

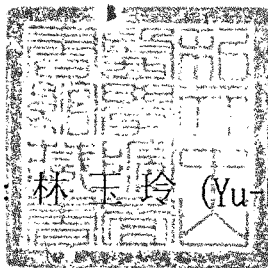
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私立中山醫學院醫學研究所碩士論文

- (I) 類胡蘿蔔素衍生物對黃麴毒素誘發DNA傷害及細胞毒性之抑制作用
Inhibition of Carotenoid Derivatives on Aflatoxin
B₁-induced DNA Damage and Cytotoxicity
in Cultured C3H10T1/2 Cells
- (II) 戊乙醯去羥甾子甘對黃麴毒素致鼠肝癌化之作用的影響
The Effect of Penta-acetyl Geniposide on Rat Liver
Carcinogenesis-induced by Aflatoxin B₁

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本論文為中山醫學院授予以理學碩士學位之必備條件之一，經中山醫學院醫學研究所碩士論文考試委員審核合格，並口試通過。

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獻 給

母親及我所懷念的父親

誌謝

寫下這頁，為論文劃下句點，但卻開啟了自己研究路程的第一頁。

非常感謝蕭松瑞院長的鼓勵支持及王朝鐘所長的悉心指導與照顧，讓我能夠在職進修完成碩士學位。

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謹以此篇論文獻給我的家人，及一個月後將為我夫的逸精先生。

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第一部份

類胡蘿蔔素衍生物對黃麴毒素

誘發DNA傷害及細胞毒性之抑制作用

縮寫表 (第一部份)

AFB ₁	:	Aflatoxin B ₁
BCT	:	Beta-carotene ; β -carotene
CCT	:	Crocetin
LCP	:	Lycopene
PE	:	Plating efficiency
RPE	:	Relative plating efficiency

第一部份

類胡蘿蔔素衍生物對黃麴毒素 誘發DNA傷害及細胞毒性之抑制作用

摘 要

黃麴毒素 (Aflatoxin-B₁) 對 C3H10T1/2 纖維母細胞株的毒性及 DNA 傷害可被三種類胡蘿蔔衍生物 (Carotenoid derivatives-Crocetin, Lycopene 及 β -carotene) 所抑制，而且被抑制的程度隨此三種衍生物的劑量升高而升高。

本研究方法中，我們利用在有老鼠肝細胞微粒體活化系統存在下，分別加入 0.5 μ g/ml AFB₁ 及不同濃度的 Carotenoid derivatives 處理一天或連續一星期後，以 C3H10T1/2 細胞株的種植效率 (Plating efficiency) 作為細胞毒性指標，則發現 Carotenoid derivatives 能抑制 AFB₁ 降低 C3H10T1/2 細胞株的種植效率。另外，以 alkaline elution 的方法檢測，發現這三種 Carotenoid derivatives 能減少因 AFB₁ 所引起的 DNA 傷害之作用。

這些結果顯示 Carotenoid derivatives 有抑制 AFB₁ 對 C3H10T1/2 之細胞毒性及 DNA damage，其抑制 AFB₁ 毒性的作用，可能與維生素 A 之機轉不同，而是經由 Carotenoid derivatives 增加 DNA 被 AFB₁ 攻擊後的修補系統 (repair system) 或減少 DNA damage，致使 DNA 的合成增加而減少 AFB₁ 產生的細胞毒性。

Inhibition of Carotenoid Derivatives on Aflatoxin B₁-induced DNA Damage and Cytotoxicity in Cultured C3H10T1/2 Cells

Abstract

Cytotoxicity and DNA damage of aflatoxin B₁ (AFB₁) on C3H10T1/2 embryo cells was suppressed by 3 carotenoid derivatives. Using the alkaline elution method, we found that the DNA damage of AFB₁-treated cells was inhibited by crocetin (CCT), Lycopene (LCP), and beta-carotene (BCT).

The decrease in the cellular DNA damage induced by AFB₁ was dependent on the dose of carotenoid derivatives. In the studies to investigate the inhibition of AFB₁-cytotoxicity, we observed that the plating efficiency of C3H10T1/2 cells in S-9 activation system was suppressed effectively with carotenoid derivatives and those compounds also can suppress the inhibition of DNA synthesis, we suggest that the inhibitory effect of carotenoid derivatives on the AFB₁-cytotoxicity in C3H10T1/2 cells might be due to the cells defense mechanisms that reduced the DNA damage or enhanced DNA repair system.

