have antiviral and antitumor activity (41), to be a macrophage activator (7) and to have a stimulating effect on T and B cells (48, 162). Clinical studies with this material await a less toxic preparation.

Isoprenosine has been shown to be an immunostimulant. It increases the mitogen stimulation of lymphocytes, induces suppressor cells and stimulates antibody formation (176). Some evidence for antitumor effect has been observed in animal tumors, but it has yet to be tested in humans.

Bestatin is another immunomodulator prepared from *Streptomyces olivoreticuli* that increases the cellular and humoral response in animals (142, 143) and humans (149). It also retards tumor growth and decreases the development of spontaneous tumors in animals (25, 51). However, chronic administration of Bestatin to animals results in a depressed immune response (26). Preliminary clinical studies of this agent are encouraging (126).

Poly IC and Poly AU are synthetic double stranded RNA preparations that act as interferon inducers, and as macrophage and natural killer cell activators. In humans, Poly IC does not induce interferon, but when stabilized with poly L lysine it does. These agents are rather toxic in humans and their effectiveness in human cancer therapy has not been demonstrated (102).

The cancer chemotherapeutic agent Mitoxantrone administered to experimental animals has been shown to suppress humoral immunity by directly abrogating helper T cell function and indirectly enhancing suppressor T cell activity by its effect on suppressor macrophages (49). Further studies of the mechanism of immunomodulation using this agent seem indicated.

CYTOKINES

Perhaps a major mechanism of action of the microbial agents and the synthetic compounds with macrophage stimulating effect is their influence on the cytokines. By stimulating the formation of these natural biological regulator substances they bring about a response that has failed to function in the cancer-bearing host. The cytokines are, for the most part, glycoproteins produced by one cell of the hematopoietic system which, by reacting with cell surface receptors of another cell, alter its metabolic activity. Their formation seems to be initiated to a significant degree by exogenous agents.

One of the most thoroughly studied of the cytokines is interferon, a glycoprotein that was discovered in the course of evaluating antiviral defense mechanism (82). There are several interferons at present classified by the cell type from which they originate and by the inducing agent. Interferon of type I, called Virus type, is induced in fibroblasts by virus, polyribonucleotides, chemicals (Tilorone, etc.), tumor or heterologous cells, and epithelial cells and is called Beta interferon. The same inducers when acting upon cells of the leucocytic series produce Alpha Interferon. Type II Interferon, also called Immune type, is induced by antigens and mitogens in T lymphocytes and is called Gamma Interferon (157).

Interferon exerts a modulating effect on the immune system by antibody suppression (32, 87), by enhancing T killer cell activity (73), by causing null lymphocytes to have killer activity against tumor cells (56, 70, 169) and by activating macrophages (80). Interferon can also enhance antibody formation (23, 86, 147) and is a modulator of differentiation of diverse cell types (50). It has been shown to inhibit both normal and tumor cell growth *in vivo* and *in vitro* (64, 98, 144), and some beneficial effects have been demonstrated on cancers in humans (66, 118, 140), although this has not been uniformly true (53). When human fibroblast interferon has been used in the treatment of human cancer an anti-interferon antibody was formed in the patient (175). If this is a common phenomenon it may limit the use of this agent.

Another glycoprotein of monocyte origin with antitumor activity is the tumor necrosing factor, which is found in the serum of BCG immunized mice subsequently treated with endotoxin (30). It has a necrotizing effect on malignant but not normal cells and is not interferon. Further studies of this cytokine will no doubt be forthcoming.

Long-term cultures of human lymphoblasts have been shown to produce lymphokines. When these were purified and administered to tumor-bearing animals prolonged survival was produced (129). In the same category are Lymphocyte Activating Factor, Interleukin-1 and T cell Growth Factor, Interleukin-2, which are currently of much interest because they can theoretically be used directly *in vivo* as cytokines or for the *in vitro* preparation of adoptive immunotherapeutic cellular reagents and because Interleukin-2 seems to correct an immune defect in the T cells of cancer patients. Preliminary studies of Interleukin-2 administered to cancer patients failed to reveal any therapeutic effect and to have mild toxic side effects (112). In a more recent study using high dose Interleukin-2 several patients showed some response (140).

