INTERFERON AND INTERFERON INDUCERS IN THE TREATMENT OF CANCER

experimental studies in mice, rats and humans

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THESIS

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born in Amsterdam

INTERFERON AND INTERFERON INDUCERS IN THE TREATMENT OF CANCER
experimental studies in mice, rats and humans

INTERFERON EN INTERFERON INDUCERS IN DE BEHANDELING VAN KANKER
experimentele studies in muizen, ratten en mensen

PROEFSCHRIFT

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To my parents
To Carola

CONTENTS

PREFACE

The most essential task of the immune system is the discrimination between "self" and "non-self" and the generation of an immune response to rid the organism of "non-self" elements, thus protecting and preserving its integrity.

Malignant transformation of normal cells may be accompanied by phenotypic changes in the involved cells. Normal cell surface antigens may be lost, new antigens may be gained or other membrane changes may occur which influence cell-to-cell interactions in the host. These changes may evoke an immune response which is a highly complex process involving the intricate interactions of lymphocytes, monocytes, cytokines, lymphokines, chemotaxis, antibodies, the complement system and many other factors so far poorly understood or unidentified.

The discovery of interferon, interferon inducers and various other agents that can augment immune responses has focused the attention on the beneficial role that manipulation of the immune response may play in the treatment of cancer.

Developments of new technologies often pave the way for key advances and new concepts in science. Tumor biology has entered a new era as a result of the advent of monoclonal antibody hybridoma techniques and DNA recombinant genetic engeneering technology. It has led to the elucidation of the concept of oncogenes, the further unraveling of the immune response and an ever increasing ability to manipulate it. This has been realized in particular by the advent of procedures that provide large quantities of various highly purified recombinant interferons, lymphokines and various other factors that play a key role in the orchestration of the immune response. It has led to the identification of the various subsets of lymphocytes and monocytes that take part in the antitumor immune response; it has revolutionized the serologic analysis of tumor-associated antigens and it has created favorable conditions for new and more refined approaches in the treatment of cancer.

This thesis will focus on the antitumor and immunomodulative activity of the interferon-inducing compound ABPP (2-amino-5-bromo-6-phenyl-4(3H)-pyrimidinone), the conditions necessary for optimal antitumor activity, the efficacy of using it in combination with lymphokines (Interferon-gamma, Interleukin-2), cytokines (Tumor Necrosis Factor) or chemotherapy (cyclophosphamide) in experimental studies with rats and mice, and finally on the use of recombinant Interferon-alphaA in the treatment of metastatic colorectal cancer in humans.

CHAPTER I

AIM AND OUTLINE OF THE THESIS

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PART I : EXPERIMENTAL STUDIES WITH THE INTERFERON-INDUCER ABPP IN RATS AND MICE

Biological response modifiers are agents that exert their antitumor effects mainly or exclusively through modulation of host immune defense mechanisms. A common feature of these agents is their capacity to induce the production of endogenous interferons and/or other lymphokines in vivo, and their capacity to activate various subsets of lymphocytes and monocytes that can lyse tumor cells in vitro and in vivo. Antiproliferative and immunomodulative effects as well as toxicity and side-effects observed in connection with the administration of these agents may differ greatly. Relatively recently the 6-aryl pyrimidinones, a series of small molecule biological response modifiers, were discovered. It was found that these agents varied greatly with respect to antiviral, antiproliferative and immunomodulative activity as well as with respect to the toxic side-effects.
Because of its effective induction of endogenous interferon, its significant immunostimulatory effects on NK cells and its low toxicity, ABPP (2-amino-5-bromo-6-phenyl-4-(3H)-pyrimidinone) appeared to be one of the most interesting and promising pyrimidinones. Most of the initial literature concerning the biological effects of ABPP dealt with NK-cell activity, assessed in vitro by ^{51}chromium release cytotoxicity assays with NK-cell sensitive target cells, and in vivo antitumor activity against non-established tumor (tumor cells in the blood born or early lodging phase). Both assessments of potential antitumor efficacy should be interpreted with great caution. It is known that most cultured tumor cell-lines are NK-sensitive and that in vitro cytotoxic activity may correspond poorly with in vivo antitumor effects, whereas fresh tumor cell preparations are usually NK-resistant and the capacity of activated killer cells to lyse these targets (LAK cell activity) corresponds well with antitumor activity of these cells in vivo. The evaluation of in vivo antitumor effects should not be based on results obtained with "non-established" (day 0-1 after inoculation of the tumor) metastatic tumor only. Established metastatic tumor (day 3 or later after inoculation of

the tumor) is quite resistant to NK-cell mediated killing and corresponds much better with the relevant clinical situation where an effective adjuvant treatment regimen in the presence of micrometastases at the time of removal of the primary tumor determines the ultimate outcome of treatment.
Because of the arguments presented above we studied : (A) the capacity of ABPP to generate LAK cell activity in vivo; (B) the importance of tumor load (nonestablished, early established and advanced tumor) and tumor site on the outcome of treatment with ABPP; (C) ways to augment immunotherapeutic effects with ABPP by combining ABPP with other biologicals (Interferon-gamma, Interleukin-2, Tumor Necrosis Factor) and with (D) cyclophosphamide in a search for treatment schedules that may hold promise in an adjuvant setting.
An outline of the approach is presented hereafter:

I(A) Immunomodulative effects of ABPP

Biological response modifiers are agents that exert their antitumor effects mainly or exclusively through modulation of host immune defense mechanisms. Cytolysis in vivo is mediated by various subsets of lymphocytes. Natural killer (NK) cells, lymphokine activated killer (LAK) cells and macrophages are believed to be of prime importance. We investigated the effects of the administration of ABPP in vivo on the NK cell activity and LAK cell activity in the peripheral blood lymphocytes, splenocytes, and peritoneal exudate cells by in vitro assessment of the levels of cytotoxicity of these lymphocytes in ^{51}Chromium release assays against various tumor cell targets. The activation of monocytes was assessed by determination of the level of phagocytosis displayed by peritoneal exudate cells after administration of ABPP.

I(B) In vivo antitumor effects of ABPP : general nature, role of tumor load and tumor site.

The in vivo antitumor effects of ABPP were studied in rats and mice. The general nature of the antitumor effects of ABPP was assessed by using a variety of tumors, differing in immunogenicity and histology, in local, local-regional and systemic tumor models in different animal species and strains. Significant antitumor effects of biological response modifiers are usually seen

only when the tumor load is small. By varying the time interval between tumor inoculation and the administration of ABPP, the antitumor effects of ABPP on small or advanced local tumor, and on nonestablished (day 0), early established (day 3) or advanced (day 7-10) metastatic tumor were investigated.

Host defense mechanisms may differ qualitatively as well as quantitatively in different body compartments and organs. The outcome of immunotherapy may therefore be related to the tumor site. The immunological "make-up" of the blood with its great variety of immune competent cells; the lungs with organ-associated NK cells and alveolar macrophages; the liver with NK-cells and Kupffer cells; and the subcutis virtually without a permanent associated population of NK cells or macrophages, differs greatly and may well determine the efficacy of immunotherapeutic agents. These aspects were evaluated by studying the effects of ABPP in local tumor models (subcutaneous, subrenal capsule assay), a local-regional model (intraperitoneal), and metastatic tumor models in lung and liver.

I(C) Potentiation of antitumor effects by combining ABPP with other biological response modifiers

Since the antitumor effects of Interferon-gamma (IFN-gamma), Interleukin-2 (IL-2) and Tumor Necrosis Factor (TNF) are mediated through at least in part different mechanisms, we investigated whether a potentiation of the antitumor effects of ABPP could be obtained by various combined treatment schedules.

I(D) Potentiation of antitumor effects by combining ABPP with cyclophosphamide in various treatment schedules

The combination of various treatment modalities may be more effective as single agent therapy as a result of the different mechanisms involved in the destruction of tumor cells. In this setting, synergistic antitumor effects may permit dose reductions of the constituents of combined treatment schedules which may result in the reduction of the toxicity of the treatment regimen. Multiple treatment cycles may therefore be tolerated and result in an effective control of tumor growth. Cyclophosphamide was chosen as the cytostatic agent to assess the hypothesis put forward above.

PART II : PHASE II STUDY WITH RECOMBINANT INTERFERON-alphaA (rIFN-alphaA) IN PATIENTS WITH METASTATIC COLORECTAL CANCER

With the advent of recombinant interferon-alpha many trials were started to evaluate the potential role of IFN-alpha in the treatment of cancer. The antitumor and immunomodulative effects of two different treatment schedules with rIFN-alphaA and their associated toxicity were evaluated in 20 patients with metastatic colorectal cancer.

II(A) Antitumor effects

Antitumor effects of rIFN-alphaA treatment were assessed by measuring metastatic lesions with CT-scan and ultrasound before, during and after treatment at 6 week intervals.

II(B) Toxicity

Toxicity of the two different treatment schedules was assessed by registration of the complaints of the patients on a weekly basis, by physical examinations and by extensive and frequent heamatological and biochemical laboratory blood tests.

II(C) Immunomodulation

Immunomodulative properties of the two treatment schedules with rIFN-alphaA were investigated by assessment of:

1. NK-cell activity of the peripheral blood lymphocytes before and at various points in time during treatment.
2. Blastogenesis of peripheral blood lymphocytes after stimulation with phytohaemagglutinin (PHA) before and during treatment.
3. Influence of treatment on the leukocyte adherence inhibition (LAI) assay with peripheral blood lymphocytes of the patients.

CHAPTER II

THE INTERFERON SYSTEM

II(1) GENERAL INTRODUCTION
II(2) BIOLOGICAL ACTIONS OF INTERFERONS
II(3) INTERFERON INDUCERS

II(1) GENERAL INTRODUCTION

Introduction

Identification of immunobiologically active cytokines and advances in DNA recombinant technology have increased the number of biological response modifiers (BRMs) that are potential candidates for immunotherapy of cancer in man. These agents exert their antitumor effect exclusively or mainly by modifying host immune responses and not by a direct effect on cancer cells. A better understanding of host responses to cancer cells and the explosive increase in ways to manipulate these responses have caused a renewed interest in tumor biology and immunotherapy. **Active specific immunotherapy**, an approach that attempts to induce specific immunity against one's own tumor by a variety of immunization procedures, has met with very little success sofar mainly because of the poor immunogenicity of most human tumors. **Active nonspecific immunotherapy**, an approach that attempts to augment the aspecific antitumor response mediated in particular by large granular lymphocytes (LGLs) and macrophages, has been more successful. One specific area involving this aspecific augmentation of host immune responses is the interferon system.

Definition

Interferons (IFNs) are a family of inducible secretory glycoproteins produced both in vitro and in vivo by virtually any eukariotic cell in response to a variety of stimuli (Ho and Amstrong, 1975). They are intercellular messengers with a major, but not absolute species specificity. Upon binding with specific cell receptors (Revel et al., 1976; Branca and Baglioni, 1981) they can direct cells to alter the expression of some of their specialized functions and bring about an IFN-induced phenotype.

History

Isaacs and Lindenmann discovered interferon in England in 1957. They demonstrated that the incubation of pieces of chicken chorioallantoic membrane with heat inactivated influenza virus induced the production of a factor by these cells that would subsequently, when added to a culture of live virus with fresh membranes, inhibit ("interfere with") the replication of the

virus. This factor was therefore called interferon. When it became clear that interferon did not only have antiviral activity but could inhibit the proliferation of various cells, especially tumor cells, great interest was raised in its potential role in the treatment of cancer. In the early 1970's Cantells' work at the Finnish blood bank led to the production of sufficient quantities of interferon-alpha (IFN-alpha) from buffy cell layers to carry out small clinical trials like the osteosarcoma trial by Strander and coworkers (1980). The development of recombinant DNA technology and the successful cloning of IFN-beta in 1979 by Taniguchi and the subsequent cloning of the IFN-alpha subtypes and IFN-gamma broadened and accelerated IFN research and created the opportunity to perform various clinical trials. One of these trials, a phase II study in patients with disseminated colorectal cancer, will be presented as a part of this thesis.

Interferon species

The IFNs are classified according to differences in antigenic, biologic and chemical properties. The three distinct antigenic types of IFN are designed alpha, beta, and gamma.

TABLE I : CLASSIFICATION AND CHARACTERISTICS OF HUMAN INTERFERONS

Type	Source	# Subspecies	Molecular Weight	pH Stability	[1]Glycosylation
IFN-alpha (Type I)	B-cell Null-cell Macrophage Recombinant	$\geq$ 15	16,000 - 25,000	+	-
IFN-beta (Type I)	Fibroblast Lymphoblast Null-cell Epithelial cell Recombinant	1	20,000	+	+
IFN-gamma (Type II)	T-lymphocytes Null-cell Recombinant	1-2	17,000	-	+

[1]refers to natural interferon; recombinant interferons are not glycoselated

The IFNs alpha and beta (also called Type I IFNs) are stable at low pH, whereas treatment of IFN-gamma (also called Type II IFN) at pH 2 neutralizes its activity. Type I IFN can be induced in cells of diverse origin, although those used in clinical trials have been induced in leukocytes or lymphoblastoid cell lines (IFN-alpha) or in fibroblasts (IFN-beta) by viruses or synthetic poly-ribonucleotides. IFN-gamma is produced by T-lymphocytes after induction by a variety of specific mitogens or foreign antigens. The classification and the main properties of the human interferons are summarized in Table I.

Induction and Production of IFNs

Production of IFN requires the interaction of an IFN inducer and a cell sensitive to it. This reaction triggers the derepression of genetic information that leads to the transscription of IFN gene(s) and the appearance of IFN-mRNA, its translation into protein and secretion of the IFN glycoprotein (Burke, 1965; Vilcek et al., 1969). The kinetics of IFN induction and release vary with different inducer-cell systems. IFN appears extracellulary within 4-12 hours, with rapid attainment of maximum levels for about 12 hours and then decreasing abruptly to return to undetectable amounts. Induction of IFN-alpha/beta is in general a more rapid process than the induction of IFN-gamma. Evidence exists that IFN biosynthesis results from a true inductive process, i.e. that it represents de novo synthesis (Stewart, 1979).

II(2) BIOLOGICAL ACTIONS OF INTERFERONS

Antiviral

The replication of a wide variety of viruses as well as microorganisms that multiply intracellularly is blocked in cells pretreated with IFN. Binding of IFN to its receptors triggers the induction of 2',5'-oligoadenylate synthetase which leads to endoribonuclease activation, which in turn inhibits RNA transcription by degrading mRNA linked to double stranded RNA (Revel et al., 1980). Thereby it inhibits DNA and RNA synthesis ands thus the replication of intracellular virus. In addition to the 2',5'-

oligoadenylate synthetase pathway, IFNs may lead to inhibition of protein synthesis by induction of a protein kinase and a phosphodiesterase pathway (Senn, 1984).

Antiproliferative

As has been discussed in the section on antiviral effects IFNs can affect nucleic acid and protein synthesis. These same mechanisms that lead to the antiviral state are probably in part responsible for the antiproliferative effect on multiplying cells. IFNs have been shown to inhibit proliferation of malignant cells preferentially or more effectively than the proliferation of normal cells (Fleischmann et al.,1984). This may be explained by the assumption that RNA's involved in an exponentially expanding process are turning over more rapidly than those involved in processes that have achieved a steady-state status (normal cellular processes) and that hence exponentially replicating tumor cells are much more vulnerable to the action of IFNs than normal cells. As IFNs inhibit the synthesis of various enzymes and other proteins a variety of mechanisms is probably involved in the direct antiproliferative effects of IFNs. For instance IFNs inhibit the synthesis of ornithine decarboxylase, an enzyme induced by mitogens, growth factors and tumor promoters and thus can counteract their effects (Sreevalsan et al, 1980). Some of the most important effects of IFNs on cell proliferation may be the result of their effects on the differentiation of cells, the expression of oncogenes and the expression of surface antigens. These mechanisms may have antiproliferative effects or lead to an increased immune response because of an increased expression of antigens as will be outlined in the following sections.

Differentiation and Antigen Expression

Interferons have been shown to have a variety of effects on cell differentiation (Burke, 1986). An important recent finding which may suggest one cellular mechanism for causing differentiation is the decreased c-myc and c-Ha-ras gene expression following IFN treatment. This effect on oncogene expression (Clemens , 1985) may be part of the antineoplastic action of IFN. The increase in cell surface antigen expression is most likely another aspect of IFN biological effects which could be important in

tumor control. This has been demonstrated for F_c-receptors on lymphocytes and for class I and II major histocompatibility complex antigens (Paulnock and Borden, 1985;Stewart and Blanchard , 1985) on lymphocytes and several other cell types. In addition to augmentation of preexisting antigen IFN has also been shown to induce HLA expression in a variety of normal cells (Rossi, 1985). In neoplastic cells IFNs have been shown to induce HLA expression and to enhance the expression of surface tumor-associated antigens (TAA) in several tumor cell lines (Giacomini et al.,1984 and 1985; Greiner et al., 1984; Carrel et al., 1985). If increased antigenicity occurs at a time when there is concurrent enhancement of macrophage-antigen-presenting functions, this could well improve endogenous antitumor activity and account for one of the many indirect antitumor effects of IFNs. Yet another possible antitumor effect of IFNs has been reported recently. Tsujimoto et al. (1986) have shown that IFN-alpha and -beta, but IFN-gamma in particular, can enhance the expression of cellular receptors for tumor necrosis factor (TNF) on tumor cells. Ruggiero an coworkers (1985) however showed that syngergism between IFN-gamma and TNF was also observed in cell lines in which TNF receptors were not significantly increased by pretreatment with IFN-gamma.

Immunomodulative

The immunomodulative properties of the IFNs may constitute one of the many mechanism by which they exert their antitumor effects. Support for this view is found in experiments in which tumors known to be IFN-resistant in vitro did regress in vivo when the animal received systemic treatment with IFN (De Maeyer-Guignard and De Maeyer, 1985). A variety of immune changes have been described. The literature is rather confusing due to the fact that timing and dose of treatment seem to be crucial as to what kind of effect is obtained. The most relevant effects appear to be those on NK cells and macrophages. In vitro work with human lymphocytes shows that IFN enhances the killing potential of NK cells both by recruitment of pre-NK cells and by increased cytotoxicity of activated cells (Herberman and Ortaldo, 1981). There is also evidence for augmentation of K cell - mediated antibody dependent cellular cytotoxicity (ADCC) (Borden et al., 1982). In vivo data from clinical studies are confusing in that

both enhancement of NK cell activity (Einhorn et al., 1978; Lucero et al., 1982) and depression (Maluish et al., 1983; Spinn et al., 1983; Tank et al., 1984) have been reported. Low dose IFN treatment results in marked enhancement of NK cell activity (Einhorn et al., 1982; Edwards et al., 1985) while high doses beyond a "threshold" may have a suppressive effect on NK cells. Results from our study indicated that the effect of IFN on NK cells was determined to a large extent by the treatment schedule. Macrophages rather than NK cells are affected by IFN-gamma as this interferon appears to be identical with the macrophage activating factor (MAF) (Vilcek et al., 1985). Enhancement of phagocytosis, antiviral and bactericidal activity as well as antitumor cytotoxicity is the result of IFN-gamma mediated macrophage activation (Steward and Blanchard, 1985; Paulnock and Borden, 1985). It appears that the antitumor effects of IFNs are based on an intricate and complex network of actions, any or all of which may be important and may depend on time dosage and type of IFN used.

II(3) INTERFERON INDUCERS

Introduction

Isaacs and Lindenmann used heat-inactivated influenza virus as interferon inducer during their classical experiments that led to the first description of interferon (1957). In addition to viruses and certain intracellular microbes a wide variety of different substances have been found to stimulate interferon production in in vitro and/or in vivo. A classification of IFN inducers, as proposed by Torrence and DeClercq in 1982 is shown in Table II. The diversity of structural classes involved, coupled with the fact that different types of IFN may be produced, precluded thus far the development of a unifying hypothesis regarding their mode of action. It is generally believed that structural similarities between DNA viruses and double stranded RNA's like Poly I:C may be responsible for the induction of mainly Type I IFNs through similar mechanisms. In the case of low molecular weight interferon inducers like substituted pyrimidines, to which ABPP belongs, interactions with nucleic acids may occur because of structural resemblance and these may be respons-

ible for induction of IFN. The differences in structure between double stranded RNA's and low molecular weight interferon inducers, as well as the differences between the various agents within this class (Table II, group III), are staggering. However some low molecular weight IFN inducers have been shown to interact with nucleic acids and it may well be that in some cases (metabolites of) these small molecule IFN inducers may modify the properties of the endogenous nucleic acids of an organism and convert these into interferon inducers (Torrence, 1982).

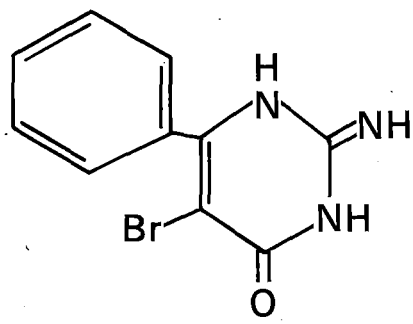
TABLE II : CLASSIFICATION OF INTERFERON INDUCERS

I.	**Viruses**
II.	**Polyanions**
	a. Nucleic acids
	b. Polyphosphates
	c. Polysulphates
	d. Polycarboxylates
III.	**Low Molecular Weight (LMW) Inducers**
	a. Tilorone and its Analogs
	b. Propaene Diamine Derivatives
	c. Pyrazolol Quinolines
	d. Acridine Derivatives
	e. Pyrimidines
	f. Mercaptoalkyl Amines
IV.	**Antibiotics**
V.	**Bacterial Products**
VI.	**Lymphocyte stimulating agents**
	a. Mitogens
	b. Specific antigens
	c. Mixed lymphocyte culture
	d. Antilymphocyte antibody
	e. Enzymes
VII.	**Microorganisms other than viruses**
	a. Bacteria
	b. Protozoa
	c. Mycoplasmas
	d. Chlamydiae
	e. Rickettsiae

Pyrimidinones

The 6-aryl pyrimidinones are a group of low molecular weight interferon inducers (Table II; group III) that can be readily synthesized by condensation of guanidine with b-ketoesters (Wierenga, 1980a). Introduction of a halogen at the 5' position is generally required for biological activity. These compounds are crystalline, have a high melting point, and they are poorly soluble in water at neutral pH. ABMP (2-amino-5-bromo-6-methyl-4(3H)-pyrimidinone) was originally identified as an active interferon inducing agent by Nichols et al. (1976). A direct relationship appeared to exist between the ability ABMP to induce interferon and mediate an antiviral state in vivo as mice were shown to be protected from the lethal consequences of infection with Semliki forest virus only by doses that induced interferon (Stringfellow, 1977). Because of ABMP's poor therapeutic index due to renal toxicity (Larsen et al., 1980) studies were initiated to identify effective analogs. Three compounds emerged as the least toxic and biologically most active. They are (1) ABPP : 2-amino-5-bromo-6-phenyl-4(3H)-pyrimidinone (shown in Figure 1); (2) AIPP : 2-amino-5-iodo-6-phenyl-4(3H)-pyrimidinone, the iodo-analog of ABPP; and (3) ABmFPP : 2-amino-5-bromo-6-m-fluoro-phenyl-4(3H)-pyrimidinone, the 6-m-fluoro-phenyl analog of ABPP (Tracey and Richard, 1986; Li et al., 1987). After some initial work with AIPP and ABPP we have restricted ourselves to experimental work with ABPP, which will constitute the main part of this thesis.

Figure 1 : Structure of ABPP



ABPP: 2-AMINO-5-BROMO-6-PHENYL-4(3H)-PYRIMIDINONE

TABLE III : IMMUNOMODULATING EFFECTS OF ABPP

Positive	(references)
- Induction of IFN Type I	(Stringfellow, 1977) (Hamilton et al.,1980; 1982)
- Induction of Interleukin-1	(Richard and Tracey, 1985)
- Induction of Interleukin-2	(Tracey et al., 1984)
- Activation of NK cells	(Lotzova et al., 1983; 1984)
- Generation of LAK cell activity	(Eggermont et al., 1987a)
- Activation of macrophages	(Li et al., 1985)
- Activation of B-cells	(Fast et al., 1982)
- Increase of antibody production	(Fast and Stringfellow, 1980)
- Increase of bone marrow colony forming units	(Taggart et al., 1980)
Negative	
- Decrease in T cell cytotoxicity in vitro	(Taggart et al., 1980)
- Decrease in T cell response to alloantigens	(Taggart et al., 1980)
- Decrease in delayed type hypersensitivity in vivo	(Pimm and Baldwin, 1985)

ABPP is a potent IFN Type I (mainly alpha) inducer in various animal species as well as in humans (Stringfellow, 1980; Earhart et al., 1985) and has strong antiviral activity in various animal species (Wierenga, 1980b). ABPP can be administered subcutaneously (s.c.), intraperitoneally (i.p.) or orally. The latter two ways of administration have the best regional and systemic biological effects. Many immunomodulating effects have been reported, most importantly the activation of NK cells (Lotzova et al., 1983; 1984), the generation of LAK cell activity in vivo (Eggermont et al., 1987a) and the activation of macrophages (Li et al., 1985). Activation of macrophages was shown to be important not only because of increasing cytotoxic activity of these cells but

mainly because of their role in the augmentation of NK-cell activity, probably via the induction or production of cytokines like IL-1 and possibly IL-2 (Tracey and Richard, 1986). Stimulatory effects of ABPP on B-cell funtions but inhibitory effects on some T-cell functions have been reported. The immunomodulating effects of ABPP are summarized in Table III.

We investigated the immunomodulating and the antitumor effects of ABPP alone and in combination with IFN-gamma, TNF, IL-2 and LAK cells and in combination with Cyclophosphamide in rats and mice. These experiments will be presented and their results discussed in the following 5 chapters of this thesis.

CHAPTER III

The content is an adapted version of the publication :

Site specific antitumor effects of 2 Pyrimidinone compounds in rats

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Site specific antitumor effects of 2 Pyrimidinone compounds in rats.

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Summary

Two pyrimidinones : 2-amino-5-iodo-6-phenyl-4(3H)-pyrimidinone (AIPP) and 2-amino-5-bromo-6-phenyl-4(3H)-pyrimidinone (ABPP) were tested for their antitumor effect on the spontaneous non-immunogenic, transplantable liposarcoma LS175 in BN rats. AIPP and ABPP are strong inducers of natural killer (NK) cell activity as was confirmed in the present communication. AIPP is known to be a weak, ABPP a strong interferon inducer. Tumor growth in the subcutaneous model was significantly ($p<0.003$) inhibited by ABPP but not by AIPP, whereas the development of lung metastases was significantly ($p<0.02$) inhibited by both compounds. Since LS175 has been shown by us in previous experiments to be sensitive to interferon, the difference between AIPP and ABPP in inhibition of subcutaneous tumor growth may be attributed to the interferon inducing properties of ABPP. NK cell activity, which is enhanced equally by both compounds, may be held responsible for the similar inhibition in the development of lung metastases after AIPP or ABPP.

INTRODUCTION

Recently a series of 5-halo-6-phenyl pyrimidinones has been found to induce interferon production in several animal species and in cultured human tissues (Stringfellow et al., 1980a;Stringfellow, 1980b). Moreover these compounds have been reported to modulate a variety of immune responses like the activation of NK cells (Lotzova et al., 1983; Taggart et al., 1980) and macrophages (Li et al., 1985). The pyrimidinones used in our experiments are 2-amino-5-iodo-6-phenyl-4(3H)-pyrimidinone (AIPP) and 2-amino-5-bromo-6-phenyl-4(3H)-pyrimidinone (ABPP). They differ greatly in their ability to induce interferon production in vitro and in vivo (Stringfellow et al., 1980). AIPP is a very weak, ABPP a very strong interferon inducer. Both are equally active in enhancing NK cell activity, which is considered an interferon independent quality of these agents (Loughman et al., 1980). In a previous communication (Marquet et al., 1983) we have shown that subcutaneous growth of liposarcoma LS175 in BN rats is inhibited

by interferon. NK cells are known to be most effective against circulating tumor cells (Hanna et al., 1980). To investigate whether the induction of interferon may play a part in the anti-tumor effects of AIPP and ABPP we compared the two agents in an assumed NK cell dependent (lung metastasis) model and in an assumed NK cell independent (subcutaneous) model using the interferon sensitive liposarcoma LS175 in BN rats.

MATERIALS AND METHODS

Rats

Male rats of the inbred BN strain were used. The animals were bred under specific-pathogen-free conditions and were 10-12 weeks old.

Tumor

LS 175 is a liposarcoma of spontaneous origin in a female BN rat. The tumor is readily transplantable in syngeneic hosts. It is nonimmunogenic as judged by the in vivo method of Prehn and Main (1957) and is NK resistant. When 2 mm cubes of tumor are implanted subcutaneously (s.c.) the tumor is readily palpable within 1 week. Tumor cell suspensions were prepared from s.c. tumor implants in RPMI-medium, enriched with 15% fetal calf serum (FCS). Tumor cells were isolated by mechanical disruption using a razor blade mounted on an overhead stirrer as described by Rein-hold (1965). Single cells were isolated from non-dispersed clumps by sieving through nylon gauze. After two washings the viability of the cells was assessed with trypan blue. This was usually 50%.