緒 論

一、總 論

許多研究顯示維生素A及其衍生物(Retinoids)有抑制癌化的產生,而胡蘿蔔素(β -carotene)也被證實能抑制由7,12-dimethyl-benz(a)anthrance誘導老鼠產生之腺腫瘤,及紫外線所引起之皮膚癌。而且自流行病學上之調查提出,攝食較多胡蘿蔔素之地區產生之癌症之機率較低,雖然Retinoids及 β -carotene之抗癌研究很多,但對於黃麴毒素B₁引起之毒性及致癌作用影響如何,則尚未詳加探討。因此本研究乃利用老鼠纖維母細胞株(C3H10T1/2 cell line)作為體外實驗環境,分別探討各種具有維生素A活性及不具維生素A活性之類胡蘿蔔素衍生物(Carotenoid derivatives: β -carotene, Lycopene, Crocetin等)對黃麴毒素B₁誘導產生之細胞毒性及DNA傷害有何效應,並藉此瞭解類胡蘿蔔素衍生物之作用機轉。

二、類胡蘿蔔素衍生物(Carotenoid derivatives)之發現背景及抑癌研究

20世紀初期維生素A被發現能夠用來預防夜盲症(night blindness)⁽¹⁾,之後,許多學者在植物中找到各種維生素A原(provitamin A-carotenoids)結構之物質。存在自然界中Vitamin A的主要型式有retinal(the al-

dehyde $C_{20}H_{28}O$, $X=O$), retinol ($H-X-OH$) 及 retinal esters 等, All-trans-Retinol 為維生素 A 中最高活化的型式 (Fig. 1), 在高等生物中扮演許多生物功能, 與細胞生長, 視覺, 粘液分泌及上皮細胞分化等有關。維生素 A 原最主要存在的型式為 β -carotene (Fig. 1), 及其它可以代謝轉換為 retinol 或無法轉換為 retinol 之各種衍生物 (總稱為 Carotenoids)。 β -carotene 帶有多不飽和碳氫鍵 ($x=x$), 人體中主要在腸道 (少部份在肝臟) 可被 dioxygenase 氧化而形成 2 mloe 的維生素 A retinol. 就在 β -carotene 及維生素 A retinol 的結構被確定之後⁽²⁾⁽³⁾, 便相繼的有學者探討維生素 A 與實驗動物及人類之間的作用關係。

一些研究指出, 當餵食含有 natural retinoids (如 retinol esters, retinoic acid) 等之維生素 A 予動物之後, 可以抑制上皮細胞癌 (epithelial cancer), 膀胱瘤, 肺癌, 胃腸道腫瘤及口腔粘膜癌症 (oral mucosa cancer) 之擴展⁽⁴⁻⁸⁾。另有流行病學調查指出, 血清中較低的維生素 A 值及飲食中 β -carotene 攝取不足可能與癌症發生率呈現某種程度的相關性^(9,10)。而在動物實驗研究結果顯示, β -carotene 可以抑制由 7,12-dimethylbenz(a)anthracene (DMBA) 所誘發引起的唾液腺瘤⁽¹¹⁾, 並且對紫外線所引發的皮膚癌也有抑制的作用⁽¹²⁾; Norman J. Temple 等人實驗餵食 inbred Swiss Webster (ICR) mince 添加有 β -carotene 之飼料 (20mg/Kg diet), 則能降低由 1,2-dimethylhydrazine (DMH) 所誘導產生直腸癌之惡化及發生比率, 而且添加 β -carotene 之劑量 (20mg/Kg diet) 相當於營養添加劑量範圍之內相當於 150-300g carrot/3,000Kcal), 因此這項結論可能適用於人類之營養狀況⁽¹³⁾; 而也更証實 Peto 等人於 1981 年提出 β -carotene 可以降低某些癌症發生率之說⁽¹⁴⁾。

在許多蔬果之中存有多類的 Carotenoids , 其中有具有維生素A活性或少量,甚至不具維生素A活性之衍生物,上述所提及之 β -carotene 為具有維生素A活性之 Carotenoid,其抗癌作用被學者相繼研究,但作用機轉則尚未明瞭,Lycopene(Fig.1)為Carotenoids中不具維生素A活性之一類, Petayev 等曾証實它能抑制自由基產生之鏈鎖反應而減少癌化之作用⁽¹⁵⁾。在梔子果實(Gardenia jasminoides)中一主要成份Crocetin (Fig.1),亦屬於 Carotenoids 之衍生物,多被用來作為糖果,甜食,冰點或飲料中之色素添加料,過去的研究顯示Crocetin 能夠抑制受病毒感染所引起之雞腫瘤產生⁽¹⁶⁾,並且能抑制癌細胞在老鼠體內的增生作用^(17,18),及抑制化學致癌物質所誘導產生的致肝癌作用⁽¹⁹⁾。

三、黃麴毒素 (Aflatoxin B₁) 的致癌作用

黃麴毒素 (Aflatoxin B₁; AFB₁) 在各種動物實驗中均被証明為一種很強的致癌物質⁽²⁰⁾,自流行病學上的研究調查結果顯示,黃麴毒素 與人類肝癌有關⁽²¹⁾,可能是造成亞洲地區和非洲地區居民肝癌發生率偏高的主因之一。

Swenson 等人⁽²²⁾為探討 AFB₁ 經過代謝之後產物的型式, 乃將 ³H 標示的 AFB₁ 注入老鼠體內, 而在肝細胞中可以找到與 DNA, rRNA 共價結合的 AFB₁; 之後再將核酸與 AFB₁ 的共價結合物以酸水解而得到 8,9-dihydrodiol, Lin 氏等人發現在活體外 DNA 以 guanine 與 AFB₁ 結合, 可得到 guanine-AFB₁ 之共價物⁽²³⁾, 若將活體外或活體內之 DNA-AFB₁ 共價物 (DNA-AFB₁ adduct) 共價物在各種不同條件下以酸水解, 則可產生四種不同產物 (Fig.2)。而由多項實驗結果證明 AFB₁ 主要的致癌機轉乃是需先經肝臟酵素系統代謝為 AFB₁-8,9-epoxide, 而成為親核性代謝物 (electrophilic metabolite) 再攻擊 DNA 而形成形成共價加成物 (DNA adduct), 此 AFB₁-DNA adduct 被認為是形成癌化的起始原因⁽²⁴⁾。