ADOPTIVE IMMUNOTHERAPY

Transfer factor (108) is a component of lysed leucocytes from sensitized animals that transfers delayed hypersensitivity type immunity to another animal. It has been shown to stimulate lymphoblast formation (5), is not RNAse sensitive and has the theoretical possibility of use in cancer im-

munotherapy. It has been reported to improve survival when given to melanoma patients (20, 59).

RNA from lymphoid tissue of immunized animals has also been shown to be capable of transferring immunity *in vitro* to lymphocytes of syngenic or xenogenic individuals (55, 115). Three studies of human renal cell carcinoma patients treated with immune RNA or autologous lymphocytes incubated with immune xenogenic RNA showed prolonged survival over controls (43, 139, 158). In animal studies the transfer of tumor immunity with xenogenic immune RNA plus RNAse inhibitor has been described (179). However, studies done in humans have been inconclusive.

Another form of adoptive immunotherapy that appears especially promising is the preparation of cytotoxic T cell clones *in vitro* using T cell growth factor (Interleukin-2) (57, 58, 137). As autologous cells with cytotoxic activity they could be administered repeatedly to the cancer patient. Preparation of lymphocyte activated killer cells (LAK) by *in vitro* treatment of patients lymphocytes with the lymphokine Interleukin-2 (IL12) has been reported (140). It has been found that administration of *in vitro* treated lymphocytes along with the IL-2 to patients has resulted in a few marked tumor regressions with complete disappearance of some tumors.

HORMONES

A number of hormones isolated from the thymus have a modulating (stimulating under some circumstances and inhibiting under others) effect on the immune system (148). Studies on tumor-bearing animals have demonstrated the favorable effect of thymic factors on survival time (95). Although few studies of the effect of these agents on human cancer have been performed, one encouraging result has been reported on oat cell carcinoma of the lung (35). Because these are normal constituents and therefore should be nontoxic they present, like interferon, a model of the ideal therapeutic agent. As immunomodulating agents the thymic hormones can probably only be used in those cases where stimulation of the effector cells is required since a depressing effect may result if a normal immune system is present. It may require that existence of a T cell deficiency be established before these agents are used.

REMOVAL OF IMMUNE SYSTEM INHIBITORS

Another approach to activating the immune system is by removal of substances that depress or inhibit its antitumor activity. A number of factors have been described which act in this fashion including immune regulating globulin (10), suppressor cell activating factor (21), prostaglandins (61) and antitumor antibody (68). Removal of these inhibitors has been attempted using plasmapheresis and immunoabsorption by Staphylococcus Protein A or other immunoabsorbants in a continuous flow system. Plasmapheresis has been shown to have an anticancer effect in human (74, 99). The therapeutic effect on breast cancer patients seemed to correlate with the presence of mitogenic activity in their plasma following perfusion of the plasma over *Staphylococcus* Protein A containing columns (16). In animal studies immunoabsorption was found to have a cancer therapeutic effect (88, 166).

Recently it has been observed that a physical agent (radiation) may have a selectively destructive effect on suppressor T cells and thus have an antitumor effect indirectly by its action on one component of the immune system (124).

ACTIVE IMMUNOTHERAPY

The original concept of immunotherapy of cancer was the immunization of an individual with a tumor specific antigen or with killed but still antigenic cancer cells. A number of studies in animals have demonstrated the effectiveness of this form of treatment. For the most part intact syngenic cancer cells have been used, often in combination with chemotherapy, surgery or microbial agents (6, 81, 106). The tumor cells have been made nonviable by several treatments that prevent their growth but presumably maintain their structural and antigenic integrity. The most popular treatments have been X-irradiation, or neuraminidase. Mitomycin C has also been used (188). In some instances extracts of the tumor have been found effective (61, 89, 90), but this has been the exception. Potentiation of these "vaccine" preparations by chemotherapeutic agents has been observed and thought to be due to the elimination of immune suppressors (91, 92). Incorporation of cholesteryl hemisuccinate into the tumor cell membrane has been reported to increase the antigenicity of the tumor associated antigens (153).