Experimental lung metastases : Lung metastases were created by injecting 1 x 10^5 tumor cells suspended in 0.5 ml RPMI in the tail vein. The number of lung colonies was counted after 14 days. The lungs were excised, and fixed in Bouin's solution. Tumor nodules on the lung surface were counted with the naked eye.

Experimental Agents

2-amino-5-iodo-6-phenyl-4(3H)-pyrimidinone (AIPP) and 2-amino-5-bromo-6-phenyl-4(3H)-pyrimidinone (ABPP) were made by published procedures (Wierenga et al., 1980) and provided by the Upjohn

Company, Kalamazoo, Michigan, USA. The agents were suspended in 0.5ml phosphate buffered saline (PBS) and injected intraperitoneally (i.p.) at a dose of 250 mg/kg.

^{51}Chromium release assay for Cytotoxicity of NK cells

Peripheral blood lymphocytes were prepared from heparinized blood samples. Mononuclear cells were seperated on a Ficoll - hypaque gradient and were used in a standard 3 hour ^{51}Cr-release NK cytotoxicity assay as described by Ortaldo et al. (1977). The target cell was Yac-1 and the cells were used at different effector to target ratios. All assays were performed in triplicate in a total volume of 0.2ml RPMI containing 10% FCS. After a 3 hr incubation the plates were centrifuged and supernatants were removed using the Titertek automatic harvesting system (Skatron, Norway) and counted in a LKB gamma counter. Specific lysis was evaluated at an effector to target cell ratio of 40:1. The amount of ^{51}Cr released from the target cells in the absence of effector cells was defined as spontaneous release.

Specific lysis was calculated as follows : % specific lysis = (mean exp. cpm - spont. cpm) / (mean max. cpm - spont. cpm) x 100

The maximum release was calculated by adding 10% Cetavlon (ICI, UK) to an aliquot of target cells.

Assessment of Macrophage activity

The effect of a single injection of ABPP i.p. at a dose of 250 mg/kg on macrophages was assessed by measuring the phagocytic activity of peritoneal exudate cells (PEC) indicated by the ingestion of latex particles at various time points after the administration of the drug.

Experimental Procedures

Subcutaneous tumor model : In the s.c. tumor model 2 mm cubes of tumor LS 175 were implanted s.c. in the left flank of BN rats. Tumor growth was assessed on day 4, 7 and 12 by measuring with calipers the two largest perpendicular diameters of the tumor. The average diameter was taken as the measure of tumor size.

Lung metastasis model : In the lung metastasis model 1 x 10^5 tumor cells were injected into the tail vein. On day 14 the rats were sacrificed and the number of lung colonies was counted.

The implantation of tumor cubes or the i.v. injection of tumor cells was preceded by i.p. injections of AIPP or ABPP at a dose of 250 mg/kg on day -3, -2 and -1. Controls were given a similar volume of phosphate buffered saline (PBS) i.p.

NK cell cytotoxicity : was measured on day 1, 3 and 9 after a single injection of AIPP or ABPP (250 mg/kg, i.p.). The controls received an equal volume of PBS i.p. In all experiments each group consisted of 5 animals.

Statistical Analysis

The difference in s.c. tumor growth or the number of lung metastases between the different groups was tested with the Student-t test.

RESULTS

Enhancement of NK cell activity

Figure 1 shows the NK-cell activity as measured on day 1, 3 and 9 after a single injection of 250 mg/kg of AIPP or ABPP i.p. Control animals recieved PBS i.p. A rapidly established and long lasting 3 fold increase of NK-cell cytotoxicity is seen after administration of either pyrimidinone.

Activation of macrophage activity

Macrophage activity was assessed by measuring the ingestion of latex particles by peritoneal exudate cells at various points in time after injection of ABPP i.p. Phagocytosis increased 4 fold (within 4 hours) and was elevated for 4 days (data not shown).

Effects on subcutaneous tumor growth

Figure 2 shows the response of tumor LS175 to administration of AIPP or ABPP in a dose of 250 mg/kg i.p. on day -3, -2 and -1. A significant ($p<0.003$) inhibition of tumor growth was observed in the ABPP treated group whereas no inhibition was seen after treatment with AIPP.

Figure 1 : Enhancement of NK-cell Activity by ABPP and AIPP

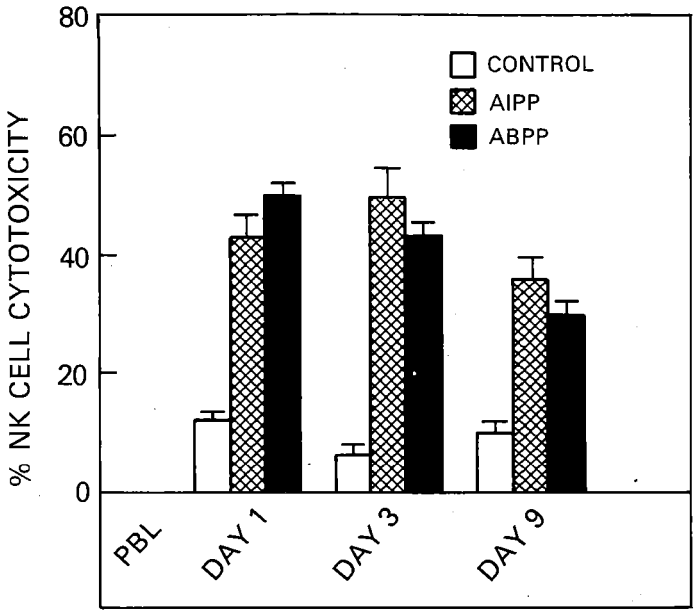

Figure 1 : Enhancement of NK cell activity in peripheral blood lymphocytes (PBL) after a single i.p. injection of 250 mg/kg of AIPP or ABPP. Control animals received PBS i.p. (N=3). The percentage of specific lysis is shown as determined in a 3 hr ^{51}Chr release assay at an effector:target (Yac-1) ratio of 40:1.

Figure 2 : Effect of AIPP and ABPP on Subcutaneous Tumor Growth

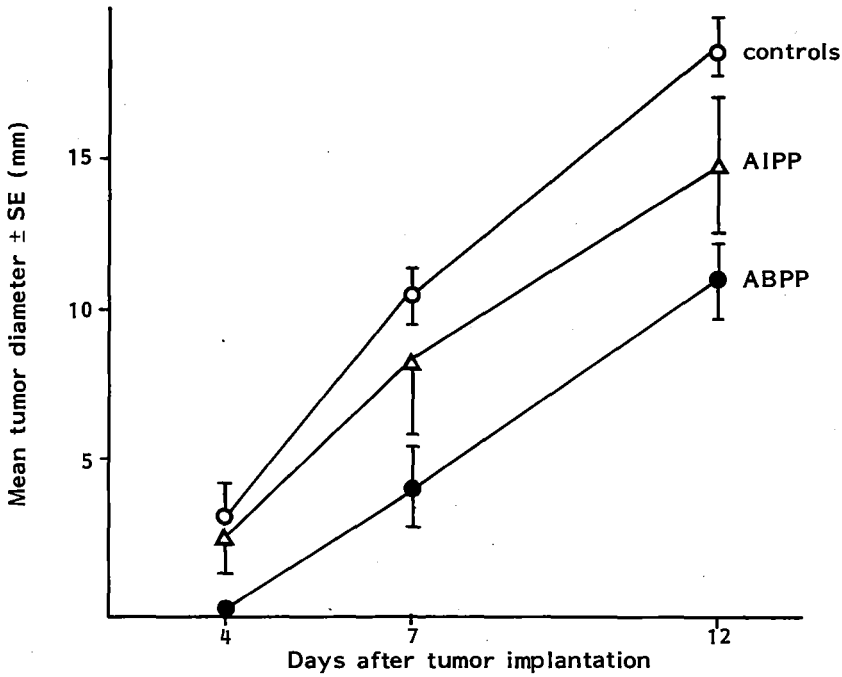

Figure 2 : Effect of treatment with AIPP and ABPP on growth of LS175. AIPP and ABPP were given at a dose of 250 mg/kg i.p. on day -3, -2 and -1. The difference between the diameter of the ABPP-treated group (●---●) and the control group (0---0) was statistically significant ($p<0.003$). There was no significant inhibition of tumor growth in the AIPP treated group (D---D). (N=5)

Effect on the development of lung metastases

Table I shows the effect of AIPP and ABPP, when given at a dose of 250 mg/kg i.p. on day -3, -2 and -1 , on the development of lung metastases. The lung colonies were counted on day 14. A significantly ($p < 0.02$) lower number of metastases was found in the rats pretreated with AIPP or ABPP. No difference existed between the two treated groups.

TABLE I : Effect of pretreatment with AIPP or ABPP on the Development of Lung Metastases

Pretreatment	Mean Number of Metastases ± SD	Range
PBS	39 ± 12	24-56
AIPP	18 ± 7	10-23
ABPP	13 ± 3	10-16

Effect of administration on day -3, -2 and -1 of 250 mg/kg of AIPP or ABPP i.p. on the development of lung metastases when counted on day 14 after injection of 1 x 10^5 cells of LS175 i.v.. A significantly ($p<0.02$) lower number of metastases were found after either AIPP or ABPP treatment.(N=5)

DISCUSSION

The inhibitory effect of both pyrimidinones on the development of lung metastases is in agreement with the results reported by Milas et al. (1982) concerning immunogenic as well as nonimmunogenic murine tumors. The activity of NK cells and macrophages at the time of i.v. tumor inoculation was strongly enhanced as a result of the pretreatment with either ABPP or AIPP. The activation of these two cell populations may well explain the results in the lung metastasis model. Hanna et al. (1980) have shown that tumor cells are most sensitive to NK cells when they are in a blood born or early lodging phase. The fact that tumor LS175 is NK resistant *in vitro* does not rule out an important role for activated NK cells *in vivo* to account for the lower number of lung metastases in the pyrimidinone treated groups : (i) *In vitro* NK resistant tumor cells may be less resistant *in vivo* to highly activated NK cells (Talmadge et al., 1984) ; (ii) Very high NK cell activity can be induced in the lungs of rats by pyrimidinones (Lotzova et al., 1984) ; (iii) activated NK cells have been shown to produce a variety of lymphokines like IFN-gamma and IL-2 (Kasahara et al., 1983). (iv) secretion of IFN-gamma by the organ associated NK cells (Wiltrout et al., 1985) or of a putative more

rapidly working macrophage activating factor (MAF) secreted by NK cells in the rat and in humans (Gomez et al., 1985) may lead to a highly effective in situ activation of alveolar macrophages.
The tumor-site-specific setting of a high concentration of macrophages, directly as well as indirectly activated by the pyrimidinones, could well explain the observed inhibition of the development of lung metastases.
The difference in tumor response to ABPP and AIPP in the s.c. tumor model may be due to the difference in IFN serum levels after the administration of these two agents. When we observe the growth curve of the tumors in the ABPP-treated group the tumor appears after 6-7 days. This is the only antitumor effect that can be observed since tumor growth follows a parallel course to the other experimental groups when the tumor has become measurable. Apparently tumor suppression is only short lived and no prolonged inhibitory effect is seen. This could be explained by the fact that high levels of IFN are induced by ABPP for a period of only 24 hours (Oku et al., 1984 ; Lotzova et al., 1984). Since LS175 has been shown to be IFN sensitive and IFN can have a direct inhibitory effect on tumor growth, high levels of interferon at the time of the s.c. tumor inoculation may have created the lag-time in tumor growth that is observed in the animals pretreated with ABPP. Since multiple injections with ABPP induce a hyporeactive state (Oku et al., 1984) as is observed with many biological response modifiers (Talmadge et al., 1985), the antitumor effect in the s.c tumor model offers little pros-pect for an effective role for ABPP in this setting.
The antimetastatic effect of these pyrimidinones is the more important observation, especially since the tumor was NK resistant. Interactions between activated NK cells and macrophages, both of which are present in high numbers in the lungs, may have overcome this resistance and may have brought about the effective lysis of tumor cells by either cell population. Since the administration of pyrimidinones is virtually non toxic (Earhart et al., 1985) these agents may hold some promise in boosting anti-metastatic mechanisms in a perioperative or otherwise adjuvant setting.

CHAPTER IV

The content is an adapted version of the publication :

Effects of the interferon-inducer ABPP on colon cancer in rats : importance of tumor load and tumor site.

Alexander M. M. Eggermont, Richard L. Marquet, Ron W. F. de Bruin and Johannes Jeekel.

From the Laboratory for Experimental Surgery, Erasmus University, Rotterdam, the Netherlands

Cancer Immunology Immunotherapy 22:217-220, 1986

Effects of the interferon-inducer ABPP on colon cancer in rats : importance of tumor load and tumor site.

Alexander M. M. Eggermont, Richard L. Marquet, Ron W. F. de Bruin, Johannes Jeekel

Summary

ABPP (2-amino-5-bromo-6-phenyl-4(3H)-pyrimidinone) is a pyrimidinone with known interferon-inducing, natural killer (NK) cell activity enhancing, antiviral and antitumor properties in several animal species. Its effect on CC531, a DMH induced, transplantable, weakly immunogenic adenocarcinoma of the colon in WAG rats, was studied. ABPP was found to have no direct cytotoxic effect on CC531 cells in vitro. When small cubes of tumor of equal weight were implanted under the renal capsule, the administration of 250 mg/kg of ABPP intraperitoneally (ip) on day 0 and +1 led repeatedly to a significant ($p<0.02$ to $p<0.001$) inhibition of tumor growth, when measured on day +7. Lower doses or a single dose of ABPP could not achieve this effect. Late administration (on day +6 and +7) of 250 mg/kg of ABPP in this model was found to have no effect on tumor growth when measured on day +13. When 5×10^5 tumor cells were injected in the portal vein, administration of 250 mg/kg of ABPP i.p. on day 0 and +1 reduced significantly ($p=0.002$) the number of liver metastases, when counted on day +30. Survival in this group was significantly prolonged ($p<0.01$). However when ABPP was given on day +6 and +7, significantly more ($p<0.02$) metastases in the liver were counted on day +30. The results show a significant antitumor effect of ABPP against tumor CC531 in the subrenal capsule assay (SRCA) model as well as in the liver metastasis model when administered at the time of tumor inoculation. Late administration of ABPP does not inhibit tumor growth in the SRCA and significantly enhances the development of liver metastases. The role of timing, tumor site and the mechanisms by which this dual outcome of immunotherapy with ABPP is mediated is discussed. The results of these experiments may have important implications for the design of clinical studies with ABPP.

INTRODUCTION

ABPP is a relatively new biological response modifier (BRM) with various effects on the immune system. It induces the production of endogenous interferon (IFN) in several animal species (Stringfellow et al., 1980a) and in man (Earhart et al., 1985). ABPP enhances NK cell activity (Taggart et al., 1980; Lotzova et al., 1983) and activates macrophages (Li et al., 1985). It has been shown to exert antitumor effects in subcutaneous (Sidky et al., 1985), lung metastasis (Milas et al., 1982) and in intra-

peritoneal tumor models (Stringfellow, 1980b). Recently we reported on its inhibiting effects on subcutaneous growth and on the development of lung metastases of a nonimmunogenic liposarcoma in BN rats (Eggermont et al., 1986a). The results suggested that the antitumor effect in the subcutaneous model was evoked by the induction of IFN, whereas the inhibition of the development of lung metastases could be accounted for by the enhancement of NK cell activity. In the present study ABPP was used in a clinically more relevant tumor model : a transplantable weakly immunogenic adenocarcinoma of the colon (CC531) in WAG rats. In a previous communication (Marquet et al., 1984) we reported on the inhibitory effects of partially purified rat interferon (RIFN) on tumor CC531 in the subrenal capsule assay and in the lung metastasis model. In the liver metastasis model however RIFN treatment appeared to be totally ineffective if not detrimental. The liver seemed to provide a protective environment against tumor immunity. The aim of the present study was to investigate the effects of ABPP on tumor CC531 in the subrenal capsule assay and in the liver metastasis model. Since tumor burden is an important factor in the clinical situation, the role of tumor load was studied with a special interest in site-specific tumor behaviour.

MATERIAL AND METHODS

Rats

Male rats of the inbred WAG/Ro strain were used. The animals were bred under specific pathogen-free conditions and were 10-12 weeks old.

Tumor

Tumor CC531 is a 1,2 dimethylhydrazine-induced, moderately differentiated adenocarcinoma of the colon in WAG rats. It is transplantable in syngeneic animals and is weakly immunogenic as determined by the method described by Prehn and Main (1957). Spontaneous lung metastases do occur if the tumor is implanted subcutaneously (s.c.). Artificial metastases can be induced in the lungs by intravenous (i.v.) injection of tumor cell suspensions, whereas liver metastases can be evoked by injection

of tumor cells into the portal vein. In the experiments discussed here, the tumor was in its 12th passage. A stationary culture of cell line CC531 has been established and is maintained in RPMI-medium enriched with 10% fetal calf serum (FCS).
Tumor cell suspensions were prepared from s.c. tumor implants in RPMI-medium, enriched with 10% FCS. as decribed in chapter III.

Tumor models

Liver metastasis model : Liver metastases were evoked by injection of 5 x 10^5 tumor cells into the portal vein. Under ether anesthesia the portal vein was exposed via a small midline incision and 0.5 ml of suspension was injected via a 16 x 0.5 mm needle. The rats were laparotomized on day +30 and the number of liver metastases was counted.

Subrenal capsule assay (SRCA) : Recipient animals were anesthesized with ether, a median laparotomy was done and both kidneys were exteriorized. A small knick was made in the capsule and a tumor cube of 6-8 mg was implanted and pushed up to the upper kidney pole. One week after the first administration of ABPP the animals were killed, the tumors were enucleated and weighed.

ABPP

ABPP (2-amino-5-bromo-6-phenyl-4(3H)-pyrimidinone) was made and provided by The Upjohn Company, Kalamazoo, Michigan, U.S.A. ABPP was suspended in phosphate buffered saline (PBS) and injected intraperitoneally (i.p.).

Experimental Procedures

To assess wether ABPP has a direct cytotoxic effect on CC531, tumor cells were obtained from the stationary cell line culture by trypsinisation for 10 minutes. The cells were counted and a dilution series ranging from 10^6 - 10^{-1} cells per ml was made in 16 mm multi-well tissue culture plates (Costar, Cambridge, Mass., USA) in a volume of 1 ml and were allowed to adhere for 1 day before ABPP was added. Of each cell concentration one well was used as a control. In the other wells 0.25 fg up to 16 fg of ABPP dissolved in 0.5 ml PBS was added. After a 6-day incubation period with ABPP the number of cells in each well was counted in the microcell counter (TOA, Medical Electronics, Japan). In the

first two SRCA's groups of 5 animals were used. After insertion of the tumor cubes under the capsule of both kidneys 250 mg/kg of ABPP was administered i.p. on day 0 and +1. The control animals recieved an equal volume (0.5 ml) of PBS i.p. The animal were killed on day +7, the tumors were enucleated and weighed. In the dose-response experiment 3 groups of 3 animals recieved 50, 150 or 250 mg/kg of ABPP on day 0 only, and 3 groups of 3 animals recieved the same dose on day 0 and +1. Control animals recieved PBS. The tumors were enucleated and weighed on day +7. The effect of late administration of ABPP in the SRCA model was assessed using groups of 8 animals. ABPP was injected in a dose of 250 mg/kg i.p. on day +6 and +7. Controls recieved PBS. The tumors were enucleated and weighed on day +13. In the liver metastasis model groups of 8 animals were used. 5 x 10^5 tumor cells were injected into the portal vein. In the first experiment 250 mg/kg of ABPP was administered i.p. on day 0 and +1, in the second experiment on day +6 and +7. The number of liver metastases was counted on day +30. Controls animals recieved PBS i.p. on the same days.

Statistical Analysis

The results were analyzed by the Student t-test and the Wilcoxon rank sum test.

RESULTS

Assessment of direct cytotoxicity of ABPP

No cytotoxic effect of any dose of ABPP against any of the various concentrations of CC531 cells was seen. The number of CC531 cells per well, counted on day +7, was virtually equal in all cases to the number of cells present in the respective control well.

Early administration of ABPP in the Subrenal Capsule Assay

Table I shows the response of CC531 to the administration of 250 mg/kg of ABPP i.p. on day 0 and +1 in the SRCA in 2 separate experiments. A significant ($p<0.02$ - $p<0.001$) inhibition of tumor growth was observed in the ABPP treated animals.

Table I : Antitumor effect early administration of ABPP in SRCA

Treatment		Mean Tumor Weight (mg) ± SD on day +7	
(1)	PBS	35.5 ± 5.5	
	ABPP	26.7 ± 8.4	($p < 0.02$)
(2)	PBS	24.8 ± 6.2	
	ABPP	12.8 ± 5.8	($p < 0.001$)

In two separate experiments (1) and (2) ABPP was administered i.p. on day 0 and +1 in a dose of 250 mg/kg. Controls received PBS. The tumors were weighed on day +7. Each experimental group consisted of 5 animals (= 10 tumors). Tumor growth was significantly inhibited by ABPP ($p<0.02$, $p<0.001$).

Different doses of ABPP in the SRCA

Table II shows the response of tumor CC531 to the administration of different doses of ABPP, when administered on day 0 only or on day 0 and +1, in the SRCA. Only when ABPP was administered in a dose of 250 mg/kg i.p. on day 0 and +1 a significant ($p<0.03$) inhibition of tumor growth was observed.

Table II : Antitumor effect of different doses of ABPP in SRCA

Treatment	ABPP on day 0		ABPP on day 0 and + 1	
	Tumor Weight ± SD		Tumor Weight ± SD	
PBS	24.3 ± 6.7		55.8 ± 12.2	
ABPP (50 mg/kg)	24.9 ± 10.1	NS	46.3 ± 13.3	NS
ABPP (150 mg/kg)	31.0 ± 6.7	NS	50.6 ± 10.9	NS
ABPP (250 mg/kg)	26.9 ± 3.7	NS	37.7 ± 10.8	($p<0.03$)

ABPP was administered i.p. on day 0 only or on day 0 and +1 at a dose of 50, 150 or 250 mg/kg. Tumors were weighed on day +7. Controls received PBS. Each group consisted of 3 animals (= 6 tumors). Tumor growth was significantly inhibited only when ABPP was administered on day 0 and +1 at a dose of 250 mg/kg ($p<0.03$).

Late administration of ABPP in the SRCA

Table III shows the response of tumor CC531 to the administration of 250 mg/kg of ABPP i.p. on day +6 and +7 in the SRCA. The tumors were enucleated and weighed on day +13. No effect on tumor growth was observed.

Table III : Antitumor effect of late administration of ABPP in the SRCA

Treatment	Mean Tumor Weight ± SD on day + 13	
PBS	67.4 ± 35.8	
ABPP	55.9 ± 26.4	NS

ABPP (250 mg/kg) was administered i.p. on day +6 and +7. Controls recieved PBS. The tumors were weighed on day +13. Each group consisted of 8 animals (= 16 tumors). No effect on tumor growth was observed.

Early administration of ABPP in the liver metastasis model

Table IV shows the response of tumor CC531 to the administration of 250 mg/kg of ABPP i.p. on day 0 and +1 in the liver metastasis model. 5 $x10^5$ Cells were injected in the portal vein. Treatment was started the same day. A laparotomy was done on day + 30 and the number of liver metastases was counted. One rat in the ABPP treated group died on day +2 due to a faulty injection on the previous day. In the ABPP treated group a highly significant ($p=0.002$) lower number of liver metastases was observed on day + 30.

The inhibition of the development of liver metastases after the early administration of ABPP was also reflected in a significant difference in survival between ABPP-treated animals and control animals. Median survival times were 46 days (range: 39-54) for the control group and 58 days (range: 47-64) for the ABPP treated group as depicted in Figure 1. This difference was statistically significant ($p<0.01$).

Table IV : Early administration of ABPP in liver metastasis model

Treatment	Number of metastases on day + 30	Mean number ± SD
PBS	18, 22, 31, >40, >40, >40, >40, >40	33.9 ± 9.2
ABPP	1, 2, 4, 7, 12, 15, >40	11.5 ± 5.1 (p=0.002)

ABPP treatment was started on the day of tumor inoculation. 250 mg/kg of ABPP was administered i.p. on day 0 and +1. Controls received PBS. ABPP significantly inhibited the development of liver metastases (p=0.002) when counted on day + 30

Figure 1 : Early administration of ABPP prolongs the survival of rats with liver metastases of tumor CC531

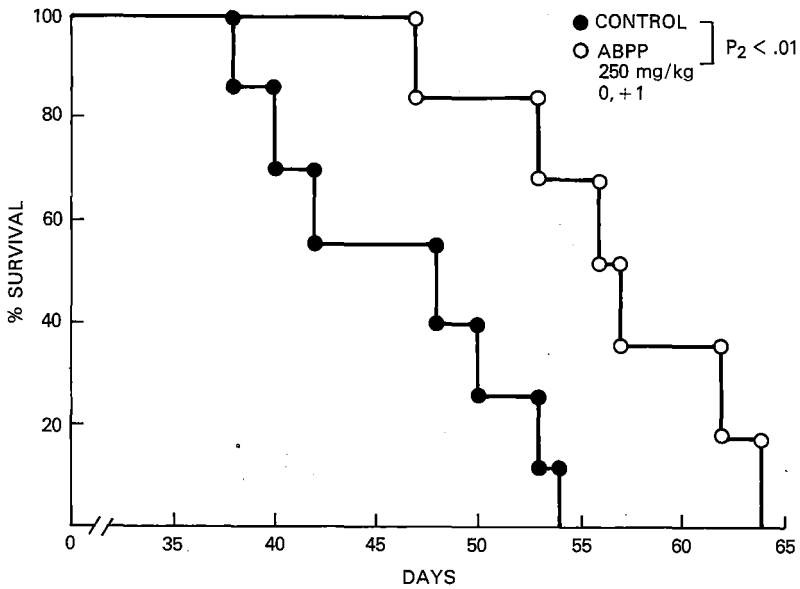

Figure 1 : Survival times of rats, injected with 5 x 10^5 CC531 tumor cells into the portal vein. 250 mg/kg of ABPP was given i.p. on day 0 and +1. Controls received PBS. There were 8 animals in the control group (●---●) and 7 in the ABPP treated group (0---0). Differences in survival times were significant, p < 0.01.

Late administration of ABPP in the liver metastasis model

Table V shows the response of tumor CC531 to the administration of 250 mg/kg of ABPP i.p. on day +6 and +7 in the liver metastasis model. The rats were killed on day +30 and the number of metastases was counted. A significantly ($p < 0.02$) higher number of metastases was observed in the ABPP treated animals.

Table V : Late administration of ABPP in liver metastases model

Treatment	Number of metastases on day + 30	Mean Number ± SD
PBS	2, 3, 5, 5, 5, 7, 8	5.0 ± 2.1
ABPP	6, 9, 9, 9, 10, 15, 25	11.9 ± 6.4 ($p<0.02$)

ABPP treatment was started 6 days after tumor inoculation. 250 mg/kg of ABPP was administered on day +6 and +7. Controls received PBS. Each experimental group consisted of 7 animals. Late ABPP treatment significantly enhanced the development of liver metastases ($p<0.02$).

