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Promotion of Hepatic Enzyme-Altered Foci in Initiated Rats by Polybrominated Biphenyls: I. Effect of Short-Term Oral Administration of Polybrominated Biphenyls. II. Effect of Dietary Vitamin A Levels. III. Effects on Intercellular Communication in Cultured Rat Liver Epithelial Cells.

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PROMOTION OF HEPATIC ENZYME-ALTERED FOCI IN INITIATED RATS BY POLYBROMINATED BIPHENYLS: I. EFFECT OF SHORT-TERM ORAL ADMINISTRATION OF POLYBROMINATED BIPHENYLS II. EFFECT OF DIETARY VITAMIN A LEVELS III. EFFECTS ON INTERCELLULAR COMMUNICATION IN CULTURED RAT LIVER EPITHELIAL CELLS

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ABSTRACT

PROMOTION OF HEPATIC ENZYME-ALTERED FOCI
IN INITIATED RATS BY POLYBROMINATED BIPHENYLS:
I. EFFECT OF SHORT-TERM ORAL ADMINISTRATION
OF POLYBROMINATED BIPHENYLS
II. EFFECT OF DIETARY VITAMIN A LEVELS
III. EFFECTS ON INTERCELLULAR COMMUNICATION
IN CULTURED RAT LIVER EPITHELIAL CELLS

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FireMaster BP-6 (FM), a commercial mixture of polybrominated biphenyls, was administered by gavage to female Sprague-Dawley rats 30 days after two-thirds partial hepatectomy and administration of 10 mg diethylnitrosamine/kg body weight, to determine if very short-term oral administration of FM would be sufficient for promotion of hepatic enzyme-altered foci. Rats were killed 120 days after gavage, and gamma-glutamyl transpeptidase-positive enzyme-altered foci were quantitated. Enzyme-altered foci were significantly increased over control values in initiated rats given 130 mg FM/kg body weight, but not in rats given 13 mg FM/kg body weight. In another experiment, initiated rats were fed diets containing 2000 IU (low A) or 200,000 IU (high A) retinyl acetate/kg feed, with either no polybrominated biphenyls, 10 ppm FM, 10 ppm 2,4,5,2',4',5'-hexabromobiphenyl, or 1 ppm 3,4,5,3',4',5'-hexabromobiphenyl (345-HBB) added from day 30 on. Rats were killed on day 180, and enzyme-altered foci were quantitated. All groups of polybrominated biphenyl-treated rats had significantly more and larger enzyme-

altered foci than the corresponding control group. The percent of the liver volume occupied by foci was significantly greater in the low A/345-HBB group than in the high A/345-HBB group. Numbers and sizes of foci were somewhat lower in the high A groups than in the corresponding low A groups, but these differences were not statistically significant. In vitro assays were used to test the ability of FM, phenobarbital, and retinyl acetate to block intercellular communication between rat liver epithelial cells. In the metabolic cooperation assay, FM, phenobarbital, and retinyl acetate all inhibited intercellular communication, and retinyl acetate did not affect blockage of metabolic cooperation by FM. In the fluorescence redistribution after photobleaching assay, only FM inhibited intercellular communication, and retinyl acetate had no effect on the ability of FM to block communication. These results suggest that retinyl acetate has little effect on promotion by polybrominated biphenyls, and that blockage of intercellular communication is a possible mechanism for promotion by polybrominated biphenyls.

Dedicated with love to

my husband, Dale

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LIST OF ABBREVIATIONS

ACAS	Anchored Cell Analysis and Sorting 470 Workstation, Meridian Instruments
BW	body weight
cAMP	3',5'-cyclic adenylic acid (cyclic AMP)
CRBP	cellular retinol binding protein
CRABP	cellular retinoic acid binding protein
DDT	1,1-bis(4-chlorophenyl)-2,2,2-trichloroethane
DEN	diethylnitrosamine
EAF	enzyme-altered foci
EGF	epidermal growth factor
FM	Firemaster BP-6
FRAP	fluorescence redistribution after photobleaching
GGT	gamma-glutamyl transpeptidase
HBSS	Hanks' balanced salt solution
HGPRT	hypoxanthine-guanine phosphoribosyl transferase
MC	metabolic cooperation
PB	phenobarbital
PBB	polybrominated biphenyl
PCB	polychlorinated biphenyl
RA	retinyl acetate
RBP	retinol binding protein
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TPA	12-O-tetradecanoylphorbol-13-acetate
WB cells	rat liver epithelial cells of WB-F344 line
245-HBB	2,4,5,2',4',5'-hexabromobiphenyl
34-TBB	3,4,3',4'-tetrabromobiphenyl
345-HBB	3,4,5,3',4',5'-hexabromobiphenyl
6-TG	6-thioguanine

INTRODUCTION

INTRODUCTION

The processes involved in the development of cancer are poorly understood. The understanding of these processes may ultimately lead to effective methods for prevention and treatment of cancer. Considering the large number of suspected carcinogens and potential target tissues, the task of defining the critical carcinogenic processes and their mechanisms is a large one. By considering a small component of the larger picture, one can approach the study of carcinogenic mechanisms and events.

The following experiments focus on one chemical carcinogen, polybrominated biphenyls (PBBs), in a single target tissue, rat liver. Polybrominated biphenyls are of concern to Michigan residents since an agricultural accident in 1973 resulted in widespread, low-level human exposure to these highly persistent compounds (Carter, 1976; Kay, 1977; Fries, 1985). The long-term human health effects of these compounds are uncertain (Landrigan et al., 1979; Stross et al., 1981). Extrapolation from animal studies to human population is imperfect, but it is the best method presently available for understanding the risks to exposed human populations. Previous studies have show that PBBs promote hepatocarcinogenesis in rats (Jensen et al., 1982, 1983,

1984; Sleight, 1985) and have a variety of toxic effects, including depletion of normal vitamin A stores in the liver (Sleight and Sanger, 1976; Gupta and Moore, 1979; Gupta et al., 1981; Akoso et al., 1982a; McCormack et al., 1982; Bernert et al., 1983; Darjono et al., 1983; Jensen et al., 1985). The mechanisms by which PBBs cause these effects are unknown. There is some evidence for the disruption of junctional intercellular communication as a possible mechanism of tumor promotion by various compounds, including PBBs (Trosko et al., 1981a, 1982; Tsushimoto et al., 1982; Williams, 1983b). Other investigators have suggested that a related compound, 2,3,7,8-tetrachlorodibenzo-p-dioxin, may act as a tumor promoter by decreasing the tissue vitamin A levels (Thunberg et al., 1979, 1980, 1984; Thunberg, 1984).

Vitamin A compounds have been shown to be effective anti-carcinogenic agents in many systems (see reviews: Lotan, 1980; Bollag and Matter, 1981; Bollag and Hartman, 1983; Hicks, 1983b; Ong and Chytil, 1983; Blunck, 1984; Moon and Itri, 1984).

The following experiments were done to further define the hepatocarcinogenic activity of PBBs in rats, to examine potential interactions between PBBs and vitamin A in hepatocarcinogenesis in rats, and to explore the <u>in vitro</u> effects of PBBs and vitamin A on junctional communication between rat liver cells.

LITERATURE REVIEW

LITERATURE REVIEW

Multistage Concepts of Hepatocarcinogenesis

The concept of carcinogenesis occurring in multiple discrete phases was first described in the early 1940's, when the sequential stages of initiation and promotion were identified in skin tumorigenesis (Berenblum, 1941; Rous and Kidd, 1941). Subsequently, the multistage concept of tumor development has been expanded and refined by many investigators, and multistage carcinogenesis has been demonstrated in various tissues including the liver, mammary gland, urinary bladder, lung and colon (see reviews: Scribner and Suss, 1978; Farber and Cameron, 1980; Pitot and Sirica, 1980; Slaga, 1983).

Many environmental factors appear to contribute to the development of cancer. Among these factors are diet, viruses, radiation, and carcinogenic chemicals (Farber, 1981; Miller and Miller, 1981; Chambers, 1985). Epidemiologic studies have implicated a number of chemicals as contributing or causative factors for human cancers (Farber, 1981).

Initiation. The first stage of the carcinogenic process is initiation, and the agents which are active in this phase are called initiators. Many structurally diverse chemicals

have been identified as initiators, but a common property of many of these diverse compounds is their metabolic conversion to highly reactive, and often highly electrophilic, metabolites (Craddock, 1976; Farber, 1981; Miller and Miller, 1981). Most of these compounds are activated in the endoplasmic reticulum of the cell by the microsomal mixed function oxygenase system, which includes the cytochrome P-450 enzymes (Farber, 1981).

The relevant target of the initiators is generally considered to be cellular DNA, where many initiators have been shown to form chemical adducts with the nucleic acids (Pitot and Sirica, 1980; Farber, 1981; Miller and Miller, 1981; Hemminki, 1983; Chambers, 1985). For example, the simple alkylating agent and initiator, diethylnitrosamine (DEN), forms adducts with guanine and adenine bases in DNA (Hemminki, 1983). These chemical-DNA interactions subsequently result in genomic alterations such as miscodings, recombinations, and transpositions of DNA (Farber, 1981; Chambers, 1985). Thus, most initiators are classified as genotoxic agents, in that they are capable of reacting with and damaging DNA (Pitot et al., 1981; Williams, 1983a). If these DNA lesions are not repaired before the cell undergoes division, the mutation is then "fixed" in the cell's genome during replication (Craddock, 1976; Farber, 1980; Pitot and Sirica, 1980; Farber, 1981; Chambers, 1985). The initiation process is considered to be relatively irreversible (Farber and Cameron, 1980; Pitot and

Sirica, 1980; Miller and Miller, 1981; Peraino, 1981; Hicks, 1983a; Hendrich et al., 1986).

Recently, some investigators have questioned whether the biochemical lesion induced by the initiator and fixed by a round of cell proliferation must necessarily occur in DNA. These authors cite those carcinogens which do not seem to interact directly or indirectly with cellular DNA, and prefer to consider initiation as an undefined biochemical or molecular lesion which may or may not be in DNA, and which is fixed by a round of cellular proliferation (Farber and Sarma, 1987). These fixed, or permanent, cellular alterations produce the initiated cells, which may then undergo promotion to express a fully neoplastic phenotype (Farber, 1981; Hicks, 1983a).

Promotion. The promotion phase of carcinogenesis is operationally defined as the process whereby previously initiated cells are induced to express an altered phenotype, different from normal cells, and undergo some further neoplastic development (Farber and Cameron, 1980; Farber, 1981; Hicks, 1983a), usually into a focal proliferation of cells such as a nodule or papilloma (Farber, 1984a; Farber and Sarma, 1987). Numerous chemicals have been identified which can provide this stimulus to the initiated cells, and these chemicals are called promoters (Farber, 1981; Pitot et al., 1981). Promoters produce a variety of effects on the target cells (Weinstein, 1980; Miller and Miller, 1981; Weinstein et al., 1982; Slaga, 1983), and supply some

selective or proliferatory stimulus to initiated cells (Farber and Cameron, 1980; Farber, 1981). Most promoters are classified as epigenetic compounds which do not damage DNA but rather produce other effects on the target cells, such as alterations in gene expression which lead to the formation of tumors (Pitot and Sirica, 1980; Miller and Miller, 1981; Weinstein et al., 1982; Hicks, 1983a; Williams, 1983b). The effects of the promoting agents on cells are, at least to some extent, reversible (Pitot et al., 1981; Hicks, 1983a; Hendrich et al., 1986).

There is evidence that two or more additional "rare events" or "hits", analogous to genomic mutations, must also occur after the initiation phase (Emmelot and Scherer, 1980; Farber, 1980; Scherer, 1984; Yokoyama and Lombardi, 1985). The promoting compound, while not being genotoxic itself, may facilitate these events by expanding the susceptible initiated cell population, increasing the error proneness of DNA replication or repair, or enhancing the effects of persistent DNA-initiator adducts (Emmelot and Scherer, 1980; Scherer, 1984).

Several compounds have been shown to be effective promoters of hepatocarcinogenesis, including phenobarbital (Peraino et al., 1971; Pitot et al., 1978), DDT (Peraino et al., 1975) and polychlorinated biphenyls (Nishizumi, 1976). Various experimental models have been developed for studying the stages of initiation and promotion in hepatocarcinogenesis in rats (see reviews: Emmelot and

Scherer, 1980; Goldsworthy et al., 1986). The models vary in the age, sex and strain of rat used, the type of carcinogens used, the type of additional treatments used (such as partial hepatectomy to act as a proliferative stimulus), and in the ability to detect initiators, promoters, or complete carcinogens. Pitot et al. (1978) developed a model for two-stage hepatocarcinogenesis which uses a single low, non-necrogenic dose of initiator (diethylnitrosamine) following partial hepatectomy. This protocol allows clear separation of the initiation and promotion phases.

The promoted cells may undergo further changes before expressing a fully malignant phenotype, and this phase of the carcinogenic process is termed the progression phase (Foulds, 1965; Pitot, 1977; Emmelot and Scherer, 1980; Farber, 1981; Pitot et al., 1981). The evolution of hepatocyte nodules to the malignant neoplasm, hepatocellular carcinoma, is an example of progression (Farber, 1984a; Farber and Sarma, 1987).

Much attention is being focused on the promotion and progression phases of carcinogenesis, since these appear to offer the best opportunity for preventive or therapeutic intervention against fatal malignant neoplastic disease (Pitot and Sirica, 1980; Weinstein, 1980; Schulte-Hermann, 1985; Weisburger, 1985; Farber and Sarma, 1987). Initiators seem to have no threshold level of exposure for action, whereas a distinct threshold level can be found for most promoters. The relatively prolonged period of at least

partially reversible action by promoters offers a better chance for intervention to reduce the exposure or effect of promoters, and is more susceptible to antineoplastic modulation by diet or hormones.

Experimental hepatocarcinogenesis in rats is highly susceptible to modulation by "non-carcinogenic" factors such as the genetic strain of rat used and the diet composition (Glauert and Pitot, 1986; Russell et al., 1987; Ura et al., 1987).

Mechanisms of Tumor Promotion

Many investigations have focused on the mechanism by which tumor promoters act, and much has been learned about the various effects and mechanisms of tumor promoters. However, no single or ultimate mechanism has been identified as yet, and it is possible that the answer will eventually be found in a variety of alternative mechanisms specific to the promoter or tissue involved. Furthermore, the existence of multiple stages of promotion, as in skin carcinogenesis, implies that multiple mechanisms of promotion exist. Several excellent reviews of current theories of the mechanism of action for tumor promoters are available (Diamond et al., 1980; Weinstein et al., 1982; Slaga, 1983; Schulte-Hermann, 1985; Farber and Sarma, 1987).

Direct Alteration of Gene Expression. One theory proposes that tumor promoters cause increased expression of certain genes which were altered during the initiation phase (Diamond et al., 1980; Kitagawa et al., 1984b). Some of the

changes in gene expression induced by promoters may result in an altered state of cellular differentiation (Diamond et al., 1980; Schulte-Hermann, 1985). In some cases the cells may become dedifferentiated to a stem cell state, while in other cases the cells may be stimulated to undergo terminal differentiation.

Promoters such as TPA act by binding to a membrane or cytosolic receptor and subsequently activating the Ca^{2+}-phospholipid dependent enzyme, protein kinase C (Hicks, 1983a; Wenner et al., 1984). Some evidence indicates that protein kinase C is actually a receptor protein for the phorbol ester tumor promoters (Blumberg et al., 1984; Miyake et al., 1984; Nishizuka, 1984). The activation of protein kinase C has many effects, including release of arachidonate via lipomodulin and phospholipases (Wenner et al., 1984). Simultaneously with TPA-induced activation of protein kinase C, other changes are seen in the cell, such as increased membrane fluidity, increased levels of ATPase, and increased phosphorylation of several proteins (Bouche et al., 1984; Wenner et al., 1984). These alterations are followed by changes in cellular adhesion, increased protein synthesis, increased ornithine decarboxylase activity, and eventually increased RNA and DNA synthesis with cellular proliferation (Wenner et al., 1984). TPA induces ornithine decarboxylase in mouse epidermis by activating protein kinase C and subsequently stimulating ornithine decarboxylase gene transcription (Verma et al., 1986).

Tumor promoters such as TPA produce cellular effects which are in many ways similar to hormones and growth factors, and act via specific cell surface receptors (Weinstein, 1980; Weinstein et al., 1982; Wenner et al., 1984). Growth factors are locally acting hormones which diffuse through intercellular spaces, bind to specific cell membrane receptors, and subsequently stimulate cellular proliferation (Goustin et al., 1986). The hormone or chemical interaction with the surface receptor produces a cascade of intracellular responses which alter the cell's pattern of gene expression (Weinstein, 1980). In the case of chemicals which are tumor promoters, this alteration leads to expression of a malignant phenotype.

It has been suggested that TPA may act as a tumor promoter by mimicking the function of an endogenous growth hormone such as epidermal growth factor (EGF) (Weinstein, 1980; Weinstein et al., 1982). One major subclass of growth factors is the family of growth factors called transforming growth factors. Both malignant and chemically transformed rat liver epithelial cells secrete transforming growth factors, including one analogous to EGF, which are different than those secreted by normal rat liver epithelial cells (Liu et al., 1988). These transforming growth factors have complex and variable effects on cell proliferation (McMahon et al., 1986; Liu et al., 1988), and appear to be involved in the proliferation of transformed rat liver epithelial cells (Liu et al., 1988). A differential sensitivity of

malignant versus normal cells to transforming growth factors has been proposed to explain the potential role of these transforming growth factors in tumorigenesis (McMahon et al., 1986; Liu et al., 1988). There is some evidence that rat hepatocellular carcinoma cells *in vitro* can produce growth factors which act on EGF receptors in normal hepatocytes to stimulate proliferation (Luetteke and Michalopoulos, 1985). Other investigators found that certain chemicals may inhibit the appearance of preneoplastic altered hepatic foci by blocking epidermal growth factor stimulation of initiated hepatocytes (DeAngelo et al., 1985).

Several links between tumor promotion, growth factors and oncogenes have been described. Some oncogene products appear to be growth factors or their membrane receptors, and some growth factors increase the transcription of certain oncogenes (Goustin et al., 1986). Growth factors and/or oncogenes have been proposed to be involved in the progression of hepatocyte nodules to tumors (Farber, 1984a, 1984b).