The demonstration that this technique is effective with some animal tumors would suggest that it might also be useful in humans. Certain logistic problems are associated with this approach in humans. It is difficult to obtain and retain enough autologous human cancer tissue for repeated injections. For any particular tumor it is not known whether the antigens are specific for that tumor or are specific for the histologic tumor type, which would permit the use of allogenic tumors. In those cases where a common tumor specific antigen is thought to exist, as in the case of melanoma, viable allogenic cells from one patient have been used as a vaccine in another patient (110). One animal study would suggest that in colon cancer the antigens may be both tumor specific and embryonic cell specific (151). Tissue culture tumors, which have the advantages of uniformity, are perhaps comprised of a single cell type and are available in quantity have in addition many disadvantages related to the potential presence of extraneous and nontumor antigens. In spite of these difficulties, a few studies have been done, particularly with acute leukemia patients using irradiated and neuraminidase treated allogenic tumor cells, often in combination with BCG or complete Freund's adjuvant. This has usually been done during remission induced by chemotherapy. Some prolongation of remission duration and increased survival time has been reported (13, 173, 182). Other types of human cancer have also been studied for the effect of active immunotherapy, again with some evidence of success (28). Regression of lung metastases from renal carcinoma have also been reported using active immunotherapy (170). The immunodepressing effect of chemotherapy may impose a limitation on the use of this method.

PASSIVE IMMUNOTHERAPY

Although the immune defense against cancer is presumably cellular in nature antibodies may play an important function in tumor specific antigen recognition and modulation of the cellular elements. The immunotherapeutic effect of serum antibodies in animal model systems has demonstrated their importance (119). Patients with a number of types of cancer have been treated with serum from patients who have recovered from cancer or their relatives. There has been some evidence of tumor regression in these cases (186).

With the development of cloned human B cells and the production of specific monoclonal antibodies the development of high titer antisera against a variety of tumor specific antigens seemed possible permitting more complete exploration of the potential for this aspect of immunotherapy. In the case of murine virus induced tumors the therapeutic effect of this method has already been demonstrated (15, 94, 145). Monoclonal antibodies to human carcinomas have been shown to specifically inhibit growth of the tumor in nude mice (72) and in humans (120).

A basic concept of present-day biology is that the biochemical components of an individual's cells are under genetic control. Thus, the presence of tumor specific antigens implies the existence of activated tumor specific antigen genes. The use of monoclonal antibodies along with molecular biologic techniques have demonstrated this relationship and the therapeutic efficacy of the specific monoclonal antibody *in vivo* and *in vitro* (44). Of interest was the observation that the antibody did not kill the cells but altered them in such a way that one of the *in vitro* malignant characteristics was abolished, suggesting that this form of therapy might prevent tumor growth without destroying the tumor. It also has implications for factors controlling differentiation. Generally speaking, however, although the monoclonal antibody technique has been available for a decade and has contributed to much information about antigens in general and to the diagnostic acumen of cancer, there has been no breakthrough in the area of cancer therapy as yet resulting from this technology. This seems in part the result of tumor specific antigens being less easily identifiable than had been expected and because to be useful such antigens need to be surface or membrane antigens.

Taking another tack, even if the specific antiserum does not have a cytotoxic effect on the cancer cells it does provide a vehicle for delivery of radioisotopes or chemotherapeutic agents directly and in general exclusively to the tumor (4, 45, 107). Antibodies to ferritin and to carcinoembryonic antigen have been shown to localize in and destroy human neoplasms when labeled with I 131 (128). Another demonstration of the potential efficiency of this system has been done with monoclonal antibody to Thyl 1.1 coupled with a ribosome inactivating agent (Gelonin) (168).

In addition to the passive transfer of antibodies the transfer of immune cells has been considered. When peripheral blood lymphocytes (PBL) from melanoma patients were administered to nude mice bearing human melanomas the survival time of the animals was prolonged compared with that of mice treated with PBL from normal individuals (2). Similarly, the use of thoracic duct lymphocytes shunted to brain tumors has been suggested (85). In animal studies, the passive transfer of immune syngenic lymphocytes in combination with chemotherapy has effectively eradicated leukemia (63).