近年來有研究指出 AFB₁ 的致癌作用可能與致癌基因 (如 ras, myc gene) 的活化或抑癌基因 (如 p53) 去活化有關⁽²⁵⁻²⁸⁾, 其作用可能來自 AFB₁ 所造成之 DNA 損傷, 導致鹼基配對錯誤。

四、研究目的

存在於天然物之中的Retinoids及 β -carotene具有抗癌作用的研究已相繼的被証實,但針對Carotenoid衍生物是否能抑制黃麴毒素引起的毒性及致癌性則較少被學者研究,由於台灣地區氣候潮濕屬於黃麴毒素易污染之地區,與肝癌發生率有極大之相關性⁽²⁹⁾,因此若能發現一些天然物來抑制黃麴毒素之致癌作用實屬重要。

雖然許多研究顯示 Retinoids 在抗癌化及抗癌細胞之作用遠大於 Carotenoid衍生物,但值得重視的是 Retinoids 具有很大的肝毒性及副作用⁽³⁰⁾;在許多動物實驗中均顯示 Carotenoid衍生物有較低的毒性作用^(31,32),因此這些天然存在的 Carotenoid衍生物在抑制癌化的角色應更為重要。

本研究以活體外環境(in vitro)比較具有維生素A活性的 β -carotene(BCT),及不具維生素A活性的Lycopene(LCP);Crocetin(CCT)分別探討對黃麴毒素誘導產生之細胞毒性及DNA損害是否有抑制作用,並探討這三類天然物中存在的物質對抑制黃麴毒素毒性的作用機轉。

材料與方法

一、化學試劑

Aflatoxin B₁ (AFB₁) 購自Moravek Biochemicals (City of Brea, CA, USA); β -carotene (BCT), Lycopene (LCP) 均購自 Sigma公司; Minimal Essential Medium (MEM) 購自Gibco Company; methyl-³H-thymidine 購自 New England Nuclear (specific activity 6.7Ci/n mole); Scintillation liquid 購自 Sigma 公司。

二、AFB₁ 及 Carotenoid derivatives之製備

將AFB₁及Carotenoid derivatives (BCT, CCT, 及LCP)分別溶於DMSO中, 濃度分別為AFB₁(2mg/ml); Carotenoid derivatives(400, 40, 4, 0.4, 0.04 mg/ml), 均放置於0°C貯存, 使用時刻則取出再以MEM培養基稀釋100倍, 各取100 μ l放入培養皿中, 使AFB₁於培養皿中之最終濃度為0.5 μ l/ml MEM; Carotenoid derivatives為100, 10, 1, 0.1, 0.01 μ l/ml MEM (LCP為2.5, 1.0, 0.1 μ l/ml MEM), DMSO最終濃度為0.025%

三、細胞培養

C3H10T1/2細胞株來自於C3H mice embryo cell分離培養而成的正常纖維母細胞⁽³³⁾，本實驗所用之C3H10T1/2 cell均在8-15代之間，培養於Eagle MEM中(內含10%胎牛血清，100 IU/ml pencillin及100 μ l/ml streptomycin)，培養條件在37°C及5% CO₂之恆溫箱中。欲取用此細胞時，以Trypsin-EDTA處理為密度均勻之懸浮液。

四、細胞毒性分析

- (1) 將培養在75cm²角瓶(Nunc, Denmark)中之C3H10T1/2 cell line 以trypsin-EDTA處理為密度均勻之懸浮液後，以MEM培養基調整細胞密度為500 cells/ml MEM，取0.5 ml (即250 cells)放入petri dish中培養24小時後，取以稀釋好之Carotenoid derivatives (BCT, CCT及LCP) 100 μ l加入，(另外以0.025% DMSO當控制組)，一起培養24小時之後移去原有之培養基，換入新的培養基，繼續培養7天。

之後移去培養基，以甲醇:醋酸(3:1, v/v)固定細胞(處理10')，移去固定液，待乾以Giemsa(5%)染色(處理30'以上)。待晾乾之後，計算直徑大於0.05cm的colonies數目。



C3H10T1/2 cell所受毒性大小之計算方式如下：
以DMSO組之種植效率(plating efficiency, PE)為100，則以藥物處理後之相對種植效率(Relative plating efficiency; RPE)為：

$$\%RPE = \frac{\text{PE of compound treated}}{\text{PE of DMSO control}} \times 100\%$$

若RPE愈小，則表示C3H10T1/2 cell受到此藥物之毒性愈大。

(2) 由上述結果，選擇 Carotenoid derivatives 對 C3H10T1/2細胞非毒性劑量，同時加入AFB₁ (0.5 μl/ml)，於存在與不存在老鼠肝細胞微粒體酵素活化系統(S-9 activation system)之下分別測定RPE值。