DISCUSSION

The effect of ABPP on the weakly immunogenic adenocarcinoma of the colon , CC531, was studied. Since it was shown that ABPP exerts no direct cytotoxicity against CC531 cells, it can be assumed that the observed tumor inhibiting or enhancing effects are due to ABPP-induced changes of the hosts immune status. When administered early (on day 0 and +1), ABPP, used in a dose of 250 mg/kg, was shown to have a significant tumor growth inhibiting effect in the subrenal capsule assay. Late administration of ABPP had no effect on tumor growth. The same response of tumor CC531 was seen after treatment with RIFN (Marquet et al., 1984). The SRCA model may be considered to be comparable to the s.c. tumor growth model. Since inhibition of s.c. growth of a liposarcoma was found to depend most likely on induction of IFN by ABPP (Eggermont et al., 1986) the effect on CC531 in the SRCA is probably mediated by ABPP-induced IFN as well. In the liver

metastasis model early administration of ABPP was also highly effective. The observed inhibition of the development of liver metastases is probably mediated the enhancement of NK cell activity. ABPP is a very potent and very rapid activator of NK cell cytotoxicity (Lotzova et al., 1983), and NK cells are known to be most effective against circulating tumor emboli (Riccardi et al., 1979; Hanna and Fidler, 1980). In our on lung metastases with the liposarcoma in BN rats the inhibition of lung metastases of a liposarcoma in rats treated with ABPP was found to depend most likely on enhanced NK cell activity and to be independent of induction of IFN (Eggermont et al., 1984). The most striking result of our experiments is the enhancement of liver metastases after late administration of ABPP. Numerous reports in the literature on a dual outcome of immunotherapy have been related to: (1) timing (Vaage, 1980), (2) dose (Ishibashi et al., 1978), (3) route of administration (Proctor et al., 1976), (4) tumor site (Ryd et al., 1979) and (5) manipulation of the tumor (Col-merauer et al., 1980). Host factors rather than direct effects of the agents are considered to determine the dual outcome. Similarly liver-specific immunological components may explain the discrepant effects of early and late administration of ABPP in the liver metastasis model. When ABPP was administrated late, the tumor cells had ample time to colonize the liver. Local factors may explain the enhancement of tumor growth, observed after the late administration of ABPP. Kupffer cells may play an important role since they may have rapidly degraded tumor-associated antigens, just like their activation can lead to prolonged graft survival of allogeneic organ transplants (Sakai, 1970) or opsonise and clear antigen reactive cells as described by Hutchinson (1980). Both mechanisms could facilitate tumor growth. Enhancement of Kupffer cell activity in rats, caused by tumor challenge, has been reported by Antikatzides and Saba (1977). It reaches its peak level after 6 days. If ABPP enhances the activity of Kupffer cells as does IFN (Huang, 1980) peak level activity may have been prolonged and may have led to the enhanced development of the liver metastases. When ABPP is administered early, the activation of Kupffer cells, wich may lead to a suppression of antitumor activity as described above by increased clearance of tumor antigens or antigen reactive T cells, is of no consequence since

the tumor cells have been adequately dealt with in the blood born and early lodging phase by activated NK cells. This would explain the observed inhibition of the development of liver metastases after early administration and the enhanced develop-ment after late administration of ABPP. An alternative or additi-ve explanation of enhanced development of liver metastases hinges on the role of suppressor T cells. Numerous publications, review-ed by Schatten et al. (1984), have established that animals bearing progressive tumors acquire suppressor T cells. Although mainly localized in the spleen they can develop in and be recruited by the liver so that one can speak of a splenohepatic suppressor axis (Vuitton et al., 1977). If ABPP stimulates suppressor T cell function in a similar fashion as can be seen after the administration of interferon-alpha (Schnaper et al., 1983), the observed enhancement of tumor development in the liver as a preferential suppressor T cell containing organ can be explained as well as the lack of enhancement observed in the SRCA model. Recent evidence for a special role of Kupffer cells has been provided by Okumura and coworker (1987). They showed that Kupffer cells can suppress NK cell activity athrough the production of prostaglandins and thus provide a good explanation for the facilitated tumor growth in the liver as observed in our experiments.

In conclusion: it has been demonstrated that ABPP can inhibit and enhance the growth of colon cancer CC531 in WAG rats. The outcome of treatment depends on timing of the administration of the drug and on tumor site-specific host factors. These findings may have serious implications for the design of clinical studies with ABPP.

CHAPTER V

The content is an adapted compilation of the publications :

Efficacy of treatment with ABPP and recombinant gamma-Interferon on colon cancer in rats depends on tumor site, tumor load and treatment schedule

Alexander M. M. Eggermont, Richard L. Marquet, Ron W. F. de Bruin and Johannes Jeekel

From the Laboratory for Experimental Surgery, Erasmus University, Rotterdam, the Netherlands

Surgical Forum 37:342-344, 1986

Combined treatment of colon adenocarcinoma in rats with Tumor Necrosis Factor (TNF) and the Interferon-inducer ABPP

Richard L. Marquet, Alexander M. M. Eggermont, Ron W. F. de Bruin, Walter Fiers and Johannes Jeekel

From the Laboratory for Experimental Surgery, Erasmus University, Rotterdam, the Netherlands

Cancer Prevention and Detection (in press, 1987)

Efficacy of combined treatment of colon adenocarcinoma in rats with the interferon-inducer ABPP and recombinant Rat Interferon-gamma (rRIFN-gamma) or recombinant Murine Tumor Necrosis Factor (rMuTNF).

Summary

We have previously shown that the early administration (day 0, 1) of the interferon (type I) inducer ABPP has significant antitumor activity against the colon adenocarcinoma CC531 in rats in the subrenal capsule assay (SRCA). The late administration (day 6, 7) of ABPP never resulted in a significant inhibition of tumor growth. Synergistic antitumor activity of combinations of Type I and Type II interferons in vitro as well as in vivo has been reported in the literature. Likewise synergistic antitumor activity has been described when Interferons (especially IFN-gamma) was used in combination with Tumor Necrosis Factor (TNF). We investigated whether the administration of recombinant rat interferon-gamma (rRIFN-gamma) synergized with in vivo ABPP-induced endogenous IFN-alpha in the SRCA tumor model. The early administration of ABPP had a significant antitumor effect in all experiments. The late administration of ABPP never reduced tumor growth significantly. rRIFN-gamma alone never had a significant antitumor effect. When given in combination with the early administration of ABPP no additional antitumor effect was seen. When the administration of rRIFN-gamma preceded the late administration of ABPP a significant inhibition of tumor growth, and therefore a synergistic antitumor effect was observed. Secondly we investigated the potential efficacy of the combined treatment with ABPP and recombinant murine tumor necrosis factor (rMuTNF) in the SRCA. In these experiments the administration of rMuTNF on day 0, 2 and 4 always led to a significant reduction of tumor growth. When administered in combination with ABPP an additive antitumor effect was seen. These experiments indicate that various biologicals can be succesfully used in combination. This may lead to dose reductions of each agent and thus deminish toxicity. Furthermore timing and proper sequencing of treatment appeared crucial and illustrates the need for further research in order to design optimal combination treatment schedules.

INTRODUCTION

With regard to the biological activities of interferon (IFN) alpha and beta (type I) and IFN-gamma (type II) differences have been observed with respect to the sensitivity of cell lines (Blalock et al., 1980) and viruses (Rubin and Gupta, 1980) to the various IFNs. In vitro and in vivo studies have shown that Type I and Type II IFNs can potentiate each other in terms of their

antiviral (Fleischman et al., 1979) and anticellular (Fleischman et al.,1980) activities. The molecular mechanism of action of IFN-gamma may be different from the other IFNs (Dianzani et al., 1980), which may be the basis for the reported synergistic activity. We have previously shown that the early administration of the interferon (IFN) inducing pyrimidinone ABPP has antitumor effect in vivo against a colon adenocarcinoma in the subrenal capsule assay (SRCA) and in a liver metastasis model in rats (Eggermont et al., 1986b). When treatment was started one week after tumor inoculation however no antitumor effect was seen in the SRCA and a detrimental effect in the liver metastasis model was observed. In order to find a way to use ABPP effectively in the advanced or established tumor setting we investigated the possible role of IFN-gamma and developed a treatment schedule with synergistic antitumor activity.

Similarly we investigated the possible benefits of combined treatment with ABPP and tumor necrosis factor (TNF). Tumor necrosis factor is a protein produced mainly by activated monocytes and probably also by activated natural killer (NK) cells. It was originally demonstrated in the serum of animals treated with BCG and endotoxin (Carswell et al., 1970). Tumor necrosis factor has been functionally defined by its cytostatic and cytolytic properties to some tumor cell lines in vitro and has the capacity to induce necrosis of solid transplantable tumors in mice (Old, 1985). Its mechanism of action is still largely unknown. There is evidence that inactivation or destruction of protein-dependent cellular function is important (Ruff and Gifford, 1981); that TNF may need to be internalized and that lyzosomal activity may be required for cell killing (Kull and Cuatrecasas, 1981); but also that components of the cell membrane may be the primary targets of TNF (Darzynkiewicz et al., 1984). In vitro synergistic anticellular activity when TNF is combined with IFNs, especially with IFN-gamma, has been reported (Williamson et al., 1981; Sugarman et al., 1985). Balkwill and coworkers (1986) reported recently on the synergistic antitumor effects observed in vivo when human xenografts in nude mice were treated with exogenous IFN-alpha or -gamma in combination with TNF. This suggests that at least in part the cytotoxic effects of IFNs and TNF are attained by different pathways. The possibly potenti-

ating antitumor effect of ABPP-induced endogenous interferon (type I) and exogenous TNF was investigated. We will demonstrate in this communication that such an effect could be obtained.

MATERIAL AND METHODS

Rats

Male rats of the inbred WAG/Ro strain were used. The animals were bred under pathogen free conditions and were 10-12 weeks old.

Tumor

Characteristics of the weakly immunogenic adenocarcinoma of the colon CC531 in WAG/Ro rats were described in chapter IV.

Subrenal capsule assay (SRCA)

The subrenal capsule assay was described in chapter IV.

Agents

ABPP was administered i.p. in all experiments at a dose of 250 mg/kg dissolved in 1 ml phosphate buffered saline (PBS). ABPP was given either "early" (day 0 and +1) or "late" (day 6 and 7).

rRIFN-gamma

Recombinant Rat IFN-gamma was a gift from dr. P. van der Meide, Primate Center TNO, Rijswijk, the Netherlands. Details of cloning , expression and purification of rRIFN-gamma have been published by Dykema et al. (1985). The preparation contained 10^6 antiviral units per mg protein and had a purity of 25%. In an earlier communication we have reported on the antiproliferative and immunomodulating properties of rRIFN-gamma (IJzermans et al., 1985). rRIFN-gamma was administered either i.p. by means of an implantable osmotic pump (Alzet) to deliver 10^6 units in 7 days or by intravenous (i.v.) injections of 2×10^5 units daily for 5 days.

rMuTNF

Recombinant murine tumor necrosis factor was a gift from Dr. W. Fiers, Laboratory of Molecular Biology, State University of Ghent, Belgium. Details of cloning, expression and purification of rMuTNF have been published elsewhere (Fransen et al., 1985). The preparation used was 99 % pure and contained 72×10^6 units

per mg protein. rMuTNFR was administered on days 0, 2 and 4 i.v. at a dose of 1 ug.

Statistical Analysis

The results were analyzed using the Student-t test. In all experiments the experimental groups consisted of at least 8 animals.

RESULTS

Early administration of ABPP in combination with rRIFN-gamma

In all SRCA experiments the early administration of ABPP inhibited tumor growth significantly ($p < 0.01$) whereas the administration of rRIFN-gamma i.p. by osmotic pump (experiment 1) and i.v. (experiment 2) had no effects at all. Combined treatment was not superior to treatment with ABPP alone. The results are summarized in Table I.

Table I : Antitumor effect of simultaneous early administration of ABPP and rRIFN-gamma in the SRCA

Treatment	Mean Tumor Weight ± SEM on day + 7	
Experiment 1		
PBS	36.4 ± 1.9 mg	
rRIFN-gamma	37.7 ± 2.4 mg	
ABPP	26.7 ± 2.7 mg	$p < 0.01$
ABPP + rRIFN-gamma	25.7 ± 2.7 mg	$p < 0.01$
Experiment 2		
PBS	27.7 ± 1.9 mg	
rRIFN-gamma	26.8 ± 2.1 mg	
ABPP	13.9 ± 1.6 mg	$p < 0.0001$
ABPP + rRIFN-gamma	14.7 ± 2.7 mg	$p < 0.001$

experiment 1:rRIFN-gamma 10^6 units, ip, via osmotic pump day 0-6
experiment 2:rRIFN-gamma 2 x 10^5 units, iv, daily on day 0-5
SEM = standard error of the mean. N = 8

Late administration of ABPP preceded by rRIFN-gamma

When ABPP was administered on day 6 and 7 no significant inhibition of tumor growth was observed. The daily administration of 2 x 10^5 units of rRIFN-gamma on day 0-5 had no antitumor effect by itself but the combined treatment with ABPP had significant antitumor effect ($p < 0.03$). This experiment is depicted in Figure 1.

Figure 1 :

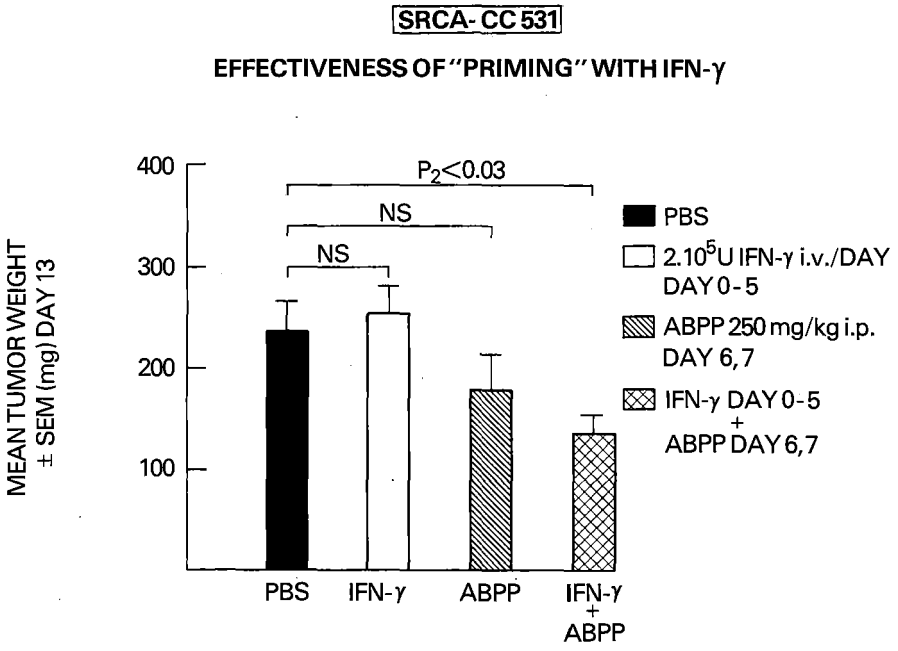

Figure 1 : In the SRCA, groups of at least 8 rats were treated either with phosphate buffered saline (PBS); rRIFN-gamma 2 x 10^5 units for five days ((0-5) i.v.; ABPP 250 mg/kg i.p. on day 6+7; or ABPP on day 6 and 7 preceded by rRIFN-gamma i.v. from day 0-5. On day 13 the tumors were enucleated and weighed. Single agent treatment was ineffective. Combination treatment with rRIFN-gamma preceding the late administration of ABPP had significant antitumor effect ($p < 0.03$). SEM = standard error of the mean.

Combined treatment with ABPP and rMuTNF

In two consecutive experiments early administration of ABPP led to a significant inhibition of tumor growth as measured on day 7 ($p < 0.001$). Administration of rMuTNF (1 ug) on day 0, 2 and 4 also had a significant antitumor effect ($p < 0.01$) A additive antitumor effect was observed when the combined treatment schedule was administered ($p < 0.0001$). The results of these experiments are summarized in Table II.

Table II : Antitumor Effects of Combined Treatment with ABPP and rMuTNF in the SRCA

Treatment	Mean Tumor Weight ± SEM on day +7	
Experiment 1		
PBS	42 ± 3.1 mg	
ABPP	26 ± 1.9 mg	$p < 0.001$
TNF	24 ± 2.1 mg	$p < 0.001$
ABPP + TNF	13 ± 2.2 mg	$p < 0.0001$
Experiment 2		
PBS	38 ± 2.7 mg	
ABPP	21 ± 1.6 mg	$p < 0.001$
TNF	27 ± 2.2 mg	$p < 0.01$
ABPP + TNF	14 ± 2.2 mg	$p < 0.0001$

experiment 1: ABPP+TNF vs ABPP: $p<0.01$; ABPP+TNF vs TNF: $p<0.01$
experiment 2: ABPP+TNF vs ABPP: $p<0.03$; ABPP+TNF vs TNF: $p<0.001$
SEM = standard error of the mean. N = 8

DISCUSSION

We have shown that synergistic or additive antitumor effects can be achieved by combining the administration of the interferon inducer ABPP with rRIFN-gamma or rMuTNF in the treatment of colon cancer CC531 in WAG rats. Early administration of ABPP had significant antitumor activity in all 4 experiments. Simultaneous administration of rRIFN-gamma never added to the antitumor effect.

This lack of in vivo antitumor effect of rRIFN-gamma is in line with previously published observations from our laboratory on the lack of in vivo antitumor activity against a nonimmunogenic liposarcoma in BN rats, in spite of proof of good activation in vitro of NK cells and macrophages (IJzermans et al., 1985). Only when rRIFN-gamma was used as a "primer", preceding the late administration of ABPP, this sequential treatment schedule inhibited tumor growth significantly, while neither ABPP nor rRIFN-gamma agent alone was effective. This synergistic antitumor effect may be due to an upregulation of the expression of interferon type I receptors by IFN-gamma. When IFN-gamma precedes treatment with ABPP maximal expression of Type I IFN receptors may render tumor cells more sensitive to the ABPP-induced endogenous IFN-alpha and explain the observed synergism. The lack of an additive or synergistic antitumor effect when ABPP is administered early and IFN-gamma simultaneously may be due to : (i) inhibition by competition with rRIFN-gamma for IFN-gamma receptors by the rapidly ABPP-induced endogenous IFN-alpha (Anderson et al., 1982) thus preventing the upregulating effects of IFN-gamma ; (ii) the fact that peak levels of ABPP-induced endogenous IFN-alpha are "short lived" (< 24 hours; Oku et al., 1984) and the upregulating effect of IFN-gamma may be too slow for the two effects to coincide and provide the opportunity for synergistic activity. It is noteworthy that Chapekar and Glazer (1985) reported similar observations in experiments assessing the anticellular activity of the combination of human rIFN-gamma and Poly(I).Poly(C) against human colon cancer cells in vitro. Only when IFN-gamma preceded treatment with Poly(I).Poly(C) a highly synergistic antiproliferative effect was observed in their experiments. Of interest are also the reports on the priming effect of IFN-gamma on the subsequent induction of cytotoxicity of macrophages by a variety of biological response modifiers derived from microorganisms (Nagao et al., 1986) or by muramyl dipeptide analog (Utsugi and Sone, 1986).

The additive antitumor effect of the combined treatment with ABPP and rMuTNF in the SRCA is comparable to the efficacy of the combination of rRIFN-gamma and rMuTNF in the same tumor model (Marquet et al., 1987 in press). This is of importance since TNF was shown to be quite toxic in that communication and combination

treatment schedules may render non-toxic doses of each agent effective. IFN-gamma has been shown to augment the cytotoxic activity of TNF in vitro (Williamsen et al., 1983; Sugarman et al., 1985; Fransen et al., 1986). There is no evidence that ABPP induces IFN-gamma. We have never been able to demonstrate in vivo induction of class II antigens, a hall mark of IFN-gamma activity as previously reported by IJzermans et al. (1986), after the administration of ABPP (Marquet, unpublished observations). Apparently IFN-alpha can also augment the antitumor effects of TNF (Balkwill et al., 1986). It has recently been demonstrated that IFN-gamma, but also IFN-alpha/beta, albeit to a lesser degree, enhance the expression of cellular surface receptors for TNF on several tumor cell lines (Tsujimoto et al., 1986a). These data seemed to suggest a causal relationship between enhanced TNF binding and increased cytotoxicity in IFN-treated cells. In an elegant study however, demonstrating the differential effects of Type I IFN and IFN-gamma on the binding of TNF to receptors in different human cell lines, Tsujimoto and coworkers (1986b) showed that these two classes of IFN can have opposite effects on the expression of TNF receptors and yet may both act synergistically with TNF in causing cytolysis. Therefore TNF receptor modulation by IFNs was not considered a major mechanism of synergism between IFN and TNF.

The experiments we have presented in this communication illustrate that the different mechanisms by which different biologicals exert their antitumor effects can be exploited by obtaining additive or even synergistic antitumor effects by administering combined treatment schedules. Further research in this field is warrented and may have clinical consequences in the forseeable future.

CHAPTER VI

In vivo generation of lymphokine activated killer cell activity by ABPP and Interleukin-2 and their antitumor effects against immunogenic and nonimmunogenic tumors in murine tumor models

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Summary

The capacity of the interferon inducer ABPP and recombinant interleukin-2 (IL-2) to generate lymphokine activated killer (LAK) cell activity in vivo was examined and compared to the cytolysis of fresh tumor cells by in vitro generated LAK cells. Various tumors differing in histology and immunogenicity were used in in vitro and in vivo experiments. The intraperitoneal administration of ABPP or IL-2 generated much higher levels of LAK cell activity in the peritoneal exudate than in the spleen. Administration of 2 injections of ABPP was as effective as a three day course of moderate doses of IL-2. Generation of LAK cell activity by IL-2 was dose dependent. Enormous numbers of highly lytic LAK cells were generated in the peritoneal exudate by high but toxic doses of IL-2. Attempts to increase in vitro generation of LAK cells by pretreatment of spleen donors with ABPP were unsuccessful. ABPP was effective against day 1 tumor but ineffective against established (day 3) tumor or advanced (day 8) tumor. Treatment of established tumor with IL-2 and LAK cells was not more effective when ABPP was given concurrently. In contrast when ABPP preceded IL-2 and LAK treatment an additional antitumor effect was seen. Immunogenic tumors were somewhat more sensitive to treatment with ABPP than nonimmunogenic tumors. Only a marginal difference in lysability in vitro existed. The antitumor effects of ABPP in vivo may therefore be mediated by other mechanisms than cytolysis by activated killer cells alone. These data taken together suggest that ABPP and IL-2 effects induce discernable levels of LAK cell activity, but do not synergize when combined.

INTRODUCTION

ABPP (2-amino-5-bromo-6-phenyl-4(3H)-pyrimidinone) is a small molecule biological that induces high levels of endogenous interferon type I and modulates a variety of immune responses (Hamilton et al., 1982; Stringfellow, 1981). Most previous reports have focused on the activation of natural killer (NK) cells and macrophages (Fast et al., 1982; Li et al., 1985; Lotzova et al.,1983; 1984 and 1986). We investigated the ability of ABPP to generate lymphokine activated killer (LAK) cell activity in vivo. LAK cell

activity has been defined as the capacity of activated lymphoid cells to kill fresh tumor cells in short term ^{51}Chromium release assays (Rosenberg, 1984; Rosenstein et al., 1984). The availability of large quantities of recombinant interleukin-2 (IL-2) has caused a renewed interest in immunotherapeutic research programs. Its use alone and in combination with adoptively transferred LAK cells has shown great promise in animal models (Lafreniere and Rosenberg, 1985; Mule et al., 1984; Ottow et al., 1987a and b; Rosenberg et al., 1985a) as well as in humans (Rosenberg et al., 1985b and 1987).
In order to evaluate the potential of ABPP as an immunotherapeutic agent we felt it appropriate to assess its capacity to induce LAK cell activity in vivo in comparison to IL-2 and to assess the potential benefit of combination treatment with these two biologicals.

MATERIAL AND METHODS

Mice

C57BL/6 (BL/6) female mice were obtained from Jackson Laboratory (Bar Harbor, ME) and used when 9-12 weeks old. They were maintained on laboratory chow and acidified water ad libitum in a pathogen free environment.

Tumors

The MCA-101, -102, -105 and -106 sarcomas, the MCA-38 colon adenocarcinoma and the B16 melanoma used in these experiments are syngeneic to BL/6 mice. MCA-101, -102, -105 and -106 sarcomas were induced in our laboratory by the intramuscular injection of 0.1 ml of 1% 3-methycholanthrene (MCA) in sesame oil as described previously (Parker and Rosenberg, 1977). A large number of vials of MCA-101, -102, -105, and -106 sarcomas from the first passage generation were cryopreserved. After thawing, these tumors were passaged subcutaneously (s.c.) in C57BL/6 mice and were used within the first six transplant generations. The MCA-105 and -106 tumors are weakly immunogenic and the MCA-101 and -102 tumors are nonimmunogenic (Papa et al., 1986). MCA-38 is a weakly immunogenic murine colon adenocarcinoma that was induced by the s.c.

injection of dimethylhydrazine (DMH) in C57BL/6 mice. This tumor was passaged s.c. and its use was not restricted to early passage generations. Cryopreserved cells from the nonimmunogenic natural killer-resistant B16 melanoma tumor line of C57/BL6 origin (Fidler, 1973) were used, thawed, placed in tissue culture and subsequently used for s.c. passage in BL/6 mice. Single cell suspensions of all tumors used were prepared as described previously (Mule et al., 1984). Briefly, fresh tumors were excised, minced with scissors and stirred in a triple enzyme solution of deoxyribonuclease, hyaluronidase and collagenase (Sigma Chemical Co., St. Louis, MO) for three hours at room temperature, filtered through 100 gauge nylon mesh (Nitex; Lawshe Industrial Co., Bethesda , MD), washed three times in Hanks balanced salt solution (HBSS; Biofluids, Rockville, MD) without calcium (Ca^{2+}) and magnesium (Mg^{2+}) and resuspended in HBSS or complete medium at the appropriate cell concentration for injection or in vitro assays.

Interleukin-2

Recombinant human interleukin-2 (IL-2) was kindly supplied by the Cetus Corporation (Emeryville, CA) (Rosenberg et al 1985a) and had a specific activity of 4 to 8 x 10^6 units/mg. IL-2 activity was measured by a standard bioassay as described previously (Donahue and Rosenberg, 1983). It was used for the preparation of LAK cells and for i.p. administration.

ABPP

ABPP was given at a dose of 250 mg/kg i.p., dissolved in 1 ml phosphate buffered saline (PBS)in all experiments.

Generation of LAK cells in vitro

LAK cells were generated as described previously (Mule et al., 1984). Briefly BL/6 spleens were harvested aseptically and mashed in HBSS with the hub of a syringe and passed through a 100 gauge nylon mesh to produce a single cell suspension. Erythrocytes were lysed osmotically with ACK buffer (Media Unit, NIH, Bethesda, MD). The remaining splenocytes were washed three times in HBSS and 5 x 10^6 cells were incubated in 750 ml culture flasks (Falcon, Oxnard, CA) containing 175 ml of complete medium and 175,000

units of IL-2 per flask. Complete medium (CM) contained RPMI 1640 with 10% fetal calf serum (FCS) (both Biofluids, Rockville, MD), 0,3% fresh glutamine, 100 ug/ml of streptomycin, 100 units/ml penicillin (all from the NIH Media Unit), 0.1 mM non-essential amino acids, 0.1 mM sodium pyruvate (all from Gibco Laboratories, Grand Island, NY), 5 x 10^{-5} M 2-mercapto-ethanol (Aldridge Chemical Co., Milwaukee, WI), 50 ug/ml gentamicin (Shearing, Henilworth, NJ), and 0.5 ug/ml of fungizone (Flow Labs, McLean, VA). The flasks were incubated for 72 hours at 37°C in a moist atmosphere with 5% CO_2. The LAK cells were then harvested, passed over Ficoll (Lympholyte-M, Cedarlane Laboratories, Hornby, Ontario, Canada) to remove dead cells, washed three times is HBSS and resuspended in CM for in vitro assays or in HBSS for i.p. injections.

^{51}Chromium release cytotoxicity assay

Spleens and peritoneal exudate cells were harvested from mice pretreated with HBSS, IL-2 or ABPP and were passed through a 100 gauge nylon mesh, the erythrocytes lysed with ACK buffer, and the remaining lymphocytes pooled and washed three times with HBSS and resuspended in CM at 1 x 10^7 cells/ml. Target cells were prepared by incubating one milliliter of one of the various fresh tumor cell suspensions (5 x 10^7 cells/ml) with ^{51}Cr (specific activity 250-2500 mci/mg) for 30 minutes. The labeled cells were washed three times and resuspended in CM at 1 x 10^5 cells/ml. 0.1 ml of labeled tumor cells and 0.1 ml of effector cells were incubated in plates of 96 round bottom wels (Linbro Chemical Co., Hamden, CT). Each well contained 10^4 labeled targets and various numbers of effector cells. After an incubation of 4 hours, supernatants were harvested employing the Titertek collecting system (Flow Laboratories, Inc., McLean, VA). The percentage of specific lysis was calculated as follows: (experimental cpm - spontaneous cpm) / (maximal cpm - spontaneous cmp) x 100. Spontaneous cpm was the amount of ^{51}Cr released from the targets in the absence of effectors.

Tumor models

In the **intraperitoneal tumor model** BL/6 mice received 1 x 10^5 MCA-105 tumor cells i.p. and were randomly allocated into a

treatment group. ABPP was administered at a dose of 250 mg/kg in 1 ml PBS i.p. unless noted otherwise. Several administration schedules were applied as is specified in the Results section. Interleukin-2 was also given i.p. at various doses depending on experimental design. In the experiments involving the administration of LAK cells and IL-2, 1 x 10^8 LAK cells were given i.p. three days after tumor inoculation and from day 3 through 7 10,000 units of IL-2 were administered i.p. twice a day (b.i.d.). Animals not receiving LAK cells and/or IL-2 were injected with identical volumes of HBSS. About 14 days after the i.p. inoculation of MCA-105 the animals were sacrified and the i.p. tumor mass scored in a blinded fashion on a scale from 0 - 3. The score was termed the Peritoneal Cancer Index (PCI) (Steller et al., 1985; Ottow et al., 1987a). The mice were ear-tagged and their numbers recorded prior to scoring. The abdomen of all mice was opened widely and scored for tumor load and mice of similar score were placed in the appropriate groups of a PCI of 0, 1, 2 or 3. A score of 0 is defined as no i.p. tumor, 1 as $\leq$ 3 pin point tumor foci (diameter $\leq$ 1 mm), 2 as moderate tumor and 3 as abundant i.p. tumor load replacing most of the peritoneal cavity. The peritoneal cancer index scoring system is illustrated in Figure 1. After all mice were scored the ear tag was read and the data were analyzed. Experimental groups in the i.p. tumor experiments consisted of at least 6 mice.