IMMUNOPREVENTION

Immunization as a means of cancer prevention has had little reason to be considered until a viral etiologic agent or tumor specific antigen was identified. Efforts to isolate antigens of this sort will continue and as illustrated in the case of viral induced tumors in animals, cancer prevention by immunization with the causative virus is possible (97).

GENERAL SUPPORTIVE METHODS

The optimal functioning of the immune system requires a certain enviroment *in vivo*. One of the important elements in this environment is the nutritional status of the individual. Anorexia and cachexia have long been associated with malignancy, but their importance for the normal function of the immune system has only recently been appreciated (141). Hyperalimentation has been shown to restore to normal the immunologic defects associated with malignancy (103), and the importance of zinc in the optimal function of the immune system has been emphasized as a part of the nutritional requirement (146). It has been known since 1965 that Vitamin A prevented carcinogen induced tumors in animals (36). Recent evidence indicates that it also affects the normal function of the immune system (164) and has been shown to have a synergistic effect on *C. parvum* immunotherapy of a mouse tumor (131).

PROSPECTS FOR IMMUNOTHERAPY

It is evident from the foregoing that both animal and clinical studies have established that tumor associated antigens do exist and that immunotherapy of cancer is possible. At the same time it has become clear that the immune system is complex. There are a number of cells that perform different functions, are stimulated or suppressed by different substances and are more or less important in tumor immunity. It is also clear that tumor antigens may be individually tumor specific. They may not all be cell surface antigens. There may be different populations of cells in any one tumor with different surface antigens. The antigens may change with time in the same tumor, perhaps by selection as the host's immune system or therapy destroy the more antigenic cells (191). The existence of tumor antigens and the successful immunotherapy of some tumors emphasize the importance of identifying and understanding these variables and the importance of carefully controlled studies.

Further effort with the augmenting agents will involve fractionation and purification in order to identify the active component, to concentrate it and to make its effects more reproducible and less toxic. No doubt, augmenting agents will be employed especially as adjuncts to other immunologic methods. Interferon and the thymic hormones hold a special promise especially as the status of the immune system is more definitively evaluated and their administration can be adjusted to optimize their effect. Studies of removing

immune system inhibitors are just beginning and it is premature to predict their impact on immunotherapy. However, the concept explains many of the questions that exist related to failure of the immune system in the control of cancer, and for that reason it is a promising approach. One might expect it would be especially valuable in combination with immunostimulatory modalities. Regardless of what immunotherapeutic techniques are used the addition of hyperalimentation to the regime would seem desirable.

The recent availability of monoclonal antibody techniques will have a particularly significant effect on cancer immunotherapy. It will permit selection from the antibodies formed of those that are tumor specific. It will also make available these specific antibodies for passive immunotherapy including delivery of radioisotopes or chemotherapeutic agents directly to the cancer cell with precise localization. The same monoclonal antibody delivery system could be used to deliver the therapeutic agents to a tumor where an exogenous antigen such as clostridium vegetative organisms were exclusively localized (114). There is much enthusiasm for the adoptive immunotherapeutic use of Interleukin-2. Certainly the most promising area at present does seem to be in the realm of the cytokines and killer cells.

The overall outlook for immunotherapy of cancer is a very promising one. It has not, as yet, proved to be an unqualified success but the fact that therapeutic effects have been achieved is most encouraging. Unfortunately, success in cancer therapy is often measured in months of remission rather than cure rate. There is a general consensus that cancer cure by immunotherapy will require treatment of the disease at an early stage when minimal tumor is present and/or by a combination of immunotherapy with surgery or chemotherapy. There is also reluctance to employ it alone on early cases so there will, no doubt, be more studies of early cases with combined treatment; all of which emphasizes the continued importance of early diagnosis of cancer regardless of the treatment offered. The only change in the forseeable future that might alter the need for early diagnosis would be the development of successful immunoprevention of cancer, and for that we must look to the combined efforts of molecular biology, virology and immunology.