(3) 另外，以上述同劑量之Carotenoid derivatives 與AFB₁同時加入250 cells/dish中，24小時之後移去培養基，再加入Carotenoid derivatives 培養7天，計算各 Carotenoid derivatives 長期作用下 C3H10T1/2 cell之RPE值。

(4) S-9活化系統之製備⁽³⁴⁾

以已知致突變物多氯聯苯混合物Aroclor 1254以500mg/Kg注入Sprague-Dawley品系雄性老鼠體內，五天後將老鼠肝臟取出，以鐵弗龍均質器均勻化後在9,000 xg離心10分鐘，取其上層液，儲存於-80℃冰箱。使用時以輔助因子配成10%混合液，包括：S-9 1ml, MgCl₂-KCl salts 0.2ml, 1M Glucose-6-phosphate 0.05ml, 0.1M NADP 0.4ml, 0.2M phosphate buffer (pH 7.4) 5ml及二次水3.35ml，每個試驗取250 μl加入。

五、DNA damage分析

(1) 細胞處理

C3H10T1/2 cell於25cm²角瓶中培養，24小時後加入0.2 μ Ci methyl-³H-Thymidine培養24小時後，移去³H-Thymidine，再加入新的MEM培養基，24小時後加入上述濃度之 Carotenoid derivatives 及 AFB₁ (0.5 μ l/ml)，培養 4-6 小時，以 Trypsin-EDTA收集細胞，於200 rpm下離心10分鐘，倒去上清液，加入1ml PBS再均勻化，置於-70°C至少48小時。

(2) DNA damage之分析方法

種植2 x 10⁵ cells/flask 24 小時之後，加入0.2 μ Ci ³H-thymidine 24 小時，再加入AFB₁ 及 carotenoid derivatives 處理24 小時，DNA damage 之分析方法依據Kohn等人⁽³⁵⁾所修飾之 alkaline elution method，方法如下述：將polyvinyl chloride filter 裝在 syringe filter holder 上，下接 peristaltic pump (0.5ml/min)，先以 1 ml PBS 潤濕 holder，再加入 2 ml PBS及 6 ml lysing solution (含2M NaCl, 0.02M EDTA, 及0.2% triton X-100 pH8.2)後，將細胞慢慢滴入，待syringe內溶液流完後，加入5 ml 1mM EDTA pH7.8，流完後再加入13.5 ml Elution Solution(含0.02M TEAH, 0.02M EDTA, pH12.2)後開始收集總量，以0.5 ml/min之速率收集。

取全量1.5ml加入13.5ml Scintillation liquid (每1000ml toluene 含30% triton X-100, 0.4% pop及 0.01% popop) 測 Single strand DNA，以 Aloka LSC-900閃爍計數儀，測得cpm值，所得之結果均為三次重覆之平均值。

將 membrane 自然乾燥後，剪成小塊狀盛裝在瓶內，先吸取 5 c.c. Elution Solution (含 0.02M TEAH, 0.02M EDTA, pH12.2) 振盪 1 分鐘後吸到另一瓶，再加 5ml Elution Solution 振盪 1 分鐘，吸到瓶內共 10 c.c.，最後取 1 c.c. 的量加入 2 c.c. Elution Solution，再加入 13.5ml Scintillation liquid-33258 測 cpm 值。

計算 Elution ratio 之公式如下：

$$\text{Elution Ratio} = \frac{(\text{Eluted cpm}) \times 9}{\text{Eluted cpm} \times 9 + \text{residue cpm} \times 30} \times 100\%$$

六、C3H10T1/2 細胞 DNA 合成影響之分析

取已稀釋好之細胞 (2×10^5 cells/ml) 0.1ml，放入 microtiter plate (96 wells, Nunc.) 中培養 24 小時後，將預先溶於 DMSO 之 Carotenoid derivatives 調配成每 ml 含 $0.02 \mu\text{g}$, $0.2 \mu\text{g}$, $2.0 \mu\text{g}$ 後，各取 $100 \mu\text{l}$ 及 $0.5 \mu\text{g}$ /ml 之 AFB_1 $10 \mu\text{l}$ 放入每個 well 中，使最終濃度為 AFB_1 $0.48 \mu\text{g}/\text{ml}$, Carotenoid derivatives 0.01 , 0.1 , $1.0 \mu\text{g}/\text{ml}$, DMSO 0.25% 及 $10 \mu\text{l}$ 之 S-9 fraction 共 0.2c.c. 及 label ^3H -Thymidine，處理 6 小時，以 Bellco harvester 收集細胞于 Bellco glass filter strip 上，加入 Scintillation liquid 3.5c.c. 後分別測 cpm 值。

結 果

由於高濃度的Carotenoid derivatives本身也可能對細胞造成毒性，因此利用各種不同濃度來處理 C3H10T1/2細胞，顯示濃度在 $1.0 \mu\text{g/ml}$ 以下之 Carotenoid derivatives對C3H10T1/2細胞不會造成毒性 (Table 1)，因此選擇 $1.0 \mu\text{g/ml}$ 以下之Carotenoid derivatives來研究對AFB₁毒性之影響。