In the **pulmonary metastasis model** BL/6 mice received 3 x 10^5 syngeneic tumor cells (MCA-102, -105 or -38) intravenously suspended in 1 ml HBSS by way of the lateral tail vein. In two experiments the mice were followed for survival. In one experiment the mice were sacrificed after each treatment group was given a letter code. The lungs of the mice were inflated with a 15% solution of india ink for enumeration of metastatic pulmonary nodules (Wexler et al., 1966). Metastases are readily countable when "bleached" by Fekette's solution up to $\pm$ 250. Lungs with metastases too numerous to count were therefore assigned an arbitrary value of 250 as described previously by Mule et al. (1984).

Statistical Analysis

Overall significance of difference in the intraperitoneal tumor experiments was examined with the Jonckheere test for trend (Hol-

lander and Wolfe, 1973). If this test showed a two sided p value (P_2) < 0.05, pairwise comparisons of differences in tumor load were examined with the Wilcoxon rank sum test with a correction for ties. Significance of differences in the numbers of pulmonary metastases or differences in survival between groups was determined by the Wilcoxon rank sum test (Gehan, 1965). Two-sided p-values are presented in all experiments.

Figure 1 : The Peritoneal Cancer Index

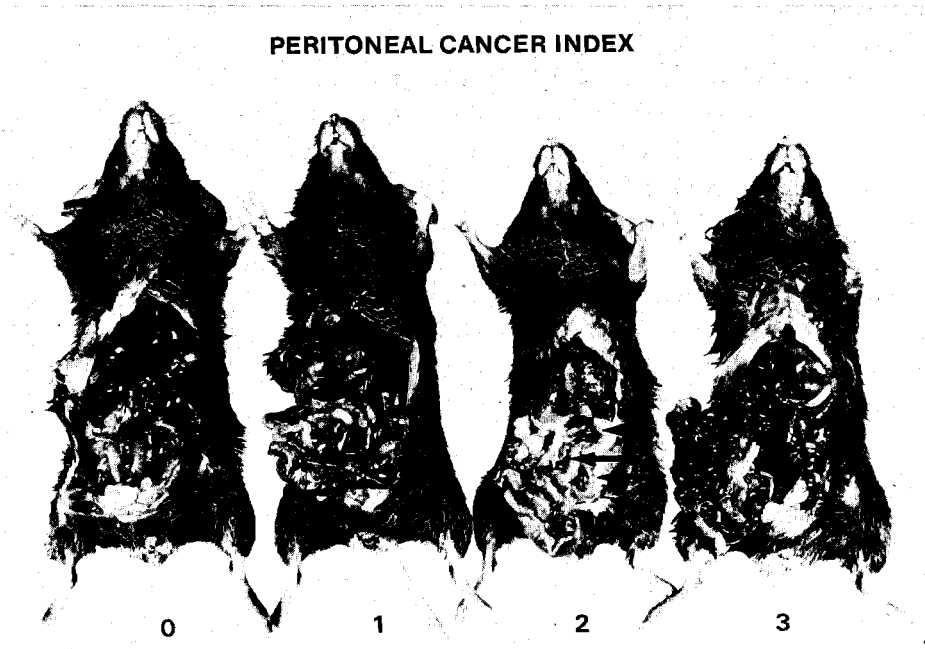


Figure 1 : The Peritoneal Cancer Index (PCI) Scoring System
Intraperitoneal tumor load is scored on a scale from 0 - 3 : 0 = no i.p. tumor; 1 = ≤ 3 pin point foci (diameter ≤ 1 mm); 2 = moderate i.p. tumor; 3 = abundant i.p. tumor replacing most of the intraperitoneal cavity. In this particular experiment the melanoma B16 was used. Small tumor foci are indicated by the black arrows.

RESULTS

In vitro cytolysis of NK-sensitive and NK-resistant tumor cells by in vivo ABPP-activated spleen cells

BL/6 mice received 250 mg/kg of ABPP in PBS i.p. on day -3 and -2. On day 0 their spleens were harvested. The enhancement of NK cell activity (Yac-1 targets) as well as the generation of LAK cell activity (fresh NK resistant tumor targets) by ABPP were assessed in ^{51}Chromium release assays. Tumors differing in histology (sarcomas, adenocarcinoma, melanoma) and in immunogenicity were used in order to determine the general nature of the cytotoxicity of the ABPP-activated lymphocytes. For comparison the cytotoxicity of *in vitro* generated LAK cells was determined in the same assays as well as the cytotoxicity displayed by splenocytes from control animals that had received PBS. The results are shown in Figure 2. Both NK cell activity and LAK cell activity induced by ABPP in the spleen was very poor. The immunogenic tumors (MCA-105, -106, and -38) seemed somewhat more easily lysed by ABPP-activated killer cells than the nonimmunogenic tumors (MCA-101, -102, and B16). This difference was marginal. Even at a 100:1 effector to target ratio specific lysis did not exceed 30%. The *in vivo* generation of LAK cell activity in the spleen by ABPP was clearly inferior to the *in vitro* generation of LAK cells by incubation of splenocytes in IL-2 for 3 days.

Figure 2 : Induction of LAK cell activity in splenocytes by ABPP

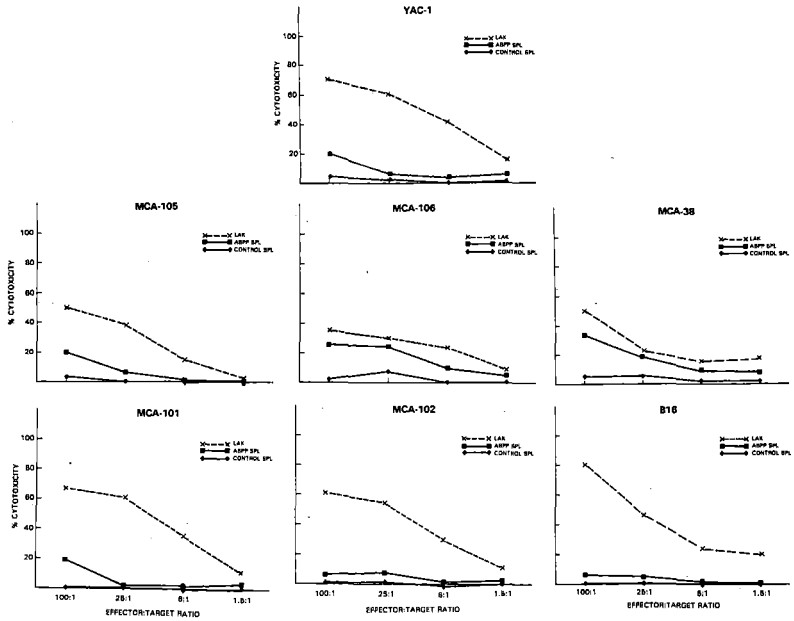


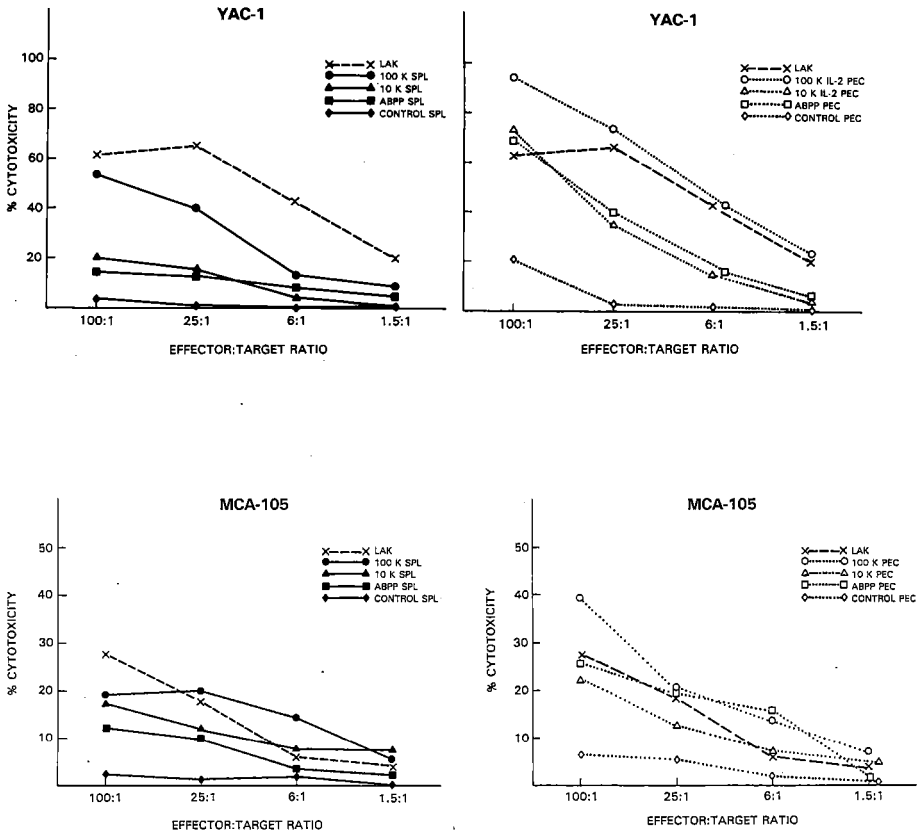
Figure 2 (top, middle, bottom) : The capacity of splenocytes (SPL) to lyse the NK cell sensitive YAC-1 target (top); the NK resistant weakly immunogenic fresh tumor cells (LAK-targets) MCA-105,-106, and -38 (middle); and the NK resistant (LAK targets) nonimmunogenic fresh tumor cells MCA-101, -102 and B16 (bottom); were assessed in 4 hour ^{51}Chromium release assays. For comparison cytotoxicity of *in vitro* generated LAK cells is shown (X---X).

Comparison of *in vivo* generation of LAK cell activity in the peritoneal exudate and the spleen by ABPP and IL-2

BL/6 mice received 100,000 (100K) or 10,000 (10K) units of IL-2 i.p., b.i.d., for three consecutive days (day -3, -2, -1) or were pretreated with 250 mg/kg of ABPP i.p. on day -3 and -2. Control animals received 1 ml of PBS i.p. only. On day 0 spleens and peritoneal exudate cells were harvested and their cytotoxicity against the fresh tumor cells (LAK-targets) MCA-105 (immunogenic) and MCA-102 (nonimmunogenic) and against the cell line NK sensitive YAC-1 cells was determined. As a positive control *in*

vitro generated LAK cells were used as is shown in Figure 3. The in vivo generation of LAK cell activity was consistently higher in the peritoneal exudate than in the spleens after the administration of either ABPP or IL-2. The level of cytotoxicity generated by two i.p. injections of ABPP was comparable to the level generated by the i.p. administration of 10,000 units of IL-2, twice a day, for three consecutive days.

Figure 3 (top, middle , bottom) :
In vivo induction of activated NK cell (Yac-1) and LAK cell (MCA-105, MCA-102) activity in the spleen and in the peritoneal exudate by ABPP and low or high doses of IL-2.



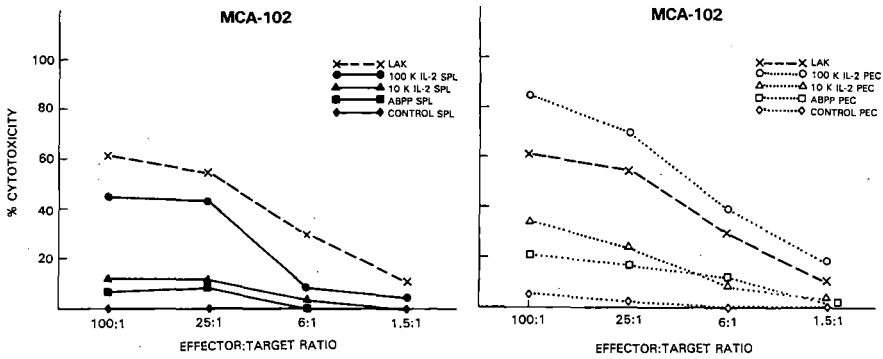


Figure 3 : BL/6 mice received either 250 mg/kg of ABPP i.p. on day -3 and -2 or a three day course of 10,000 (10K) or 100,000 (100K) units of IL-2 i.p., b.i.d. The cytotoxicity of splenocytes (SPL) and peritoneal exudate cells (PEC) against YAC-1 (top), MCA-105 (middle) and MCA-102 (bottom) tumor cells was assessed in 4 hour ^{51}Cr release assays on day 0 and compared with the cytotoxicity displayed by in vitro generated LAK cells. The generation of activated killer cells in the peritoneal exudate was clearly more effective than in the spleen. The IL-2 effect was dose dependent and the in vivo generation of LAK cell activity by high doses of IL-2 (0---0) was even more effective than the standard in vitro generation of LAK cell activity (X---X).

In vivo generation of LAK cell activity by IL-2 is dose dependent

High doses of IL-2 i.p. generated much higher levels of LAK cell activity in both the spleen and the peritoneal exudate than low doses. This is also illustrated in Figure 3. After 3 days of i.p. administration of 100,000 (100K) units of IL-2, b.i.d., the LAK cell activity in the peritoneal exudate was very high and even superior to in vitro generated LAK cell activity.

Generation of LAK cells *in vitro* after pretreatment of spleen donors with ABPP *in vivo*

BL/6 mice received 250 mg/kg of ABPP, i.p., 3 and 2 days prior to splenectomy for LAK cell generation *in vitro*. Cytolysis of fresh tumor cells (MCA-105, -102 and -38) by these LAK cells was compared to the cytolysis seen with standard LAK cells. Similar levels of cytotoxicity were observed in 4 hour ^{51}Chromium release asssays with either LAK cell population. It was thus concluded that pretreatment with ABPP did not effect the *in vitro* generation of LAK cell activity (data not shown).

Effects of ABPP on immunogenic and nonimmunogenic tumors in an intraperitoneal tumor model

BL/6 mice were inoculated with 1 x 10^5 tumor cells i.p. on day 0. ABPP was given at 4 different schedules as indicated in Table I. The mice were sacrificed on day 14 and the i.p. tumor load was scored blindly according to the peritoneal cancer index (PCI) system. Table I shows that a significant reduction in i.p. tumor load was observed after the administration of ABPP on day 1 and 2 with the immunogenic tumors MCA-105, -106 and MCA-38, but not with the nonimmunogenic tumors MCA-101 and -102. When ABPP was administered on day 3 and 4 a small reduction in i.p. tumor load was noted in all experiments with the immunogenic tumors. In half of the experiments this difference was statistically significant. This was not observed with the nonimmunogenic tumors. The administration on day 8 and 9 did not result in a significant reduction of i.p. tumor. These results show that the antitumor effects of ABPP are clearly related to tumor load. Further more the results suggest that to a lesser degree they may also be related to the immunogenicity of the tumor.

TABLE I : ANTITUMOR EFFECTS OF ABPP ON IMMUNOGENIC AND NONIMMUNOGENIC TUMORS IN AN INTRAPERITONEAL TUMOR MODEL

MEAN PCI ± SEM

EXP	TUMOR	CONTROL	ABPP1,2	ABPP3,4	ABPP8,9	ABPP3,4-8,9
Immunogenic tumors						
1	MCA-105	3.00 ± 0.00	2.17 ± 0.40 (0.02)	2.33 ± 0.33 (0.02)	2.33 ± 0.33 (0.02)	2.67 ± 0.21
2	MCA-105	3.00 ± 0.00	2.17 ± 0.30 (0.009)	2.50 ± 0.34	3.00 ± 0.00	2.50 ± 0.34
3	MCA-105	2.87 ± 0.09	2.17 ± 0.31 (0.02)	2.83 ± 0.17	2.83 ± 0.17	2.50 ± 0.21
4	MCA-106	2.87 ± 0.13	1.89 ± 0.26 (0.009)	2.00 ± 0.37 (0.05)	2.33 ± 0.31	-
5	MCA-38	2.80 ± 0.13	1.67 ± 0.28 (0.003)	2.42 ± 0.15 (0.03)	2.58 ± 0.15	2.42 ± 0.15 (0.03)
6	MCA-38	2.60 ± 0.13	1.86 ± 0.20 (0.004)	2.32 ± 0.15 (0.04)	2.67 ± 0.11	2.17 ± 0.31 (0.05)
Nonimmunogenic tumors						
7	MCA-101	1.90 ± 0.31	1.40 ± 0.40	2.17 ± 0.40	-	2.17 ± 0.17
8	MCA-102	2.58 ± 0.15	1.90 ± 0.28 (0.06)	1.90 ± 0.28 (0.06)	2.50 ± 0.21	2.33 ± 0.33
9	MCA-102	2.67 ± 0.15	2.40 ± 0.16	2.42 ± 0.19	2.83 ± 0.17	2.67 ± 0.31

PCI = peritoneal cancer index ; SEM = standard error of the mean ; P_2-values of pair-wise comparisons with control animals are indicated between brackets when differences were significant. ABPP was administered at a dose of 250 mg/kg, i.p., on two consecutive days at different time intervals after i.p. tumor inoculation as is indicated at the top of the table. Fourteen days after i.p. tumor inoculation the mice were sacrificed and their i.p. tumor load was scored in a blinded fashion with the PCI-scoring system.

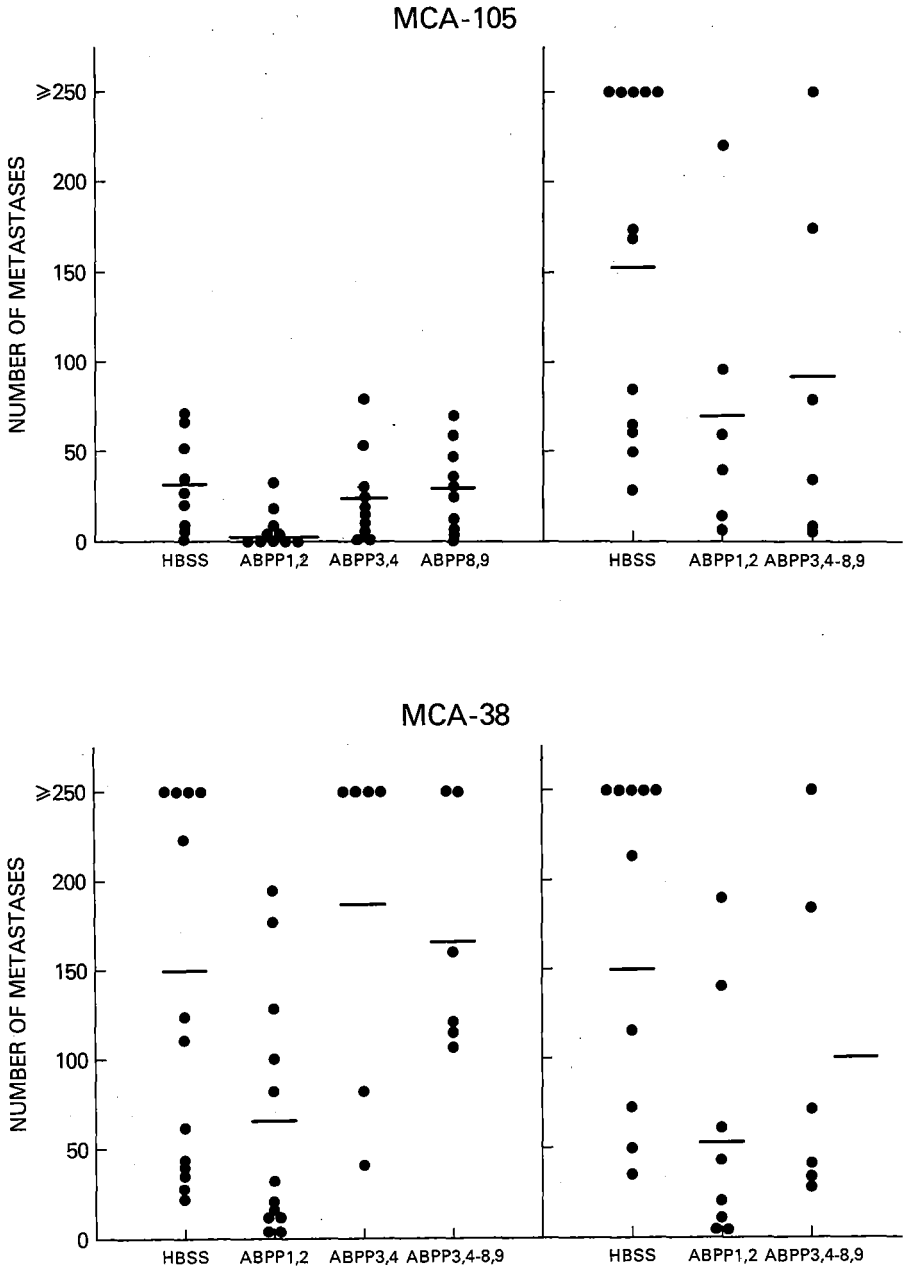
Effects of ABPP in a pulmonary metastasis model

BL/6 mice received 3 x 10^5 tumor cells i.v. on day 0 (In the first experiment with MCA-105 1 x 10^5 cells were given i.v.). Treatment with ABPP was given in the same manner as in the intraperitoneal tumor experiments. On day 14 the mice were sacrificed, the lungs inflated with Indian ink and harvested and put in Fekette's Solution. After this procedure the tumor colonies in the lung were enumerated in a blinded fashion. The results are depicted in Figure 4. In the pulmonary metastasis model the results were similar to those obtained in the intraperitoneal model. Again the antitumor effects of ABPP were primarily influenced by tumor load. Again the immunogenic tumors MCA-105 and MCA-38 seemed more sensitive to the effects of ABPP than the nonimmunogenic tumor MCA-102.

Effects of ABPP in combination with LAK cells and IL-2

BL/6 mice were inoculated i.p. with 1 x 10^5 MCA-105 tumor cells and were treated with 10,000 units of IL-2 from day 3 through 7. ABPP was administered before or at the same time as the IL-2 + LAK treatment cycle. LAK cells ($1x10^8$) were given i.p. on day 3 in 2 experiments. The mice were sacrificed on day 14 and scored for i.p. tumor. When ABPP was administered early (day 1, 2) an additional tumor reducing effect was seen. The results of the experiments in which the administration of ABPP preceeded the administration of IL-2 and LAK are summarized above in Table II. Administration of ABPP concurrently with a course of IL-2 and LAK (on day 3 and 4) or afterwards (on day 8 and 9) did not effect the efficacy of treatment with IL-2 and LAK cells (these data are not shown).

Figure 4 : Antitumor effects of ABPP in the pulmonary metastasis models against immunogenic and nonimmunogenic tumors



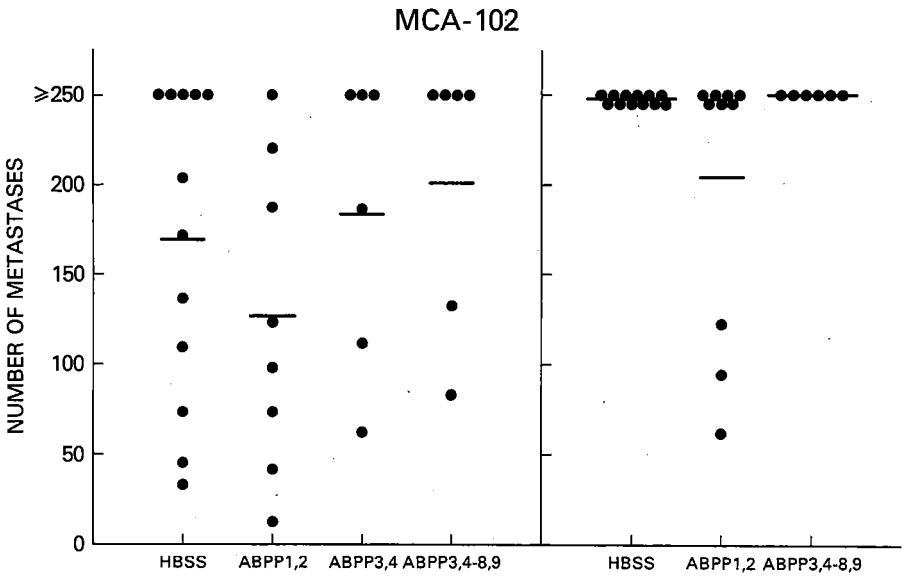


Figure 4 (top, middle, bottom) : Antitumor effect of ABPP on pulmonary metastases. BL/6 mice were inoculated with 1 x 10^5 MCA-105 tumor cells i.v. (top-left) and with 3 x 10^5 cells in all other experiments. ABPP given on day 1,2 or 3,4 and/or 8,9 as shown at the bottom of the scatter diagram. The number of pulmonary metastases was scored on day 14. Early administration of ABPP reduced the number pulmonary metastases of the immunogenic tumors significantly in all experiments (MCA-105: $p < 0.05$; MCA-38: $p < 0.01$) but had no clear antitumor effect on the nonimmunogenic tumor MCA-102. ABPP had no significant effect against established tumor regardless of its immunogenicity.

TABLE II

EFFECT OF EARLY ADMINISTRATION OF ABPP ON DAY 1 AND 2 FOLLOWED BY IL-2 + LAK

MEAN PCI ± SEM

EXP	CONTROL	ABPP 1-2	IL-2	ABPP1-2/IL-2	IL-2+LAK	ABPP1-2/IL-2+LAK
1	3.0 ± 0	2.17 ± 0.40 (0.02)	2.67 ± 0.34 (NS)	1.00 ± 0.37 (0.00008)	2.17 ± 0.31 (0.003)	0.83 ± 0.40 (0.00008)
			-(0.008)-		-(0.04)-	
2	3.0 ± 0	2.17 ± 0.30 (0.009)	2.50 ± 0.34 (NS)	1.33 ± 0.33 (0.0004)	-	-
			-(0.04)-			
3	2.87 ± 0.09	2.17 ± 0.31 (0.02)	2.33 ± 0.33 (NS)	1.83 ± 0.40 (0.008)	2.00 ± 0.26 (0.002)	2.00 ± 0.26 (0.002)
			- NS -		- NS -	

PCI = peritoneal cancer index ; SEM = standard error of the mean ; ABPP was administered at a dose of 250mg/kg, i.p., on day 1 and 2 after i.p. tumor inoculation. 1 x 10^8 LAK cells were administered i.p. on day 3 and IL-2 was given at a dose of 10,000 units, i.p., twice a day, from day 3 through 7. The mice were sacrificed at day 14 and their i.p. tumor load was scored blindly with the PCI scoring system. P_2-values of pair-wise comparisons with the control animals are shown between brackets.

DISCUSSION

Lymphokine activated killer cell activity has been defined as the capacity of lymphoid cells to lyse fresh tumor cells in 4 hour ^{51}Chromium release assays (Rosenberg, 1984; Rosenstein et al, 1984). Interleukin-2 has been shown to be the crucial lymphokine in inducing this capacity (Grimm et al, 1983). The experiments described in this communication are the first to demonstrate that the i.p. administration of the interferon inducer ABPP generates lymphoid cells in the peritoneal exudate, and to a much lesser degree in the spleen, that can lyse fresh tumor targets and thus by definition display LAK cell activity. Apparently the induction of endogenous interferon type I leads to a cascade of events that may well include the production of endogenous IL-2 by activated NK cells (Kasahara et al, 1983) and the subsequent induction of LAK cell activity. Even though this activation process by way of the induction of endogenous lymphokines quite effectively generated a level of LAK cell activity comparable to the effects of a 3 day course of moderate doses of IL-2 b.i.d., only a limited level of LAK cell activity can be induced by ABPP. This is due to the fact that repetitive doses of ABPP do not further increase the endogenous production of lymphokines and induce a state of hyporesponsiveness (Oku et al, 1984). This is commonly seen after multiple administrations of several biological response modifiers (Talmadge et al., 1985) and may be due to a subsequent decrease in the number of large granular lymphocytes (Saito et al., 1985). The observation that the level of LAK cell activity as well as the numbers of LAK cells generated in vivo depended on the dose of IL-2 used clearly underlines the therapeutic potential of this lymphokine. This proliferation of activated lymphoid cells by high doses of IL-2 , as demonstrated by Ettinghausen and collegues (1985), is evidently an important aspect of the efficacy of treatment of intraperitoneal tumor with moderately high doses of IL-2 alone (Ottow et al., 1987b; Eggermont et al.,1987c; 1987h). The toxicity associated with these doses of IL-2 however is quite serious (Chang et al., 1984; Matory et al., 1985; Lotze et al., 1986) and is the main obstacle to exploit the antitumor effects of IL-2 maximally.