Oncogenes appear to be normal cellular genes that are expressed at an inappropriate time or an inappropriate level for normal cellular activity (Bartram, 1984; Chambers, 1985). More than 20 oncogenes have been identified, and many have been shown to be normal cellular genes carried by retroviruses (Chambers, 1985; Barbacid, 1986). Carcinogenesis may involve oncogene activation via

hypomethylation, since the methylation state of the gene is a factor in controlling transcription (Vorce and Goodman, 1987). Oncogene products can be found both in the cytoplasm and the nucleus of the cell. Those localized in the cytoplasm regulate levels of critical second messenger molecules, such as guanosine triphosphate (GTP), whereas those oncogene products found in the nucleus regulate the transcriptional machinery of the cell (Weinberg, 1985). The specific gene products regulated by retroviral oncogenes appear to a play a major role in the process of neoplastic transformation; however, no analogous gene products or markers have been identified for chemical carcinogens (Pitot et al., 1985).

Expression of some oncogenes is commonly increased during hepatocarcinogenesis in rats (Hsieh et al., 1987), and this pattern of gene expression differs from that seen in normal proliferating rat liver cells (Hsieh et al., 1988). One study found that transcription products of two oncogenes were not increased in carcinogen-induced preneoplastic altered hepatic foci, but were increased in some hepatic nodules and carcinomas (Beer et al., 1986). The authors suggested that this may represent a secondary alteration in gene expression occurring during the later stages of hepatocarcinogenesis.

Some authors have suggested that tumor promoters cause a myriad of effects on the cell by direct or indirect production of oxygen radicals within the cell (Kensler and

Trush, 1984; Cerutti, 1985; Troll and Wiesner, 1985). Other investigators report that protease induction may be a critical event in hepatic tumor promotion (Enomoto et al., 1987).

Selective toxicity. Tumor promoters seem to confer some selective proliferative advantage on the initiated cells over the normal surrounding cells. As opposed to a theory of selective or differential stimulation of the initiated cells by the promoters, several models for hepatocarcinogenesis are based on the apparent resistance of initiated cells to the toxic or growth-inhibiting effects of some promoters (Emmelot, 1971; Farber, 1976; Solt and Farber, 1976; Solt et al., 1977; Farber, 1980; Cameron and Farber, 1981; Schulte-Hermann, 1985; Farber and Sarma, 1987). These "resistant hepatocyte" models are based on the theory of "selective toxicity" or "differential inhibition" by promoters. These promoters appear to be selectively toxic or "mito-inhibitory" for normal, but not initiated, cells. It is possible that the resistant phenotype expressed by these initiated cells represents a genomically programmed form of physiological adaptation to the promoter (Farber, 1984b; Farber and Sarma, 1987).

An example of the resistant hepatocyte phenomenon has been described with promotion by PBBs, where the hepatocyte nodules found in the later stages of promotion appear to be resistant to the lipid accumulation produced within normal hepatocytes by PBBs (Jensen et al., 1983).

Inhibition of Intercellular Communication. Gap junctional intercellular communication refers to the process by which small molecules pass between cells through gap junctions, which are specialized zones of contact between adjacent cells (see reviews: Loewenstein, 1979; Larsen, 1983; Pitts and Finbow, 1986). Gap junctions are plaques composed of arrays of particles, called connexons, embedded in the plasma membrane and extending out into the intercellular space to meet with connexons from adjacent cells (Larsen, 1983; Revel et al., 1984; Pitts and Finbow, 1986). Each connexon is a cylindrical structure about 6-10 nm in diameter, composed of six protein subunits in a ring-like arrangement surrounding a central permeable channel about 2 nm in diameter (Zampighi, 1980; Larsen, 1983; Revel et al., 1984; Pitts and Finbow, 1986). Gap junctions can shift between open and closed configurations, and act as a molecular sieve, allowing relatively small molecules up to about 1000 daltons in molecular weight to diffuse through from one cell to another (Pitts, 1980; Hooper, 1982; Pitts and Finbow, 1986). Junctional permeability is at least partially controlled by intracellular concentrations of Ca^{2+}, H^{+} and cAMP (Hooper, 1982; Trosko et al., 1983; DeMello, 1984; Trosko and Chang, 1984b; Spray and Bennett, 1985; Neyton and Trautmann, 1986; Pitts and Finbow, 1986). Increased intracellular concentrations of Ca^{2+} or H^{+} decrease gap junction permeability, whereas increased cAMP levels enhance junctional communication. Cyclic AMP can

prevent the blockage of intercellular communication and/or restore junctional communication after blockage by compounds such as phenobarbital, DDT (Klaunig and Ruch, 1987b), and TPA (Enomoto et al., 1984). Cyclic AMP appears to affect intercellular communication by inducing gap junction protein synthesis (Enomoto et al., 1984; Kanno, 1985).

Gap junctional intercellular communication is important in the regulation of differentiation and proliferation of cells (Loewenstein, 1979; Pitts, 1980; Finbow and Yancey, 1981; Hooper, 1982; Trosko and Chang, 1984a). One suggested mechanism is that gap junctions allow the transfer and subsequent dilution of growth-stimulatory substances (endogenous growth factors or specific essential metabolites required for growth) to below threshold levels (Loewenstein, 1979; Finbow and Yancey, 1981). Thus in cells with inhibited intercellular communication, these growth-stimulating substances would be undiluted and result in uncontrolled proliferation. Others have suggested that gap junctions allow the transfer of substances such as chalones, which induce differentiation and/or inhibit proliferation (Potter, 1980; Williams, 1981, 1983b). Thus in cells with inhibited junctional communication, the intercellular transfer of these exogenous growth controls would be prevented, again allowing uncontrolled proliferation. By either of these mechanisms, the partial or complete blockage of intercellular communication by tumor promoters could release quiescent tumor cells (initiated cells) from the growth

control of surrounding normal cells (Emmelot, 1971; Yotti et al., 1979; Potter, 1980; Williams, 1981, 1983a, 1983b; Peraino et al., 1983; Trosko et al., 1983; Trosko and Chang, 1984b; Wenner et al., 1984; Yamasaki et al., 1984).

Tumor promoters could inhibit junctional communication by altering the ability of cells to form gap junctional channels, or by affecting the function or structure of gap junctions (Trosko and Chang, 1984b). Promoters may affect the function of gap junctions by modifying cell membranes or by directly or indirectly increasing intracellular calcium concentrations. Activation of protein kinase C has been suggested as a common mechanism for blockage of intercellular communication by both tumor promoters and oncogenes (Trosko et al., 1984; Yamasaki, 1986). Recent studies have associated the activation of protein kinase C with blockage of junctional communication by the tumor promoter TPA (Oh et al., 1988). Some oncogene-transformed cells have increased protein kinase C activity along with reduced junctional communication (Chang et al., 1985), and cells transformed by viral oncogenes have decreased junctional permeability (Azarnia and Loewenstein, 1984a, 1984b).

Many tumor promoters inhibit gap junctional intercellular communication <u>in vitro</u> (Murray and Fitzgerald, 1979; Yotti et al., 1979; Trosko et al., 1981b; Williams et al., 1981; Lawrence et al., 1984; Warngard et al., 1985; Zeilmaker and Yamasaki, 1986; Jone et al., 1987; Rolin-

Limbosch et al., 1987; Ruch et al., 1987). Some tumors have decreased numbers of gap junctions as compared to their non-neoplastic tissue of origin (Weinstein et al., 1976; Finbow and Yancey, 1981; Swift et al., 1983; Janssen-Timmen et al., 1986). The loss of intercellular communication alone is not a sufficient event for the onset of malignant growth, but it may be one of several steps required for such growth (Trosko et al., 1983), or alternatively it may be merely a consequence of malignant transformation (Finbow and Yancey, 1981).

Many assays have been developed to study a compound's ability to disrupt intercellular communication. One type of assay is the metabolic cooperation assay, which depends on the exchange of specific metabolites between cells, some of which are genetically unable to form these metabolites themselves (Subak-Sharpe et al., 1969; Hooper, 1982). In this assay wild-type cells containing the enzyme hypoxanthine guanine phosphoribosyltransferase (HGPRT+ cells) are co-cultured with mutant cells ($HGPRT^-$ cells) which lack the enzyme (Trosko et al., 1981b, 1982; Hooper, 1982). The HGPRT+ cells can metabolize the purine analog 6-thioguanine to a toxic nucleotide which is subsequently incorporated into nucleic acids, resulting in cell death. $HGPRT^-$ cells cannot metabolize 6-thioguanine, but can receive the toxic nucleotide via gap junctional communication with adjacent HGPRT+ cells. Transfer of the toxic nucleotide can be prevented by chemicals which inhibit

intercellular communication, allowing HGPRT$^-$ cells to survive. The relative number of HGPRT$^-$ survivors corresponds to the degree of inhibition of metabolic cooperation, and thus intercellular communication, by the chemical.

Another type of assay is the fluorescent dye transfer assay, which depends on the exchange of dye molecules between cells through gap junctions (Loewenstein and Kanno, 1964; Loewenstein, 1979). Many of these assays use fluorescein or carboxyfluorescein dye as tracer molecules, which can be injected into a cell using a micropipette and then monitored for spread into adjacent cells through gap junctions. Some assays have been developed to avoid the need for microinjection procedures (Goodall and Johnson, 1982; Wade et al., 1986). In these assays, the dye molecules are introduced into the culture medium in nonpolar, hydrophobic ester form, such as carboxyfluorescein diacetate. These esters will diffuse into a cell across the intact plasma membrane. Once inside the cell, the dye molecules are quickly hydrolyzed by esterases into hydrophilic free carboxyfluorescein, which cannot readily diffuse across the intact plasma membrane to leave the cell (Rotman and Papermaster, 1966). In this manner cells can be labelled with fluorescein molecules for intercellular communication studies.

An obvious limitation of <u>in vitro</u> assays for tumor promoters is that a single assay can only include one specific set of conditions, whereas tumor promotion <u>in vivo</u>

involves compounds which are tissue- and species-specific and are influenced by the hormonal, immunological, and environmental status of the animal. Cells in culture are not necessarily analogous to intact cells in a living organism. Metabolic activation and overall cellular metabolic processes can vary greatly between in vitro and in vivo conditions. Furthermore, since the development of cancer is presumably the response of a cell population to certain internal and external stimuli, it is difficult to know if the cellular responses seen in vitro are comparable to those occurring in vivo (Farber and Sarma, 1987).

Preneoplastic Lesions in Rat Liver

Morphologic changes during hepatocarcinogenesis represent a relatively continuous spectrum from the earliest recognizable cellular alterations to highly undifferentiated carcinomas.

Microscopic islands or foci of altered hepatocytes, which are often referred to as "preneoplastic", are the first recognizable proliferation of phenotypically altered cells seen in hepatic carcinogenesis (Scherer and Hoffmann, 1971; Pitot and Sirica, 1980; Scherer, 1984; Farber and Sarma, 1987). These foci appear to be monoclonal in origin, meaning they are clones of cells derived from a single precursor cell (Rabes et al., 1982; Weinberg et al., 1987).

Foci of altered hepatocytes can be classified in at least four distinct phenotypes using routine histologic stains such as hematoxylin and eosin: clear cell foci,

acidophilic (eosinophilic or ground glass) cell foci, basophilic cell foci, and mixed cell foci (Squire and Levitt, 1975; Institute of Laboratory Animal Resources, 1980; Peraino et al., 1983; Bannasch et al., 1985). There is some evidence for sequential development of foci from clear and acidophilic cell types to mixed cell types and finally to basophilic cell types (Enzmann and Bannasch, 1987).

Many specific biochemical alterations can be used as markers for these foci, including alterations in carbohydrate metabolism, decreased ability to store iron, decreases in glucose-6-phosphatase, ATPase, and beta-glucuronidase, as well as increases in alpha-fetoprotein, gamma glutamyltranspeptidase (GGT), and increased glycogen storage after fasting (Farber, 1976; Farber, 1980; Pitot and Sirica, 1980; Scherer, 1984). However, altered foci are not a homogeneous population in terms of the specific markers, and more advanced neoplastic lesions such as nodules and carcinomas show a similar individual variability in their pattern of biochemical alterations (Pitot, 1977, 1980; Emmelot and Scherer, 1980; Pitot et al., 1982). Furthermore, the phenotypic heterogeneity expressed by altered foci may very well represent different developmental stages in an ordered sequence of metabolic changes occurring during the carcinogenic process (Bannasch, 1986; Enzmann and Bannasch, 1987). No single critical biochemical marker has been identified for preneoplastic lesions in rat liver (Pitot et al., 1985).

Environmental factors such as diet may alter the expression of these markers. Rats fed a purified diet after the promotion phase had a lower number of foci, suggesting that crude nonpurified diets may contain some agents which influence foci persistence or the stability of markers (Pitot et al., 1985; Hendrich et al., 1986).

The enzyme GGT is the most efficient marker for altered hepatic foci produced in the Pitot protocol for two-stage hepatocarcinogenesis, identifying over 80% of the foci (Pitot et al., 1978, 1985). This efficiency is partly due to direct induction of GGT by phenobarbital, however GGT also appears to be the most efficient marker for other promoters such as dioxin (Pitot et al., 1985).

Hepatic enzyme-altered foci (EAF) are used as the endpoint in many hepatocarcinogenesis bioassays. The enhancement of EAF in these assays correlates well with the appearance of hepatic tumors in long-term studies (Scherer and Emmelot, 1975; Squire and Levitt, 1975; Watanabe and Williams, 1978; Emmelot and Scherer, 1980), although not in a direct 1:1 ratio. Numbers of EAF produced are several orders of magnitude greater than the numbers of tumors which eventually occur (Peraino et al., 1983). Altered foci may undergo redifferentiation to normal hepatic parenchyma if the promoting stimulus is removed (Scherer, 1984), which suggests that some foci may be nontumorigenic or else that they simply require further action by the promoter to express their neoplastic potential (Peraino et al., 1983).

Hepatocyte nodules are the next visible stage in the sequence of preneoplastic lesions, and appear to be derived from the progression of EAF (Emmelot and Scherer, 1980; Institute of Laboratory Animal Resources, 1980). These nodules are variously termed "hyperplastic nodules", "neoplastic nodules", or "adenomas" (Institute of Laboratory Animal Resources, 1980). Farber and Sarma (1987) have proposed that these lesions be referred to as "hepatocyte nodules", since this is a more neutral term without implications regarding their fate or significance.

Grossly, hepatocyte nodules can be identified as grayish-white spots in the liver (Farber, 1976). Histologically, hepatocyte nodules are distinct proliferations of altered hepatocytes which are larger than EAF and compress the surrounding normal liver (Squire and Levitt, 1975; Institute of Laboratory Animal Resources, 1980; Farber and Sarma, 1987).

There are two types of hepatocyte nodules: those that remodel or differentiate into apparently normal liver after exposure to a promoter ceases, and those that remain as persistent nodules and have spontaneous cell proliferation (Farber, 1980; Pitot and Sirica, 1980; Farber, 1984a, 1984b; Farber and Sarma, 1987). Once exposure to the promoting influence is terminated, about 95-98% of the nodules undergo phenotypic reversion or differentiation into normal liver and in effect "disappear", while 2-5% remain as persistent nodules, which may undergo further progression into

hepatocellular carcinomas (Tatematsu et al., 1983; Farber, 1984a, 1984b; Farber and Sarma, 1987).

Hepatocellular carcinomas occur in several forms, ranging from differentiated trabecular carcinomas and adenocarcinomas to highly undifferentiated, anaplastic carcinomas (Squire and Levitt, 1975; Farber, 1976; Institute of Laboratory Animal Resources, 1980). Gross or microscopic areas of hemorrhage and necrosis are common in these tumors (Farber, 1976).

A sequence of neoplastic progression consisting of normal liver, EAF, hepatocyte nodule, differentiated carcinoma and finally anaplastic carcinoma has been widely accepted (Newberne, 1976; Farber, 1980; Goldfarb and Pugh, 1982; Scherer, 1984), although this is not necessarily an obligate progression. For example, carcinomas may develop directly from EAF, without passing through a nodule stage (Williams, 1980b; Enzmann and Bannasch, 1987).

Proliferation of a population of cells described as biliary ductular epithelial cells or oval cells has been described as a common feature of the early stages of hepatocarcinogenesis induced by many chemicals (Farber, 1976; Williams, 1980b). These oval cells appear to be a heterogeneous population of epithelial cells, and evidence indicates that they can transform or differentiate into hepatocytes (Inaoka, 1967; Pitot and Sirica, 1980; Evarts et al., 1987; Germain et al., 1988). Thus oval cells appear to be hepatocytic precursors or stem cells. The role of oval

cells in the development of EAF is unclear (Williams, 1980b).

Cultured rat liver epithelial cells appear to be oval cells (Tsao et al., 1984a), and after *in vitro* transformation these cells can be transplanted into neonatal rats or nude mice and produce a variety of hepatic tumors, including hepatocellular carcinomas (Sinha et al., 1987; Tsao and Grisham, 1987). One study found that nonparenchymal liver epithelial cells isolated from the livers of carcinogen-treated rats could be transfected *in vitro* with an oncogene and produce hepatocellular carcinomas when transplanted into nude mice (Braun et al., 1987). Thus these cells appear to be multipotent stem cells (Grisham, 1980; Braun et al., 1987; Sinha et al., 1987; Tsao and Grisham, 1987).

Polybrominated Biphenyls

Chemistry. Polybrominated biphenyls (PBBs) are halogenated aromatic hydrocarbons which are structurally and functionally similar to polychlorinated biphenyls (PCBs), chlorinated dibenzofurans and dibenzodioxins (McConnell and Moore, 1979; Rappe et al., 1979; Goldstein, 1980). FireMaster BP-6 (FM) is a commercial mixture of about 12-14 major PBB congeners (Moore and Aust, 1978; Moore et al., 1978a; Aust et al., 1981; Dannan et al., 1982a) which accidentally contaminated livestock feed in Michigan in 1973 (Dunckel, 1975; Carter, 1976; Kay, 1977; Fries, 1985). Approximately 90% of persons who were Michigan residents in

1973-1974 have detectable tissue residues of PBB (Brilliant et al., 1978; Wolff et al., 1982).