在沒有活化系統 (S-9 activation system) 下， $0.5 \mu\text{g/ml}$ 之AFB₁產生細胞毒性很小 (Table 2)，但若以S-9活化作用下細胞之plating efficiency (PE; 種植效率值) 降為2% (Table 3)，顯見AFB₁須經過代謝活化才對細胞產生毒性作用。若同時以不同之Carotenoid derivatives在各種濃度同時處理一天或一週後，其PE值顯著增加，其中以Crocetin (CCT) 效果最好。而連續處理一週者，降低AFB₁毒性除CCT外，其它 (BCT, LCP) 均比處理24小時的效果顯著 (Table 3)。由以上結果顯示除BCT在 $0.01 \mu\text{g/ml}$ 之濃度外，其他兩種 (CCT, LCP) 在各種不同之濃度均可降低AFB₁對C3H10T1/2細胞之毒性。

AFB₁代謝活化後會對細胞DNA產生損害作用，受損害的DNA在鹼性處理下會斷裂成單鏈的DNA，此單鏈的DNA容易穿透濾膜而被沖洗出來 (Elution)，利用此方法我們研究Carotenoid derivatives ($0.01 \mu\text{g/ml}$ - $1.0 \mu\text{g/ml}$) 在降低AFB₁對細胞DNA產生傷害之能力。結果顯示C3H10

T1/2細胞DNA在S-9活化系統下被AFB₁傷害達76% (無S-9活化下為42%)，細胞若同時經Carotenoid derivatives及AFB₁在活化系統下處理24小時，則DNA傷害度減少，即被Eluted的DNA減少，尤其在有S-9活化下作用更顯著，其濃度在0.1 μg/ml-1.0 μg/ml，與AFB₁組比較p值均小於0.001-0.005，而Crocetin(CCT)在無S-9活化系統下也有相同作用(Table 4)。因此Carotenoid derivatives可能有抑制AFB₁攻擊DNA的能力或是增加DNA之修補作用。

利用標示的Thymidine(³H-thymidine)為DNA合成的材料，從³H-thymidine嵌入(incorporation)DNA的多寡可預知DNA合成的增加與減少，當AFB₁在有S-9或無S-9活化作用下，其³H-thymidine嵌入的量比正常組少，表示DNA的合成受阻(Table 5)，若增加Carotenoid derivatives在10.0 μg/ml時則³H-thymidine嵌入DNA的量增加，此結果顯示Carotenoid derivatives可以減少AFB₁抑制DNA合成之作用。

並由結果發現具維生素A活性之β-carotene (BCT)抑制AFB₁毒性之作用較小，而不具維生素A活性之Lycopene (LCP)及Crocetin(CCT)其活性較大。

討 論

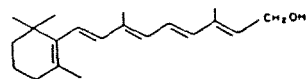
綜合以上結果顯示AFB₁(0.1 μg/ml)在S-9活化系統下與細胞DNA共價結合，造成對C3H10T1/2細胞DNA產生傷害而造成細胞毒性在3種Carotenoid derivatives (BCT, LCP, CCT)存在下，均能減少DNA受損程度，而減少AFB₁產生之細胞毒性。而不具維生素A活性之LCP及CCT均比具維生素A活性之BCT有較強之抑制作用，顯示過去認為Carotenoid derivatives的抗癌作用係因能轉化為維生素A，而有相似於維生素A之作用^(36, 37)似乎不是主要的抑癌作用。

雖然許多研究顯示Retinoids在抗癌化及抗癌細胞之作用均遠大於Carotenoid derivatives，但值得重視的是Retinoids 具有很大的肝毒性及副作用⁽³⁸⁾，而在許多動物實驗研究中均顯示Carotenoid derivatives的低毒性作用，因此這些天然物存在的物質在抑制癌化角色應更重要。

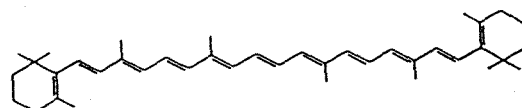
AFB₁代謝活化為AFB₁-8,9-epoxied後與DNA共價結合，雖然沒有直接的證據顯示此種共價結合與DNA受損之程度平行(因為大部份受損之DNA會被修補)，但在in vivo及in vitro的試驗中顯示N-hydroxy-2-acetylaminofluorene與DNA共價結合後會造成DNA受損的程度是平行的⁽³⁹⁾，在我們的研究中也顯示以AFB₁處理C3H10T1/2細胞，造成DNA受損(以Alkaline elution測定)，此種受損而未修補的DNA可能無法複製，因此我們發現處理AFB₁後，也造成C3H10T1/2細胞DNA的合成減少，因而造成AFB₁的細胞毒性。在以Carotenoid derivatives 添加處理

的實驗組則顯示AFB₁引起的DNA受損程度降低,增加了DNA的合成,因而減低了細胞的毒性。

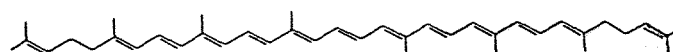
綜合上論,BCT,CCT及LCP這三種Carotenoid derivatives均能抑制AFB₁在S-g活化系統下引起的細胞毒性,其抑制細胞毒性之機制為這些Carotenoid derivatives減少AFB₁引起的DNA傷害(或增加DNA修補作用),使C3H10T1/2細胞DNA合成增加,進而抑制AFB₁引起之細胞毒性。



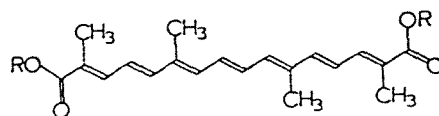
All-trans Retinol



β -Carotene (BCT)



Lycopene (LCP)



Crocetin R = H (CCT)

Fig.1 The structures of All-trans-retinol, β -carotene, Lycopene, and Crocetin.