Since ABPP may upregulate the expression of IL-2 receptors (Brrideau and Wolcott, 1985) we investigated its potential use as a "primer" in order to increase LAK cell generation by IL-2. Splenocytes from mice that were pretreated with ABPP however were no better source for the in vitro generation of LAK cells than splenocytes from untreated mice.

In intraperitoneal tumor experiments ABPP, when administered early (day 1,2), prior to IL-2 and LAK cells, had an additional antitumor effect. This effect most likely reflects the tumor reduction achieved by ABPP on a day 1 tumor load which subsequently rendered the IL-2 plus LAK cell treatment more effective. The lack of potentiation of IL-2 plus LAK effects when ABPP was given concurrently as well as the lack of "priming effect" of ABPP on the in vitro generation of LAK cells as desribed above does not suggest that a synergistic effect between endogenously induced lymphokines and exogenously administered IL-2 exists. In view of recent reports on the negative effects that the administration of exogenous IFN-alpha can have on the activation of lymphocytes by IL-2 (Brunda et al., 1986) it must be stressed that no detrimental effect was seen as a result of the concurrent or prior administration of this interferon inducer.

Initial tumor load was crucial as to whether tumor reduction was achieved by treatment with ABPP. This observation is in line with results reported earlier (Eggermont et al., 1986b). In contrast to our experience with immunogenic and nonimmunogenic tumors in rats (Eggermont et al., 1986a; 1986c) and results in murine models reported by Milas et al., (1982) a difference in sensitivity to treatment with ABPP between immunogenic and nonimmunogenic tumors was seen. The immunogenic tumors, especially the murine adenocarcinoma MCA-38, were more sensitive than the nonimmunogenic sarcomas MCA-101 and -102. This difference in vivo was bigger than the marginal difference in lysability that seemed to exist in vitro. These findings were corroborated by a remarkable efficacy of combined treatment schedules of ABPP and cyclophosphamide of the weakly immunogenic tumors (MCA-105 and MCA-38) as opposed to the much less spectacular results of this combination against the nonimmunogenic tumor MCA-102, as we reported recently in this journal (Eggermont et al., 1987b). This observation may reflect a difference in rate of tumor growth between

the nonimmunogenic and weakly immunogenic tumors. The antitumor effects of ABPP may however be mediated by more mechanisms than by LAK-like mediated cytolysis alone. It is concievable that the polyclonal activation of B cells or the increased production of antibodies (Fast et al., 1982) induced by ABPP enhances the anti-tumor effects of ABPP through antibody dependent cellular cyto-toxicity mediated tumor reduction (manuscript in preparation). This mechanism then could well play a part in the efficacy of ABPP against immunogenic tumors but would be absent in the setting with nonimmunogenic tumors and explain its inefficacy against them.

CHAPTER VII

The content is an adapted version of the publication :

Synergistic antitumor activity of cyclophosphamide and ABPP in the treatment of established and advanced tumors in murine tumor models

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Synergistic antitumor activity of cyclophosphamide and ABPP in the treatment of established and advanced tumors in murine tumor models

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Summary

We have previously shown that the in vivo administration of ABPP, an interferon inducing pyrimidinone, generates activated killer cells that can lyse fresh tumor cells in vitro in 4 hour ^{51}Chromium release assays. The administration of this agent however has no effect on established tumor. In this communication we show that ABPP, when used in combination with low or moderate doses of cyclophosphamide, can be quite effective against early established (day 3) tumor as well as against far advanced, grossly visible (day 8-10) tumor in both the intraperitoneal (i.p.) and the pulmonary metastasis model. The synergistic antitumor activity of this chemoimmunotherapeutic regimen is very strong against immunogenic tumors but rather weak against nonimmunogenic tumors. Two treatment cycles were significantly more effective than one and multiple cycles even cured the majority of mice with established i.p. tumor. These experiments demonstrate the potential of chemoimmunotherapeutic regimens and highlight the efficacy of multiple treatment cycles.

INTRODUCTION

We have shown previously that the intraperitoneal administration of ABPP in BL/6 mice can generate high levels of activated killer cells in the peritoneal exudate (Eggermont et al., 1987a). These cells are capable of lysing fresh tumor cells in 4 hour ^{51}Chromium release assays. They display therefore the lymphokine activated killer cell phenomenon (Rosenstein et al., 1984). Several pyrimidinones have been shown to be potent interferon inducers (Hamilton et al., 1982, Stringfellow et al., 1980a) that modulate a variety of immune responses, in particular the activation of natural killer (NK) cells and macrophages (Lotzova et al., 1983; 1984; 1986; Li et al., 1985; Eggermont et al., 1986a). In various tumor models in mice as well as rats, we have shown that the administration of ABPP alone has no significant antitumor effect against established tumor (Eggermont et al., 1986b; 1987a). This is in line with observations made by others on the

antitumor effects of biological response modifiers (BRM) (Bast and Bast., 1976). The goal of this study was to investigate whether ABPP in combination with cyclophosphamide could be used successfully against established tumor. Li and colleagues (1984) have shown that the antitumor effect of ABPP against leukemia and the B16 melanoma can be greatly enhanced by early administration of cyclophosphamide preceding ABPP. We will show that significant reduction in tumor load and a survival benefit can be achieved when ABPP is administered in combination with cyclophosphamide in the treatment of established and even macroscopically visible metastatic tumor. Furthermore, we will show that the degree of synergism in antitumor effects is related to the immunogenicity of the tumors.

MATERIAL AND METHODS

Mice

C57BL/6 (BL/6) female mice were obtained from Jackson Laboratory (Bar Harbor, ME) and used 9-12 weeks old. They were maintained on laboratory chow and acidified water ad libitum in a pathogen free environment.

Tumors

The MCA-101, -102, -105, and -106 sarcomas and the MCA-38 colon adenocarcinoma used in these experiments are syngeneic to BL/6 mice and were described in chapter VI. Single cell suspensions of all tumors used were prepared as described previously (Mule et al., 1984).

AGENTS

ABPP : ABPP was administered i.p. at a dose of 250 mg/kg dissolved in 1 ml phosphate buffered saline (PBS) at various time intervals after tumor inoculation depeding on experimental design.

Cyclophosphamide (CYTOXAN, CY) : was purchased from Mead and Johnson and Co. (Evansville, IN). It was dissolved in sterile water to a concentration of 200 mg/ml and further diluted with HBSS to a concentration depending on the experimental design.

Tumor models

Intraperitoneal tumor model : BL/6 mice received 1 x 10^5 tumor cells i.p. and were randomly allocated into a treatment group. Treatment with ABPP was started on day 1, 3 or 8 and consisted of one i.p. injection of 250 mg/kg on two consecutive days. Treatment schedules are shown in Tables and the legends to the figures. CY was administered i.p. or i.v. at various doses depending on experimental design. In combination treatment regimens CY was given 12 hours before the first of two ABBP injections. Control animals received equal volumes of HBSS needed for the administration of CY of ABPP. Fourteen days after the i.p. inoculation of tumor the animals were sacrified and their intraperitoneal tumor load was assessed in a blinded fashion on a scale from 0 - 3. The score was termed the Peritoneal Cancer Index (PCI). Teh procedure was described in chapter VI. Each experimental group in the i.p. tumor experiments consisted of 6 - 12 mice.

Pulmonary metastasis model : BL/6 mice received 3 x 10^5 syngeneic tumor cells intravenously by way of the lateral tail vein. Treatment schedules were similar to those used in the intraperitoneal tumor experiments. The mice were sacrificed after each treatment group was given a letter code. The lungs of the mice were inflated with a 15% solution of india ink for enumeration of metastatic pulmonary nodules (Wexler, 1966). Metastases are readily countable when "bleached" by Fekette's solution up to $\pm$ 250. Lungs with metastases too numerous to count were therefore assigned an arbitrary value of 250. In both tumor models some experiments were scored for survival.

Statistical Analysis

Overall significance of difference in the intraperitoneal tumor experiments was examined with the Jonckheere test for trend (Hollander and Wolfe, 1973). If this test showed a two sided p value $\leq$ 0.05, pairwise comparisons of differences in tumor load were examined with the Wilcoxon rank sum test with a correction for ties. Significance of differences in the numbers of pulmonary metastases or differences in survival between groups was determined by the Wilcoxon rank sum test (Gehan, 1965). Two-sided p values are presented in all experiments. In all intraperitoneal tumor experiments where the end point was scored with the peri-

toneal cancer index, the size of the experimental groups varied from 6 to 12 animals. In all pulmonary metastasis experiments the experimental groups consisted of 12 animals. In all survival experiments size varied from 12 - 24 animals per group.

RESULTS

Effect of different doses of cyclophosphamide on intraperitoneal tumor growth

BL/6 mice where inoculated intraperitoneally (i.p.) with 1 x 10^5 MCA-tumor cells. On day 3, 8, or on day 3 and day 8, the mice received 10 mg/kg up to 75 mg/kg of CY i.p. or i.v. depending on experimental design. Around day 14, the mice were sacrified and the intraperitoneal tumor load was assessed in a blinded way using the peritoneal cancer index scoring system. The results are summarized in Table I. It is clearly shown that reduction of i.p. tumor load by CY is a dose-related phenomenon. When doses of 50 mg/kg or higher are used, a consistently significant reduction in i.p. tumor load is achieved when CY is administered on day 3. The administration of this dose on day 8 only seems to have little if any effect. However, the administration of 50 up to 75 mg/kg of CY i.p. or i.v. had a consistent impact on i.p. tumor load. In most experiments that will be discussed, CY was given i.p. at a dose of 50 mg/kg. As is shown in Table I there was no difference in antitumor effect after i.p. or i.v. administration.

The antitumor effect of cyclophosphamide is related to the immunogenicity of the tumor

BL/6 mice were inoculated with 1 x 10^5 tumor cells of the immunogenic MCA-105 or -38, or the nonimmunogenic MCA-101 or 102. They were treated with CY (50 mg/kg) on day 3 ,8 or 3 and 8. The results are summarized in Table II. The immunogenic tumors appeared to be quite sensitive to treatment with CY in contrast to the nonimmunogenic tumors. A single dose of CY on day 8 was ineffective in virtually all experiments regardless of the tumor. In contrast when preceded by a dose on day 3 a strong additive antitumor effect was seen.

Table I.

EFFECT OF DIFFERENT DOSES OF CYCLOPHOSPHAMIDE ON INTRAPERITONEAL TUMOR GROWTH

MEAN PCI ± SEM

TUMOR	EXP.	DOSE	CONTROL	DAY 3	DAY 8	DAY 3-8
MCA-105	1 (A)	10 mg/kg (iv)	2.75 ± 0.13	2.67 ± 0.21	2.67 ± 0.21	3.00 ± 0
MCA-105	1 (B)	10 mg/kg (ip)	2.75 ± 0.13	2.67 ± 0.21	2.50 ± 0.22	2.83 ± 0.17
MCA-105	2	25 mg/kg (ip)	2.80 ± 0.09	2.67 ± 0.21	-	2.50 ± 0.34
MCA-105	3	25 mg/kg (ip)	2.67 ± 0.14	-	2.50 ± 0.34	1.83 ± 0.40 (0.07)
MCA-105	4	25 mg/kg (ip)	2.75 ± 0.11	2.67 ± 0.21	2.50 ± 0.34	2.17 ± 0.27
MCA-105	5	50 mg/kg (ip)	2.83 ± 0.11	2.50 ± 0.22	-	1.83 ± 0.31 (0.006)
MCA-105	6	50 mg/kg (ip)	2.87 ± 0.09	1.83 ± 0.31 (0.002)	-	0.50 ± 0.21 (0.00005)
MCA-105	7 (A)	50 mg/kg (iv)	2.86 ± 0.10	2.08 ± 0.23 (0.02)	-	1.08 ± 0.19 (0.00002)
MCA-105	7 (B)	50 mg/kg (ip)	2.86 ± 0.10	2.00 ± 0.26 (0.004)	-	1.33 ± 0.33 (0.0003)
MCA-105	8	50 mg/kg (ip)	2.80 ± 0.09	2.50 ± 0.22	2.83 ± 0.17	1.17 ± 0.31 (0.0002)
MCA-105	9	50 mg/kg (ip)	2.67 ± 0.14	-	2.17 ± 0.40	0.50 ± 0.22 (0.0004)
MCA-105	10	75 mg/kg (ip)	2.80 ± 0.09	1.50 ± 0.43 (0.002)	-	0.50 ± 0.22 (0.00004)
MCA-105	11	75 mg/kg (ip)	2.67 ± 0.14	-	1.83 ± 0.31 (0.03)	0.17 ± 0.17 (0.0004)
MCA-105	12	75 mg/kg (ip)	2.75 ± 0.11	1.50 ± 0.43 (0.005)	2.17 ± 0.40	0.33 ± 0.14 (0.000005)

Table II

EFFECT OF CYCLOPHOSPHAMIDE (50 MG/KG) ON INTRAPERITONEAL TUMOR GROWTH OF IMMUNOGENIC AND NONIMMUNOGENIC TUMORS

MEAN PCI ± SEM

TUMOR	EXP.	CONTROL	DAY 3	DAY 8	DAY 3-8
MCA-105	1	2.83 ± 0.11	2.50 ± 0.22	-	1.83 ± 0.31 (0.006)
MCA-105	2	2.87 ± 0.09	1.83 ± 0.31 (0.002)	-	0.50 ± 0.21 (0.00005)
MCA-105	3(iv)	2.86 ± 0.10	2.08 ± 0.23 (0.2)	-	1.08 ± 0.19 (0.00002)
MCA-105	3(ip)	2.86 ± 0.10	2.00 ± 0.26 (0.004)	-	1.33 ± 0.33 (0.0003)
MCA-105	4	2.80 ± 0.09	2.50 ± 0.22	2.83 ± 0.17	1.17 ± 0.31 (0.0002)
MCA-105	5	2.67 ± 0.14	-	2.17 ± 0.40	0.50 ± 0.22 (0.0004)
MCA-38	6	2.80 ± 0.13	1.67 ± 0.22 (0.002)	2.08 ± 0.23 (0.03)	0.33 ± 0.14 (0.00004)
MCA-38	7	2.47 ± 0.17	1.40 ± 0.22 (0.004)	2.20 ± 0.23	0.60 ± 0.22 (0.0002)
MCA-101	8	2.10 ± 0.23	-	-	1.60 ± 0.43
MCA-102	9	2.58 ± 0.15	2.00 ± 0.21 (0.05)	2.60 ± 0.22	2.00 ± 0.25 (0.08)
MCA-102	10	2.67 ± 0.19	2.00 ± 0.25 (0.05)	-	-

Early chemoimmunotherapy of immunogenic and nonimmunogenic intraperitoneal tumors

BL/6 mice received 1 x 10^5 MCA-105, 38, or 102 tumor cells i.p. and were subsequently treated on day 3 with 10 mg/kg up to 75 mg/kg of CY i.p. or i.v. depending on experimental design as is shown in **Table III**. 12 Hours after the administration of CY, 250 mg/kg of ABPP was administered i.p. The antitumor effects achieved by CY alone, ABPP on day 3 and 4 or by the combination of CY and ABPP are shown in Table III. It is clearly demonstrated that when CY was given prior to ABPP the most powerful antitumor effect was seen which led consistently to a highly significant reduction in i.p. tumor load. Even when only 10 mg/kg of CY was administered, the combination with ABPP reduced i.p. tumor significantly. It must further be noted that the administration of ABPP alone had very little effect and that the administration of various doses of CY alone resulted in a dose dependent reduction of i.p. tumor. Furthermore, the immunogenic tumors MCA-105 and MCA-38 seemed to be more sensitive to the antitumor effects of CY. In 4 out of 6 experiments, when a dose of 50 mg per kg of CY was used, the combined treatment with ABPP was significantly better than the treatment with CY alone and always more effective than the treatment with ABPP alone.

Effects of late chemoimmunotherapy on immunogenic and nonimmunogenic intraperitoneal tumors

The antitumor effects of CY (50 mg/kg, i.p.) and the administration of 250 mg/kg of ABPP i.p. on day 8 and 9 or the combination of these two drugs against far advanced tumor (day 8 represents grossly visible i.p. tumor) was assessed. The results are shown in **Table IV**. A significant reduction in i.p. tumor load was achieved in all experiments by combination treatment. There seemed to be a difference in sensitivity to this treatment between the different tumors. The nonimmunogenic MCA-102 showed hardly any tumor reduction after treatment. The tumor reduction achieved with combination treatment against MCA-105 was consistent but marginal. Against MCA-38, however, a highly significant tumor reduction was obtained in all three experiments and combination treatment was clearly better than treatment with CY alone.

Table III

EARLY CHEMOIMMUNOTHERAPY OF IMMUNOGENIC AND NONIMMUNOGENIC INTRAPERITONEAL TUMORS

MEAN PCI ± SEM

TUMOR	DOSE CY (mg/kg)	CONTROL	ABPP 3-4	CY 3	CY 3/ABPP 3-4
MCA-105	10	2.75 ± 0.13	2.67 ± 0.21	2.67 ± 0.21	1.83 ± 0.40 (0.06)
MCA-105	10	2.75 ± 0.13	2.67 ± 0.21	2.67 ± 0.21	2.00 ± 0.26 (0.02)
MCA-105	50	2.87 ± 0.09	2.83 ± 0.17	1.83 ± 0.31 (0.002)	0.50 ± 0.34 (0.00007)
				0.004	
MCA-105	50	2.86 ± 0.10	3.00 ± 0.0	2.08 ± 0.23 (0.02)	1.00 ± 0.37 (0.0003)
				0.003	
MCA-105	25	2.80 ± 0.09	2.67 ± 0.17	2.67 ± 0.21	2.33 ± 0.21 (0.04)
	50			2.50 ± 0.22	2.00 ± 0.37 (0.02)
	75			1.50 ± 0.43 (0.002)	1.17 ± 0.31 (0.0002)
				0.008	
MCA-38	50	2.80 ± 0.13	2.42 ± 0.15	1.67 ± 0.22 (0.002)	0.75 ± 0.25 (0.0001)
				0.0002	
MCA-38	50	2.47 ± 0.17	2.20 ± 0.29	1.40 ± 0.22 (0.004)	1.00 ± 0.52 (0.02)
				0.06	
MCA-38	50	2.60 ± 0.13	2.32 ± 0.15 (0.04)	1.55 ± 0.16 (0.00004)	0.83 ± 0.23 (0.000004)
				0.0003	
MCA-102	50	2.58 ± 0.15	1.90 ± 0.28 (0.06)	2.00 ± 0.21 (0.05)	1.92 ± 0.23 (0.04)

Table IV

LATE CHEMOIMMUNOTHERAPY OF IMMUNOGENIC AND NONIMMUNOGENIC INTRAPERITONEAL TUMORS

MEAN PCI ± SEM

EXP	TUMOR	CONTROL	ABPP 8,9	CY(50mg/kg) day 8	CY 8/ABPP8,9
1	MCA-105	2.75 ± 0.11	2.83 ± 0.17	2.50 ± 0.23	2.33± 0.19 (0.07)
2	MCA-105	2.80 ± 0.09	2.83 ± 0.17	2.83 ± 0.17	2.67 ± 0.21
3	MCA-105	2.67 ± 0.14	2.83 ± 0.17	2.17 ± 0.40	2.00 ± 0.26
			¦ 0.03 ¦		
4	MCA-38	2.60 ± 0.13	2.67 ± 0.11	2.14 ± 0.17 (0.03) - 0.03	1.44 ± 0.23 - (0.0003)
			¦ 0.0003 ¦		
5	MCA-38	2.80 ± 0.13	2.58 ± 0.15	2.08 ± 0.23 (0.03) - 0.08	1.33 ± 0.31 - (0.002)
			¦ 0.004 ¦		
6	MCA-38	2.47 ± 0.19	2.83 ± 0.17	2.20 ± 0.25	1.67 ± 0.33 (0.05)
			¦ 0.03 ¦		
7	MCA-102	2.58 ± 0.15	2.50 ± 0.21	2.60 ± 0.22	2.25 ± 0.25

Effects of two courses of chemoimmunotherapy on early established intraperitoneal tumor

ABPP was administered on days 3 and 4, and days 8 and 9 after i.p. tumor inoculation. CY was given either alone on day 3 and 8 or on day 3 and 8, twelve hours prior to the administration of ABPP. An important and consistent highly significant reduction of i.p. tumor was seen in those mice treated with the combination of CY and ABPP. The results are shown in the **Tables V_a and V_b.**

Table Va : EFFECT OF 2 COURSES OF CHEMOIMMUNOTHERAPY ON INTRAPERITONEAL MCA-105

MEAN PCI ± SEM

EXP	TUMOR	DOSE CY (mg/kg)	CONTROL	ABPP 34-89	CY 3-8	CY 3-8/ABPP 34-89
1 ip	MCA-105	50	2.87 ± 0.09	2.50 ± 0.21	0.50 ± 0.21	0.00 ± 0.00
				(0.08)	(0.00005) - 0.8 - (0.00004)	
					0.002	
1 iv	MCA-105	50	2.86 ± 0.10	3.00 ± 0.00	1.08 ± 0.19	0.83 ± 0.31
					(0.00002)	(0.0002)
					0.003	
2 iv	MCA-105	10	2.75 ± 0.13	2.67 ± 0.21	2.83 ± 0.17	1.40 ± 0.37
					(NS) - 0.02 - (0.004)	
					0.04	
2 ip		10	2.75 ± 0.13	2.67 ± 0.21	3.00 ± 0.00	1.17 ± 0.31
					(NS) - 0.003 - (0.0009)	
					0.008	
3 ip	MCA-105	25	2.80 ± 0.09	2.67 ± 0.21	2.50 ± 0.34	2.33 ± 0.33
		50			1.17 ± 0.31	0.33 ± 0.21
					(0.0002) -0.07- (0.00004)	
					0.003	
		75			0.50 ± 0.22	0.00 ± 0.00
					(0.00004)-0.08-(0.00003)	
					0.002	
4 ip	MCA-105	50	2.67 ± 0.14	2.83 ± 0.17	0.50 ± 0.22	0.00 ± 0.00
					(0.0004)-0.08-(0.0003)	
					0.002	

TABLE Vb

EFFECT OF 2 COURSES OF CHEMOIMMUNOTHERAPY ON INTRAPERITONEAL MCA-38, -101 AND -102

MEAN PCI ± SEM

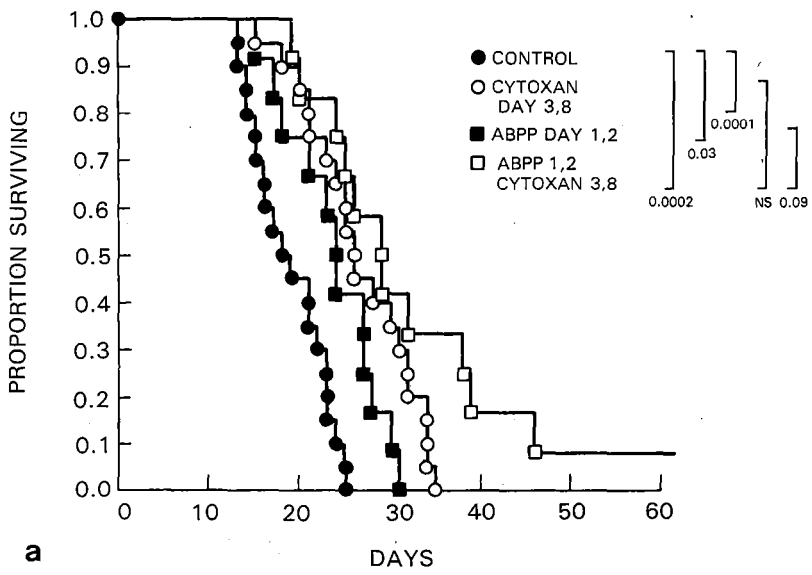
EXP	TUMOR	DOSE CY (mg/kg)	CONTROL	ABPP 34-89	CY 3-8	CY3-8/ABPP 34-89
5 ip	MCA-38	50	2.80 ± 0.13	2.42 ± 0.15 (NS)	0.22 ± 0.14 (0.00004)	0.00 ± 000 -0.05-(0.00002) 0.0009
6 ip	MCA-38	50	2.60 ± 0.13	2.60 ± 0.16 (NS)	0.45 ± 0.13 (0.000003)	0.09 ± 0.06 -0.02-(0.000003) 0.000003
7 ip	MCA-101	50	2.10 ± 0.23	2.83 ± 0.17	1.60 ± 0.43	1.00 ± 0.37 (0.04) 0.05
8 ip	MCA-102	50	2.58 ± 0.15	2.33 ± 0.22 (NS)	2.00 ± 0.25 (0.08)	2.00 ± 0.21 - NS - (0.05) NS

The antitumor effect of this combination treatment was especially powerful against the immunogenic tumors MCA-105 and MCA-38. A modest reduction in i.p.tumor load was obtained against the non-immunogenic tumors MCA-101 and 102. The combination treatment of immunogenic tumors was consistently better than treatment with ABPP alone or with CY alone. These differences were much less pronounced with the nonimmunogenic tumors.

Efficacy of chemoimmunotherapy with cyclophosphamide and ABPP prolongs survival in intraperitoneal tumor model

As is shown in **Figures 1A, 1B and 1C**, the combination of ABPP and CY , administered at different intervals after the i.p. inoculation of MCA-105 tumor was always superior to single agent therapy. The combination treatment enhanced antitumor effects and prolonged survival. These results confirmed the results described above in the PCI experiments with tumor MCA-105.

Figure I (a-b-c) : Efficacy of Chemoimmunotherapy on i.p. MCA-105



a

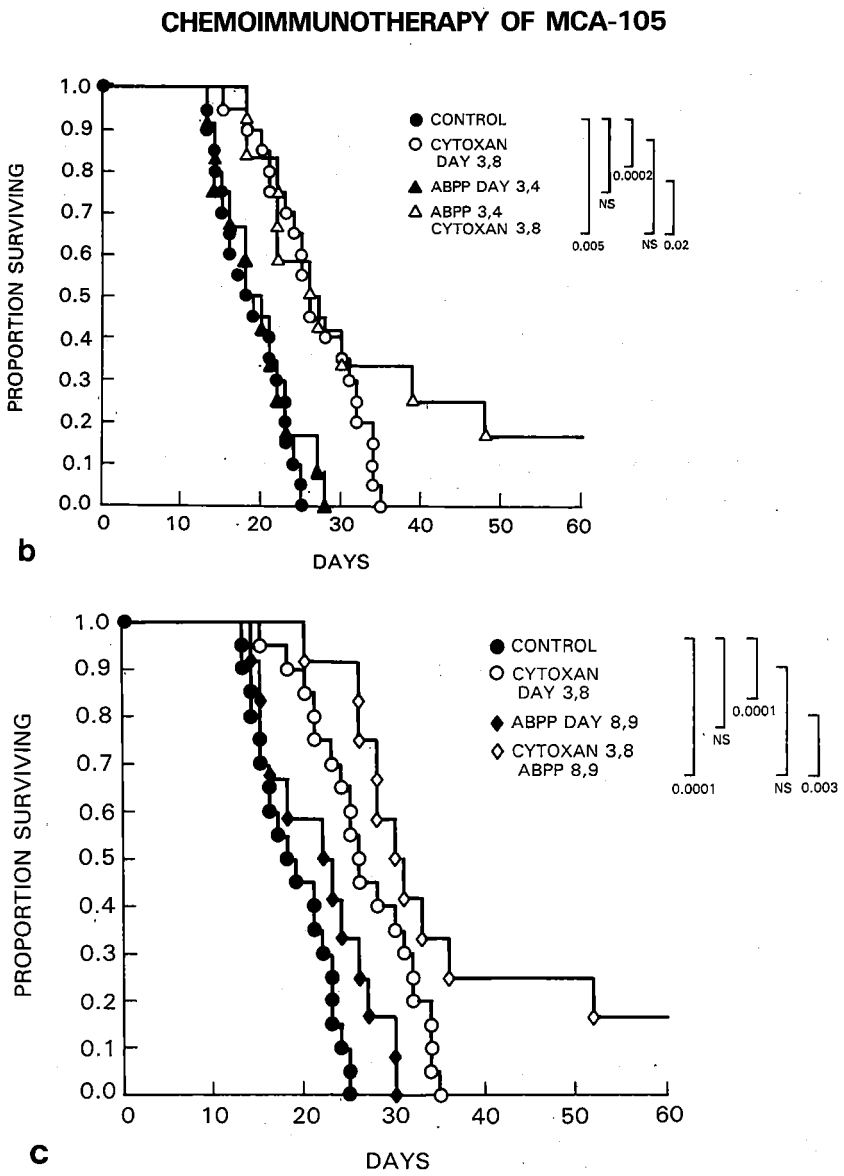


Figure 1 : Superiority of combination treatment with CY and ABPP over single agent treatment. BL/6 mice received 1 x 10^5 MCA-105 tumor cells ip and were treated with different treatment schedules with CY and/or ABPP as indicated in the right upper corners of the survival curves. CY always enhanced the antitumor effect of ABPP especially against established (middle)(B) and advanced ip tumor (bottom)(C).