The most abundant congener in the FM mixture is 245-HBB, which comprises about 50-70% of the mixture on a weight basis (Sundstrom et al., 1976; Aust et al., 1981; Robertson et al., 1984). The next most abundant congener is 2,3,4,2',4',5'-hexabromobiphenyl, which comprises about 12% of the mixture (Aust et al., 1981; Robertson et al., 1983, 1984). About 25 different congeners have been identified in the FM mixture, including 3,4,3',4'-tetrabromobiphenyl (Orti et al., 1983; Robertson et al., 1983, 1984).

The FM mixture is classified as a mixed microsomal enzyme inducer, since it induces both 3-methylcholanthrene (cytochrome P448) and phenobarbital (cytochrome P450) types of microsomal enzymes (Dent et al., 1976a, 1976b). The individual congeners are classified as either phenobarbital, 3-methylcholanthrene, or mixed type inducers. For example, 245-HBB is a pure phenobarbital-type inducer, which induces cytochrome P450 isozymes such as NADPH-cytochrome P-450 reductase, aminopyrine demethylase and epoxide hydratase (Moore et al., 1978b). Phenobarbital-type induction is generally associated with low toxicity of the congener, and a structural pattern including bromination of one or both <u>ortho</u> carbons on each of the rings (Dannan et al., 1983). The congener 345-HBB, which is present in FM in very small quantities (Orti et al., 1983; Robertson et al., 1984), is a pure 3-methylcholanthrene-type inducer (Aust et al., 1981;

Robertson et al., 1982, 1984), inducing cytochrome P448 isozymes such as aryl hydrocarbon hydroxylase. This type of induction is generally associated with greatest toxicity and a structural pattern which allows a coplanar configuration similar to TCDD and binding to the Ah (TCDD) receptor protein. The absence of ortho bromines and the presence of at least two adjacent lateral bromines (meta and para) appears to allow the coplanar configuration and binding to the Ah receptor. The congener 2,4,5,3',4',5'-hexabromobiphenyl is a mixed-type inducer (Dannan et al., 1978a).

Polybrominated biphenyls are very poorly soluble in water and highly resistent to degradation, making them very stable compounds in animal tissues and the environment (Fries, 1985). The half-life of FM in the adipose tissue of rats is about 69 weeks (Miceli and Marks, 1981). The estimated half-life of 245-HBB in humans is 6.5 years (Tuey and Matthews, 1980).

The rate of metabolism of a given PBB congener apparently depends on the degree of bromination and the positions of the bromines (Mills et al., 1985). Increased bromination is generally associated with a decreased rate of metabolism of the congener. Most congeners which can be metabolized to any appreciable extent have adjacent nonhalogenated carbons in the ortho and meta or meta and para positions. Others suggest that a nonhalogenated para carbon is required for metabolism (Dannan et al., 1978b).

Toxicity and Pathology in the Rat. Polybrominated biphenyls cause a delayed toxicity syndrome in many species, including rats (see reviews: Kay, 1977; Kimbrough et al., 1978a; Damstra et al., 1982; Fries, 1985). The toxic syndrome includes weight loss, porphyria, thymic and splenic atrophy, hepatic enlargement, fatty degeneration of the liver (Sleight and Sanger, 1976; Gupta and Moore, 1979; Gupta et al., 1981, 1983a; Render et al., 1982), thyroid gland changes resembling hyperplastic goiter (Sleight et al., 1978; Akoso et al., 1982b), and depletion of hepatic vitamin A stores (Akoso et al., 1982a; McCormack et al., 1982; Darjono et al., 1983).

The liver changes described in PBB-intoxicated rats include gross hepatic enlargement, hepatocellular swelling, disruption of hepatic architecture, individual hepatocellular necrosis, fatty vacuolation of hepatocytes, and bile duct proliferation (Sleight and Sanger, 1976; Gupta and Moore, 1979; Gupta et al., 1981, 1983a).

The relatively nontoxic congener 245-HBB produces primarily hepatocellular swelling, lipid vacuolation and proliferation of smooth endoplasmic reticulum, whereas the more highly toxic congener 345-HBB additionally produces bile ductule proliferation, myelin body formation, and disorganization of rough endoplasmic reticulum (Render et al., 1982).

Role in Carcinogenesis. Chronic toxicity studies with PBBs in rats have shown an increased incidence of hepatic

nodules and carcinomas (Kimbrough et al., 1978b, 1981; Gupta et al., 1983b; Groce and Kimbrough, 1984). Polybrominated biphenyls act as hepatic tumor promoters in initiated rats (Jensen et al., 1982, 1983, 1984; Jensen and Sleight, 1986). The FM mixture and 245-HBB both promote hepatic EAF in initiated rats, although FM appears to be a more potent promoter on a weight basis than 245-HBB (Jensen et al., 1982). This may be due to the presence of very potent individual minor congeners in the FM mixture (Jensen et al., 1982), or to an additive or synergistic effect of the mixture of congeners (Jensen et al., 1982; Jensen and Sleight, 1986).

The metabolizable congener, 3,4,3',4'-tetrabromobiphenyl (34-TBB), which is a moderately toxic 3-methylcholanthrene-type enzyme inducer, promotes hepatic EAF at nontoxic doses in initiated rats (Dixon et al., 1988). In contrast to 245-HBB and 34-TBB, the congener 345-HBB promotes hepatic EAF only at doses which produce decreased body weight gain and hepatotoxicity (Jensen et al., 1983; Jensen and Sleight, 1986).

The mechanism of promotion by PBBs is unknown. Some authors (Weinstein et al., 1982) have proposed that the tumor-promoting activity of polycyclic aromatic hydrocarbons is mediated by the cellular aromatic hydrocarbon receptor, called the Ah or TCDD receptor. Thunberg and coworkers have suggested that TCDD might act as a promoter by decreasing tissue vitamin A levels (Thunberg et al., 1979, 1980, 1984;

Thunberg, 1984).

The hepatic tumor promoting activity of 345-HBB does not appear to be related to hepatic microsomal enzyme induction by the chemical (Jensen et al., 1983), since a dose sufficient to induce microsomal enzymes is not necessarily sufficient to promote EAF. Jensen et al. (1983) proposed that 345-HBB may act as a promoter by producing chronic toxicity and necrosis of noninitiated hepatocytes, thus producing a prolonged regenerative stimulus and proliferation of initiated cells.

It has been suggested that 345-HBB has a different mechanism of hepatic tumor promoting activity than 245-HBB (Jensen et al., 1983; Jensen and Sleight, 1986). In contrast to 345-HBB, 245-HBB is an effective promoter at nontoxic doses (Jensen et al., 1982). Furthermore, a combination of a promoting dose of 245-HBB and a nonpromoting dose of 345-HBB produces a synergistic enhancement of hepatic EAF in initiated rats, again implying different mechanisms for the promoting effects of these two congeners (Jensen and Sleight, 1986).

Effects on Intercellular Communication. FireMaster BP-6 and 245-HBB block metabolic cooperation at nontoxic concentrations in cultured V79 fibroblasts (Trosko et al., 1981a; Tsushimoto et al., 1982, 1983) and in human teratocarcinoma cells (Kavanagh et al., 1987). The congener 245-HBB also blocks junctional communication in dye transfer assays using rat liver epithelial cells (Evans et al.,

1988). In contrast, 345-HBB does not block metabolic cooperation at nontoxic doses (Tsushimoto et al., 1982; Kavanagh et al., 1987).

These results correspond well with the hepatic tumor promoting activity of PBBs seen *in vivo*, where FM and 245-HBB are effective promoters at nontoxic doses while 345-HBB promotes only at hepatotoxic doses (Jensen et al., 1982, 1983; Jensen and Sleight, 1986). The congener 34-TBB, which is a hepatic tumor promoter *in vivo*, does not block metabolic cooperation at nontoxic doses in human teratocarcinoma cells (Kavanagh et al., 1987). One explanation offered for this inconsistency is that the teratocarcinoma cells apparently lack the ability to metabolize 34-TBB by microsomal enzyme pathways, and a metabolite of 34-TBB is believed to be the active agent for tumor promotion (Kavanagh et al., 1987).

Vitamin A Compounds (Retinoids)

Chemistry, Metabolism and Homeostasis. Retinoids are fat-soluble compounds with vitamin A bioactivity. The major retinoids encountered in nature include retinol (the alcohol), retinal (the aldehyde), retinoic acid, and the retinyl esters.

Animals are not capable of *de novo* synthesis of retinoids, but require exogenous sources of carotenoid precursor forms of vitamin A, such as beta-carotene, or preformed retinoids themselves for vitamin A function (see reviews: Wolf, 1980, 1984; Goodman, 1984a, 1984b; Goodman

and Blaner, 1984; Underwood, 1984). While some species of animals absorb a portion of their dietary carotenoids into the general circulation, generally the ingested carotenoids are converted to retinol in the intestinal mucosa. Likewise, dietary retinyl esters are hydrolyzed in the intestinal lumen to free retinol which passes into the intestinal mucosa. Retinol in the intestinal mucosa is esterified, incorporated into chylomicrons, and transported into the general circulation via the lymphatics (Goodman, 1984a, 1984b; Goodman and Blaner, 1984; Underwood, 1984).

The retinyl esters in chylomicrons are preferentially removed from the general circulation fairly quickly by hepatocytes, where they are hydrolyzed to retinol (Wolf, 1980, 1984; Goodman, 1984a; Goodman and Blaner, 1984; Underwood, 1984). This retinol may then bind with plasma retinol binding protein (RBP) and be released back into the circulation, or it may be reesterified and stored in the liver. The liver appears to have two distinct storage pools for vitamin A (Goodman, 1984a, 1984b; Wolf, 1984; Hendriks et al., 1985; Blomhoff, 1987). One is a large, long-term storage pool in nonparenchymal cells of the liver, and the other is a smaller, short-term storage pool in hepatocytes which is utilized in vitamin A-deficient states. The nonparenchymal storage cells are referred to as "perisinusoidal stellate cells," "lipocytes," or "Ito cells" (Goodman, 1984a, 1984b; Blomhoff, 1987).

Stored retinyl esters in the liver are mobilized by

retinyl ester hydrolases (primarily retinyl palmitate hydrolase) to produce free retinol, which then binds to apo-RBP (the binding protein without retinol) to form the retinol-RBP complex, holo-RBP (Wolf, 1980, 1984; Goodman, 1984c; Goodman and Blaner, 1984; Underwood, 1984). Holo-RBP combines with prealbumin (also known as transthyretin) and is released into the blood where it circulates as a retinol-RBP-prealbumin complex (Muto and Goodman, 1972; Wolf, 1980; Goodman, 1984a, 1984b, 1984c). It is believed that this elaborate transport system functions to help protect the unstable retinol molecules, as well as to protect the tissues from the surfactant and toxic actions of free retinol (Wolf, 1980, 1984). Furthermore, the prealbumin complex cannot pass through the glomeruli as holo-RBP alone can, thus preventing loss of the holo-RBP complex in the glomerular filtrate. Uncomplexed holo-RBP which does pass into the glomerular filtrate is metabolized in the renal tubules, releasing retinol which can be esterified and stored in the kidney. Relatively small amounts of retinyl esters are stored in the kidney, and may be recycled to the liver via plasma lipoprotein complexes.

Each individual has a steady-state or homeostatic level of circulating plasma retinol, which tends to be maintained under all conditions except a state of extreme hepatic vitamin A depletion or excess (Olson, 1984; Underwood, 1984; Wolf, 1984). Release of vitamin A from the liver and into the circulation appears to controlled by the vitamin A needs

of extrahepatic tissues (Wolf, 1984).

Upon arrival at the target cell, the holo-RBP-prealbumin complex binds to a cell surface receptor and releases retinol into the cell (Wolf, 1980, 1984; Goodman, 1984c). Many cells contain cellular (cytosolic) retinol binding protein (CRBP) and/or cellular retinoic acid binding protein (CRABP) in their cytoplasm (Ong and Chytil, 1975, 1978a, 1978b; Sani and Banerjee, 1978; Saari et al., 1978; Ross et al., 1978). Cellular retinoic acid binding protein can bind several other vitamin A derivatives, but not retinol or retinal (Wolf, 1980, 1984). Nuclear binding of CRBP has been shown to occur, and supports the theory that vitamin A can act like steroid hormones to regulate gene expression (Zile and Cullum, 1983; Goodman, 1984b; Wolf, 1984). Vitamin A also appears to have direct effects on cytoplasmic components such as glycoproteins, endoplasmic reticulum and cell membranes, particularly the plasma membrane.

Retinol and retinal can be reversibly oxidized and reduced *in vivo*, but the oxidation of retinol or retinal to retinoic acid is irreversible (Underwood, 1984; Wolf, 1984). Retinoic acid is a highly active metabolite of retinol in some tissues (Wolf, 1980, 1984). Many other metabolites of retinol have been identified, but the functional significance of these derivatives is unknown.

Metabolism of retinol in the target peripheral tissues eventually results in the formation of polar catabolic end

products, including some formed from retinoic acid intermediates, which are finally excreted in the bile and urine (Wolf, 1980, 1984; Underwood, 1984).

Deficiency and Toxicity in the Rat. Animals require exogenous vitamin A sources to maintain several normal functions: overall body growth, differentiation and integrity of epithelial tissues, bone remodeling, reproduction and vision (see reviews: Wolf, 1980; Underwood, 1984).

Many clinical signs can be observed in vitamin A-deficient rats. Impaired growth rate in young animals is the first clinical manifestation and a sensitive indicator of vitamin A deficiency (Wolf, 1980; Zile and Cullum, 1983; Underwood, 1984).

Keratinization and/or squamous metaplasia of various epithelial tissues is seen in vitamin A-deficient rats (Wolf, 1980; Underwood, 1984). Epithelial tissues such as the cornea, conjunctiva, salivary glands, and the mucosa of the respiratory, urinary and genital tracts have decreased numbers of goblet cells and squamous metaplasia of the epithelium. The corneal changes cause a roughened, wrinkled and nonreflecting appearance of the cornea, termed xerophthalmia. Decreased numbers of goblet cells are seen in the intestinal mucosa. Squamous metaplasia is seen in the epithelium of the intrahepatic bile ducts. The epidermis becomes hyperkeratotic, thickened, dry and scaly. These altered epithelial tissues are compromised by loss of the

protective mucous secretions, and are susceptible to microbial infections.

Chronic vitamin A deficiency in growing rats produces alterations in bone formation, such as loss of bone density, thickened bones, and bone deposition in abnormal locations (Wolf, 1980; Underwood, 1984). These lesions are due to decreased osteoclastic activity (Wolf, 1980). Elevated cerebrospinal fluid pressure is believed to be due to impaired absorption of cerebrospinal fluid by vitamin A-deficient epithelial surfaces such as the arachnoid villi and dura mater (Wolf, 1980; Underwood, 1984). Vitamin A deficiency produces decreased levels of some hepatic drug-metabolizing enzymes, including cytochrome P450 (Dogra et al., 1983).

Sterility is seen in vitamin A-deficient adults, due to impaired spermatogenesis in males and a lack of the vitamin A required in females for normal estrous cycling, conception, implantation, maintenance of gestation and embryonic development. While retinoic acid can fulfil the vitamin A requirement for many functions, spermatogenesis apparently has a specific requirement for retinol, and visual function requires retinal (Wolf, 1980; Zile and Cullum, 1983; Goodman, 1984b; Underwood, 1984).

The general signs of hypervitaminosis A include anorexia, weight loss, hair loss and thickening of the skin, anemia, and cachexia (Kamm et al., 1984). Vitamin A toxicity is characterized by lesions in bone and cartilage,

hemorrhages in muscle and various tissues, teratogenic effects on exposed fetuses, and fatty change in the liver (Wolf, 1980; Kamm et al, 1984). Pharmacologic doses of retinyl acetate produce a low incidence of mild hepatomegaly, hepatic periportal fibrosis and bile duct hyperplasia (McCormick et al., 1986, 1987b). The bony and cartilaginous changes are characterized by osteoporosis and fractures, exostosis, and destruction of epiphyseal cartilage (Wolf, 1980). These bony changes are due to decreased osteogenesis resulting from decreased osteoblastic activity. Vitamin K deficiency secondary to excess vitamin A causes the hemorrhages seen in various tissues.

Role in Carcinogenesis. Dietary factors are one of the major risk determinants for the development of cancer (Kroes et al., 1986), and are known modifiers of the process of hepatocarcinogenesis (Pitot and Sirica, 1980). The promotion and progression phases of carcinogenesis are particularly susceptible to modulation by dietary and hormonal factors (Farber and Sarma, 1987). Many different nutritional components have been shown to influence tumor development, and among the most studied of these are the vitamin A compounds.

The interaction between retinoids and carcinogenesis appears to be a complex one. Retinoids are generally considered to be anticarcinogenic agents, particularly during the promotion phase of tumorigenesis in skin, lung, mammary gland and urinary bladder (see reviews: Lotan, 1980;

Bollag and Matter, 1981; Bollag and Hartmann, 1983; Hicks, 1983b; Ong and Chytil, 1983; Blunck, 1984; Moon and Itri, 1984). However, in some systems, retinoids have no effect on (Wenk et al., 1981; Silverman et al., 1981; Birt et al., 1983; Decaens et al., 1983; Longnecker et al., 1983; Beems, 1984; Gensler and Bowden, 1984; Welsch et al., 1984; Ohshima et al., 1985), or even enhance (Silverman et al., 1981; Stinson et al., 1981; Welsch et al., 1981; Hennings et al., 1982; Stinson and Reznik, 1982; Verma et al., 1982; Birt et al., 1983; Longnecker et al., 1983; Mian et al., 1984; Fischer et al., 1985; Kurokawa et al., 1985; McCormick et al., 1987a), the carcinogenic process.

Most reports indicate that vitamin A deficiency increases susceptibility to cancer (Newberne and Rogers, 1973; Rogers et al., 1973; Nettesheim and Williams, 1976; Newberne and Suphakarn, 1977; Dogra et al., 1985). However, others report no effect (Zile et al., 1986). Epidemiologic studies of human cancer incidence show some correlation between decreased vitamin A levels or intake and an increased incidence of cancer (see reviews: Kummet and Meyskens, 1983; Goodman, 1984b; Hennekens et al., 1986). Clinical trials of retinoids for therapy in human cancer patients have met with some success (see reviews: Bollag and Hartmann, 1983; Kummet and Meyskens, 1983; Ong and Chytil, 1983; Peck, 1983; Goodman, 1984b).