Table.1 Effects of carotenoid derivatives on cytotoxicity in cultured C3H10T1/2 cells.

Treatment*	Dose ($\mu\text{g/ml}$)	Plating efficiency	% RPE**
Normal		29 \pm 3***	
DMSO	0.025%	28 \pm 3	100
BCT	1.0	31 \pm 5	115
	10.0	3 \pm 1 ^b	11
	100.0	0 \pm 0 ^b	0
LCP	0.1	26 \pm 6	95
	1.0	21 \pm 3 ^a	78
	10.0	24 \pm 6	86
CCT	1.0	24 \pm 9	89
	10.0	27 \pm 2	99
	100.0	6 \pm 0 ^b	0

* Cells were seeded at 250 cells/dish and treated with indicated concentration of carotenoid derivatives in DMSO for 1 week.

** % of relative plating efficiency = $\frac{\text{Colonies of treatment}}{\text{Colonies of DMSO control}} \times 100\%$

*** mean \pm SD, values are the averages of triplicate determinations.

^a $p < 0.05$; ^b $p < 0.001$; compared with DMSO control.

Table.2 Inhibitory effects of carotenoid derivatives on cytotoxicity induced by AFB₁ in cultured C3H10T1/2 cells without S-9 activation system.

Treatment*	Dose(μ g/ml)	Plating efficiency	%RPE**
Normal	0	24 \pm 8***	98
DMSO	0.025%	25 \pm 4	100
AFB ₁	0.5	24 \pm 4	84
AFB ₁ plus:			
BCT	0.01	21 \pm 4	85
	0.1	21 \pm 4	85
	1.0	24 \pm 2	96
LCP	0.001	27 \pm 1	110
	0.01	20 \pm 0	81
	0.1	28 \pm 5	114
CCT	0.01	27 \pm 2	111
	0.1	29 \pm 1	116
	1.0	24 \pm 6	99

* Cells were seeded at 300 cells/dish and treated with 0.5 μ g/ml AFB₁ and various concentration of carotenoid derivatives for 24 hrs.

** See Table 1.

*** Mean \pm SD, vlaues are the averages of triplicate determinations.

Table 3. Inhibitory effects of continuous or discontinuous exposure of carotenoid derivatives on cytotoxicity induced by AFB₁ in cultured C3H10T1/2 cells with S-9 activation system.

Treatment*	Dose (μg/ml)	Carotenoid derivatives (24 hr)		Carotenoid derivatives (1wk)	
		colonies	%RPE	colonies	%RPE
Normal	0	19 ± 1**	119	19 ± 1	119
DMSO	0.025%	16 ± 7	100	16 ± 7	100
AFB ₁	0.5	0 ± 1	2	0 ± 1	2
AFB ₁ plus:					
BCT	0.01	0 ± 0	0	6 ± 4	37
	0.1	2 ± 1	13	7 ± 3 ^a	43
	1.0	7 ± 2 ^a	44	12 ± 3	73
LCP	0.001	1 ± 1	6	6 ± 4	39
	0.01	6 ± 0 ^c	38	10 ± 1	63
	0.1	12 ± 3 ^b	69	11 ± 3	69
CCT	0.01	10 ± 2 ^b	63	8 ± 2 ^b	52
	0.1	12 ± 1 ^c	72	11 ± 1 ^c	67
	1.0	14 ± 2 ^c	88	13 ± 4 ^b	83

* Cells were seeded at 250 cells/dish and treated with AFB₁ and carotenoid derivatives for 24hr in DMSO (0.025%) with activation system, then removed AFB₁. In continuous experiments, carotenoid derivatives were added to culture and keep for one week.

** Mean ± SD, n=3.

^a p<0.02; ^b p<0.01; ^c p<0.001; compared with AFB₁-treated group.

Table.4 Protection of carotenoid derivatives on AFB₁-induced single strand DNA breaks in cultured C3H10T1/2 cells.

Treatment*	Dose (μg/ml)	% of DNA-eluted**	
		-(S-9)	+(S-9)
Normal	0	6 ± 1	10 ± 2
DMSO	0.025%	12 ± 2	15 ± 3
AFB ₁	0.5	42 ± 4	76 ± 8
AFB ₁ plus :			
BCT	0.01	45 ± 5	53 ± 6
	0.1	40 ± 3	47 ± 6 ^b
	1.0	43 ± 7	32 ± 4 ^c
LCP	0.001	41 ± 5	65 ± 12
	0.01	44 ± 8	56 ± 8 ^a
	0.1	40 ± 4	41 ± 7 ^c
CCT	0.01	50 ± 6	51 ± 7 ^b
	0.1	30 ± 2 ^c	47 ± 6 ^c
	1.0	19 ± 4 ^c	32 ± 6 ^c

* Cells were seeded 2×10^5 cells/flask for 24 hr. The DNA labelled by adding 0.2μ Ci ³H-thymidine for 24hr and non-radioactive medium incubated for an additional 24hr then exposed to carotenoid derivatives and AFB₁ for 24 hr. DNA damage assay by alkaine elution method.