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INDEX

POSTSCRIPT TO CHAPTER 2. BREAST CANCER DIAGNOSTIC IMAGING BY R. H. MATALLANA

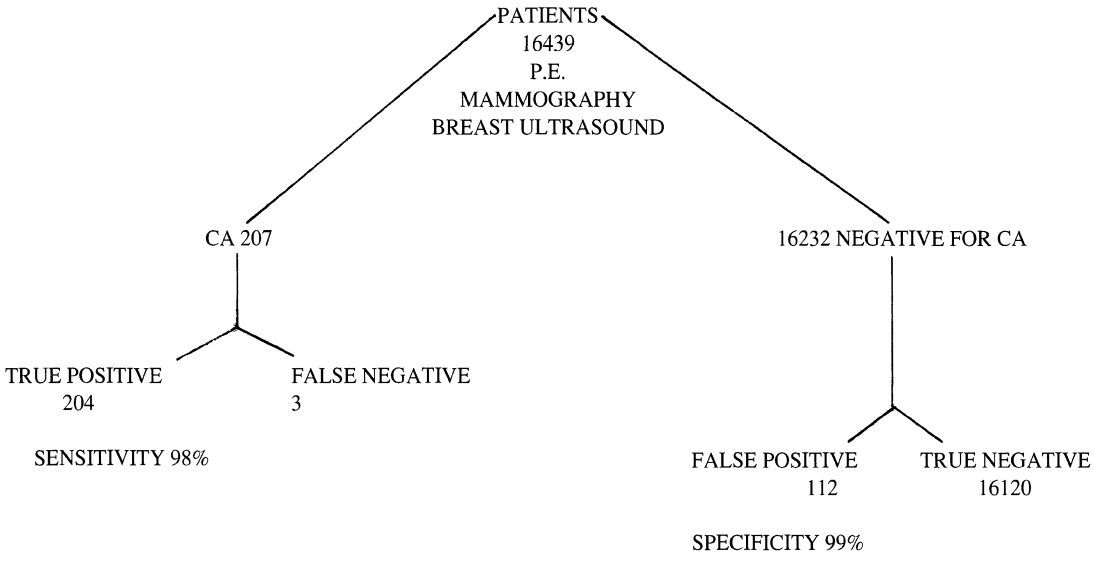
A comprehensive diagnostic approach to breast cancer was reported by Matallana. Between November 4, 1984 and September 1, 1988 a total of 16.439 patients were evaluated by clinical examination, state-of-the-art mammography, and, when indicated, complementary ultrasound mammography. As a result of this comprehensive diagnostic approach 547 patients underwent biopsy and 229 carcinomas were detected in 207 patients, age 29 to 91 years.

Our yield of malignanacies were one carcinoma every 2.6 biopsies; 114 or 62% nonpalpable lesions were identified with mammography and ultrasound. With mammography alone 66 or 36% were diagnosed; 3 or 1.6% were identified by ultrasound only; 87 or 47% subclinical carcinomas were identified because of macrocalcifications. The sizes of 64 or 86% subclinical masses were 1 cm or smaller. Of 183 subclinical carcinomas, 179 had preoperative localization as follows: under mammographic guidance 112; under ultrasonic guidance 67; no information 2; no preoperative localization 2. Axillary lymph node metastases occurred in 22 or 10%. Nonpalpable lesions of 22 or 10% were related to a diameter of: 0.5, 0.8, 1 (2), 1.2, 1.4, 1.5 (3) cm; 13 were palpable masses of: 1 (2), 1.1, 1.4 (2), 2, 2.5 (3), 3.5 (2), 4 (2). No axillary metastases were reported in the noninvasive group. The sensitivity was 98.5%, the specificity 99%, the positive predictive value 65%, the negative predictive value 99%, the prevalence 1.2%, the accuracy 99%. (Table 1).

POSTSCRIPT REFERENCE

Mattallana RH: Comprehensive diagnostic approach to breast cancer. Vth International Congress of Senology, Buenos Aires, Argentina, 1988.

Table 1. Comprehensive breast diagnosis



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