High cure rate by weekly administration of combination treatment with cyclophosphamide and ABPP in mice with early established intraperitoneal tumor

An extraordinary potentiation of treatment was achieved by giving CY (50 mg/kg) weekly followed by one injection of ABPP 12 or 24 hours later. The results of this drug combination schedule are shown in Figure 2. This treatment resulted in more than 60% long term survivors. These mice all lived for 100 days at which point in time they were sacrificed and an autopsy was performed. None of the mice had tumor in the peritoneal cavity upon examination.

Figure 2 : Cure of early established intraperitoneal tumor by weekly administration of cyclophosphamide and ABPP

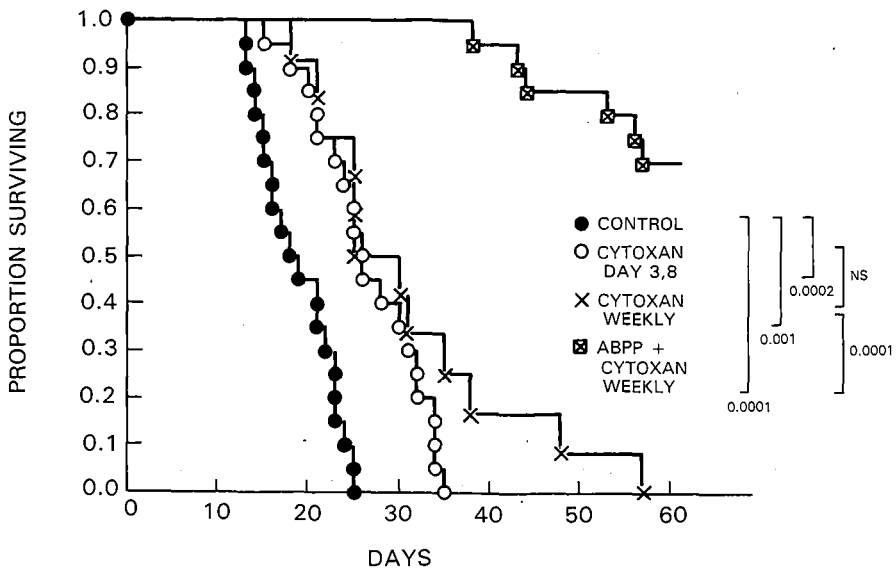


Figure 2 : Cure by weekly administration of combination treatment Treatment of established MCA-105 tumor ip in BL/6 mice with a weekly administration of CY and ABPP demonstrated highly synergistic antitumor activity and cured the majority of mice.

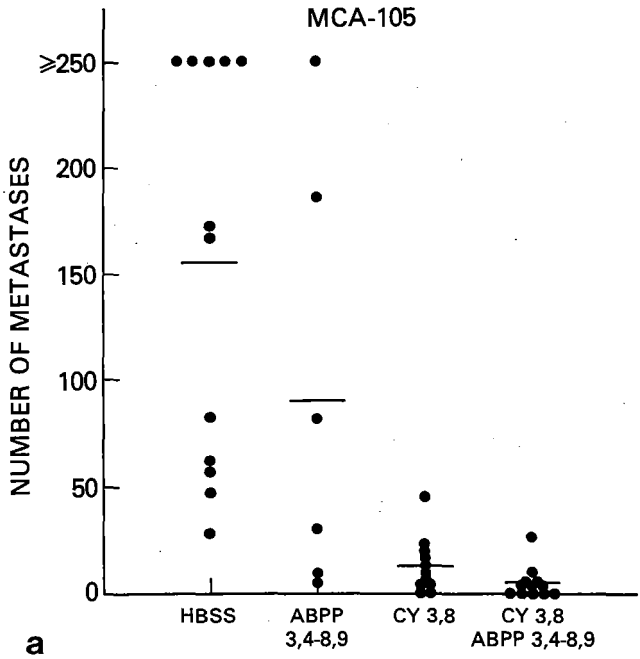
Significant reduction of pulmonary metastases of immunogenic tumors but not of nonimmunogenic tumors after chemoimmunotherapy with cyclophosphamide and ABPP

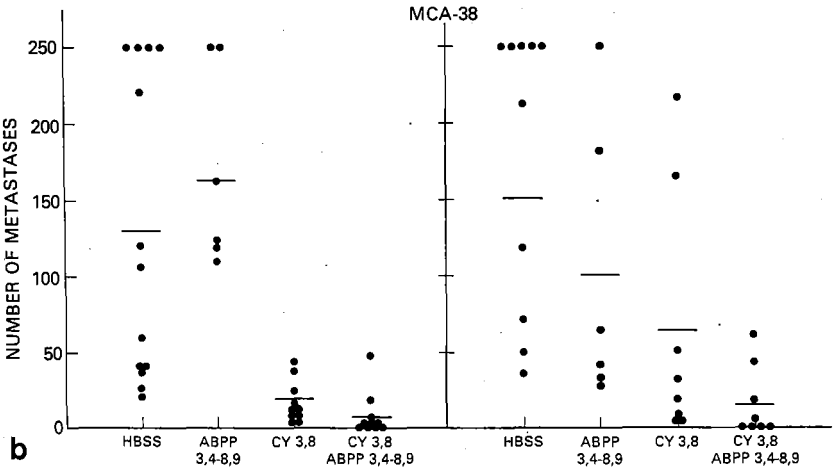
BL/6 mice were inoculated with 3 x 10^5 tumor cells of either the weakly immunogenic tumors MCA-105 or MCA-38 or the nonimmunogenic tumor MCA-102, intravenously on day 0. CY (50 mg/kg) was given on day 3 and ABPP was administered either alone or 12 hours after the administration of CY. As is demonstrated by the scatter diagrams in **Figures 3A, 3B and 3C**, a dramatic reduction in the number of pulmonary metastases was seen after treatment with the combination of CY and ABPP in the mice with the immunogenic tumors MCA-105 and MCA-38, but not so in the mice inoculated with the nonimmunogenic tumor MCA-102.

Effects of cyclophosphamide, ABPP or the combination treatment on advanced pulmonary metastases

In the next series of experiments, the impact on grossly visible lung metastases was evaluated. BL/6 mice were inoculated with 3 x 10^5 tumor cells of either MCA-105, MCA-38, or MCA-102. Treatment was started on day 10. The mice either received HBSS or one injection of CY (100 mg/kg) i.p. or one injection of ABPP 250 mg/kg on day 10 and 11 alone or 12 hours after the administration of CY. The survival curves in **Figure 4 a, b and c** show a significant survival benefit as a result of the treatment with 100 mg/kg of CY i.p. This effect was significantly enhanced if the treatment with CY was followed by two injections of ABPP on days 10 and 11 in the mice inoculated with the immunogenic tumors. With MCA-102 this phenomenon was much weaker. These experiments were all repeated with virtually identical results.

Figure 3 (a,b,c) : Efficacy of combination treatment of established pulmonary metastases with CY and ABPP depends on immunogenicity of the tumor. BL/6 mice received 3 x 10^5 tumor cells iv and were treated with CY (50 mg/kg) and/or ABPP as indicated on the x-axis of the scatter diagrams. Each dot represents one mouse and indi-cates the number of pulmonary metastases in this mouse as scored on day 14. Treatment of the immunogenic tumors MCA-105 (3A) and MCA-38 (3B) with CY and especially with the combination of CY and ABPP was very effective. MCA-105 : HBSS vs CY : $p < 0.0001$; HBSS vs CY+ABPP : $p < 0.0001$; CY vs CY+ABPP : $p < 0.05$); MCA-38 : Left: HBSS vs CY : $p < 0.0005$; Right : $p < 0.01$; HBSS vs CY+ABPP : Left: $p < 0.0005$; Right : $p < 0.005$; left: CY vs CY+ABPP: $p < 0.05$; right : N.S. No significant reduction was seen after treat -ment with ABPP on days 3,4 and 8,9 in any experi-ment. CY+ABPP was significantly better in all experiments than treatment with ABPP only. No significant reduction of tumor was seen with the nonimmunogenic MCA-102 (3C) with any treatment schedule.




MCA-38
NUMBER OF METASTASES
250
200
150
100
50
0
HBSS
ABPP 3,4-8,9
CY 3,8
CY 3,8 ABPP 3,4-8,9
HBSS
ABPP 3,4-8,9
CY 3,8
CY 3,8 ABPP 3,4-8,9

b

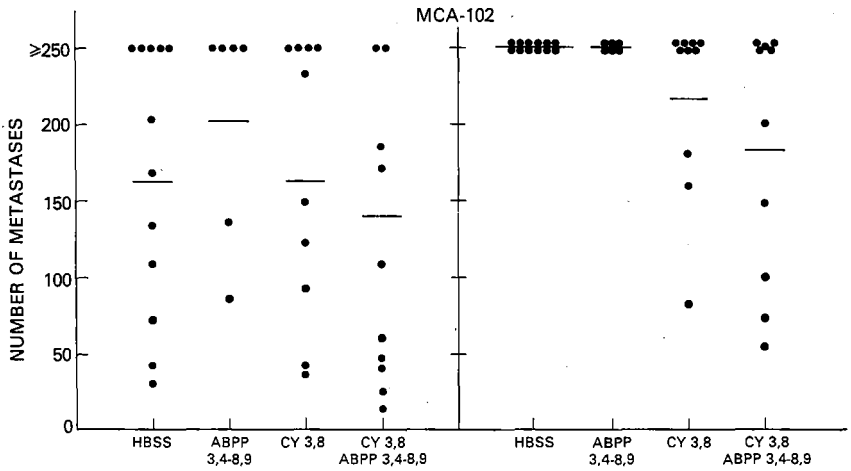
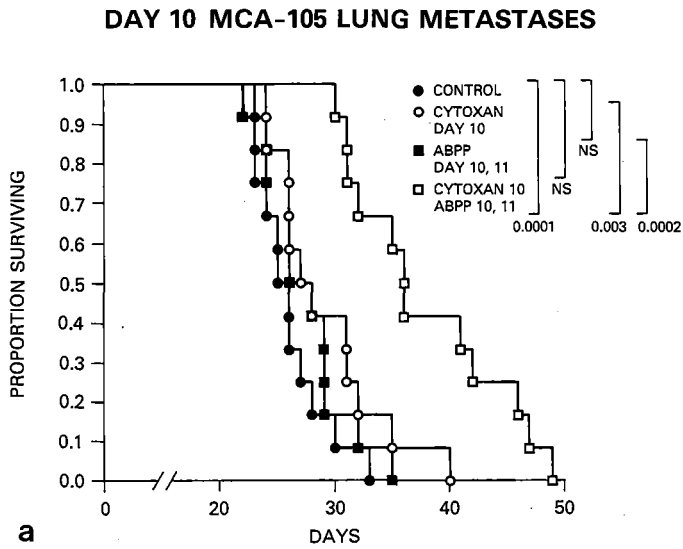
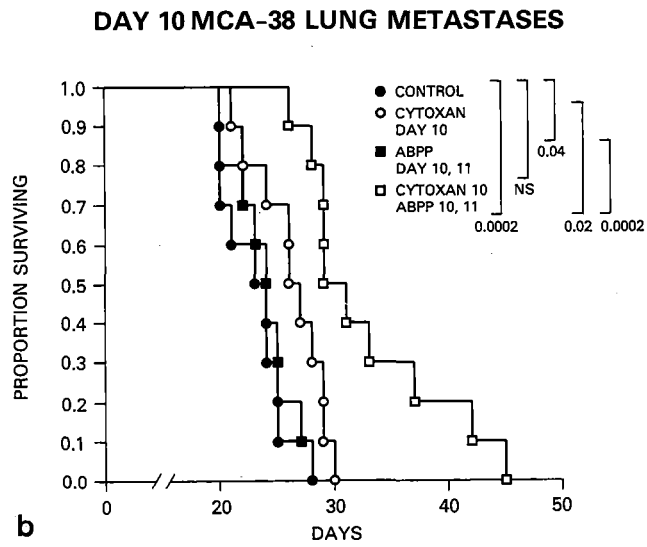

MCA-102
NUMBER OF METASTASES
≥250
200
150
100
50
0
HBSS
ABPP 3,4-8,9
CY 3,8
CY 3,8 ABPP 3,4-8,9
HBSS
ABPP 3,4-8,9
CY 3,8
CY 3,8 ABPP 3,4-8,9

Figure 4 (a-b-c) :
Effect of Chemoimmunotherapy on advanced pulmonary metastases



a



b

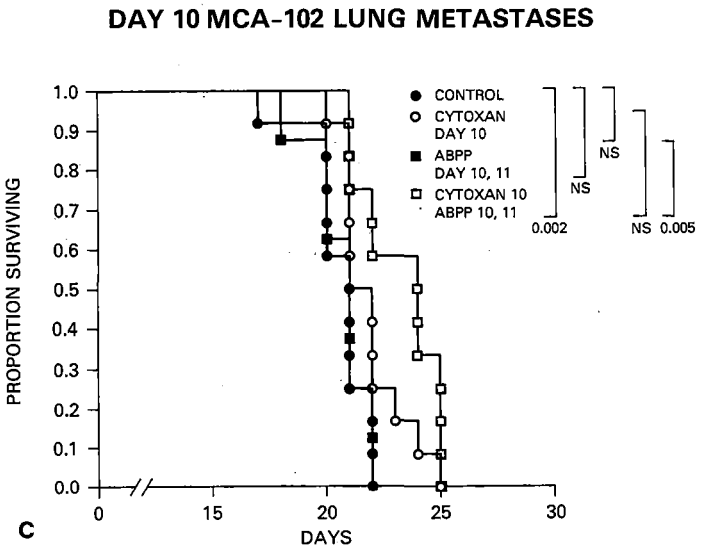


Figure 4 (a,b,c) : Efficacy of treatment of advanced pulmonary metastases with CY and ABPP depends on immunogenicity of the tumor. The survival curves of mice with advanced (day 10) pulmonary metastases of MCA-105 (A), MCA-38 (B) and MCA-102 (C) treated with CY and/or ABPP demonstrate the efficacy of CY and especially of the combined treatment of CY+ABPP against the immunogenic tumors (A and B). MCA-102 appears much less sensitive. ABPP alone has no antitumor effect against day 10 metastases regardless of tumor immunogenicity.

DISCUSSION

The experiments presented here are the first to show that ABPP can be used effectively in the treatment of established and advanced tumor. The administration of ABPP alone was totally ineffective in this setting. The administration of low or moderate doses of CY had a moderate effect against early established tumors, but very little against advanced tumors. In contrast, combination treatment with CY and ABPP was quite effective and displayed a clearly synergistic antitumor effect. Similar synergism has been observed with combined treatment of early established intraperitoneal tumor with cyclophosphamide and IL-2 and LAK cells (Eggermont and Sugarbaker., 1987g). Multiple cycles of the combined treatment modality were very effective and cured the majority of mice with early established i.p. tumor.

Several explanations for the synergism may be offered: (i) The administration of cyclophosphamide may reduce early established (day 3) tumor load to a level that corresponds with day 1 tumor load. ABPP has a significant antitumor effect against this tumor load (Eggermont et al., 1987a) and is therefore likely to contribute to the increased tumor reduction after combination therapy. This mechanism may explain the synergism observed in the treatment of day 3 tumor. Since the administration of CY has virtually no effects on day 8 i.p. tumor or day 10 pulmonary metastases a different mechanism may be involved in this setting. (ii) Removal of suppressor cells has been suggested by several authors to be the main mechanism by which CY may enhance the antitumor effect of immunotherapeutic regimens (Bear, 1986; Evans, 1983; Greenberg et al., 1985; North, 1982). It has been shown that suppressor cells emerge about one week after tumor inoculation and play an increasingly important role in the immune status of the tumor bearing host (North, 1984). Removal of these cells by CY may well be the most important mechanism by which synergism between CY and ABPP in the treatment of advanced tumors in our experiments was achieved. (iii) A third factor may play a role in both the early established and the advanced tumor setting. Papa and coworkers (1987) have shown that tumor cells from a tumor bearing mouse, treated with CY 24-36 hours earlier, are more easily lysed in vitro by lymphokine activated killer cells than tumor cells from

untreated mice. We have shown previously (Eggermont et al., 1987a) that ABPP induces lymphokine activated killer cell like activity in vivo and the activity of these cells may well be facilitated in vivo as well by the administration of CY prior to ABPP.

Chemoimmunotherapy was much more effective against immunogenic tumors than against nonimmunogenic tumors. The immunogenic tumors have been shown more sensitive to treatment with ABPP alone (Eggermont et al., 1987a) and displayed a greater sensitivity than nonimmunogenic tumors to CY in the experiments reported in this communication. This observation suggests that the elimination of suppressor cells that may be elicited by immunogenic but not by nonimmunogenic tumors may in part be responsible for difference in the degree of synergy that is observed with the combined treatment. Alternatively one may hypothesize that immunogenic tumors induce cytotoxic T lymphocytes (CTL) in numbers large enough to competitively inhibit LAK function by consumption of IL-2 as we have shown previously (Eggermont et al., 1986e; Sugarbaker et al., 1987a; 1987b). Cyclophosphamide can abrogate the primary immune response (Eggermont et al., 1987c; Peppeloni et al., 1986) and thereby remove the for IL-2 competing population of CTLs and consequently enhance LAK cell function (Anderson et al., 1986; Eggermont et al., 1986e). The greater sensitivity of the immunogenic tumors may also be due to an ABPP-induced increase in antibody production and the subsequent increased clearance of tumor cells (Fast et al., 1982; Li et al., 1985).

Important is the efficacy of repetitive cycles of the combined treatment. An interval of 5-7 days was chosen in order to avoid hyporesponsiveness to the administration of ABPP. Oku et al. (1984) have shown that the induction of IFN by ABPP, when administered repetitively, is effective only when an interval of at least 5 days is observed. When treatment cycles were administered at weekly intervals a majority of mice with established MCA-105 tumor i.p. was cured. This observation shows that there may be a place for chemoimmunotherapeutic regimens in the adjuvant treatment setting of solid tumors and that further research is warranted and should be directed at the optimalization of treatment schedules based on a better understanding of the kinetics and the temporary effects of its constituents.

CHAPTER VIII

The content is a modified version of the publications :

Phase II trial of high-dose recombinant leukocyte alpha-2 interferon for metastatic colorectal cancer without previous systemic treatment

Alexander M. M. Eggermont[1], Willem Weimar[2], Richard L. Marquet[1], Johannes S. Lameris[3] and Johannes Jeekel[1].

From the departments of General Surgery[1], Internal Medicine-I[2] and Radiology[3] of the University Hospital Rotterdam - Dijkzigt, Rotterdam, the Netherland.

Cancer Treatment Reports 69:185-187, 1985

Clinical and immunological evaluation of 20 patients with advanced colorectal cancer treated with high dose recombinant leukocyte interferon-alphaA (rIFN-alphaA)

Alexander M. M. Eggermont[1], Willem Weimar[2], Buphendra Tank[3], Amelie M. Dekkers-Bijma[3], Richard L.Marquet[3], Johannes S. Lameris[4], Dick L. Westbroek[1] and Johannes Jeekel[1].

From the departments of General Surgery[1], Internal Medicine-I2, and Radiology[4] of the University Hospital Rotterdam-Dijkzigt and from the Laboratory for Experimental Surgery of the Erasmus University[3], Rotterdam, the Netherlands.

Cancer Immunology and Immunotherapy 21:81-84, 1986

Leukocyte adherence inhibition (LAI), Natural Killer (NK) cell activity, Gamma-Interferon Production Capacity (GIPCA) and Phytohaemagglutinin (PHA) response in patients treated with recombinant leukocyte A interferon.

Buphendra Tank[1], Amelie M. Bijma[1], Richard L. Marquet[1], Alexander M. M. Eggermont[2], Willem Weimar[3], Johannes Jeekel[2] and Dick L. Westbroek[2].

From the Laboratory for Experimental Surgery of the Erasmus University, Rotterdam and the department of General Surgery[2] and Internal Medicine-I[3] of the University Hospital Rotterdam-Dijkzigt, Rotterdam, the Netherlands.

Oncology 42:157-163, 1985

Clinical and immunological evaluation of 20 patients with metastatic colorectal cancer treated with high dose recombinant leukocyte interferon-alphaA (rIFN-alphaA)

Alexander M. M. Eggermont, Willem Weimar, Bhupendra Tank, Amelie M. Dekkers - Bijma, Richard L. Marquet, Johannes S. Lameris, Dick L. Westbroek and Johannes Jeekel.

Summary

Twenty patients with advanced colorectal cancer were treated with high dose recombinant leukocyte interferon-alphaA (rIFN-alphaA). Patients were treated either chronically (Group I: twice a week up to 20 x 10^6I.U./m^2 i.m.) or cyclically (Group II: 1-4 periods of 8 consecutive days up to 20 x 106 I.U./m^2 i.m. daily at 20 days intervals) during a period of 12 weeks. Growth of marker lesions was monitored by physical examination, computerized tomography and ultrasonography. For immunological monitoring the following tests were performed sequentially: tube leukocyte adherence inhibition (LAI), natural killer (NK) cell activity, interferon-gamma production capacity upon induction by Concanavalin A and the phytohaemagglutinin (PHA) induced blastogenesis. There was one patient with a partial response, one with stable disease and one with a mixed response. Seventeen patients showed growth of metastatic marker lesions but only seven patients qualified for having progressive disease (>25% increase in tumor mass). Overall mean survival was 14.5 months, median survival was 15.5 months. Follow up was determined by survival and ranged from 3 - 20 months after stopping treatment. Survival was significantly shorter when the extent of hepatic disease was >25% ($p=0.05$), when extensive extra-hepatic disease was present ($p<0.005$), when alkaline phosphatase level serum levels were >2x normal ($p<0.02$) or when the performance status was <100% ($p<0.001$). Toxicity consisting mainly of fever, fatigue, anorexia and weight loss was serious in Group I and minimal in Group II. There was virtually no haematological toxicity in Group I whereas transient leukocytopenia and mild thrombocytopenia occurred at the end of each cycle in Group II. There were no drug related deaths. CEA-levels corresponded to stage and course of disease in 18 out of 20 patients. LAI assays remained unaltered irrespective of the mode of therapy. In both chronically and cyclically treated patients rIFN-alphaA led to a "short lived" augmentation of NK cell activity. In the cyclically treated group this was are current phenomenon at the start of each cyle whereas a significant depression of NK cell activity was seen in chronically treated patients throughout the rest of the course of treatment. Interferon-gamma production capacity upon stimulation with concanavalin-A was impaired in most patients when they entered the study and was significantly stimulated during rIFN-alphaA therapy whereas a reduction in the response to phythaemagglutinin (PHA) was seen. It was concluded from this study that rIFN-alphaA has no significant antitumor activity in patients with metastatic colorectal cancer. The differences in toxicity and in immunomodulative effects between the two treatment schedules may have important implications for the design of future studies with rIFN-alphaA in humans in a different setting.

INTRODUCTION

Colorectal cancer is second only to lung cancer as the most common malignancy in the western hemisphere and causes 10-15% of all cancer deaths. Five year survival has not improved over the last 30 years as the tumor proved refractory to most chemotherapeutic agents. Five-fluorouracil (5-FU), the standard agent in this disease, has no effect on long term survival in metastatic colorectal cancer when given alone or in combination with other drugs (Davis and Kisner, 1978; Falkson and Falkson, 1981; Yorkshire Gastrointestinal Study Group, 1984). Adjuvant therapy with 5-FU alone (Grage and Moss, 1981) or in combination with MeCCNU (Higgins et al., 1984) and/or MER (GITSG, 1984) showed only a minor benefit in patients with Dukes C disease and in patients with rectal cancer, or showed no benefit at all.

In view of the absence of an effective adjuvant therapy regimen for colorectal cancer one was justified to perform phase II trials in patients with metastatic colorectal cancer as soon as interferon became available in large enough quantities to perform these studies. With the advent of recombinant interferon preparations it became possible to perform clinical studies.

Interferons are proteins with anti-viral, anti-proliferative and immunomodulatory activity (Ho and Amstrong, 1975). Amongst the group of solid tumors interferon-induced tumor regression had been reported in metastatic breast cancer (Gutterman et al., 1980; Borden et al., 1982), renal cell carcinoma (Quesada et al., 1983) and in malignant melanoma when combined with cimetidine (Flodgren et al., 1983) at the onset of our study. In the present phase I-II study the potential antitumor efficacy, the toxicity and the immunomodulatory effects of high dose rIFN-alphaA therapy in patients with advanced metastatic colorectal cancer were evaluated.

PATIENTS, MATERIALS AND METHODS

Patients

Entry criteria

Patients with histologically confirmed resected colorectal cancer, without invasion into neighbouring organs and with histolog-

ically or cytologically confirmed measurable metastatic lesions were eligible for treatment; ascites, pleural fluid and bone metastases were not considered evaluable lesions. Patients who entered the study could not have other malignant disease nor previous or concurrent chemo-, immuno- or radiotherapy. Other entry criteria included: age < 80 years, Karnofsky index > 70%, bilirubin < 20 mmol/l, creatinine clearance > 50 ml/min, no uncontrolled infections, no symptomatic cardiovascular disease, white blood cell count > 3 x 10^9/l and no non-steroid antiinflammatory therapy.

Patients characteristics

Twenty patients, 11 females and 9 males entered the study. Their median age was 62.5 years (range 39-78). The median interval between surgical resection of the primary tumor and interferon treatment was 5 months (range 1-49). Metastatic lesions were clearly measurable with CT-scan and ultrasonography in all cases. CT and ultrasonography were done every 6 weeks. CEA-levels were determined every 6 weeks. In 18 patients liver metastases provided the measurable lesions, in one patient retroperitoneal and supraclavicular lymphnodes and in one patient retroperitoneal lymphnodes and peritoneal lesions. Extent of hepatic and extrahepatic disease are summarized in table I.

Interferon

Recombinant leukocyte interferon-alphaA (rIFN-alphaA , Ro-22-8181 made by Hoffmann - LaRoche, Basel) was used. The material has been purified up to > 96% homogeneity and is free of detectable endotoxin when checked with the limulus test.

Treatment schedules

Ten patients were treated chronically (Group I) and recieved twice weekly up to 20 x 10^6 I.U./m^2 of rIFN-alphaA i.m. for a period of 12 weeks on an outpatient basis. The other 10 patients were treated cyclically (Group II) and recieved for 1-4 periods of 8 consecutive days up to 20 x 10^6 I.U./m^2 i.m. daily at intervals of 20 days. The total dose strived at in 12 weeks was 24 x 20 x 10^6 I.U./m^2 per patient.

Response catagories

The usual response catagories were used: complete response (CR) was defined as disappearance of all evidence of disease lasting > 6 weeks; partial response (PR) was defined as > 50% decrease in the sum of the product of diameters of marker lesions lasting > 6 weeks, without appearance of new lesions; progressive disease (PD) was defined as an increase of > 25% in the diameters of marker lesions and/or the appearance of new lesions; stable disease (SD) was defined as failure to qualify for CR, PR or PD. Patients receiving at least 8 x 20 x 10^6 I.U./m^2 were considered evaluable.

Immunological monitoring

For immunological monitoring the following tests were performed sequentially : tube leukocyte adherence inhibition (LAI) assays were performed in 10 patients on days 0, 28, 56 and 77 using colon cancer extracts of proved specificity as specific antigen and breast cancer extracts as non-specific antigen as is routine in our laboratory (22). A non-adherence index (NAI) > 30 was considered as positive, a NAI < 30 as negative; NK cell activity was sequentially evaluated in 8 chronically treated patients on days 0, 2, 7, 28, 56 and 77 and in 2 cyclically treated patients on days 0, 2 and 7 of each cycle while simultaneously assays using in vitro rIFN-alphaA pretreated effector cells were performed to determine boostability; on the same days gamma-Interferon production capacity upon stimulation by Concanavalin A and the blastogenesis response upon stimulation by PHA were determined in these patients. Details of the methods used are described by Tank et al.(1984; 1985).