** % of DNA damage eluted ratio = $\frac{\text{eluted DNA}}{\text{eluted DNA} + \text{residues DNA}} \times 100\%$

^a p<0.005; ^b p< 0.02; ^c p< 0.01;
group.

compared with AFB₁-treated

Table 5. Increased effects of carotenoid derivatives on DNA synthesis by treatment of AFB₁ in cultured C3H10T1/2 cell.

Treatment*	Dose ($\mu\text{g/ml}$)	without S-9		with S-9	
		cpm	% of AFB ₁	cpm	% of AFB ₁
Normal		2409 \pm 132	-	2981 \pm 251	
DMSO	0.025 %	2367 \pm 155	-	2842 \pm 201	-
AFB ₁	0.5	1888 \pm 123	100	2318 \pm 367	100
AFB ₁ plus:					
BCT	0.01	1823 \pm 236	96	2368 \pm 489	102
	0.1	2115 \pm 107	112	2493 \pm 290	107
	1.0	2139 \pm 83 ^a	113	2678 \pm 127	116
LCP	0.001	1907 \pm 314 ^c	101	2367 \pm 128	102
	0.1	2080 \pm 185	110	2782 \pm 511	119
	1.0	2102 \pm 302	111	2838 \pm 344	123
CCT	0.01	2193 \pm 339	116	2246 \pm 326	97
	0.1	2129 \pm 198	113	2615 \pm 510	112
	1.0	2434 \pm 41 ^b	129	2678 \pm 176	116

* Cells were seeded 2×10^4 cells/well for 24 hr then $0.5 \mu\text{g/ml}$ of AFB₁, 0.01, 0.1, $1.0 \mu\text{g/ml}$ of carotenoid derivatives, $0.01 \mu\text{Ci}$ ³H-thymidine and S-9 with or without were added to the culture and incubated for an additional 6 hr. DNA synthesis is estimated by ³H-thymidine incorporation as described in materials and methods. Values are the averages of triplicate determinations.

^ap<0.05; ^bp<0.01; ^cp<0.001; compared with AFB₁-treated group.

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第二部份

戊乙醯去羥梔子甘

對黃麴毒素致鼠肝癌化之作用的影響

縮寫表(第二部份)

(AC) ₅ GP	:	Penta-acetyl geniposide
AFB ₁	:	Aflatoxin B ₁
AST	:	Alanine aminotransferase (GPT)
ALT	:	Asparate aminotransferase (GOT)
CDNB	:	1-Chloro-2,3-dinitrobenzene
GSH	:	Glutathione
GST	:	Glutathione S-transferase
γ -GMNA	:	Gamma-glutamyl acid-4-methoxy- β -naphthlamide
γ -GTase	:	Gamma-glutamyl transpeptidase(γ -GTase; γ -GT;GGT)
H & E.	:	Hematoxylin and eosin
OPT	:	o-phthadialdehyde
T.Bili.	:	Total Bilirubin

第二部份

戊乙醯去羥梔子甘對黃麴毒素致鼠肝癌之作用的影響

摘 要

戊乙醯去羥梔子甘 (Penta-acetyl geniposide; (AC)₅GP) 對黃麴毒素 (Aflatoxin B₁; AFB₁) 引起老鼠急性肝毒性有促進之作用; 而對 AFB₁ 引起老鼠慢性致癌作用則有抑制之趨向。

(AC)₅GP 預先以胃管餵食三天或三小時, 在以腹腔注射 AFB₁ (2mg/Kg body Weight), 則發現 (AC)₅GP 的添加會加強由 AFB₁ 所引起老鼠肝毒性之強度, 血清中 Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), γ -glutamyl transpeptidase (γ -GT) 及 ALP 之活性均較 AFB₁ 單獨處理組明顯上升, 表示老鼠肝臟受到傷害而釋放這些酵素至血清中。而肝臟中 Glutathione (GSH) 之含量均因預先處理 (AC)₅GP 三小時或三天而降低; 預先處理 (AC)₅GP 三天則造成 Glutathione-S-transferase 之活性下降, 顯示以 (AC)₅GP 之處理對於 AFB₁ 造成之急性肝毒性並沒有抑制現象, 反而有增強之效應。

而以 (AC)₅GP 預先處理三天, 再同時處理 AFB₁ (每星期5次, 共8星期) 之後, 則發現由 AFB₁ 所引起老鼠肝臟中 γ -GTase larger foci 可被抑制為 γ -GTase smaller foci, 表示老鼠受 AFB₁ 長期注射處理之後在 liver carcinoma promotion 階段可被 (AC)₅GP 所抑制。

The Effect on Penta-acetyl Geniposide on the Rat Liver carcinogenesis-induced by Aflatoxin-B₁

Abstract

Penta-acetyl geniposide ((AC)₅GP) has potentiation effect on the acute rat liver carcinogenesis-induced by AFB₁; but it can suppress the rat liver chronic carcinogenesis-induced by AFB₁.