The critical mechanisms by which retinoids influence the carcinogenic process are unknown, although many possible

mechanisms have been suggested. These include inhibition of ornithine decarboxylase activity, stimulation of the synthesis of certain glycoproteins, alteration of gene expression via steroid hormone-like receptors, stimulation of immune system surveillance, simulation of gap junctional intercellular communication, altering plasma or lysosomal membrane fluidity, stimulation of various protein kinase systems, and effects on the state of cellular differentiation and/or proliferation (see reviews: Lotan, 1980; Sporn and Roberts, 1983; Blunck, 1984; Mandani and Elmongy, 1986).

Retinoids have profound effects on differentiation of a variety of neoplastic and nonneoplastic cells in culture, and counteract the effects of tumor-promoting phorbol esters in several systems (Goodman, 1984b).

Retinoic acid has variable and inconsistent effects on the growth and biochemical properties of transformed rat liver epithelial cells in culture (Tsao et al., 1987). The biochemical enzymatic changes induced in rat liver epithelial cells by RA are the opposite of some of the changes observed during chemical transformation of these cells by N-methyl-N'-nitro-N-nitrosoguanidine (Tsao et al., 1984b).

Effects on Intercellular Communication. Similar to their inconsistent effects on *in vivo* carcinogenesis, the retinoids have variable effects on gap junctions and intercellular communication. Topical retinoic acid

application to basal cell carcinomas in human patients produced proliferation of gap junctions between the neoplastic cells (Elias et al., 1980). However, retinoic acid had no effect on the disappearance of gap junctions produced by TPA in mouse epidermis (Kalimi and Sirsat, 1984).

Retinoic acid has been reported to enhance (Yamasaki et al., 1984) or have no significant inhibitory effect on intercellular communication (Trosko et al., 1982; Radner and Kennedy, 1984; Morel-Chany et al., 1986). However, others report that retinoic acid does inhibit intercellular communication, at least in high concentrations (Shuin et al., 1983; Walder and Lutzelschwab, 1984; Davidson et al., 1985; Pitts et al., 1986). Less work has been described with other retinoids, such as retinol or retinyl acetate. One study found that retinol, retinyl acetate and retinyl palmitate had no effect on intercellular communication (Pitts et al., 1986), while another study found that retinol, retinal and retinyl acetate did inhibit intercellular communication, although not as strongly as retinoic acid (Davidson et al., 1985).

Biphasic effects on intercellular communication, depending on the retinoid concentration, have been observed (Rutten et al., 1988). In this study, physiologic (low) concentrations of retinoic acid and retinol actually increased intercellular communication, whereas pharmacologic (high) concentrations inhibited intercellular communication.

Some studies have found that retinoic acid protects against TPA-induced blockage of intercellular communication (Shuin et al., 1983; Yamasaki et al., 1984), while others found that retinoic acid enhanced the blockage of intercellular communication by TPA (Shuin et al., 1983; Radner and Kennedy, 1984; Davidson et al., 1985; Morel-Chany et al., 1986). A recent study found that retinol can prevent the blockage of intercellular communication produced by cigarette-smoke condensate (Rutten et al., 1988).

The effects of vitamin A on intercellular communication *in vitro* seem to vary greatly with retinoid concentration, cell type, culture conditions, and the type of communication assay.

Halogenated Aromatic Hydrocarbons and Vitamin A

Halogenated aromatic hydrocarbons, such as PBBs, PCBs and TCDD, cause marked disturbances in vitamin A homeostasis. Significant reduction of hepatic vitamin A stores is seen in animals given PCBs (Villeneuve et al., 1971; Bitman et al., 1972; Cecil et al., 1973; Innami et al., 1974, 1976, 1982; Saito et al., 1982, 1983; Brouwer and van den Berg, 1984; Brouwer et al., 1985; Spear et al., 1986), TCDD (Thunberg et al., 1979, 1980, 1984; Thunberg and Hakansson, 1983; Thunberg, 1984; Hakansson and Ahlborg, 1985), or PBBs (Akoso et al., 1982a; McCormack et al., 1982; Bernert et al., 1983; Darjono et al., 1983; Jensen et al., 1985). Both FM (Akoso et al., 1982a; McCormack et al., 1982; Bernert et al., 1983; Darjono et al., 1983), and 345-HBB

(Akoso et al., 1982a; Jensen et al., 1985, 1987) cause marked depletion of hepatic vitamin A stores, whereas 245-HBB does not decrease hepatic vitamin A stores (Akoso et al., 1982a).

The mechanism by which these chemicals cause alterations in vitamin A homeostasis is unknown. Two enzymes which are involved in vitamin A metabolism, acyl-CoA:retinol acyltransferase and retinyl palmitate hydrolase, are decreased in the livers of rats treated with 345-HBB (Jensen et al., 1987). Acyl-CoA:retinol acyltransferase levels in the kidney are unaffected by 345-HBB treatment (Jensen et al., 1987). Changes in the vitamin A distribution in serum and kidney are seen in animals given PCBs (Brouwer and van den Berg, 1984, 1986; Brouwer et al., 1985; Powers et al., 1987), TCDD (Thunberg et al., 1979; Thunberg, 1984; Hakansson and Ahlborg, 1985), or PBBs (Bernert et al., 1983; Darjono et al., 1983; Jensen et al., 1985, 1987). Increased vitamin A excretion in urine and feces has been reported in rats given TCDD (Hakansson and Ahlborg, 1985) or PBB (Cullum and Zile, 1985; Jensen et al., 1985, 1987). It has been proposed that increased renal metabolism of vitamin A and deregulation of hepatic retinoid metabolism, leading to increased retinoid catabolism (Jensen et al., 1987), are responsible for the changes in vitamin A homeostasis produced by PBBs (Cullum and Zile, 1985). Others have proposed that PCBs interfere with vitamin A transport in the serum (Brouwer and van den Berg, 1986).

Although the mechanism by which they act is unclear, it is apparent that the halogenated aromatic hydrocarbons, including PBBs, cause major alterations in the regulation of vitamin A metabolism and subsequently in vitamin A homeostasis. Such observations led to the interesting, but unproven, proposal by Thunberg and coworkers that TCDD might promote tumorigenesis by producing vitamin A deficiency (Thunberg et al., 1979, 1980, 1984; Thunberg, 1984).

CHAPTER 1

SHORT-TERM ORAL ADMINISTRATION OF POLYBROMINATED BIPHENYLS ENHANCES THE DEVELOPMENT OF ENZYME-ALTERED HEPATIC FOCI IN RATS

CHAPTER 1

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Abstract

Firemaster BP-6 (FM), a commercial mixture of polybrominated biphenyls (PBBs), has been shown to act as a tumor promoter in hepatocarcinogenesis assays in rats. Most hepatic tumor promoters must be administered for many weeks or months. Because FM is highly persistent in animal tissues, it was hypothesized that very short-term administration of FM would result in tumor promotion. Female Sprague-Dawley rats weighing 185-215 g were partially hepatectomized, and injected with 10 mg diethylnitrosamine/kg body weight (BW) intraperitoneally 24 hours later. Thirty days later rats were gavaged with FM in corn oil, at total doses of 0, 13, or 130 mg FM/kg BW. Half the dose was given on day 30, and the remaining half was given 24 hours later. At 120 days after gavage the rats were killed and necropsied. Five liver sections from each animal were histochemically stained for gamma-glutamyl transpeptidase-positive enzyme-altered foci (EAF). EAF were significantly increased over control values in initiated

rats given 130 mg FM/kg, but not in animals given 13 mg FM/kg. Enhancement of these EAF in initiated rats reflects tumor promoting activity. In this study, 24 hour administration of FM in initiated rats was sufficient to enhance hepatic EAF measured 120 days later in an initiation-promotion protocol, and a dose of 13 mg FM/kg was apparently below the no-effect threshold level for enhancement of EAF.

Introduction

Experimental hepatocarcinogenesis can be regarded in at least two distinct stages: initiation and promotion. The initiation phase is generally considered to be analogous to a mutation-like event in the target cell resulting from a single exposure to the initiator, and is essentially irreversible (Farber, 1980; Pitot and Sirica, 1980; Scherer, 1984; Schulte-Hermann, 1985). The events occurring during promotion are not well defined, but are believed to involve epigenetic alterations in the expression of genetic material and/or indirect action on the genome, and are reversible to some extent. Hepatic tumor promotion requires relatively long-term action by the promoter (Pitot and Sirica, 1980; Pitot et al., 1982; Schulte-Hermann et al., 1983a; Schulte-Hermann, 1985). Mechanisms such as prolonged hyperplastic stimuli, or selective cytotoxicity for non-initiated cells, have been implicated in providing selective pressure for the proliferation of initiated cells during the promotion phase (Pitot and Sirica, 1980; Barbason

et al., 1983; Peraino et al., 1983; Schulte-Hermann, 1985). Alternatively, promotion may result from the specific induction of phenotypic expression of the molecular lesion produced during initiation, without external selection for initiated cells (Peraino et al., 1983; Schulte-Hermann, 1985).

Various initiation-promotion assays for hepatocarcinogenesis in rats have been developed in the past 15 years (Peraino et al., 1971; Solt and Farber, 1976; Cayama et al., 1978; Pitot et al., 1978; Herren et al., 1982). These protocols allow separation of the initiation and promotion phases, and permit the testing of a chemical's ability to specifically initiate or promote liver tumors. Most of these bioassays use hepatic enzyme-altered foci (EAF) as the endpoint. EAF are strongly implicated as preneoplastic lesions, and the enhancement of EAF in these bioassays correlates well with subsequent tumor production in long-term studies (Scherer and Emmelot, 1975; Squire and Levitt, 1975; Watanabe and Williams, 1978; Emmelot and Scherer, 1980).

Polybrominated biphenyls (PBBs) are highly lipophilic, persistent, halogenated aromatic hydrocarbons with many structural and functional similarities to polychlorinated biphenyls, chlorinated dibenzofurans and dibenzodioxins (McConnell and Moore, 1979; Rappe et al., 1979; Goldstein, 1980). Firemaster BP-6 (FM) is a commercial mixture of PBBs mistakenly added to livestock feed in Michigan in 1973. FM

subsequently contaminated livestock and poultry, and thus entered the human food chain (Carter, 1976; Kay, 1977). Approximately 90% of persons who were Michigan residents in 1973-1974 have detectable tissue residues of PBB (Brilliant et al., 1978; Wolff et al., 1982). FM is composed of 12-14 major PBB congeners (Moore and Aust, 1978; Moore et al., 1978a; Dannan et al., 1982a). FM causes mixed-type (both phenobarbital- and 3-methylcholanthrene-type) induction of hepatic microsomal enzymes (Dent et al., 1976a, 1976b; Dannan et al., 1978a, 1982b; Dent, 1978).

FM causes a delayed toxicity in rats, including weight loss and hepatomegaly with fatty degeneration (Sleight and Sanger, 1976; Gupta and Moore, 1979; Gupta et al., 1981, 1983), thyroid follicular cell hyperplasia and hypertrophy with decreased colloid (Sleight et al., 1978; Akoso et al., 1982b), and depletion of hepatic stores of vitamin A (Akoso et al., 1982a; McCormack et al., 1982; Darjono et al., 1983). Development of hepatic carcinomas and neoplastic nodules have been observed in chronic toxicity studies (Kimbrough et al., 1978, 1981; Gupta et al., 1983; Groce and Kimbrough, 1984).

Previous studies in our laboratory have shown FM to act as a hepatic tumor promoter in an initiation-promotion bioassay (Jensen et al., 1982, 1984). Due to the highly persistent nature of PBB in animal tissues and the lack of appreciable metabolism, it was hypothesized that even very short-term administration of FM would result in tumor

promotion.

Methods

Female Sprague Dawley rats (Charles River, Portage, MI) weighing 185-215 g were housed three per polypropylene cage and given food (Certified Rodent Chow #5002, Ralston Purina Company, St. Louis, MO) and water ad libitum.

An initiation-promotion protocol for experimental hepatocarcinogenesis in rats developed by Pitot et al. (1978) was used. Rats were initiated by a two-step process. Two-thirds partial hepatectomy on day zero was followed by intraperitoneal administration of 10 mg diethylnitrosamine (DEN) (Sigma Chemical Co., St. Louis, MO) per kg body weight (BW) 24 hours later. After 30 days, initiated rats were randomly assigned to treatment groups of six animals each, and non-initiated rats were randomly assigned to treatment groups of three animals each.

FM (Firemaster BP-6, Michigan Chemical Co., St. Louis, MI) was administered in corn oil vehicle by gavage. Each rat received a total volume of 5 ml corn oil, containing 0, 13 or 130 mg FM/kg BW. Rats were gavaged with half the dose on day 30 and half the dose on day 31.

One hundred and twenty days after gavage, the rats were fasted overnight, then anesthetized with ether, decapitated, and necropsied. Sections of liver, brain, spleen, kidney, adrenal gland, thyroid gland, pituitary gland, lung, heart, stomach, small intestine, pancreas, colon and urinary bladder were fixed in 10% buffered formalin and stained with

hematoxylin and eosin for histopathologic examination.

Five representative samples (approximately 10 x 10 x 4 mm) from each liver were immediately mounted and frozen on corks in O.C.T. embedding medium (Miles Scientific, Naperville, IL), which was cooled in isopentane in a liquid nitrogen bath. These samples were stored at -70°C until used. A cryostat was used to obtain an 8 u section from each of the five samples for each animal, and these sections were stained for gamma-glutamyl transpeptidase (GGT) according to Rutenburg et al. (1969) with minor modifications (Kalengayi et al., 1975), and examined at 90x magnification using a projection microscope. GGT-positive EAF were traced, and the area of each focus was measured using a planimeter. The number of EAF per cm^3 of liver (Scherer et al., 1972; Scherer, 1981) and the mean diameter of the EAF for each treatment group were calculated. GGT-positive EAF were circular to slightly oval in shape, ranged from 80 u to 700 u in diameter, and were readily distinguishable from nonspecific staining for GGT in periportal (zone 1) hepatocytes.

Microsomes were isolated as described by Moore et al. (1978b) from the livers of 3 animals per group. Cytochrome P-450 levels were assayed by the method of Omura and Sato (1964), aminopyrine demethylase levels were analyzed as described by Moore et al. (1978b), and ethoxyresorufin-O-deethylase levels were assayed using the methods of Burke and Mayer (1974). Samples of liver and fat

were analyzed for PBB content by gas chromatography as described by Jensen et al. (1984).

For statistical analysis, the data were log transformed, subjected to a one-way analysis of variance, and the Student-Neuman-Keuls' test for multiple comparisons was applied (Steel and Torrie, 1980), using a significance level of $p \leq 0.05$.

Results

Body weight gain from the time of gavage to necropsy 120 days later was somewhat less in rats given 130 mg FM/kg than in rats given no PBB (see Table 1.1), however the difference was not statistically significant. PBB concentrations in liver and fat increased proportionately with FM dose (see Table 1.1). Hepatic microsomal enzyme levels were increased in all groups of PBB-treated rats (see Table 1.2), indicating persistent enzyme-inducing activity of FM.

Histologic changes related to treatment were seen in the livers of rats given FM, and consisted of hepatocellular swelling and vacuolation as described in previous studies (Sleight and Sanger, 1976; Jensen et al., 1982). A section of liver from a control group rat is illustrated in Figure 1.1. Rats given 13 mg FM/kg had mild hepatocellular swelling and vacuolation, primarily in the centrilobular regions. Rats given 130 mg FM/kg had moderate hepatocellular swelling and vacuolation and mild multifocal proliferation of bile ductules (see Figure 1.2). Foci and areas of altered

Table 1.1. Body Weight Gains and PBB Concentrations in Initiated Rats[a]

Dose (mg FM/Kg BW)	Wt. gain (gavage to day 120)	PBB concentration (ppm)[b]	
		Liver	Adipose tissue
0	61 ± 13	1.2	0.9
13	71 ± 8	30.1	50.6
130	41 ± 4	350.6	509.3

[a] - N=6. Data expressed as x ± S.E.
[b] - Values are expressed on a lipid basis

Table 1.2. Hepatic Microsomal Enzyme Induction in Initiated Rats[a]

Dose (mg FM/Kg BW)	Cytochrome P-450 (nmol/mg protein)	Aminopyrine demethylase (nmol/mg protein-min)	Ethoxyresorufin-o-deethylase (nmol/mg protein-min)
0	1.18 ± 0.05	5.72 ± 0.44	0.24 ± 0.02
13	1.20 ± 0.10	16.35 ± 0.43	5.30 ± 0.12
130	3.25 ± 0.05	18.90 ± 0.12	21.20 ± 0.35

[a] - N=3. Data expressed as x ± S.E.

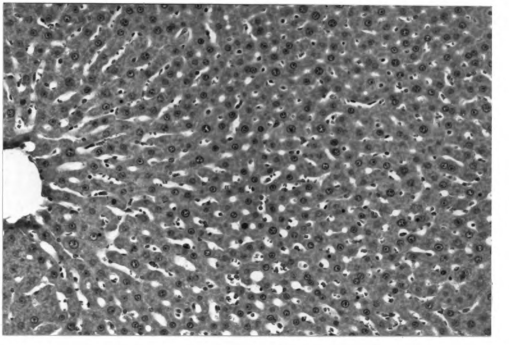

Figure 1.1. Photomicrograph of a section of liver from a rat in the control group. Notice the normal lobular architecture, with cords of hepatocytes radiating from a central vein and distinct sinusoidal spaces between the cords. (H & E stain, X 160).

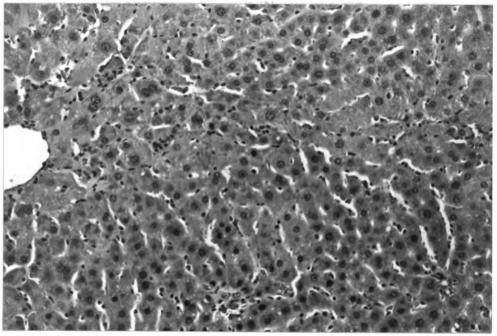

Figure 1.2. Photomicrograph of a section of liver from a rat given 130 mg FM/kg BW 120 days earlier. Notice the moderate hepatocellular swelling with subsequent narrowing of sinusoidal spaces, disruption of architecture, and the mild vacuolation of hepatocytes. (H & E stain, X 160).