RESULTS

All patients were evaluable for tumor response and toxicity. Follow up ranged from 3 - 20 months after stopping treatment as was determined by survival. The characteristics of the patients and their response to the chronical (A) or cyclical (B) treatment with rIFN-alphaA are summarized in **table I(A) and table I(B).**

TABLE I (A) : Characteristics of patients treated with rIFN-alphaA twice a week (Chronically)

Patient (sex, age)	Extend Hepatic Disease	Extend Extrahepatic Disease	CEA start - end	Total dose of rIFN in U/m^2	Tumor Response	Survival (months)
1. M, 59	multiple R + L 25% < X < 50%	-------	> 600 - > 600	260 x 10^6	PD*	20
2. M, 66	multiple R + L X > 50%	retroperitoneal lnn.	> 600 - > 600	360 X 10^6	PD	20
3. F, 76	multiple R + L X < 25%	retroperitoneal lnn.	96 - 423	205 X 10^6	PD*	18
4. M, 62	multiple R + L 25% < X < 50%	retroperitoneal lnn.	12 - 12	600 X 10^6	PR	26
5. F, 48	-----	retroperitoneal lnn. perit. carcinomatosis	175 - 398	480 X 10^6	PD*	12.5
6. M, 39	----	retroperitoneal lnn. supraclavicular lnn.	49 - 94	280 X 10^6	PD	5
7. F, 61	solitary central X < 25%	------	354 - 228	180 X 10^6	PD*	8
8. M, 69	multiple R + L X < 25%	------	14 - 95	400 X 10^6	PD*	16
9. F, 60	multiple R + L X < 25%	------	471 - > 600	380 X 10^6	PD*	22
10. M, 62	multiple R + L X < 25%	------	116 - 269	325 X 10^6	PD*	18.5

TABLE I (B) : Characteristics of patients treated with rIFN-alphaA in cycles of 8 consecutive days (Cyclically)

Patient (sex, age)	Extend Hepatic Disease	Extend Extrahepatic Disease	CEA start - end	Total dose of rIFN in U/m^2	Tumor Response	Survival (months)
11. M, 66	solitary R X < 25%	------	2.4 - 3.6	480 X 10^6	PD	28
12. F, 57	multiple R + L X < 25%	------	19.9 - 19.5	480 X 10^6	SD	16
13. M, 64	multiple R + L X < 25%	------	11.9 - 30	480 X 10^6	PD*	11.5
14. F, 65	diffuse R + L X > 50%	Retroperitoneal lnn. Lung metastases	210 - > 600	160 X 10^6	PD	3.5
15. F, 72	diffuse R + L x > 50%	Lung metastases	48 - > 600	160 X 10^6	MR	6.5
16. M, 54	multiple R + L 25% < X < 50%	Lung metastases	288 - > 600	480 X 10^6	PD	9
17. F, 78	solitary L X < 25%	Retroperitoneal lnn. Lung metastases	120 - 318	640 X 10^6	PD*	12
18. F, 78	multiple R + L X < 25%	------	98 - 120	240 X 10^6	PD*	15
19. F, 65	multiple R + L 25% < X < 50%	Retroperitoneal lnn.	2.5 - 2.5	160 X 10^6	PD	4.5
20. F, 73	multiple R X < 25%	------	132 - 258	640 X 10^6	PD*	17

M = male; F = female; PD = progressive disease (>25%); PD* = progressive disease (<25%); SD = stable disease; MR = mixed response; PR = partial response; X = % of liver replaced by tumor; lnn. = lymphnodes.

Tumor response

The tumor response to treatment of the 20 patients is listed in table I. One patient (no.4, group I) had a partial remission at 12 weeks after starting treatment. Treatment had to be discontinued at that point because of severe fatigue and weakness. The partial remission however progressed to a near total remission (> 90% tumor reduction) during the following months. His performance status was rapidly reestablished at 100% and previously severely disturbed liver functions became normal. Seven months after stopping treatment, CEA-levels rose and progressive disease compared to the state of near total remission was demonstrated by CT and ultrasonography. A second course of 10×10^6 I.U./m^2 twice weekly was given for 6 weeks at which point it had to be stopped because of intolerable toxicity. Disease was slowly progressive from then on and the patient died 26 months after the initial diagnosis of metastatic disease. One patient (no 12, group II) had no tumor growth at all over a period of 5 months while CEA levels remained unchanged. She was classified as having stable disease. One patient (no 15, group II) developed a partial remission of her liver metastases after one course of treatment. Three weeks later a brain metastasis became clinically evident and was confirmed by CT. Her performance status deteriorated rapidly and further treatment was not given. She was classified as having a mixed response (MR). Ten patients showed continuous but minimal growth (increase < 25%) of their marker lesions throughout treatment. All these patients but one (no 7, group I) had a marked concomittant increase in CEA-levels and had slowly progressive disease after stopping treatment. Patient no 7 developed obstructive jaundice and died 6 months after stopping treatment. Although the measured increase in tumor mass was < 25% these patients are listed in table I as having progressive disease (PD*). Seven patients had unequivocal progressive disease throughout treatment and are listed as PD.

Overall mean survival from the date of diagnosis of metastatic disease was 14.5 (SD $\pm$ 7.0) months. Median survival was 15.5 months. Mean survival in Group I was 16.6 ($\pm$ 6.4) months vs. 12.3 ($\pm$ 7.2) months in Group II (NS). Using the student-t test the following factors, as determined at the date of diagnosis of metastatic disease, were found to correlate with survival: Extent

of hepatic disease (x) : x < 25% vs. x > 25% ; 17.3 (± 5.8) vs 10.6 (± 7.5)mts , p = 0.05. Extent of extra hepatic disease : E_0 (none), 17.2 (± 5.5), E^1 (only retroperitoneal lymphnodes), 15.9 (± 8.0), E_2 (retroperit. lnn. + lung or carcinosis peritonei), 7.5 (± 4.4) : E_0 vs. E_1 : NS, E_0 vs. $E_1 + E_2$: NS ($p < 0.08$), E_0 vs E_2 : $p = 0.005$. Liver Metastases (LM) only vs. LM + E_1-E_2 : 17.2 (± 5.5) vs. 12.4 (± 8.1), NS ; LM only vs. LM + E_2 : 17.2 (± 5.5) vs. 6.7 (± 4.6), $p < 0.02$. Alkaline Phosphatase (AlkPhos) levels : Normal (N) or < 2 x N vs. AlkPhos > 2 x N : 15.7 (± 6.5) vs. 6.0 (± 2.3), $p < 0.02$. Performance Status (PS) of the patient at the time of diagnosis of metastatic disease correlated best with survival : PS = 100% (16 pts.) vs PS <100% (4 pts., 80-90%) , 16.8 (± 5.6) vs. 4.9 (± 1.3) : $p < 0.001$. Survival from the date of diagnosis of metastatic disease in these patients did not show a correlation with : patients sex ; site, diameter and histologic grade of the primary tumor nor with the stage of disease at the time of diagnosis of the primary tumor. The correlation between the different factors at the time of diagnosis of metastatic disease and survival are summarized in table II.

Table II :

Factors correlating with survival in metastatic colorectal cancer

- Extend of hepatic disease < 25% vs > 25%	p = 0.05
- Extend of extrahepatic disease E_0 vs E_2	p < 0.005
- Liver Metastases (LM) only vs LM + E_2	p < 0.02
- Alk. Phos. Normal (N) vs Alk. Phos. > 2 x N	p < 0.02
- Performance Status (PS) 100% vs PS < 100%	p < 0.001

(Factors as determined at the time of diagnosis of metastatic disease)

CEA-levels were elevated in all but two (no 11 and 20) patients. CEA-levels remained unchanged in the patients with a partial response and with stable disease. A steep rise or a maximal level was seen in 17 patients which corresponded well with their course of disease. Only in one patient (no 7) a fall in CEA-level occur-

red which did not reflect her course of disease. Overall performance of CEA as a monitoring device was excellent.

Toxicity

High dose rIFN-alphaA treatment when administered twice weekly caused far more and heavier side effects than expected. High fever with chills after each dose, prolonged nausea, anorexia, fatigue and weight loss were pronounced as is indicated in table III. Hallucinations, confusion and lethargy occurred in 3 patients but disappeared upon reducing the dose. In all but two patients (no 4 ,5) in group I dose adjustment had to be made as can be deducted from the total dose recieved by each patient as indicated in table I. Cyclic treatment was very well tolerated. High fever with chills and nausea occurred only on day one of each cycle. Anorexia, fatigue and weight loss were absent or much less pronounced (table III). Six patients recieved 3 - 4 complete full dose cycles. One patient (no 18) withdrew from treatment after 1.5 cycles in spite of only mild side effects. Three patients (no 14, 15, 19) with extremely advanced disease recieved only 1 cycle because of rapidly progressive disease (no 14,19) and brain metastasis (no 15). Other side effects were relatively benign. Eleven patients complained of getting numb fingers for a period of 1/2 - 2 hours after each dose of interferon given. A bitter taste was a side effect in 12 patients. Objective neurotoxic effects or permanent sequelae were not found by a consultant neurologist at the end of treatment in any of the patients. Four patients developed Herpes Hominis lesions of the lips in the first week of treatment which disappeared while continuing treatment. Mild alopecia was seen in six patients and lasted for about 2 months. Virtually no haematological toxicity was seen in Group I. Daily injections had a significantly more pronounced ($p < 0.01$, Wilcoxon) depressive effect on white blood cell and platelets count than did twice weekly administration. Transient mild disturbance of liver functions was seen only in one patient in Group II. Renal functions were not affected in any of the patients but in one (no 7, group I) who developed transient proteinuria. No cardiotoxicity was observed in any of the patients when electrocardiograms made at the start and at the end of treatment were compared.

Table III : TOXICITY of rIFN-alphaA

SYMPTOMS	GROUP I (10pts)	GROUP II (10pts)
Flue-like symptoms	10	10*
Weight loss		
- none	4	7
- mild (<5%)	2	1
- moderate (5-10%)	1	2
- severe (>10%)	2	0
Anorexia	8	4
Nausea	3	3*
Fatigue		
- none	0	3
- mild	3	3
- moderate	3	3
- severe	4	1
Numb Fingers (1/2-2hrs)	6	5
Bitter Taste	8	4
Hallucinations	3	0
Lethargy	4	1
Herpes Facialis	3	1
Alopecia (mild)	3	3
Haemoglobin > 7.5 mmol/l	8	9
< 7.5 mmol.l	2	1
WBC (cells/mm^3) > 4000	7	1
3000 - 4000	3	3
1500 - 3000	0	6
Platelets (cells/mm^3)		
> 150,000	9	1
100,000 - 150,000	1	7
50,000 - 100,000	0	2
Rise in Liver Enzymes	0	1
Proteinuria	1	0

* = only on first day of treatment cycle; WBC = white blood cell count; hrs = hours; pts = patients

Immunological monitoring

Sequential **tube LAI assays** were performed in 10 patients. In 7 patients the LAI was positive and negative in three. The reactions before, during and after treatment with rIFN-alphaA did not change.

Sequential **NK cell activity tests** showed in all chronically treated patients tested a 2 fold increase of NK cellactivity on day 2 after the start of treatment. Continuation of twice weekly injections resulted in a rapid decline of activity (on day 7), followed by a permanent marked two fold depression and loss of boostability by rIFN-alphaA in vitro for the rest of the treatment period as is illustrated in Figure 1.

Figure 1

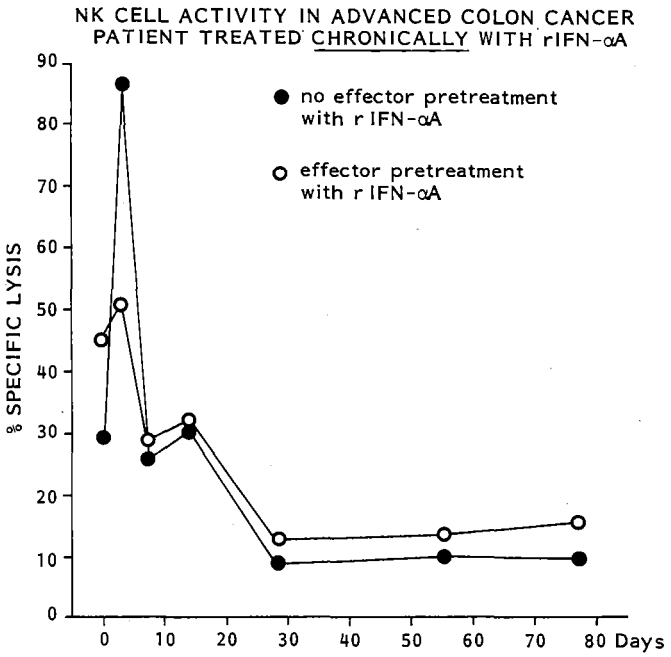


Figure 1 : Typical NK cell cytotoxicity profile at effector : target ratio of 40 : 1 of patients treated with high dose rIFN-alphaA twice a week. ●---● No effector pretreatment with rIFN-alphaA; 0---0 effector pretreatment with rIFN-alphaA. A 2-3 fold increase of NK cell cytotoxicity is seen at the start of treatment. This is followed by a 2-fold depression of NK cell cytotoxicity during the rest of the treatment period. Also the loss of boostability of NK cell activity by effector pretreatment with rIFN-alphaA is noted.

The cyclically treated patients tested showed a two fold increase of NK cell activity on day 2 of each cycle. NK cell activity levels at the start of each cycle were normal and there was no loss of boost-ability in vitro as is depicted in Figure 2.

Figure 2 :

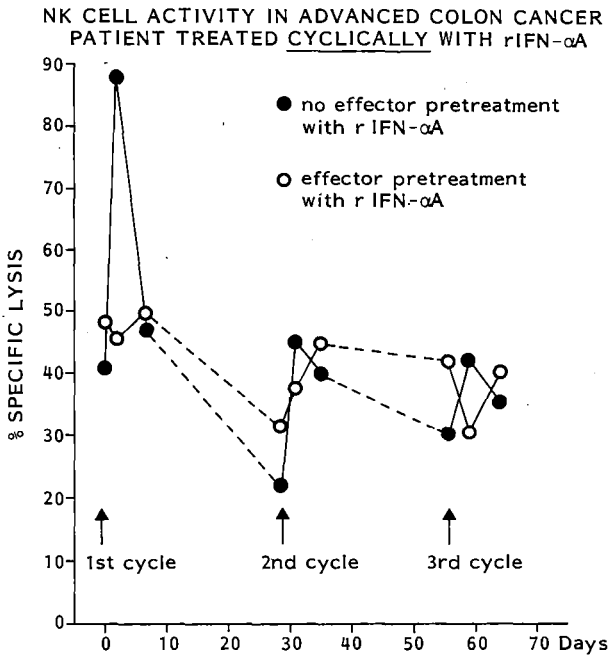


Figure 2 : Typical NK cell cytotoxicity profile at effector : target ratio of 40 : 1 of patients treated cyclically (3 cycles of 8 consecutive days) with high dose rIFN-alphaA. ●---● no effector cell pretreatment with rIFN-alphaA. 0---0 effector cell pretreatment with rIFN-alphaA. A ± two fold increase of NK cell cytotoxicity is seen at the start of each treatment cycle. No chronic depression of NK cell activity is seen. No loss of boostability of the effector cells is seen when pretreated with rIFN-alphaA.

Gamma-Interferon Production Capacity upon induction with Con A proved to be extremely low (< 10 umits/ml) as was the PHA response (60% of the normal) in the patients when tested as the entered the study. RIFN-alphaA therapy resulted in most of them (6 out of 8) in a significant production of IFN-gamma. In the only two patients that had a base line GIPCA > 0 units/ml a depression was noted following rIFN-alphaA treatment. The most consistent finding in the chronically treated patients was an inverse correlation between the GIPCA and PHA response. This pattern is shown in Figure 3.

Figure 3 :

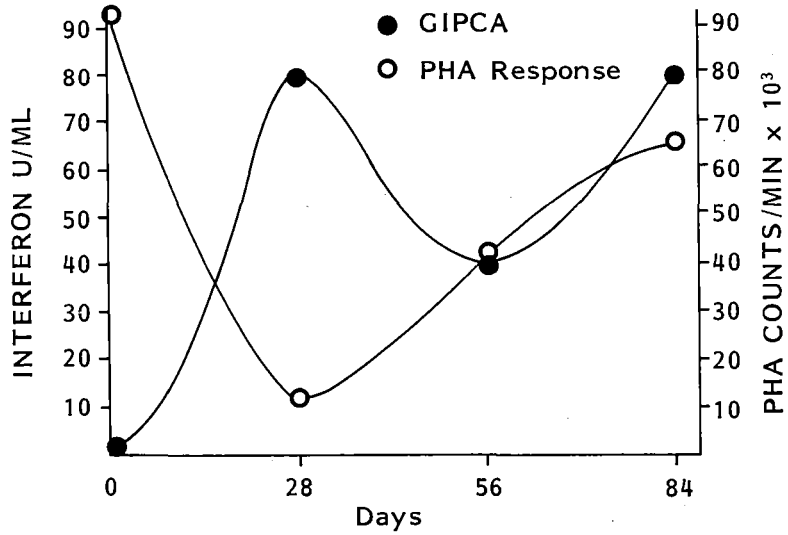


Figure 3 : Typical response pattern of the Gamma-Interferon Production Capacity (GIPCA) and blastogenesis response to PHA in a patient treated twice weekly with high doses of rIFN-alphaA. ●----● GIPCA ; 0---0 PHA response. The GIPCA level before the first dose of rIFN-alphaA is undetectable but is boosted to normal levels upon administration of rIFN-alphaA. There is an inverse correlation bedtween the GIPCA and the PHA response.

In 2 cyclically patients the GIPCA and PHA responses were determined. In 1 patient a cyclic in-crease was registered in the GIPCA after rIFN-alphaA adminis-tration as is depicted in Figure 4. In the other patient GIPCA remained undetectable.

Figure 4 :

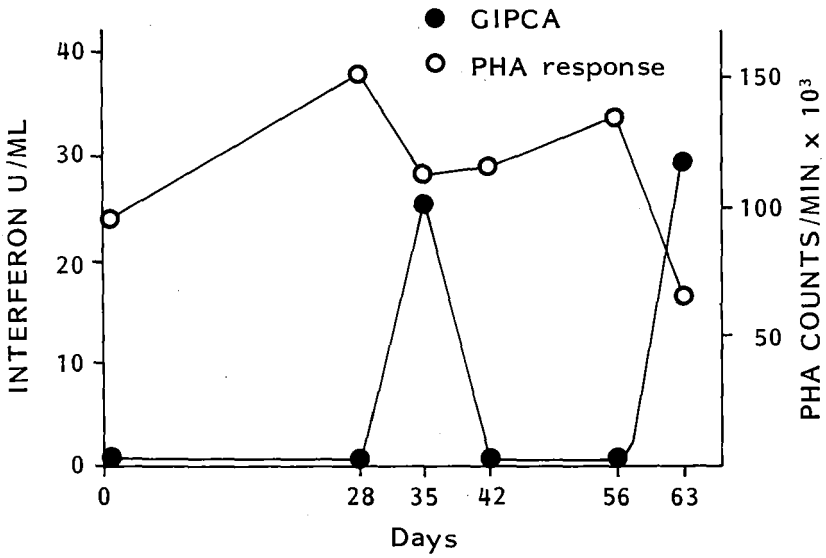


Figure 4 : GIPCA and PHA response pattern in a patient treated with high doses of rIFN-alphaA for 3 cycles of 8 consecutive days. ●---● GIPCA; 0---0 PHA response. GIPCA levels were undetectable before rIFN-alphaA treatment and boosted to normal levels by the administration of rIFN-alphaA. An inverse correlation seems to exist between the GIPCA and the PHA response.

DISCUSSION

High dose rIFN-alphaA treatment when administered twice weekly proved to be quite toxic. Especially anorexia and fatigue, reflected in considerable weight loss were pronounced. High dose

cyclic treatment appeared ,except for transient leukocyto- and thrombocytopenia, comparatively nontoxic and was well tolerated. Paresthesias, taste disturbances, fatigue, lethargy and hallucinations suggest a certain degree of neural involvement. Interferon-induced, dose-dependent, reversible central nervous system toxicity with alterations in electroencephalograms and electroneuromyograms has been reported by Smedley et al.(1983) and by Mattson and Niiranen (1983). We did not perform these investigations but thorough neurological examinations revealed no objectively demonstrable damage in any of our patients. Reactivation of endogenous Herpes virus Hominis was observed in 4 patients. Apparently the classic inhibitory effect of interferon on virus replication is "overruled" by non-antiviral effects in the high doses used. Only one partial response in 20 patients was seen. This is in agreement with Neefe et.al.(1984), who reported one partial remission in 18 patients treated with the same rIFN-alphaA preparation for previously untreated colorectal cancer. Studies with low dose natural IFN-alpha (Figlin et al., 1983; Oberg et al., 1983), low dose lymphoblastoid IFN (Chaplinski et al., 1983), and another recombinant IFN-alpha preparation given in large doses (Lundell et al., 1984; Silgals et al., 1984) were not able to demonstrate antitumor effects in this disease. The results and some details of the trials performed in metastatic colorectal cancer are summarized in **table IV**. Wrigley et al. (1984) reported 2 patients with a partial response out of 20 patients treated with either high or low dose rIFN-alpha when combined with 5-FU.

One of our patients had a mixed response and in one patient unequivocal stable disease lasted for 5 months. Growth of the marker lesions was very slow (increase < 25%) in 10 patients. According to the official criteria they all could have been classified as having stable disease during their period of treatment. CEA-levels rose markedly over the same period and no acceleration but continuous slowly progressive disease was seen after stopping treatment. In our opinion these patients should not be classified as stable disease since most likely they suffered from slowly progressive disease before, during and after treatment.

It remains to be seen wether stable disease is a usefull response category in phase-II studies since reporting that interferon

induced stable disease in 11 out of 20 patients would leave a falsely positive impression about the effectiveness of treatment with rIFN-alphaA in advanced colorectal cancer. In 7 patients measurements of marker lesions showed unequivocal progressive disease.

Table IV : Phase II trials with IFN in patients with metastatic colorectal cancer

Reference	Interferon Type	Dose 10^6 Units	No evaluable patients	Response CR/PR	%
Chaplinski 1983	HuIFN(Ly)	3 (i.m) tiw 6 wks	18	0/0	0
Figlin 1983	HuIFN(Le)	3 (i.m.) qd 5 wks	18	0/0	0
Oberg 1983	HuIFN(Le)	3 (i.m.) qd 4 wks	9	0/0	0
Silgals 1984	rIFN-alpha$_2$	30,50 (i.v.) qd x 5 d q 2-3 wks	21	0/0	0
Lundell 1984	rIFN-alpha$_2$	50 (i.v.) qd x 5 d q 6 wks	18	0/0	0
Neefe 1984	rIFN-alphaA	50 (i.m.) tiw	21	0/1	5
Flodgren 1985	rIFN-alphaA	7.5 (i.m) tiw 6 wks $\pm$ cimetidine	16	0/0	0
Eggermont 1986	rIFN-alphaA	20 (i.m.) biw 12 wks	10	0/1	10
		qd x 8 d q 3 wks	10	0/0	0

HuIFN(Ly) = human lymphoblastoid IFN; HuIFN(Le) = Human leukocyte IFN; CR = complete response; PR = partial response; q = every; qd = daily; biw = twice a week; tiw = three times a week; wk = week; i.m. = intramuscularly; i.v. = intravenously.

Survival in metastatic colorectal cancer was found to correlate with extent of hepatic disease, extent of extrahepatic disease, level of alkaline phosphatase and with performance status. Similar correlations have been reported by others (Goslin et al., 1982; Petrelli et al, 1984; Wagner et al., 1984). Survival in metastatic disease did not show a correlation with patients sex, site, diameter and histologic grade of the primary tumor or with the stage of disease at the time of diagnosis of the primary tumor. Alkaline phosphatase levels were normal (N) in 9 patients and < 2 x N in 7 patients when metastatic disease was diagnosed. Other liver function tests were even less sensitive which is consistent with the report by Schreve et al.(1984). For diagnosis as well as monitoring of metastatic colorectal cancer there seems hardly any place left for liver function tests since CEA-levels were found to be very reliable.

Tube LAI test results were not affected by interferon treatment and the test was found to be of little value in monitoring metastatic disease in these patients. The negative LAI results in 3 patients could be attributed to an inherent insensitivity of the assay or to a tumor overload phenomenon as described by Thomson et al. (1982). Monocytes play a central role in triggering the cascade of events leading to non-adherence in the tube LAI . Our results indicate that the administration of rIFN-alphaA does not affect this monocyte activity. Modulation of human NK cell activity by exogenous administration of human leukocyte interferon is well documented (Einhorn et al., 1978; Herberman et al., 1979; Huddlestone et al., 1979; Lindal et al., 1972). After an initial "short lived" two fold augmentation of NK cell activity this cytotoxicity was markedly depressed during interferon treatment when administered chronically. This confirms the work of Herberman's group (Maluish et al., 1983) and could be explained by an interferon-induced exhaustion of the precursor NK cell population. Restoration of this precursor population apparently occurred during the 20-day-interferon-free intervals in the cyclically treated patients since a normal level of NK cell activity was found at day 0 of each cycle. A significantly augmented peak level was seen on day 2 of each cycle which tapered off to normal levels on day 7 of each cycle. In vitro tests with rIFN-alphaA - pretreated lymphocytes showed significantly enhanced NK cell

activity only on day 0 of the chronically treated patients and only on day 0 of each cycle in the cyclically treated patients. This indicates that in vivo administration of rIFN-alphaA leads to a maximum attainable NK cell activity as has been reported by Einhorn et. al. (Einhorn et al., 1980 and 1984). RIFN-alphaA treatment resulted in normalisation of initially impaired interferon-gamma production in our patients. NK cells have the ability to produce interferons (Ratliff et al., 1982) and Interleukine-2 (IL-2) is known to increase interferon-gamma production (Kawade et al., 1983). These findings suggests a network of interactions between IFN-alpha , IL-2 , IFN-gamma and NK cells that remains to be elucidated. The mode of action by which interferon treatment can result in an antitumor effect in vivo has not been clarified. The odds for clinically relevant succes should however be more in favor for interferon therapy when less tumor cells are present than in the situation of advanced metastatic cancer. With one partial response, one mixed response and one stable disease , rIFN-alphaA treatment was shown to exert an antitumor effect in some patients in spite of the overwhelming number of tumor cells present. Cyclic treatment appeared the best scheme in view of its low clinical toxicity and its immunostimulative properties. It deserves further investigation in a setting where less tumor cells are present.

CHAPTER IX

GENERAL DISCUSSION

GENERAL DISCUSSION

The experimental work in rodents with the interferon inducer ABPP, presented in this thesis, has demonstrated the limited antitumor effects of ABPP when administered alone. The results of our experiments with ABPP were in line with the general observation that biological response modifiers are effective only when the tumor load is small. ABPP was only effective when administered within 24 hours after tumor inoculation, when tumor load is small and tumor cells are still in their highly vulnerable blood born and early lodging phase. No significant effect was noted in the clinically much more important experimental setting e.g. against early established (day 3) or advanced (day 7 - 10) tumor. When ABPP was administered in combination with other biologicals like IFN-gamma or TNF however, significant antitumor activity was seen also against established tumor. This is an encouraging observation since it shows the usefulness of combining biologicals that may exert their antitumor effects through different mechanisms. Further more it may lead to the development of effective treatment schedules with reduced toxicity as a result of dose reductions of the individual biologicals in combinations with synergistic antitumor activity. Superior activity was demonstrated when chemoimmunotherapeutic treatment schedules, in which the administration of cyclophosphamide preceded ABPP were applied to early established as well as far advanced metastatic tumors. Repetitive treatment cycles were highly effective and even cured the majority of mice with established intraperitoneal tumor.
Given the fact that the administration of ABPP is associated with very little toxicity it can be concluded that ABPP may be used clinically with success in an adjuvant setting, when administered in combination with cyclophosphamide. Special care should be taken to design a treatment schedule that consists of repetitive cycles at time intervals avoiding the hyporesponsiveness observed characteristically when multiple doses of biologicals are given within a short period of time.
The observation that <u>in vivo</u> generation of LAK cell activity after the intraperitoneal administration of ABPP was superior at the local-regional level, in the peritoneal cavity, special attention should be given to the possibilities to use ABPP as an

adjunct in intracavitary treatment protocols. It is our feeling that ABPP has shown enough potential in these experiments that justify clinical studies, preferably in an adjuvant setting in which ABPP is administered systemically (orally) or intracavitary as an adjunct to another treatment modality.

The set up and the results of the Phase II trial with rIFN-alphaA that we performed in patients with metastatic colorectal cancer are typical examples of the "interferon experience". It is rather appalling to see with how little success the enormous number of phase II trials, performed with various interferon preparations and tested in a wide array of malignancies over the last 5 years, has met (Goldstein and Laszlo, 1986). With exception of the big success in the treatment of hairy cell leukemia (Quesada et al., 1985a; Thompson et al., 1985) and the modest success rates in the treatment of lymphoma (Foon et al., 1984), in Kaposi's sarcoma (Real et al., 1984), in multiple myeloma (Costanzi et al., 1984), and in renal cell cancer (Quesada et al., 1985b), no significant effect against any of the solid tumors has been observed. This situation may in part be due to the fact that too few phase I studies were performed before the "big boom" in phase II trials. Too little was known about optimal doses, routes of administration, local-regional versus systemic effects, induction of hyporesponsiveness etc. when most phase II studies were embarked upon. Too many similar phase II studies were performed, generating many overlapping data, answering few new scientific questions. Illustrative of this situation is that the most interesting result of our phase II trial was the phase I aspect of the study by applying two treatment schedules that appeared to differ greatly with respect to the associated toxicity and immunomodulative properties. This observation is indicative of the fact that with the same amount of effort, time and money that was invested in the big wave of phase II trials with IFN conducted up till now, much more valuable information could have been gathered and might have been successfully applied in those areas where interferons have proven to be clinically successful.