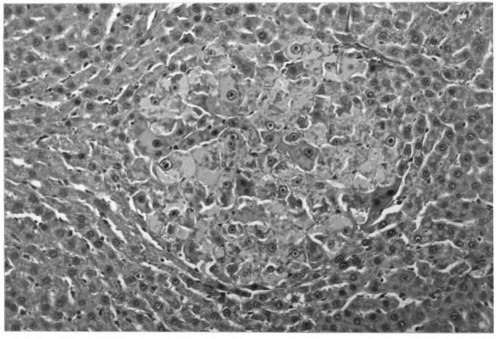

Figure 1.3. Photomicrograph of a focus of altered hepatocytes in the liver of an initiated rat given 130 mg FM/kg BW 120 days earlier. Notice the roughly circular area of proliferated hepatocytes. These cells are variable in size, and have an enlarged nucleus with a prominent nucleolus. Most of the cells have abundant acidophilic cytoplasm, although a few cells with basophilic cytoplasm are intermixed. The cells composing the focus merge with the surrounding normal hepatocytes, without a sharp line of demarcation. (H & E stain, X 160).

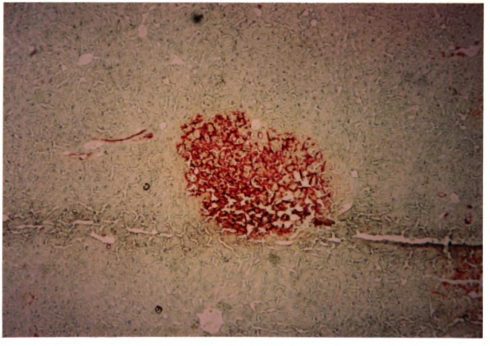

Figure 1.4. Photomicrograph of a histochemically stained enzyme-altered focus in the liver of an initiated rat given 130 mg FM/kg BW 120 days earlier. (Gamma-glutamyl transpeptidase stain, X 64).

Table 1.3. Enzyme-Altered Foci Per Cubic Centimeter of Liver in Initiated and Noninitiated Rats 120 Days After Gavage[a]

Initiation	N	Dose (mg FM/kg BW)	EAF/cm^3 liver	Mean Diameter of EAF (u)
PH-DEN	6	0	64 ± 16	168 ± 18
None	3	0	0	-
PH-DEN	6	13	155 ± 58	162 ± 9
None	3	13	30 ± 22	144 ± 35
PH-DEN	6	130	561 ± 225[b]	255 ± 7[b]
None	3	130	73 ± 24	188 ± 25

[a] - Data expressed as x ± S.E.
[b] - Significantly different from initiated rats which received 0 or 13 mg FM/kg ($p \leq 0.05$).

hepatocytes (Institute of Laboratory Animal Resources, 1980) were seen in initiated rats given FM, and consisted primarily of acidophilic cell foci with occasional mixed cell foci composed of acidophilic and clear cells (see Figures 1.3 and 1.4). All other tissues examined had no significant changes.

Numbers of GGT-positive EAF per cm^3 of liver and the mean diameter of EAF were significantly increased in initiated rats given 130 mg FM/kg, as compared to initiated rats given 0 or 13 mg FM/kg (Table 1.3). Noninitiated rats given FM had relatively low numbers of EAF/cm^3 liver.

Discussion

Two oral doses of FM 24 hours apart were sufficient to result in enhancement of DEN-initiated EAF in an initiation-promotion assay. Enhancement of EAF in this bioassay is considered evidence for tumor promoting activity (Pitot et al., 1978; Emmelot and Scherer, 1980). Hepatic tumor promoters accelerate the appearance of EAF, and have been shown to increase the number and size of EAF at a given time point (Kunz et al., 1982, 1983; Herren and Pereira, 1983; Schulte-Hermann et al., 1983a).

Generally, tumor promotion is considered to be a relatively long-term phenomenon, requiring weeks or months of administration of the promoting agent. Continuous administration of the chemical (or intermittent dosing at frequent intervals), for one to two months or longer is generally required for hepatic tumor promotion to occur

(Pitot et al., 1982; Ward, 1984). Jensen et al. (1982) reported that 10 or 100 ppm FM in the diet for 180 days promoted the appearance of gamma glutamyl transpeptidase(GGT)-positive EAF in the liver of DEN-initiated rats. Subsequently, Jensen et al. (1984) demonstrated that 15 days of administration of 100 ppm FM in the diet at the beginning of the promotion phase was as effective in enhancing EAF as 140 days of administration of 10 ppm FM in the diet. Thus it is not surprising that results in the current study indicate that two oral doses of FM in corn oil given 24 hours apart were sufficient to promote the development of EAF in an initiation-promotion bioassay.

PBB are very poorly metabolized and slowly excreted (Matthews et al., 1977; Miceli and Marks, 1981), thus a single administered dose results in persistent internal exposure to PBB. The major components of FM remained at significant levels in rats killed at 120 days after gavage. This persistence of the chemicals in non-metabolized states can explain the persistent activity of these compounds in the induction of microsomal enzymes and promotion of EAF observed in this study. The polychlorinated biphenyls (PCB) are close chemical relatives of PBB and are also highly persistent in animals. Pereira et al. (1982a) found a single dose of PCB was sufficient to enhance the incidence of hepatic EAF in DEN-initiated rats.

The lack of significant enhancement of EAF by 13 mg

FM/kg is consistent with a no-effect threshold level observed for other hepatic tumor promoters (Kunz et al., 1982, 1983; Ward et al., 1983; Goldsworthy et al., 1984; Kitagawa et al., 1984a; Oesterle and Deml, 1984). Oesterle and Deml (1984) found a no-effect level and a dose-dependent increase in the number of EAF in initiated rats promoted with weekly doses of PCB.

The non-initiated rats given FM had low numbers of EAF. Enhancement of foci in non-initiated animals has been seen with PBB in previous studies (Jensen et al., 1982, 1984), and with other hepatic tumor promoters (Pereira et al., 1982b; Kitagawa et al., 1984a; Oesterle and Deml, 1984). This enhancement of EAF may result from promotion of spontaneously initiated cells or reflect some initiating activity of the promoter (Peraino et al., 1983; Schulte-Hermann et al., 1983b; Schulte-Hermann, 1985). Although there is no evidence that PBBs have mutagenic or genotoxic activity (Ficsor and Wertz, 1976; Dannan et al., 1978b; Wertz and Ficsor, 1978; Kavanagh et al., 1985), it is possible that FM has some ability to act as a complete carcinogen, with both initiating and promoting activity. FM has not been tested for initiating activity in a two-stage bioassay, since FM would persist in the animals' tissues beyond the initiation phase and throughout the promotion phase. However, 3,4,3',4'-tetrabromobiphenyl, which is a minor congener in FM that can be metabolized and is therefore nonpersistent, was tested and some evidence for

weak initiating activity was found (Dixon et al., 1988).

The results in the current study indicate that 130 mg FM/kg, administered over a 24 hour period one month after initiation, is capable of significantly enhancing EAF in a hepatic initiation-promotion assay. A lower dose of 13 mg FM/kg does not significantly enhance EAF, thus implying that a threshold dose for enhancement of EAF exists.

CHAPTER 2

EFFECTS OF DIETARY RETINYL ACETATE ON PROMOTION OF HEPATIC ENZYME-ALTERED FOCI BY POLYBROMINATED BIPHENYLS IN INITIATED RATS

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Abstract

Vitamin A inhibits development of some chemically-induced tumors. Polybrominated biphenyls (PBBs) are hepatic tumor promoters which affect vitamin A homeostasis in rats. We hypothesized that dietary vitamin A levels would influence tumor promotion by PBBs. Female Sprague-Dawley rats were initiated with diethylnitrosamine. Beginning seven days later, the rats were fed diets containing 2000 IU (low A) or 200,000 IU (high A) retinyl acetate/kg feed. From day 30 on, diets contained either no PBB, 10 ppm FireMaster BP-6 (FM), 10 ppm 2,4,5,2',4',5'-hexabromobiphenyl (245-HBB), or 1 ppm 3,4,5,3',4',5'-hexabromobiphenyl (345-HBB). Rats were killed on day 180. Numbers of gamma-glutamyl transpeptidase-positive foci/cm^3 liver and the mean volumes of these foci were somewhat lower in the high A groups than in the corresponding low A groups, but these differences were not statistically significant. The percent of the liver volume occupied by foci was significantly greater in the low A/345-

HBB group than in the high A/345-HBB group. Thus high dietary vitamin A levels had some inhibitory effect on the promotion of hepatic altered foci in initiated rats by 345-HBB.

Introduction

Experimental hepatocarcinogenesis in rats can be considered in two sequential phases: initiation and promotion. Initiation is a rapid, permanent and heritable alteration in the genome of a subpopulation of susceptible cells, and is not expressed phenotypically (Farber, 1980; Pitot and Sirica, 1980; Scherer, 1984; Schulte-Hermann, 1985). The promotion phase is the subsequent period of clonal expansion of the initiated cell population, with concurrent expression of an altered phenotype in these cells (Pitot and Sirica, 1980; Hicks, 1983a; Schulte-Hermann, 1985; Farber and Sarma, 1987). This phase results in the appearance of morphologically recognizable preneoplastic or neoplastic lesions, such as hepatic enzyme-altered foci (EAF), neoplastic nodules, hepatomas, and eventually hepatocellular carcinomas (Farber, 1976; Institute of Laboratory Animal Resources, 1980; Pitot and Sirica, 1980; Williams, 1980; Schulte-Hermann, 1985; Farber and Sarma, 1987).

The mechanisms of hepatic tumor promotion are not well defined. Polybrominated biphenyls (PBBs) have been shown to act as hepatic tumor promoters in an initiation-promotion bioassay (Jensen et al., 1982, 1983, 1984; Jensen and

Sleight, 1986), but the mechanism by which they act is unknown. It has been suggested (Jensen et al., 1983; Jensen and Sleight, 1986) that the PBB congener, 2,4,5,2',4',5'-hexabromobiphenyl (245-HBB) has a different mechanism of hepatic tumor promoting activity than another PBB congener, 3,4,5,3',4',5'-hexabromobiphenyl (345-HBB). 245-HBB is a relatively nontoxic, phenobarbital-type inducer of hepatic microsomal enzymes (Moore et al., 1978), and has been shown to be a potent hepatic tumor promoter (Jensen et al., 1982; Jensen and Sleight, 1986). In contrast, 345-HBB is a toxic, 3-methylcholanthrene-type inducer of microsomal enzymes (Aust et al., 1981), and promotes tumors only at or above doses which cause significant hepatotoxicity (Jensen et al., 1983). FM is a commercial mixture of about 12 major PBB congeners (Moore and Aust, 1978; Aust et al., 1981), and causes mixed (both phenobarbital- and 3-methylcholanthrene-type) induction of hepatic microsomal enzymes (Dent et al., 1976a, 1976b; Aust et al., 1981). The congener 245-HBB comprises about 50-70% of the FM mixture (Sundstrom et al., 1976; Aust et al., 1981; Robertson et al., 1984), and the congener 345-HBB is found in very small quantities in the FM mixture (Orti et al., 1983; Robertson et al., 1984).

Polybrominated biphenyls and related compounds, such as polychlorinated biphenyls (PCBs) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), cause profound alterations in vitamin A homeostasis. Significant reduction of hepatic vitamin A stores is seen in animals given PCBs

(Villeneuve et al., 1971; Bitman et al., 1972; Cecil et al., 1973; Innami et al., 1974, 1976, 1982; Saito et al., 1982, 1983; Brouwer and van den Berg, 1984; Brouwer et al., 1985; Spear et al., 1986), TCDD (Thunberg et al., 1979, 1980, 1984; Thunberg and Hakansson, 1983; Thunberg, 1984; Hakansson and Ahlborg, 1985), or PBBs (Akoso et al., 1982; McCormack et al., 1982; Bernert et al., 1983; Darjono et al., 1983; Jensen et al., 1985). Both the FM mixture (Akoso et al., 1982; McCormack et al., 1982; Bernert et al., 1983; Darjono et al., 1983) and 345-HBB (Akoso et al., 1982; Jensen et al., 1985) cause marked depletion of liver vitamin A stores, whereas 245-HBB does not decrease the amount of vitamin A stored in the liver (Akoso et al., 1982).

The mechanism by which these chemicals cause alterations in vitamin A homeostasis is unknown. Changes in the vitamin A distribution in serum and kidney are seen in animals given PCBs (Brouwer and van den Berg, 1984, 1986; Brouwer et al., 1985; Powers et al., 1987), TCDD (Thunberg et al., 1979; Thunberg, 1984; Hakansson and Ahlborg, 1985), or PBBs (Bernert et al., 1983; Darjono et al., 1983; Jensen et al., 1987). Increased vitamin A excretion in urine and feces has been reported in rats given TCDD (Hakansson and Ahlborg, 1985) or PBB (Cullum and Zile, 1985; Jensen et al., 1985), and it has been proposed that increased renal metabolism of vitamin A and deregulation of hepatic retinoid metabolism are responsible for the changes in vitamin A homeostasis produced by PBBs (Cullum and Zile, 1985). Others

have proposed that PCBs interfere with vitamin A transport in the serum (Brouwer and van den Berg, 1986). Regarding the mechanism of tumor promotion by TCDD, Thunberg and coworkers have proposed that TCDD may act as a promoter by decreasing the tissue vitamin A levels (Thunberg et al., 1979, 1980, 1984; Thunberg, 1984).

Retinoids are compounds with vitamin A bioactivity. They have been extensively studied as potential antineoplastic agents, and many retinoids have been shown to inhibit the carcinogenic process in a variety of tissues particularly during the promotion phase (see reviews: Lotan, 1980; Bollag and Matter, 1981; Bollag and Hartmann, 1983; Hicks, 1983b; Ong and Chytil, 1983; Blunck, 1984; Moon and Itri, 1984). Vitamin A-deficient animals show an increased susceptibility to cancer in some studies (Newberne and Rogers, 1973; Rogers et al., 1973; Nettesheim and Williams, 1976; Newberne and Suphakarn, 1977; Dogra et al., 1985), but not in others (Zile et al., 1986). Treatment with retinoids has had variable effects on the carcinogenic process. According to some reports, retinoids have no effect on (Wenk et al., 1981; Silverman et al., 1981; Decaens et al., 1983; Longnecker et al., 1983b; Beems, 1984; Gensler and Bowden, 1984; Welsch et al., 1984; Ohshima et al., 1985) or even enhance the carcinogenic process (Silverman et al., 1981; Stinson et al., 1981; Welsch et al., 1981; Hennings et al., 1982; Stinson and Reznik, 1982; Verma et al., 1982; Birt et al., 1983; Longnecker et al., 1983b; Mian et al., 1984;

Fischer et al., 1985; Kurokawa et al., 1985; Ohshima et al., 1985; McCormick et al., 1987). Epidemiologic studies of humans show some correlation between decreased vitamin A and increased cancer incidence (see reviews: Kummet and Meyskens, 1983; Hennekens et al., 1986), and clinical trials with retinoids as therapeutic agents in human cancer patients have met with some success (see reviews: Bollag and Hartmann, 1983; Kummet and Meyskens, 1983; Meyskens, 1983; Ong and Chytil, 1983; Peck, 1983). The mechanisms by which retinoids influence the carcinogenic process are unknown, although many possible mechanisms have been suggested (see reviews: Lotan, 1980; Sporn and Roberts, 1983; Blunck, 1984; Mandani and Elmongy, 1986).

The current study was done to determine the effect of dietary vitamin A levels on hepatic tumor promotion by PBBs in the rat. Firemaster BP-6 and 345-HBB, which have profound effects on vitamin A homeostasis in rats, as well as 245-HBB, which is the major congener present in FM and has no significant effect on vitamin A homeostasis, were used in this experiment. It was hypothesized that if dietary vitamin A levels influence the tumor promoting actions of PBB, this effect would be most pronounced in rats given FM or 345-HBB.

Methods

Animals and experimental protocol - Female Sprague-Dawley rats (Charles River Breeding Laboratories), initially weighing 180-200 g, were housed in polypropylene cages and

given food and water ad libitum. Body weights were monitored weekly.

An initiation-promotion protocol for experimental hepatocarcinogenesis in rats (Pitot et al., 1978) was used. Rats were initiated on day 1 by intraperitoneal administration of 10 mg diethylnitrosamine (DEN; Eastman Kodak) per kg body weight, given 24 hours after two-thirds partial hepatectomy. Beginning on day 7, rats were fed vitamin A-deficient basal diet (TD83158, Teklad) with either low or high vitamin A supplementation. These diets were prepared by adding either 2000 IU (low A) or 200,000 IU (high A) retinyl acetate in gelatin coated beadlet form (Hoffmann-LaRoche) per kg feed.

The promotion phase of the experiment began on day 30 after initiation. The low or high vitamin A diet was supplemented with either no PBB (control), 10 ppm FM (Michigan Chemical Co.), 10 ppm 245-HBB, or 1 ppm 345-HBB. These diets were continued through day 180. Treatment groups contained 12 initiated rats. Control groups consisted of 12 initiated rats fed low or high vitamin A diets without PBB, For each dietary treatment, a group of 3 noninitiated rats served as an additional control.

On day 180, following an 18 hour fast, each rat was anesthetized with ether, a blood sample was obtained by cardiac puncture, and the animal was decapitated and examined. Sections of liver, lung, kidney, thymus, thyroid gland, salivary gland, spleen, trachea, eyelid, and uterus

were fixed in 10% buffered formalin, and subsequently stained with hematoxylin and eosin for histopathologic examination. Additional samples of liver were collected for evaluation of hepatic enzyme-altered foci and vitamin A analyses as described below.

Evaluation of hepatic enzyme-altered foci (EAF) - At the time of necropsy, five representative samples (each measuring approximately 10 x 10 x 4 mm) were obtained from each liver and immediately mounted on corks in O.C.T. embedding medium (Miles Scientific). The samples were immediately frozen by placing them in isopentane cooled in a liquid nitrogen bath, and were stored at -70^{o}C until used. A cryostat was used to obtain a section 8 u thick from each of the five samples for each animal, and these sections were stained for gamma-glutamyl transpeptidase (GGT) according to Rutenburg et al. (1969) with minor modifications (Kalengayi et al., 1975). These sections were examined at 90x magnification using a projection microscope. GGT-positive EAF were traced and measured with a planimeter. The number of EAF/cm^3 liver, the mean volume of the EAF, and the percent of the liver volume occupied by EAF were calculated for each animal using the methods of Saltykov as described by Campbell et al. (1982).