A more fundamental question is whether the classical phase II trial in patients with advanced, measurable metastatic disease is an appropriate and justified method to reach conclusions

concerning the efficacy, potential clinical value and biological activity of biological response modifiers. It is well known and has been shown again in this thesis that biologicals are effective only when the tumor load is small. From a theoretical point of view and as a result of the overwhelming evidence from experimental work in animals it is not logical to perform these phase II trials and look for tumor response rates in advanced cancer, as is done in the standard screening procedure for chemotherapeutic drugs.

A different approach in the clinical evaluation of biological response modifiers may well be justified by focusing on extensive phase I studies aimed at arriving at treatment schedules with optimal immunostimulatory effects and minimal toxicity. Once such a schedule is obtained its clinical value would be best tested in an adjuvant setting, alone and in combination with other treatment modalities in patient populations that have a particularly poor prognosis like "radically operated" pancreas, gastric of esophageal cancer patients, Dukes C_{II} colorectal cancer patients, stage II melanoma patients etc. If results would be encouraging with these poor risk populations various groups of patients with a greater variety of malignancies could then be treated in phase III trials in order to establish the efficacy and the broad clinical value of the agent.

Although "the interferon experience" has been a sobering one, especially for an overly optimistic laic society, it would be wrong to conclude that immunotherapy has no future.

Breakthroughs in biotechnology have created unprecedented opportunities to do more basic, more refined, quantitatively and qualitatively superior research unraveling the complexity of the immune response to cancer and increasing the possibilities to manipulate it.

It remains extremely difficult to predict immunotherapeutic effects since so many different factors influence the immune status of the host, determine the outcome of immunotherapy and influence tumor growth. We have shown elsewhere that surgery and blood-transfusions can profoundly influence tumor growth and determine the effect of immunotherapy (Jeekel et al., 1982; Marquet et al., 1986; Eggermont et al., 1987e). Repetitive treatment cycles with lymphokines may rapidly become ineffective when serum inhibiting

factors emerge and block the crucial lymphokines or neutralize the effector cells of immunotherapeutic regimens (Eggermont and Sugarbaker, 1987f). Actively ongoing immune responses in the tumor bearing host may consume lymphokines and thus competitively inhibit the action of killer cells and thus abrogate their anti-tumor effects (Eggermont et al., 1987c; Sugarbaker et al.,1987b). On the other hand alloimmune responses at the site of the tumor may generate IL-2 and activated killer cells in situ and thus augment the efficacy of immunotherapeutic regimens (Eggermont et al.,1987h;). The generation of an inflammatory reaction within tumor tissue recruits inflammatory cells to the site of the tumor and may instigate aspecific killing as well as augment the population of killer cell precursors at the tumor site. Immunotherapy may be augmented by such a favorable concentration of effector cells (Steller et al., 1986; 1987). The above illustrates that systemically ongoing immune responses may have immunosuppressive effects and thus facilitate tumor growth or render immunotherapy ineffective, while similar processes at the local-regional level or at the site of the tumor can potentiate the antitumor effects of immunotherapy.

Effective routing of lymphokines but especially of the effector cells to the tumor site may well be the crucial step towards successful immunotherapy. Local-regional treatment concepts (Sugarbaker et al., 1987a; Eggermont et al.,1987i; 1987j), adoptive immunotherapy regimens improved by specificity of the effector cells and the use of monoclonal antibodies may improve the efficacy of immunotherapeutic regimens in the future by increasing effector cell - tumor cell contact and effector cell to tumor cell ratios.

In few scientific fields has the increase in knowledge been as spectacular as in immunology over the last two decades. It is therefore impossible to predict which developments lay ahead, but most likely immunotherapy will develop further and establish itself as the fourth modality in the treatment of cancer.

CHAPTER X

SUMMARY

SAMENVATTING

SUMMARY

The first part of this thesis consists of a series of experiments, conducted in rats and mice, with the low molecular weight interferon-inducer ABPP (2-amino-5-bromo-6-phenyl-4(3H)-pyrimidinone). Its antitumor and immunomodulating effects were evaluated in vitro and in vivo using a variety of tumors differing in immunogenicity and histology. In various tumor models the role of tumor load and tumor site in the outcome of immunotherapy with ABPP were investigated. Further more the efficacy of combining ABPP with other biologicals like recombinant Rat Interferon-gamma (rRIFN-gamma) and recombinant Murine Tumor Necrosis Factor (rMuTNF) and recombinant Human Interleukin-2 (IL-2) or with the cytostatic agent cyclophosphamide (CY) was studied.

In **chapter III and IV** we showed that ABPP has no direct antiproliferative or cytotoxic effect in vitro and that therefore the antitumor effects in vivo were most likely mediated by its immunomodulating properties : the induction of IFN and the activation of natural killer (NK) cells and macrophages. It was demonstrated that pretreatment of BN rats with the strong interferon-inducer ABPP significantly retarded tumor growth of the nonimmunogenic liposarcoma LS175 in the subcutaneous (s.c.) tumor model, whereas the administration of the very weak interferon-inducing homologue AIPP did not. Since we subsequently showed that both agents enhanced NK cell activity equally effectively it was concluded that the difference between the two agents in antitumor activity in the s.c. model might reflect their capacities to induce IFN. Further more these results suggested that the capacity to enhance NK cell activity might not depend on the prior induction of IFN. Also we demonstrated that both agents inhibited equally effectively the development of lung metastases with LS175 in BN rats. Since both agents are equally potent activators of NK cells and as NK cells are known to be most effective against tumor cells in their blood born or early lodging phase the result in the lung metastasis model strongly suggested that the antitumor effects in this tumor model were mediated by NK cells.

In experiments with the weakly immunogenic colon adenocarcinoma (CC531) in WAG rats we further demonstrated the role of tumor load and tumor site on the outcome of treatment with ABPP.

In the subrenal capsule assay (SRCA) as well as in the liver metastasis model significant antitumor effects were obtained when ABPP was administered early (day 0,1) but not when given late (day 6,7). This was in line with the general observation that biologicals are effective provided tumor load is small. In the liver metastasis model administration of ABPP 1 week after tumor inoculation even enhanced the development of liver metastases. This tumor site-related phenomenon was reminiscent of earlier observations in the liver metastasis model after treatment with IFN-alpha and suggested that the immunological "make-up" of the liver could be responsible for the observed enhancement of tumor growth. In the setting of 7 day old established liver metastases activation of NK cells is known to have no significant impact on tumor growth. Other mechanisms may also be involved at this stage in containing tumor growth like antibody dependent cellular cytotoxicity (ADCC) and cytotoxic T cell mediated killing of the immunogenic tumor CC531. Since ABPP enhances phagocytosis by macrophages we speculated that the facilitated growth of liver metastases after the late administration of ABPP might be the result of an increased clearance of tumor antigens and antigen reactive T cells by activated Kupffer cells in the liver. Recently others have shown that activated Kupffer cells inhibit NK cell activity by way of secretion of prostaglandins. These data support a crucial role for ABPP-activatyed Kupffer cells in this tumor site specific phenomenon of enhanced growth of liver metastases. The potentially different outcome of immunotherapy in different organs or body compartments should prompt us to pay attention to this aspect in the evaluation of biologicals in clinical studies and shows the need for strict criteria that define enhanced tumor growth as a response catagory, a catagory often not included in phase II trials.

In chapter V we described the experiments exploring the benefit that can be obtained by combining different biologicals. Reports in the literature have claimed synergistic antitumor activity in vitro of combinations of Type I and Type II IFNs as well as combinations of IFNs with TNF. We investigated whether ABPP-induced endogenous IFN might synergize with exogenous rRIFN-gamma or rMuTNF. It was found that rRIFN-gamma alone had no antitumor effect, in spite of its previously demonstrated immunomodu-

lative activity. When administered simultaneously with ABPP no additional antitumor effect was observed. However when rRIFN-gamma was administered prior to the otherwise ineffective late administration of ABPP a synergistic antitumor effect was seen. We speculated that this " priming effect " of rRIFN-gamma was due to an upregulation of Type I IFN receptors on the tumor cells, thereby significantly increasing their sensitivity to the ABPP induced Type I IFN. Combination treatment with rMuTNF had clearly additive antitumor effects. These experiments showed that different biologicals can be used effectively when combined. Timing of treatment appeared crucial and illustrates the need for further investigations in this area.

In **chapter VI and VII** the experimental work with ABPP in mice was described. First we showed that the in vivo administration of ABPP intraperitoneally (i.p.) enhanced NK-cell activity in the spleen and more effectively in the peritoneal exudate, as assessed in ^{51}Chromium release assays with Yac-1 target cells. More importantly, by using various fresh tumor cell preparations as targets, we were able to demonstrate that the administration of ABPP generated LAK cell activity in the spleen and especially in the peritoneal exudate. The level of LAK cell activity generated in vivo as assessed in vitro 3 days after the administration of 250 mg/kg of ABPP i.p. was comparable to the effect seen after the administration of 10,000 units of IL-2, i.p., twice a day for three consecutive days. The in vivo generation of LAK cells by IL-2 appeared dose dependent and was so effective in the peritoneal exudate that cytotoxicity levels after 3 days of i.p. administration of 100,000 units of IL-2 twice daily resulted in cytotoxicity levels equal to or even superior to those obtained by standard 3 day in vitro culture techniques. It can be concluded from these experiments that IL-2 as well as ABPP may hold promise for clinical application in particular in an intraperitoneal local-regional treatment setting. Subsequently we investigated whether ABPP and IL-2 might potentiate each others effects. Because of the potential upregulating effects on IL-2 receptors by ABPP we investigated whether in vivo pretreatment of spleen donors would augment subsequent in vitro generation of LAK cells from their spleens. No beneficial or detrimental effect was seen. In vivo experiments in the i.p. as well as the pulmonary metasta-

sis model showed that early administration of ABPP (day 1,2) inhibited tumor growth significantly but that ABPP when given in the setting of early established (day 3) tumor or far advanced (day 8-10) tumor was ineffective. The weakly immunogenic sarcomas MCA-105 and -106 and the weakly immunogenic murine colon adenocarcinoma MCA-38 were more sensitive to treatment with ABPP than the nonimmunogenic sarcomas MCA-101 and -102. Further more we showed that combined treatment with ABPP and IL-2 alone or with ABPP and IL-2 + LAK cells did not augment the therapeutic effects of IL-2 + LAK treatment unless ABPP was administered early, prior to IL-2 + LAK, which led to an additive ABPP-mediated antitumor effect. These experiments showed that ABPP and IL-2 both induced discernable levels of LAK cell activity in vivo and that their effects did not synergize. This suggested that these are attained by at least in part common pathways.

In **chapter VII** we explored the therapeutic potential of the combination of two different treatment modalities e.g. : chemotherapy with cyclophosphamide (CY) and immunotherapy with ABPP. After a dose-finding study with (CY) the effect of a moderate dose of CY (50 mg/kg), with a moderate effect on day 3 tumor and little if any effect on day 8-10 tumor, was evaluated when given 12 hours prior to the administration of ABPP i.p.. A remarkable synergistic antitumor effect of the combined treatment schedule was seen in both early established and far advanced tumors in the i.p. as well as the pulmonary metastasis model. This effect was much stronger against the weakly immunogenic tumors than the nonimmunogenic tumors. The efficacy of various treatment schedules was evaluated and it could be shown that a second treatment cycle, given at a 5 day interval in order to avoid hyporesponsiveness induced by ABPP, was highly effective. Repetitive treatment cycles at weekly intervals were able to even cure over 70% of the mice with early established i.p. tumor. The synergistic antitumor activity of combined treatment with CY and ABPP could be the result of various effects : (i) tumor reduction caused by CY and thus enabling ABPP to be effective; (ii) selective eradication of suppressor-T cells by CY, a mechanism that might at the same time explain the difference in efficacy of the combined treatment against the immunogenic tumors and the nonimmunogenic tumors; (iii) the CY-induced increased lysability of the tumor;

(iv) abrogation of the primary response by CY and thus removal of a population of CTLs that would have competed with LAK cells for IL-2 and possibly other lymphokines and thus could have abrogated their effect. These experiments highlighted the important potential that combined modality treatments may have and focus on the efficacy of repetitive treatment cycles and the importance of further research in this field.

In **chapter VIII** we described the toxicity, the antitumor effects and the immunomodulating effects of two different treatment schedules with high doses of recombinant Human Interferon-alphaA (rIFN-alphaA) in 20 patients with advanced metastatic colorectal cancer. Interferon was administered either chronically e.g. 20 x 10^6 units/m^2 intramuscularly, twice a week over a period of 12 weeks; or cyclically, e.g. 20 x 10^6 units/m^2 i.m. daily for 3 periods of 8 consecutive days at 3 week intervals. The chronical treatment schedule appeared to be far more toxic than the cyclical schedule. The main toxic side effects that were often dose limiting were fever with chills, fatigue, malaise, anorexia and weight loss, whereas the toxicity of the cyclical schedule was virtually restricted to a mild leukocytopenia at the end of each treatment cycle.

The response rate was rather poor. Only 1 partial remission was seen in 20 patients. One patient had stable disease for a period of 5 months and one patient had a mixed response. In 7 patients progression of disease was so slow that on the basis of measurements of their marker lesions they could have been qualified as stable disease. A concomittant sharp rise in CEA levels and the continued slow progressive natural course of disease after rIFN-alphaA was stopped prompted the decision to classify these patients as having progressive disease throughout treatment. The remaining 10 patients had unequivocal progressive disease throughout treatment. It was concluded therefore that rIFN-alphaA has no antitumor effect against advanced colorectal cancer.

The immunomodulative effects of rIFN-alphaA appeared to differ depending on the type of treatment schedule. NK-cell activity was enhanced by rIFN-alphaA only at the beginning of the chronic treatment schedule and depressed from day 7 on throughout the rest of the treatment. In patients treated cyclically, NK-cell

activity was enhanced by rRIFN-alphaA at the beginning of each treatment cycle and was not found to be depressed by rIFN-alphaA at any point throughout this treatment schedule. The outcome of the leukocyte adherence assay (LAI) was not influenced by the administration of rIFN-alphaA. The Gamma-Interferon Production Capacity (GIPCA) was found to be extremely depressed at the start of treatment in most patients and was restored by the rIFN-alphaA in most patients to normal levels. A decreased blastogenesis in response to stimulation with Phytohaemagglutin (PHA) was observed after the administration of rIFN-alphaA.

It was concluded that in view of the few toxic side effects and in view of the favorable immunomodulative properties of the cyclical treatment schedule this schedule deserved to be tested in a different setting in a disease with greater sensitivity to treatment with IFN.

SAMENVATTING

In het eerste deel van dit proefschrift worden experimenten met de interferon(IFN)-inducer ABPP (2-amino-5-bromo-6-phenyl-4(3H)-pyrimidinone) in ratten en muizen beschreven. De antitumor en immunomodulerende effecten van ABPP werden geëvalueerd in vitro en in vivo. Hierbij werd gebruik gemaakt van een verscheidenheid aan tumoren die qua immunogeniciteit en histologie van elkaar verschillen. In diverse tumor modellen werd de invloed van massa en localisatie van de tumor op de effectiviteit van immunotherapie onderzocht.

In **hoofdstuk III en IV** toonden wij aan dat ABPP geen direct antiproliferatief of cytotoxisch effect heeft in vitro en dat derhalve de antitumor effecten in vivo waarschijnlijk het gevolg zijn van de immunomodulatieve effecten van ABPP : inductie van IFN, activatie van natural killer (NK) cellen en macrophagen. Voorbehandeling van BN ratten met de krachtige IFN-inducer ABPP remde de groei van het niet-immunogene liposarcoma LS175 in het subcutane (s.c.) tumor model. Toediening van de zwakke IFN-inducer AIPP had geen effect op s.c. tumor groei. Wij concludeerden derhalve dat de verschillen in antitumor effect in het s.c. tumor model het verschil in IFN-inducerend vermogen van ieder agens weerspiegelde en dat het vermogen om NK cellen te activeren mogelijk onafhankelijk was van voorafgaande inductie van IFN. Vervolgens toonden wij aan dat beide pyrimidinonen in dezelfde mate NK cellen activeerden en in het longmetastase model de ontwikkeling van longmetastasen in gelijke mate tegengingen. Deze resultaten suggereerden dat de inhibitie van longmetastasen gerelateerd was aan de activatie van NK cellen waarvan bekend is dat hun antitumor effect het grootst is op circulerende tumor cellen en op metastasen in de vroege innestelings fase (<24 uur).

In **hoofdstuk IV** wodt in experimenten met het zwak immunogene colon adenocarcinoma (CC531) in WAG ratten, de invloed van de tumor massa en tumor localisatie op het resultaat van immunotherapie met ABPP beschreven. Zowel in het tumor model waarbij stukjes tumor onder de nierkapsel worden geimplanteerd (subrenal capsule assay = SRCA) als in het levermetastase model was vroege toediening van ABPP (op dag 0 en 1; dag 0 is dag van implantatie van de tumor) effectief. Geen significante antitumor

activiteit werd gezien als ABPP laat (op dag 6 en 7) werd toegediend. Deze resultaten komen overeen met de algemene waarneming dat agentia met immunomodulatieve activiteit (biological response modifiers = BRM) alleen significante antitumor activiteit tonen als de tumor massa klein is. In het levermetastase model werden na late toediening van ABPP zelfs significant meer levermetastasen waargenomen. Dit aan de tumor-localisatie gerelateerde verschijnsel was al eerder in ons laboratorium waargenomen bij de behandeling van levermetastasen met IFN-alpha. Deze resultaten suggereerden dat de immunologische "infrastructuur" van de lever verantwoordelijk zou kunnen zijn voor de versnelde tumorgroei. Het is bekend dat NK cellen geen significant effect hebben op de groei van 7 dagen oude "gevestigde" levermetastasen. Andere immunologische processen kunnen mogelijk tumorgroei in dit stadium beinvloeden zoals antilichaam afhankelijke cellulaire cytotoxiciteit (ADCC : antibody dependent cellular cytotoxicity) en T-cel cytotoxiciteit gericht tegen de zwak immunogene tumor CC531. Aangezien ABPP phagocytose door macrophagen verhoogt, suggereerden wij dat de versnelde groei van 7 dag oude levermetastasen na de late toediening van ABPP het gevolg zou kunnen zijn van een verhoogde eliminatie van tumor antigenen en antigeen reactieve T-cellen door de door ABPP geactiveerde Kupffer cellen in de lever en dat dit zou leiden tot een verlaging van ADCC en T-cel antitumor activiteit. Recent werd door anderen aangetoond dat geactiveerde Kupffer cellen NK cel activiteit kunnen remmen middels productie van prostaglandinen. Deze gegevens steunen de hypothese dat Kupffer cellen een centrale rol spelen in dit aan de lever gerelateerde phenomeen van versnelde tumor groei na behandeling met ABPP of IFN. Deze observatie dat het resultaat van immunotherapie sterk kan afhangen van de localisatie van de tumor heeft ons inziens consequenties ten aanzien van de evaluatie van immunotherapie in de kliniek. Strikte criteria zijn nodig om versnelde tumorgroei te definieren als een respons catagorie die tot op heden niet routinematig bij phase II trials wordt gehanteerd.

In **hoofdstuk V** worden de experimenten beschreven waarin de effectiviteit van combinatie behandeling met verschillende BRMs werd bestudeerd. Verscheidene auteurs hebben synergistische antitumor activiteit <u>in vitro</u> beschreven van combinaties van type I en type II IFNs. Wij toonden aan dat de combinatie van ABPP

(inductie van endogeen IFN type I) met exogeen recombinant ratten IFN-gamma (rRIFN-gamma) en met recombinant muizen tumor necrosis factor (rMuTNF) een synergistische respectievelijk additieve antitumor activiteit vertoonde afhankelijk van tijdstip en volgorde van toediening van de BRMs. Monotherapie met rRIFN-gamma bleek ondanks eerder aangetoonde immunomodulatieve effecten geen antitumor activiteit te hebben. Bij gelijktijdige toediening in combinatie met ABPP werd geen additioneel antitumor effect waargenomen. Echter als rRIFN-gamma werd toegediend voorafgaand aan de late toediening van ABPP (dag 6,7), werd een synergistische antitumor activiteit gezien. Wij speculeerden dat dit "priming effect" van rRIFN-gamma mogelijk het gevolg was van een door IFN-gamma geinduceerde verhoogde expressie van IFN-type I receptoren op tumor cellen, met als gevolg een verhoogde gevoeligheid van de tumor cellen voor het door ABPP geinduceerde IFN type I. ABPP in combinatie met rMuTNF had een duidelijk additief antitumor effect. Deze experimenten toonden aan dat verschillende BRMs mogelijk met succes in combinatie kunnen worden toegediend en dat veel van de effectiviteit blijkt af te hangen van de opzet van de behandelingsschemas.

In **hoofdstuk VI en VII** wordt het experimentele werk met ABPP in muizen beschreven. Met behulp van ^{51}Chromium release assays met Yac-1 target cellen toonden wij aan dat de intraperitoneale (i.p.) toediening van ABPP NK activiteit in vivo induceert in milt en vooral in peritoneale exudaat cellen (PEC). Door gebruik te maken van verscheidene NK-cell-resistente verse tumor cellen als target, toonden wij vervolgens aan dat ABPP LAK (lymphokine activated killer) cel activiteit induceert in vivo. Het niveau van LAK cel activiteit in miltcellen en PEC 3 dagen na 1 injectie van 250 mg/kg ABPP, kwam overeen met de LAK cel activiteit die na toediening van 2 X 10,000 eenheden IL-2, gedurende drie dagen, werd waargenomen in dezelfde cel populaties. De in vivo inductie van LAK cel activiteit door IL-2 bleek dosis-afhankelijk en was zo effectief dat de toediening van 2 X 100,000 eenheden IL-2, gedurende 3 dagen even effectief (miltcellen) of zelfs effectiever (PEC) was dan de standaard in vitro LAK cel kweek methoden. Wij concludeerden derhalve dat ABPP en IL-2 mogelijk van bijzondere klinische waarde kunnen zijn als zij in een locoregionale setting (bv. intracavitair) aangewend worden. Vervolgens onder-

zochten wij of ABPP en IL-2 elkaars antitumor effecten konden potentieren. Aangezien ABPP de expressie van IL-2 receptoren kan verhogen onderzochten wij of voorbehandeling van miltdonoren met ABPP de effectiviteit van de daarop volgende in vitro kweek methode van LAK cellen kon vergroten. Dit bleek niet het geval. In vivo experimenten met zowel het i.p. tumor model als het longmetastase model toonden aan dat alleen vroege toediening van ABPP (dag 1,2) tumorgroei significant vertraagde. Toediening van ABPP had vrijwel nooit een significant antitumor effect op dag 3 gevestigde tumoren en nooit op ver voortgeschreden tumorgroei (dag 8-10). De zwak immunogene tumoren : de sarcomen MCA-105,-en 106 and het coloncarcinoom MCA-38, bleken gevoeliger voor behandeling met ABPP dan de niet immunogene sarcomen MCA-101 en ,-102. Combinatie behandeling met ABPP en IL-2 of ABPP en IL-2 met LAK cellen vergrootte het therapeutisch effect van IL-2 met of zonder LAK cellen niet, tenzij ABPP werd toegediend op dag 1 en 2, voorafgaand aan de standaard behandeling met IL-2 en LAK cellen. In dat geval werd een additief antitumor effect waargenomen. Deze experimenten toonden aan dat ABPP en IL-2 beide duidelijke LAK cel activiteit in vivo kunnen induceren maar dat hun effecten elkaar niet versterken.

In **hoofdstuk VII** onderzochten wij de waarde van het combineren van 2 verschillende behandelings modaliteiten, te weten : chemotherapie met cyclophosphamide (CY), en immunotherapie met ABPP. Het effect van een bescheiden dosis CY (50 mg/kg), toegediend 12 uren eerder dan ABPP, op dag 3 tumor en op dag 8-10 tumor in het i.p. en in het longmetastase tumor model werd geevalueerd. In beide tumor modellen werd een sterk synergistisch antitumor effect waargenomen bij het toedienen van de combinatie therapie zowel ten aanzien van dag 3 tumor als ten aanzien van ver voortgeschreden dag 8-10 tumor. Dit effect was veel sterker t.a.v. de immunogene tumoren dan de niet immunogene tumoren. De effectiviteit van verschillende behandelingsschemas werd geëvalueerd en het kon worden aangetoond dat een tweede behandelings cyclus, toegediend 5 dagen na de eerste cyclus om door ABPP geinduceerde hyporeactiviteit te vermijden, bijzonder effectief was. Herhaalde behandelingen met tussenpozen van 1 week waren zelfs in staat om > 70% van de muizen met dag 3 i.p. tumor te genezen. De synergistische antitumor activiteit van cyclophos-

phamide in combinatie met ABPP zou het gevolg kunnen zijn van : (i) tumor reductie veroorzaakt door CY waarna ABPP effectief kan zijn; (ii) selectieve eliminatie van suppressor-T cellen door CY, een mechanisme dat tevens het verschil in effectiviteit van de combinatie behandeling van immunogene t.o.v. die van niet immunogene tumoren kan verklaren; (iii) een door CY geinduceerde verhoogde kwetsbaarheid van de tumor cellen; (iv) het te niet doen van de primaire immuunrespons door CY en de verwijdering derhalve van een populatie cytotoxische T-cellen die met LAK cellen om het binden van interleukine-2 en mogelijk ook andere lymphokinen zouden hebben gestreden, en derhalve hun effect te niet hadden kunnen doen. Deze experimenten toonden aan dat de combinatie van verschillende behandelingsmodaliteiten veelbelovend kan zijn en vestigden de aandacht op de effectiviteit van cyclische behandelingsschemas en het belang van verder onderzoek op dit gebied.

In **hoofdstuk VIII** beschreven wij de toxiciteit, de antitumor activiteit en de immunomodulatieve effecten van twee verschillende behandelingsschemas met hoge doses recombinant humaan interferon-alphaA (rIFN-alphaA) in 20 patienten met gemetastaseerd colon-/rectumcarcinoom. IFN werd chronisch (20 X 10^6 units/m^2, i.m., 2 maal per week, gedurende 12 weken) of cyclisch (20 X 10^6 units/m^2 i.m., gedurende 8 opeenvolgende dagen, met tussenpozen van 3 we-ken). Het chronische behandelingsschema bleek veel toxischer dan het cyclische schema. De belangrijkste, vaak dosisbeperkende, bijwerkingen waren koorts met koude rillingen, vermoeidheid, malaise, anorexie en gewichtsverlies. Toxiciteit van het cyclische schema bleef vrijwel beperkt tot het optreden van een matige leukocytopenie aan het einde van iedere behandelings cyclus.

De tumorrespons was slecht. Slechts 1 partiële remissie werd waargenomen in 20 patienten. Een patient had stable disease gedurende 5 maanden en bij een patient werd een mixed response waargenomen. In 7 patienten verliep het voortschrijden van de ziekte zo traag dat zij op grond van metingen van de metastasen als stable disease konden worden geclassificeerd. Een gelijktijdig scherpe stijging van serum CEA spiegels en het voortgezette langzaam progressive beloop van de ziekte nadat de behandeling met IFN was gestaakt, deed ons besluiten deze patienten toch te

classificeren als progressive disease. De overige 10 patienten werden op grond van de response criteria als progressive disease geclassificeerd. Derhalve moest worden geconcludeerd dat rIFN-alphaA geen antitumor effect heeft in patienten met ver voortgeschreden gemetastaseerd colon-/rectumcarcinoom.

De immunomodulatieve effecten van rIFN-alphaA bleken af te hangen van het behandelingsschema. Bij het chronische behandelingsschema bleek NK cel activiteit alleen de eerst dagen door rIFN-alphaA te worden verhoogd en gedurende de rest van de behandeling lager te zijn dan de uitgangswaarde. Bij cyclische behandeling werd de NK cel activiteit aan het begin van iedere cyclus verhoogd en was zij tijdens de behandeling niet lager dan normaal.

De resultaten van de leukocyte adherence assay (LAI) werden door IFN niet beinvloed. De Gamma-IFN Productie Capaciteit (GIPCA) bleek aan het begin van de behandeling bij de meeste patienten sterk verlaagd te zijn. Zij werd door de toediening van rIFN-alphaA in de meeste patienten op het normale niveau gebracht. De blastogenese als gevolg van stimulatie met phytohaemagglutin (PHA assay) daalde bij de meest patienten na de toediening van rIFN-alphaA.

Gezien de geringe toxiciteit en de goede immunostimulatieve eigenschappen van het cyclische behandelingsschema met rIFN-alphaA concludeerden wij dat de effectiviteit van dit schema in een andere setting, bij een aandoening die gevoeliger is voor behandeling met IFN, dient te worden geëvalueerd.

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