Vitamin A content of liver and serum - Samples of liver and serum were stored frozen until analyzed. Vitamin A analyses were performed under amber lights. The liver retinoids were extracted using the procedure of Olson

(1979), and serum retinol was extracted using the method of Bieri et al. (1979). The amount of retinoids in the liver and serum was determined by reverse phase-high pressure liquid chromatography (Cullum and Zile, 1986), using 13-cis-ethylretinamide as the internal standard.

Statistical analyses - Relevant comparisons between groups were made using the Wilcoxon-Mann-Whitney two sample test (Steel and Torrie, 1980) at $p \leq 0.01$.

Chemicals - All chemicals were obtained commercially as noted above, with the exception of 245-HBB and 345-HBB. 245-HBB was separated and purified to >99% purity from FM by crystallization and alumina chromatography (Moore and Aust, 1978). 345-HBB was purchased from the RFR Corporation (Hope, RI), and purified to >99% purity by repeated alumina chromatography (Aust et al., 1981).

Results

Body weight gains from day 30-180 are summarized in Table 2.1. No significant differences were seen between rats given low vitamin A and high vitamin A diets for any of the PBB treatments. 245-HBB had no significant effect on weight gain, as compared to controls. In rats fed the low A diet, FM and 345-HBB caused significantly decreased weight gains as compared to low A/control rats. In rats fed the high A diet, 345-HBB caused significantly decreased weight gains as compared to high A/control rats. Rats fed high A/FM had lower weight gains than the high A/control rats, but the difference was not significant.

Table 2.1. Body Weight Gains (Grams) in Initiated Rats From Day 30-180[a]

Vitamin A Level in Diet	Polybrominated Biphenyls in Diet			
	Control (no PBB)	FM	245-HBB	345-HBB
Low	105 ± 10	73 ± 10[b]	92 ± 9	38 ± 5[b]
High	86 ± 11	58 ± 5	90 ± 6	46 ± 5[c]

[a] - N=12, except for the low vitamin A/345-HBB group where N=11. Data expressed as x ± S.E.
[b] - Significantly different from the low vitamin A/control group ($p \leq 0.01$)
[c] - Significantly different from the high vitamin A/control group ($p \leq 0.01$)

At postmortem examination, the initiated rats given PBB had variable numbers of small pale foci on the surfaces of their livers. Rats given 345-HBB had enlarged, pale livers with rounded edges. One rat in the low A/345-HBB group had numerous raised nodules, approximately 0.5-2.0 mm in diameter, scattered throughout the liver.

On histopathologic examination, moderate lymphoid depletion in the thymus and spleen, hyperplastic goiter in the thyroid glands, and mild squamous metaplasia of the thyroglossal duct, Meibomian glands of the eyelid, and uterine endometrium were seen in some of the rats treated with FM or 345-HBB. No difference in incidence or severity of these lesions was seen between low and high vitamin A treatments.

On histopathologic examination of the liver, hepatic abnormalities were seen which were similar to those described with PBBs by others (Sleight and Sanger, 1976; Gupta et al., 1981; Jensen et al., 1982, 1983). The livers of rats given FM had mild to moderate disruption of hepatic architecture, hepatocellular swelling, centrilobular vacuolation, as well as mild scattered biliary proliferation and variable numbers of altered islands of hepatocytes. Hepatocyte nodules were seen only in one rat in the high A/FM group. The livers of rats given 245-HBB had mild disruption of hepatic architecture, hepatocellular swelling, centrilobular vacuolation and mild biliary proliferation. Altered islands of hepatocytes were few in number, and

hepatocyte nodules were seen only in one rat in the high A/245-HBB group. The livers of rats in the low A/345-HBB group had moderate to severe disruption of hepatic architecture, moderate hepatocellular swelling, moderate to severe centrilobular vacuolation and mild biliary proliferation. Numbers of altered islands of hepatocytes were variable, and hepatocyte nodules were seen in four rats (see Figure 2.1). The livers of rats in the high A/345-HBB group had mild to moderate disruption of hepatic architecture and hepatocellular swelling, moderate to severe centrilobular vacuolation, and mild biliary proliferation. Numbers of altered islands of hepatocytes were variable, and hepatocyte nodules were seen in only one rat.

Numbers of EAF/cm^3 liver in the initiated rats are shown in Table 2.2. All PBB treatment groups had significantly greater numbers of EAF/cm^3 liver than the corresponding low A or high A control group. The numbers of EAF/cm^3 liver were lower in each high A group than the corresponding low A group, but none of these differences were statistically significant. The mean volumes of the EAF in the initiated rats are shown in Table 2.3. The mean volume of EAF in the low A/345-HBB group was significantly greater than in the low A/control group. The mean volumes of the EAF in the high A/FM and high A/345-HBB groups were significantly greater than in the high A/control group. No significant differences were seen between corresponding low A and high A groups. The percent of the liver volume

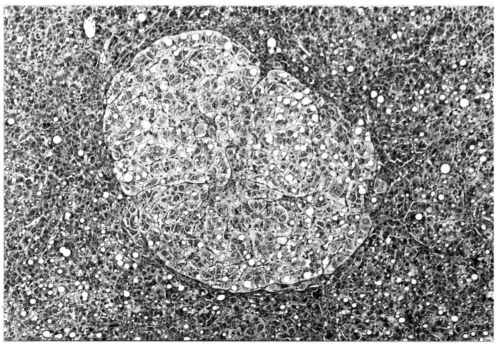

Figure 2.1. Photomicrograph of a hepatocyte nodule in the liver of an initiated rat in the low A/345-HBB group. Notice the large and roughly circular area of proliferated hepatocytes. These cells are variable in size, have acidophilic or basophilic cytoplasm, and frequently have an enlarged nucleus with a prominent nucleolus. The nodule is sharply demarcated from and compressing the surrounding hepatic parenchyma. Marked disruption of hepatic architecture and vacuolation of hepatocytes can be seen in the hepatic parenchyma surrounding the nodule. (H & E stain, X 64).

Table 2.2. Hepatic Enzyme-Altered Foci/cm^3 Liver in Initiated Rats[a]

Vitamin A Level in Diet	Polybrominated Biphenyls in Diet			
	Control (no PBB)	FM	245-HBB	345-HBB
Low	25 ± 15	710 ± 179[b]	86 ± 9[b]	396 ± 58[b]
High	6 ± 2	604 ± 193[c]	53 ± 14[c]	309 ± 39[c]

[a] - N=12, except for the low vitamin A/345-HBB group where N=11. Data expressed as x ± S.E.
[b] - Significantly different from the low vitamin A/control group ($p \leq 0.01$)
[c] - Significantly different from the high vitamin A/control group ($p \leq 0.01$)

Table 2.3. Mean Volume (mm^3) of Hepatic Enzyme-Altered Foci in Initiated Rats[a]

Vitamin A Level in Diet	Polybrominated Biphenyls in Diet			
	Control (no PBB)	FM	245-HBB	345-HBB
Low	.0142 ± .0101	.0235 ± .0037	.0373 ± .0172	.0867 ± .0289[b]
High	.0095 ± .0009	.0165 ± .0016[c]	.0126 ± .0019	.0233 ± .0040[c]

[a] - N=12, except for the low vitamin A/345-HBB group where N=11. Data expressed as x ± S.E.
[b] - Significantly different from the low vitamin A/control group ($p \leq 0.01$)
[c] - Significantly different from the high vitamin A/control group ($p \leq 0.01$)

Table 2.4. Percent of the Liver Volume Occupied by EAF in Initiated Rats[a]

Vitamin A Level in Diet	Polybrominated Biphenyls in Diet			
	Control (no PBB)	FM	245-HBB	345-HBB
Low	0.012 ± 0.006	2.078 ± 0.718[b]	0.322 ± 0.162[b]	2.633 ± 0.711[b,d]
High	0.005 ± 0.002	1.203 ± 0.514[c]	0.066 ± 0.019[c]	0.777 ± 0.210[c,e]

[a] - N=12, except for the low vitamin A/345-HBB group where N=11. Data expressed as x ± S.E.
[b] - Significantly different from the low vitamin A/control group ($p \leq 0.01$)
[c] - Significantly different from the high vitamin A/control group ($p \leq 0.01$)
[d] - Significantly different from the high vitamin A/345-HBB group ($p \leq 0.01$)
[e] - Significantly different from the low vitamin A/345-HBB group ($p \leq 0.01$)

Table 2.5. Serum Retinol Concentration (ng Retinol/ml Serum) in Initiated Rats[a]

Vitamin A Level in Diet	Polybrominated Biphenyls in Diet			
	Control (no PBB)	FM	245-HBB	345-HBB
Low	333 ± 33	283 ± 23	312 ± 30[b]	194 ± 39
High	242 ± 12	208 ± 20	203 ± 14[c]	313 ± 31

[a] - N=12, except for the low vitamin A/345-HBB group where N=11. Data expressed as x ± S.E.
[b] - Significantly different from the high vitamin A/245-HBB group ($p \leq 0.01$)
[c] - Significantly different from the low vitamin A/245-HBB group ($p \leq 0.01$)

Table 2.6. Total Liver Retinyl Esters (mg Retinyl Esters per Liver) in Initiated Rats[a]

Vitamin A Level in Diet	Polybrominated Biphenyls in Diet			
	Control (no PBB)	FM	245-HBB	345-HBB
Low	1.27 ± 0.20	0.08 ± 0.01[b]	1.40 ± 0.24	0.54 ± 0.19
High	161.00 ± 24.53	85.72 ± 5.12	167.44 ± 14.68	59.09 ± 5.35[c]

[a] - N=12, except for the low vitamin A/345-HBB group where N=11. Data expressed as x ± S.E. Each low vitamin A group was significantly different from the corresponding high vitamin A group receiving the same polybrominated biphenyl treatment.
[b] - Significantly different from the low vitamin A/control group ($p \leq 0.01$)
[c] - Significantly different from the high vitamin A/control group ($p \leq 0.01$)

occupied by EAF in the initiated rats is shown in Table 2.4. All PBB treatment groups had a significantly greater percent of liver volume occupied by EAF than the corresponding low A or high A control group. The percent of the liver occupied by foci was significantly greater in the low A/345-HBB group than in the high A/345-HBB group. Numbers and volumes of EAF in non-initiated rats were very small (data not shown).

Serum retinol levels are shown in Table 2.5. The only significant difference was between the low A/245-HBB and high A/245-HBB groups. The total retinyl ester content in the livers is shown in Table 2.6. The rats fed the low A diet had much lower liver retinyl ester stores than the rats fed the high A diet. As compared to the low A/control group, both the low A/FM and low A/345-HBB groups had decreased total liver retinyl esters, although the difference was statistically significant only for the low A/FM group. As compared to the high A/control group, both the high A/FM and high A/345-HBB groups had decreased total liver retinyl esters, although the difference was statistically significant only for the high A/345-HBB group. The values for liver retinol and retinyl ester concentrations and total liver retinol content had a similar pattern (data not shown).

Discussion

The levels of dietary vitamin A in this study do not appear to have been inadequate or toxic, as the body weight gains in the control groups were normal, and no gross or

histologic evidence of vitamin A deficiency or toxicosis was seen. Both FM and 345-HBB were toxic as evidenced by a decrease in body weight gain, and this correlated with the degree of hepatotoxicity seen histologically. The increased dietary levels of vitamin A in the high A groups did not protect against these toxic effects of the PBBs. Some authors report that supplemental vitamin A in the diet can partially protect against decreased body weight gains seen in PBB-treated (Darjono et al., 1983) or PCB-treated rats (Innami et al., 1974). Supplemental dietary vitamin A can delay, but not prevent, the weight loss and death produced by TCDD (Stohs et al., 1984; Hassan et al., 1985). Thus it is not surprising that vitamin A did not prevent the decreased weight gain seen in PBB-treated rats over a five month period in the current study.

Vitamin A can actually enhance the toxicity of some chemicals. Daoud and Griffin (1985) found that retinoic acid increased the hepatotoxic effects of 2-acetylaminofluorene in rats, and theorized that this might be due to increased liberation of lysozymes by the retinoic acid. Dietary retinyl acetate and the phenolic antioxidant, butylated hydroxytoluene, can act synergistically to produce a high incidence of hepatic fibrosis and bile duct hyperplasia in rats (McCormick et al., 1986). The authors speculated that this might represent potentiation by butylated hydroxytoluene of a vitamin A toxicity normally seen only at higher doses of vitamin A.

The high vitamin A dietary levels were likewise not able to prevent the decrease in hepatic vitamin A stores caused by FM and 345-HBB, relative to the high A/control group. Others have reported that vitamin A supplementation does not prevent the depletion of hepatic vitamin A in PBB-treated rats (Darjono et al., 1983).

Although both FM and 345-HBB caused marked reductions in total liver retinyl esters, it appears that even in the low A groups these reduced liver vitamin A stores were still sufficient to maintain normal serum retinol levels, as none of the PBB treated groups had significantly reduced serum retinol levels as compared to their respective vitamin A control groups. Serum levels of vitamin A are homeostatically controlled over a wide range of hepatic vitamin A concentrations, and tend to be maintained within normal concentrations except at very low or high liver levels of vitamin A (Underwood et al., 1979; Hicks et al., 1984; Olson, 1984).

Thunberg et al. (1979, 1980, 1984; Thunberg, 1984) proposed that TCDD may act as a tumor promoter by producing a relative or local vitamin A deficiency. Presumably this mechanism would also apply to related compounds such as FM and 345-HBB. The results of the current study are consistent with this hypothesis to a limited extent. All the groups of initiated rats which were treated with PBBs had enhanced numbers and sizes of hepatic EAF compared to the corresponding control groups, and the numbers and sizes of

EAF were consistently, although not significantly, greater in the low A groups than in the corresponding high A groups. There was a significantly greater percent of the liver volume occupied by EAF in the low A/345-HBB group than in the high A/345-HBB group. Thus, there is some indication for an inhibitory effect of dietary vitamin A supplementation on the promotion of hepatic EAF by PBBs. A stronger inhibitory effect of vitamin A on the development of these EAF might have been observed if the foci were evaluated at additional time points, or if different concentrations of dietary vitamin A had been used.

Previous studies have found variable effects of retinoids on hepatic carcinogenesis. Both retinoic acid (Daoud and Griffin, 1980) and abscisic acid (Shearer, 1983), which is a carotenoid and natural vitamin A analog, have a strong inhibitory effect on azo-dye hepatocarcinogenesis in rats. Another study found that both deficient and excess levels of dietary vitamin A could inhibit the growth of subcutaneously transplanted hepatocellular carcinomas, as compared to rats receiving adequate vitamin A (Morre et al., 1980). These authors also reported that excess dietary vitamin A (400,000 IU retinyl acetate/kg feed) prevented the metastasis of these transplanted hepatocarcinomas (Morre et al., 1980). Rats given the carcinogen aflatoxin B_1 and fed either low, normal or high dietary levels of vitamin A do not develop significantly different numbers of liver tumors (Newberne and Rogers, 1973; Newberne and Suphakarn, 1977),

indicating that dietary vitamin A levels do not influence aflatoxin-induced hepatocarcinogenesis. Evidence for vitamin A-induced enhancement of hepatocarcinogenesis can also be found, as dietary supplementation with some synthetic retinoids produced a dose-related increase in the incidence of hepatocellular carcinomas in rats given azaserine (Longnecker et al., 1983a).

DeLuca et al. (1984) reported that the cell membrane fractions in Morris rat hepatomas were deficient in vitamin A as compared to surrounding normal liver, and they concluded that the neoplastic tissue has lost the ability to take up and store vitamin A, with subsequent reduction in vitamin A-related functions. They felt that this was consistent with the hypothesis that tumor promotion may involve the deficiency of an essential nutritional factor, such as vitamin A, or of its functions (DeLuca, 1983). This is similar to the proposal by Thunberg and coworkers (Thunberg et al., 1979, 1980, 1984; Thunberg, 1984) that TCDD may promote tumorigenesis by producing a vitamin A-deficient state. Some support for this hypothesis is found in the results of the current study with the related PBB compounds.

In summary, the current study found that both FM and 345-HBB decreased body weight gains, decreased hepatic vitamin A stores, and enhanced hepatic EAF in initiated rats. Hepatic EAF were also enhanced by 245-HBB. The numbers and sizes of EAF were somewhat lower in animals given a high

vitamin A diet than in animals given a low vitamin A diet with the same PBB treatment, but these differences were not statistically significant. However, the total percent of the liver volume occupied by EAF was significantly greater in rats given low A/345-HBB than in rats given high A/345-HBB. Thus, dietary retinyl acetate supplementation had some inhibitory effect on the tumor-promoting activity of PBBs, but did not prevent hepatotoxicity or depletion of hepatic vitamin A stores produced by FM and 345-HBB.

CHAPTER 3

EFFECTS OF HEPATIC TUMOR PROMOTERS PHENOBARBITAL AND POLYBROMINATED BIPHENYLS ON INTERCELLULAR COMMUNICATION BETWEEN RAT LIVER EPITHELIAL CELLS

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Abstract

Firemaster BP-6 (FM), a mixture of polybrominated biphenyls, and phenobarbital (PB) promote hepatic carcinogenesis in rats. Inhibition of intercellular communication is a possible mechanism of tumor promotion. Vitamin A compounds, such as retinyl acetate (RA), have been shown to antagonize the carcinogenic process in some systems. In this study, FM, PB and RA were tested *in vitro* in two intercellular communication assays using a rat liver epithelial cell line (WB-F344). One assay measured inhibition of metabolic cooperation (MC) between cells containing the enzyme hypoxanthine-guanine phosphoribosyl transferase and mutant cells lacking the enzyme. At nonlethal concentrations, FM, PB and RA all inhibited MC. Nonlethal concentrations of RA did not affect blockage of MC by FM. The other intercellular communication assay evaluated the inhibition of fluorescence redistribution after photobleaching (FRAP), which occurs through gap

junctions between cells loaded with a fluorescent dye. At nonlethal concentrations, PB allowed dye transfer and return of fluorescence in photobleached cells, whereas FM inhibited FRAP. Nonlethal concentrations of RA allowed dye transfer and return of fluorescence in photobleached cells, and had no effect on the ability of FM to block FRAP. In summary, the hepatic tumor promoter PB inhibited MC, but did not block junctional communication in the FRAP assay. The hepatic tumor promoter FM inhibited MC and also blocked FRAP. Retinyl acetate blocked MC but did not inhibit FRAP, and had no effect on the ability of FM to block junctional communication in the MC or FRAP assays.

Introduction

Tumor promoting agents act by mechanisms which are currently unknown. One of the mechanisms which has been proposed is the disruption of gap junctional intercellular communication (Yotti et al., 1979; Potter, 1980; Williams, 1981; Trosko et al., 1983; Trosko and Chang, 1984b; Yamasaki et al., 1984). Many tumor promoters have been shown to inhibit gap junctional communication *in vitro* (Murray and Fitzgerald, 1979; Yotti et al., 1979; Trosko et al., 1981b; Williams et al., 1981; Lawrence et al., 1984; Warngard et al., 1985; Zeilmaker and Yamasaki, 1986; Jone et al., 1987; Rolin-Limbosch et al., 1987; Ruch et al., 1987). Gap junctions are specialized zones of contact between adjacent cells, through which small molecules can pass from cell to cell (see reviews: Loewenstein, 1979; Larsen, 1983; Pitts

and Finbow, 1986). Gap junctional communication between cells is important in the control of differentiation and proliferation of cells (Loewenstein, 1979; Pitts, 1980; Hooper, 1982). Inhibition of normal intercellular communication may prevent the normal exchange of regulatory signals between cells, and thus lead to disturbances of differentiation and/or proliferation (Loewenstein, 1979; Trosko and Chang, 1984a; Loch-Caruso and Trosko, 1985). The process of carcinogenesis is considered to be a disorder of differentiation and/or proliferation of cells (Farber, 1976; Potter, 1978, 1980).

Many assays for detecting the inhibition of junctional communication have been described. Among these are MC assays (Trosko et al., 1981b; Gupta et al., 1985; Miller et al., 1986; Jongen et al., 1987) and dye transfer assays (Friedman and Steinberg, 1982; Fitzgerald et al., 1983; Rivedal et al., 1985). Metabolic cooperation assays depend on the exchange of specific metabolites between cells, some of which are genetically unable to produce these metabolites (Subak-Sharpe et al., 1969; Hooper, 1982). Fluorescent dye transfer assays depend on the exchange of hydrophilic dye molecules which diffuse poorly across intact cell membranes but can be readily transferred between cells via gap junctions (Loewenstein, 1979).

Phenobarbital is well established as a tumor promoting agent in <u>in vivo</u> hepatocarcinogenesis assays in rats (Peraino et al., 1971, 1973; Lans et al., 1983; Schulte-

Hermann, 1985), and has been shown to block MC between Chinese hamster V79 fibroblasts (Jone et al., 1985a), mouse and rat hepatocytes (Klaunig and Ruch, 1987a; Ruch et al., 1987), human hepatoma cells (Rolin-Limbosch et al., 1986), and between co-cultivated primary rat hepatocytes and ARL strain rat liver epithelial cells (Williams, 1980a). Firemaster BP-6 is a mixture of polybrominated biphenyls which promotes preneoplastic lesions and tumors in the livers of initiated rats (Jensen et al., 1982, 1984), and blocks MC in vitro in V79 fibroblasts (Trosko et al., 1981a) and in human teratocarcinoma cells (Kavanagh et al., 1987). The current study was undertaken to evaluate the effects of these hepatic tumor promoters on junctional communication between rat liver epithelial cells in vitro, using both MC and fluorescent dye transfer assays.

We further wanted to assess the effect of a vitamin A compound, retinyl acetate, on junctional communication in these cells. Vitamin A compounds (retinoids) are generally considered to be anticarcinogenic agents, particularly during the promoting phase of carcinogenesis in skin, lung, mammary gland and urinary bladder (see reviews: Lotan, 1980; Hicks, 1983b; Ong and Chytil, 1983; Moon and Itri, 1984). However, this anticarcinogenic activity of retinoids is not consistent. In some systems, retinoids have no effect on (Birt et al., 1983; Gensler and Bowden, 1984), or even enhance (Hennings et al., 1982; Fischer et al., 1985; Kurokawa et al., 1985; McCormick et al., 1987a), the

carcinogenic process *in vivo*. Results of previous studies in our laboratory indicated that dietary RA levels had no significant effect on the ability of FM to promote hepatic enzyme-altered foci in initiated rats (see chapter 2).

One mechanism by which retinoids might influence the carcinogenic process is by altering junctional intercellular communication. One study found that topical retinoic acid treatment of basal cell carcinomas in humans resulted in proliferation of gap junctions between cells in the tumor tissue (Elias et al., 1980). *In vitro* studies of retinoid effects on intercellular communication have shown variable results. Some reports indicate that retinoids can protect against the blockage of intercellular communication produced by the classic tumor promoter TPA (Shuin et al., 1983; Yamasaki et al., 1984). However, other reports indicate that retinoids enhance the effect of TPA on intercellular communication (Shuin et al., 1983; Radner and Kennedy, 1984; Morel-Chany et al., 1986), and that retinoids alone can inhibit intercellular communication (Pitts et al., 1986; Shuin et al., 1983; Walder and Lutzelschwab, 1984; Davidson et al., 1985).

The objectives of the current study were to determine the ability of PB, FM, and RA to block junctional communication in cultured rat liver epithelial cells, and to determine the effect of RA on the ability of FM to block junctional communication in these cells.

Methods

Cells and culture conditions. A diploid hepatic epithelial cell line (designated WB-F344), isolated from the liver of an adult rat and having the phenotype of oval cells (Tsao et al., 1984a), was obtained from the laboratory of J. W. Grisham, University of North Carolina. Cells were grown in modified Eagle's medium with Earle's balanced salts, supplemented with a 50% increase of vitamins and essential amino acids except glutamine, a 100% increase of nonessential amino acids, 5% fetal bovine serum, 1 ug/ml insulin, and either 50 ug/ml gentamicin or 100 units/ml penicillin G with 100 ug/ml streptomycin. The cells were kept in humidified incubators with 5% CO_2 at 37°C, and were propagated in plastic tissue culture flasks (25, 75 or 150 sq cm). During experimental procedures, the cells were plated in either 35 or 60 mm diameter round plates with 2 or 5 ml of media.

Chemicals. All chemicals tested in the assays were prepared in stock solutions in dimethyl sulfoxide or ethanol, and stored in a freezer. Fresh dilutions were made at the time of each experiment. Aldrin (>99% purity) was obtained from the Pesticide Research Center at Michigan State University. Phenobarbital was obtained from Sigma Chemical Co. (St. Louis, MO). Firemaster BP-6 was manufactured by Michigan Chemical Co. (St. Louis, MI). Crystalline retinyl acetate was kindly supplied by Hoffmann-LaRoche, Inc. (Nutley, NJ).

Metabolic cooperation assay. This assay has been described by Jone et al. (1987). Wild type WB cells contain the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT+). Mutant HGPRT$^-$ WB cells were previously isolated following irradiation and selection in medium containing 6-thioguanine (Jone et al., 1987). For these experiments, 60 mm tissue culture plates were seeded with 3 or 4 x 10^5 HGPRT+ cells and 100 HGPRT$^-$ cells. Plating efficiency was determined for each experiment by seeding 100 HGPRT$^-$ cells per plate with no added chemical and counting the surviving colonies at the end of the experiment. Cytolethality of the test chemicals was determined by seeding 100 HGPRT$^-$ cells per plate, adding the corresponding amount of test chemical, and counting the surviving colonies relative to plating efficiency at the end of the experiment. Background levels of HGPRT$^-$ cell recovery were determined in MC plates with no test chemical added. Aldrin was added at a concentration of 10 ug/ml in another group of MC plates to serve as a positive control for blockage of MC. The test chemicals were added two hours after the cells were plated. 6-thioguanine (6-TG) was added to each plate at a concentration of 500 ug/ml media one hour after the test chemicals were added. Three days later, medium was replaced with fresh medium containing 6-TG but no test chemical. Medium was replaced again 4 days later. After 1-2 days additional growth, the colonies were fixed, stained and counted. Five or ten plates were used for each treatment

group, and each experiment was repeated at least twice. Data were statistically analyzed using the Wilcoxon-Mann-Whitney two-sample test ($p \leq 0.05$).

Fluorescence redistribution after photobleaching assay. This assay has been described by Wade et al. (1986), and offers the advantage of not requiring complex microinjection techniques. Tissue culture plates (35 mm in diameter) were seeded with HGPRT+ cells at low density. The test chemicals were added to the plates 1.5 to 2 hr later. Twenty-four hr later, the plates were briefly rinsed three times with modified Hanks' balanced salt solution (HBSS) with Ca^{++}, Mg^{++} and HCO_3 added, then incubated at 37°C for 15 min with 7 ug carboxyfluorescein diacetate/ml modified HBSS. The plates were then rinsed three times with the modified HBSS, and maintained in the modified HBSS during the remainder of the experiment. The cells were examined in an inverted phase contrast and epifluorescence microscope (ACAS 470 Workstation, Meridian Instruments, Okemos, MI). Selected cells were photobleached and then monitored for return of fluorescence at 6 min intervals for a total of 18 min. Each experiment was repeated at least twice, with at least 20 cells photobleached and monitored for fluorescent return in each treatment group.

Results

Metabolic cooperation studies. Phenobarbital was toxic, as measured in the cytolethality plates, only at the highest concentration of 800 ug/ml (see Figure 3.1).

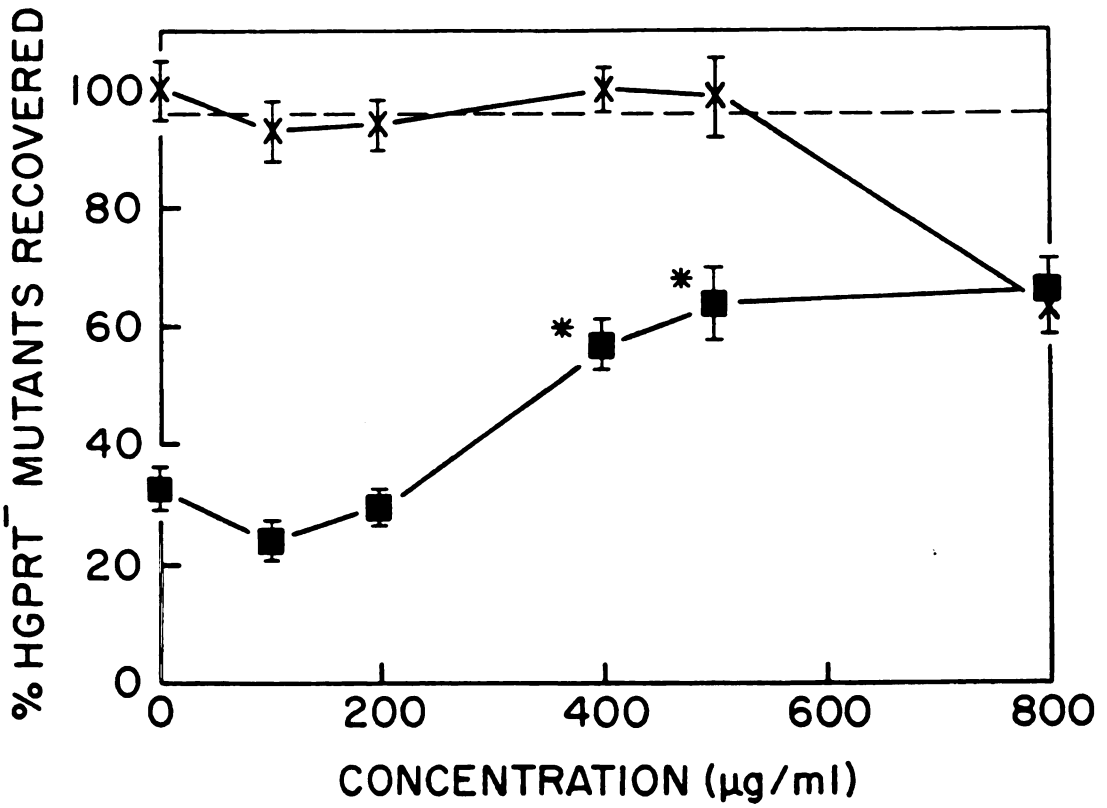

Figure 3.1. Dose-response curves for PB in cytolethality (—✕—) and MC (—■—) plates. Aldrin was the positive control for blockage of MC (--------). Data are plotted with bars representing the standard error of the mean. (*) indicates values which were significantly different from background values (MC plates without PB) at non-cytolethal concentrations of PB.

Significant increases in HGPRT$^-$ colony survival above background were seen at PB concentrations of 400 and 500 ug/ml, indicating blockage of metabolic cooperation at these nontoxic concentrations. Firemaster BP-6 was toxic at the highest concentration of 50 ug/ml (see Figure 3.2). Significant increase in HGPRT$^-$ colony survival above background was seen at the nontoxic FM concentration of 25 ug/ml. Retinyl acetate was lethal at a concentration of 50 uM, with no colonies surviving (see Figure 3.3). However, a concentration of 10 uM produced no apparent toxicity. Retinyl acetate increased HGPRT$^-$ colony survival above background at concentrations of 1 and 10 uM.

Combination studies with 25 ug/ml FM and various concentrations of RA showed some toxicity at all concentrations of RA tested (see Figure 3.4), with only 40% colony survival in the cytolethality plates at 10 uM RA, and no surviving colonies at 50 uM RA. Thus, it appears that there is some potentiation of toxicity when RA and FM are combined. There was a significant increase in HGPRT$^-$ colony survival (as compared to that with FM alone) only at 10 uM RA.

FRAP studies. The cells were examined with phase contrast photomicroscopy immediately prior to photobleaching as well as at the conclusion of the experiment; no significant morphologic changes indicating cytotoxicity were noted. Control groups with no test chemical added had rapid return of fluorescence after photobleaching in cells

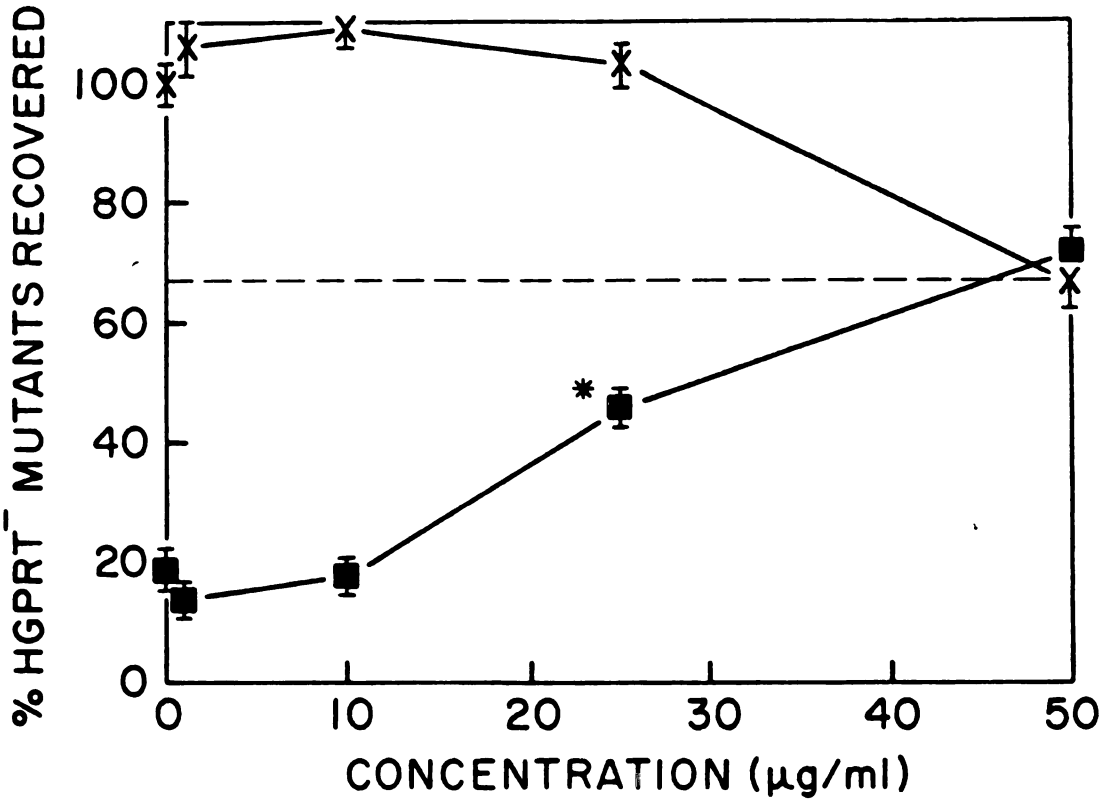

Figure 3.2. Dose-response curves for FM in cytolethality (—X—) and MC (—■—) plates. Aldrin was the positive control for blockage of MC (--------). Data are plotted with bars representing the standard error of the mean. (*) indicates values which were significantly different from background values (MC plates without FM) at non-cytolethal concentrations of FM.

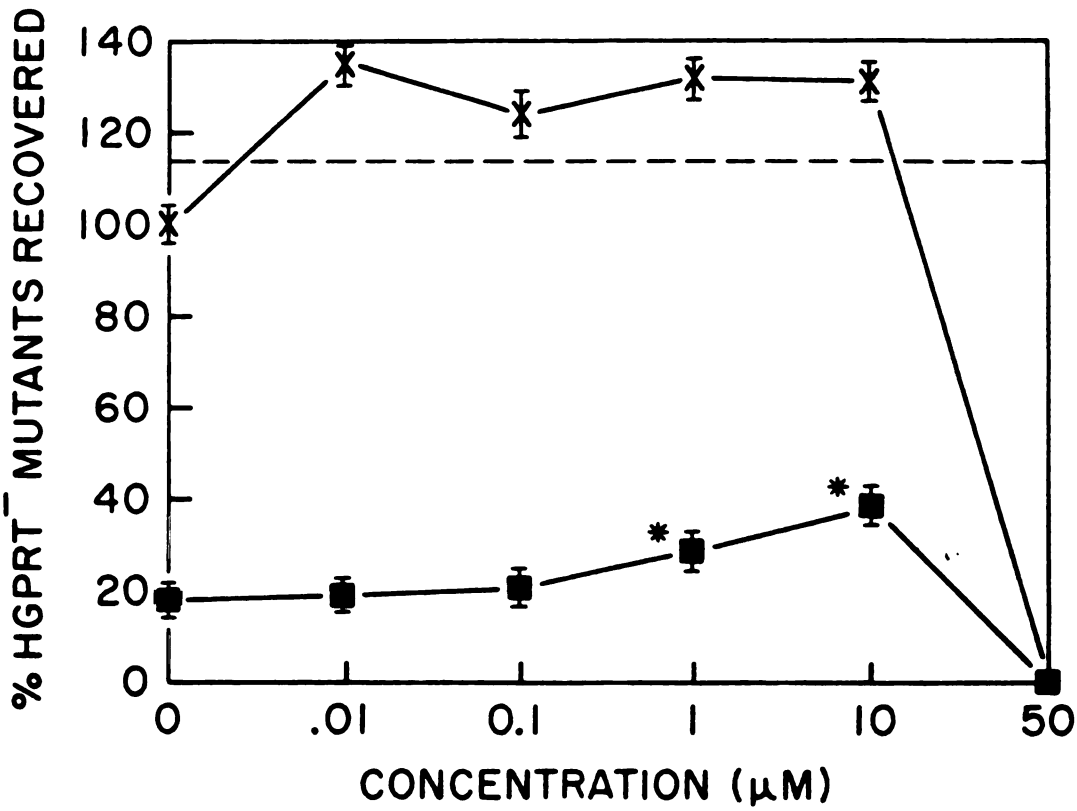

Figure 3.3. Dose-response curves for RA in cytolethality (—✕—) and MC (—■—) plates. Aldrin was the positive control for blockage of MC (--------). Data are plotted with bars representing the standard error of the mean. (*) indicates values which were significantly different from background values (MC plates without RA) at non-cytolethal concentrations of RA.

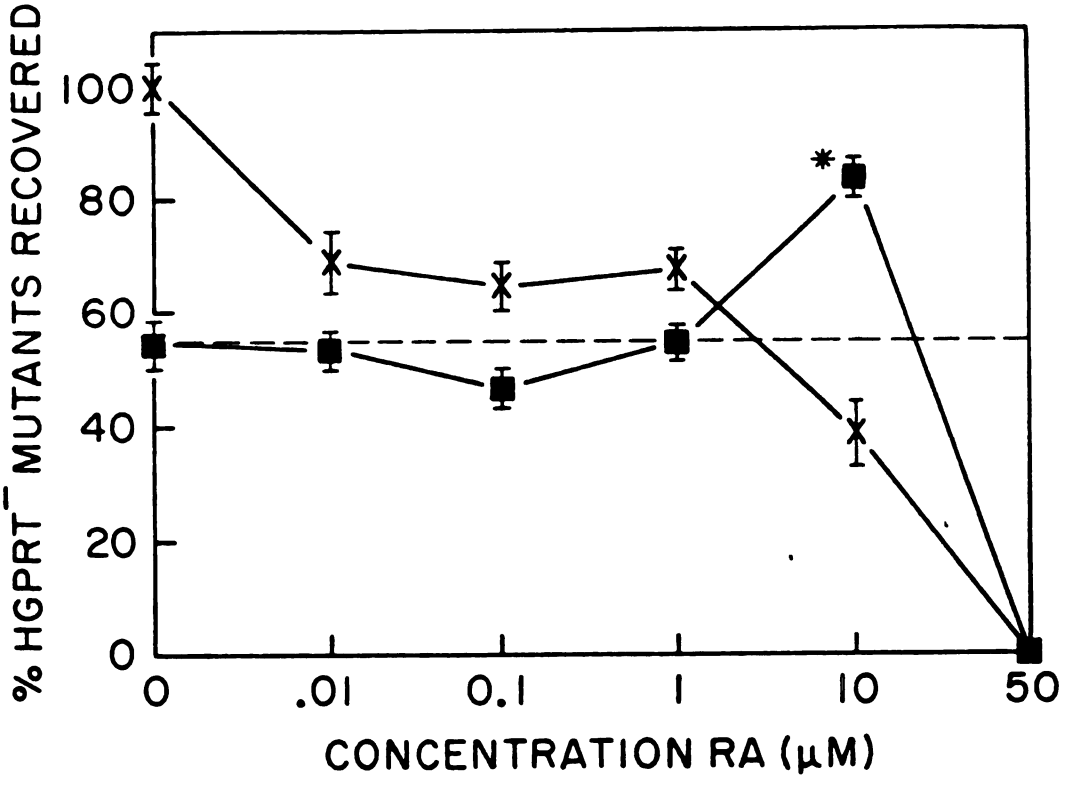

Figure 3.4. Dose-response curves for RA when used in combination with 25 ug FM/ml media. Cytolethality (—✕—) and MC (—■—) results are shown, as well as the result in MC plates with FM alone (--------). Data are plotted with bars representing the standard error of the mean. (*) indicates values which are significantly different from the result in MC plates with FM alone.

Figure 3.5. ACAS images of fluorescent emissions from cells treated with 500 ug PB/ml media. The emitted intensities are color-coded and displayed on the computer video screen as a pseudo-color contour map of the cells.

(A) 1 isolated pair of cells, 1 isolated single cell, and 2 cells in contact with other cells which have been selected for photobleaching.

(B) Immediately after photobleaching, notice there is a very small amount of fluorescence remaining in the isolated pair of cells, indicating incomplete photobleaching.

(Continued)

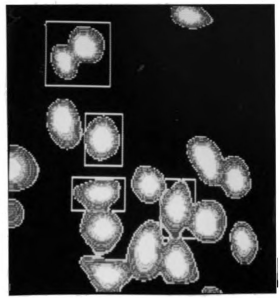

Figure 3.5 (A)

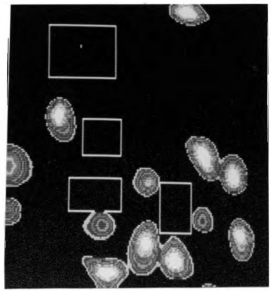

Figure 3.5 (B)

Figure 3.5. (cont'd.)

(C) 18 min later, the two photobleached cells which were in contact with other unbleached cells have considerable return of fluorescence, the isolated single cell has no return of fluorescence, and the isolated pair of cells has a small amount of fluorescence (due to the incomplete photobleaching).

Figure 3.5 (C)

Figure 3.6. ACAS images of fluorescent emissions from cells treated with 25 ug FM/ml media.

(A) 1 isolated cell and 2 cells in contact with other cells which have been selected for photobleaching.

(B) Immediately after photobleaching.

(Continued)

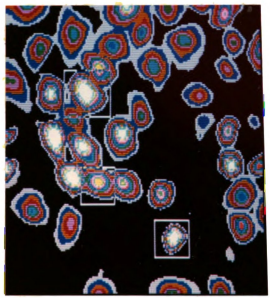

Figure 3.6 (A)

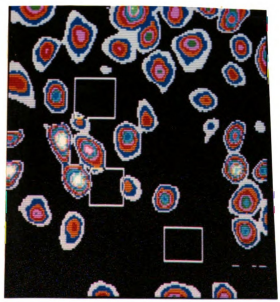

Figure 3.6 (B)

Figure 3.6. (cont'd.)

(C) 18 min later, no return of fluorescence is seen in either the isolated cell or in the cells which were in contact with unbleached cells.

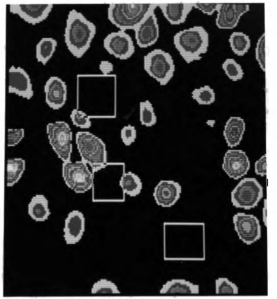

Figure 3.6 (C)

which were in contact with neighboring unbleached cells (not shown). When WB cells were treated with 500 ug PB/ml media for 24 hours, photobleached cells in contact with neighboring unbleached cells showed return of fluorescence, thus indicating no blockage of the junctional transfer of dye between cells (see Figure 3.5). In contrast, 25 ug/ml FM blocked junctional transfer between cells, as evidenced by a complete lack of return of fluorescence in photobleached cells in contact with neighboring unbleached cells (see Figure 3.6). With 10 uM RA, return of fluorescence was seen in photobleached cells which were in contact with neighboring unbleached cells, indicating that RA did not inhibit junctional transfer of dye molecules under the conditions of this experiment. A combination of 25 ug/ml FM and 1 uM RA blocked the return of fluorescence in photobleached cells, indicating that RA did not inhibit or reverse the blockage of junctional communication caused by FM.

Discussion

The results of these two assays for inhibition of junctional intercellular communication varied for the three chemicals tested. Phenobarbital and FM, both hepatic tumor promoters *in vivo* in rats, blocked junctional communication between WB rat liver epithelial cells in the MC assay. However, only FM blocked junctional communication as measured in the FRAP assay. Retinyl acetate, a vitamin A analog, inhibited junctional communication in the MC assay,

but did not block junctional communication in the FRAP assay. In addition, RA had no ability to prevent or reverse the blockage of junctional communication caused by FM in either assay.

Although there is a report that phenobarbital did not block MC in V79 cells (Umeda et al., 1980), others have reported that phenobarbital inhibited MC in V79 cells (Trosko et al., 1982; Jone et al., 1985a), as well as in mouse hepatocytes (Ruch et al., 1987), human hepatoma cells (Rolin-Limbosch et al., 1986), and between co-cultivated primary rat hepatocytes and ARL strain rat liver epithelial cells (Williams, 1980a). Zeilmaker and Yamasaki (1986) found that phenobarbital blocked dye transfer between V79 cells, but only after 72-96 hr exposure to the chemical. These results are consistent with our findings that phenobarbital blocks MC (a protocol involving 3 days of exposure to the chemical), but does not inhibit FRAP (a protocol involving only 24 hr exposure to the chemical) in a rat liver epithelial cell line.

Previous studies with FM found that it blocked MC in V79 cells (Trosko et al., 1981a) and in human teratocarcinoma cells (Kavanagh et al., 1987). In addition, 2,2',4,4',5,5'-hexabromobiphenyl, a major component of the FM mixture, has been shown to inhibit gap junctional communication in a FRAP assay (Evans et al., 1988). The current study shows that FM has a similar effect in the WB rat liver epithelial cell line.

The effects of retinoids on intercellular communication appear to be highly variable. Retinoic acid has been reported to have no significant inhibitory effect on intercellular communication (Trosko et al., 1982; Radner and Kennedy et al., 1984; Yamasaki et al., 1984; Morel-Chany et al., 1986), but others, using either different cells or conditions, have reported retinoic acid does block intercellular communication (Pitts et al., 1986; Shuin et al., 1983; Walder and Lutzelschwab, 1984; Davidson et al., 1985). Retinoic acid has been reported to prevent TPA-induced blockage of intercellular communication (Shuin et al., 1983; Yamasaki et al., 1984), but in other studies using other cell types or different concentrations, retinoic acid has been reported to additively or synergistically enhance TPA-induced blockage of intercellular communication (Shuin et al., 1983; Radner and Kennedy, 1984; Morel-Chany et al., 1986). The effect seems to vary greatly with retinoic acid concentration, cell type, culture conditions and type of communication assay. Less work has been described with other retinoids, such as retinol or RA. One study found these had no effect on intercellular communication (Pitts et al., 1986), whereas another study showed they are less potent inhibitors of intercellular communication than retinoic acid (Davidson et al., 1985).

In this study, PB and RA blocked junctional communication in the MC assay but not in the FRAP assay. Discrepant results have been reported for other chemicals

(Zeilmaker and Yamasaki, 1986; Miller et al., 1987), however these discrepancies are usually noted between studies done in different laboratories and therefore possibly under different conditions. It does appear that junctional communication assays are exquisitely sensitive to very subtle differences in cellular conditions and environment (Yamasaki et al., 1984; Enomoto and Yamasaki, 1985; Hartman and Rosen, 1985; Jone et al., 1985b; Miller et al., 1987). Yamasaki et al. (1984) have noted that the MC assay assesses the transfer of relatively large substances through gap junctions, and thus concluded that the blockage of MC is not proof that a chemical is causing complete communication incompetence.

There are several differences between the MC and FRAP assays which may account, at least in part, for the discrepant results with PB and RA in this study. The MC assay assesses the indirect result of communication between cells in culture media over a 3-4 day interval, whereas the FRAP assay assesses the direct result of communication between cells in modified HBSS over an 18 min interval. Furthermore, MC is a complex process, involving the transfer of 6-TG metabolites which may exert their toxic effects by incorporation into DNA or by interfering with nucleic acid synthesis and coenzyme function (Berman et al., 1985). In contrast, dye transfer in the FRAP assay is a relatively simple process of diffusion of carboxyfluorescein through gap junctional channels between cells. Zeilmaker and

Yamasaki (1986) found that 5 chemicals which had been reported to block MC in V79 cells failed to inhibit dye transfer in V79 cells. They attributed this discrepancy to the inherent differences between MC assays and dye transfer assays, including the differing time intervals and the methods of assessing cytotoxicity in the two assays. It is interesting to note that by using a microinjection protocol, they saw inhibition of dye transfer by phenobarbital (Zeilmaker and Yamasaki, 1986), in contrast to the results with phenobarbital in the current FRAP study.

The MC assay involves a high population density of cells exposed to the test chemical for 3 days. In contrast, the FRAP assay involves a low population density of cells which are exposed to the test chemical for 24 hours. Cell density is closely interrelated with the growth phase of the cells. The effect of TPA on intercellular communication is highly dependent on the growth stage of the cells; TPA-induced inhibition of intercellular communication is transient in some cell types when they are in subconfluent conditions, whereas the inhibition is continuous when these cells are confluent (Enomoto and Yamasaki, 1985). The growth stage of the cells may be different in the MC and FRAP assays. A transient inhibitory effect of PB and RA in the FRAP protocol may have disappeared by the time the cells are examined 24 hr later since the cells are still in an active growth phase at that time. It is unclear why FM would not show a similar transient effect, but it is

entirely possible that FM is acting via a different mechanism than PB or RA to cause blockage of intercellular communication.

The sensitivities of the two assays may be quite different. Stedman and Welsch (1985) concluded that MC and dye transfer assays using V79 cells had approximately equal sensitivities. In contrast, Pitts and Kam (1985) found that an MC assay was more sensitive than either dye transfer or electrical coupling assays in co-cultured rat liver epithelial cells and hamster fibroblasts. If the MC assay is more sensitive in the WB rat liver epithelial cell line as well, this could explain the positive results for PB and RA in the MC assay despite negative results in the FRAP assay.

The results in this study indicate that while intercellular communication assays are potentially useful indicators for tumor promoting compounds, caution must be used in interpreting the results since differences in cells, culture conditions, laboratory techniques, and type of assay may affect the results in any given assay. Further investigations of the mechanisms by which tumor promoters inhibit junctional communication should help increase the applicability of these assays for identifying potential tumor promoting compounds.

SUMMARY

SUMMARY

The results of the experiments described in this dissertation may be summarized as follows. Two oral doses of Firemaster BP-6 (FM) 24 hours apart were sufficient to result in enhancement of hepatic enzyme-altered foci (EAF) in initiated rats during a two-stage hepatocarcinogenesis assay. Although tumor promotion is considered to be a relatively long-term phenomenon which requires weeks or months of exposure to the promoting agent, short-term administration of FM is sufficient for promotion to occur because FM is very slowly metabolized and excreted (Matthew et al., 1977; Miceli and Marks, 1981), thus resulting in long-term activity from a single dose. In the current study, FM remained at significant levels in the rat tissues 120 days after gavage, and persistent induction of hepatic microsomal enzymes was also observed. The low dose of 13 mg FM/kg body weight did not significantly enhance EAF in initiated rats, consistent with a no-effect threshold dose which has been observed with other tumor promoters (Kunz et al., 1982, 1983; Ward et al., 1983; Goldsworthy et al., 1984; Kitagawa et al., 1984; Oesterle and Deml, 1984).

In the second experiment, both FM and the polybrominated biphenyl (PBB) congener, 3,4,5,3',4',5'-

hexabromobiphenyl (345-HBB), inhibited normal body weight gain, produced histologic lesions in the liver, and decreased the hepatic vitamin A stores in treated rats. High dietary levels of vitamin A did not prevent the above changes. The PBB congener 2,4,5,2',4',5'-hexabromobiphenyl (245-HBB) produced only mild histologic lesions in the liver. Firemaster, 245-HBB and 345-HBB all significantly enhanced hepatic EAF in initiated rats. Rats given PBB and low dietary levels of vitamin A had somewhat greater numbers and sizes of EAF than rats given the same PBB and high dietary vitamin A levels, although these differences were not statistically significant. However, the total percent of the liver volume occupied by EAF was significantly greater in rats given 345-HBB with low dietary vitamin A than in rats given 345-HBB with high dietary vitamin A levels. Thus, dietary vitamin A supplementation had some inhibitory effect on the tumor-promoting activity of PBBs.

In the third experiment, FM, the vitamin A compound retinyl acetate (RA), and the liver tumor promoter phenobarbital (PB) blocked junctional intercellular communication between rat liver epithelial cells in metabolic cooperation assays. However, only FM inhibited junctional communication in dye transfer assays using fluorescence redistribution after photobleaching techniques. In addition, RA did not prevent or reverse the blockage of junctional communication caused by FM in either assay. The discrepant results for RA and PB between the metabolic

cooperation assays and the dye transfer assays may be due to inherent differences between the two types of assays.

The role of inhibition of intercellular communication by tumor promoters in the carcinogenic process must be definitively established before these assays can be relied upon as *in vitro* screening procedures for tumor promoting compounds. While supportive evidence is strong, inhibition of communication must be mechanistically linked to the process of tumor promotion to prove a cause and effect relationship. Since tumor promotion is essentially an *in vivo* concept, the gulf between cell culture and whole animal effects must be bridged. One approach would be the development of a technique to specifically and quantitatively assess junctional intercellular communication *in vivo*. Some have suggested double labelling of cultured hepatocytes with both fluorescent beads too large to pass through gap junctions and with another marker small enough to pass through junctional channels. These cells could then be transplanted back into the liver of an initiated rat. Following a promotion regimen, the liver could be evaluated for the appearance of single-labelled hepatocytes, indicating junctional transfer of the smaller markers to unlabelled cells. Another approach would be the development of specific probes to block gap junction formation or function, without extraneous cellular effects (Warner et al., 1984).

Until such time, *in vitro* assays to measure chemicals'

effects on intercellular communication appear to be useful indicators of potential tumorigenic properties, but must be used and interpreted with caution.

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