After gastric intubation for (AC)₅GP either 3 days or 3hr, the rats were i.p. administration for AFB₁, 24hr later the sacrificed rats were found liver cytotoxicity increasing extensively than the AFB₁ control group. And the serum enzymes: Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), γ -glutamyl transpeptidase (γ -GTase) activities were remarkably increasing. Either the groups pretreating (AC)₅GP 3 days or 3hr then i.p. AFB₁, the Glutathione (GSH) concentrations in the livers were reduced, and the Glutathione-S-transferase activity were not increased to enhance the detoxication pathway. It means that pretreating (AC)₅GP 3 days or 3hr cannot suppress the acute liver toxicity-induced by AFB₁, but enhance the toxicity.

On the chronic i.p. administration of AFB₁ 8 weeks (5 days/week), (AC)₅GP pretreating 3 days then gastric intubation for 8 weeks (5 days/week) can suppress the formation of γ -GTase larger foci induced by AFB₁. (AC)₅GP seems to suppress liver carcinogenesis on the liver carcinoma promotion stage-induced by AFB₁.

總 論

梔子 (*Gardenia jasminoids* Ellis) 為茜草科 (Rubiaceae) 植物 (Fig.1), 在中國屬於中品藥, 漢方中如梔子羌湯 (梔子、乾羌), 茵陳蒿湯 (梔子、茵陳、大黃)、黃蓮解毒湯 (梔子、黃蘆、黃芩、黃柏) 皆含有梔子, 臨床上有消炎、解熱、利尿等療效, 民間廣為使用, 因此在生藥上佔有相當重要之地位。梔子中主要成份有黃酮類梔子素 (Gardenin), 藏紅花素 (Crocic acid), 藏紅花酸 (Crocetin), 梔子甘 (Garderoside), 去羥梔子甘 (Geniposide), D-甘露醇 (D-mannitol) 等。過去有研究指出 Crocin 對 AFB₁ 及 dimethylnitrosamine 誘導之肝損傷具有保護作用⁽¹⁾, 另一成份去羥梔子甘 (Geniposide) 為一種 iridoidglycoside, 具有利膽作用, 及保肝作用^(2,3)。

而自梔子乾燥果實之甲醇粗萃取液經過乙酰反應, 再進行簡易分離步驟即可得到大量戊乙酰去羥梔子甘 (Penta-acetyl geniposide; (AC)₅GP) 之新化合物, 在過去研究顯示 (AC)₅GP 對於 AFB₁ 引起 C3H10T1/2 細胞之毒性有抑制作用⁽⁴⁾; 而且對於腫瘤細胞 C6 Glioma cell line 有抑制生長之作用⁽⁵⁾, 因此本研究乃以 (AC)₅GP 作為材料以探討對黃麴毒素引起老鼠之肝毒性有何影響。實驗分析 AFB₁ 引起急性肝毒性及慢性肝毒性兩大部份, 藉由血清肝功能酵素之測定, 肝臟中 GSH 含量及 GST 活性之測定, 並配合組織冷凍切片 γ -GTase foci 之鑑定等方面來加以探討對肝癌化之影響。

一、戊乙醯去羥梔子甘之製備及來源

梔子甘 (Geniposide) 為梔子果實中一主要成份，其藥理方面研究證明梔子甘具有刺激膽汁分泌，具有利膽的作用⁽⁶⁻¹⁰⁾。同時亦有報告指出梔子甘 (240 mg/kg) 或梔子粗萃取物 (1.5 g/kg) 連續處理 S.D. rat 三天後，肝臟中 α -naphthol glucuronosyltransferase 及 Glutathione S-transferase 之活性顯著增加⁽¹¹⁾，但對 phase I enzyme 如 arylhydrocarbohydrorylase 則不受影響。

基於 Geniposide 之藥理上保肝之作用，而且自梔子果實中純化 Geniposide 過程中可得到大量之 Acetylgeniposide，因此我們自梔子中萃取出 Penta-acetyl geniposide，其製備過程需先將果實中脂質去除，之後再以 Butanol 及 H₂O 來萃取、分層，取得 Butanol 層後進行乙醯化，最後以 Ethanol 再結晶，則可以得到配醣體上接上五個 acetyl group 之 Penta-acetyl geniposide。製備流程及 Penta-acetyl geniposide ((AC)₅GP) 結構見 Fig.2

二、戊乙醯去羥梔子甘相關之抑癌研究

本研究室曾以前述之方法自梔子果實中所得之Penta-acetyl geniposide ((AC)₅GP)對AFB₁引起C3H10 T1/2纖維母細胞之毒性有抑制作用⁽⁴⁾;而且對C6 Glioma cell 腫瘤細胞的生長有抑制之現象⁽⁵⁾;另外,將C6 Glioma cell transplant至老鼠皮膚之tumor生長也可以被(AC)₅GP所抑制⁽¹²⁾。

三、黃麴毒素之致癌研究

AFB₁經由phase I enzyme (cytochrome P450 monooxygenase)代謝之後形成Aflatoxin B₁-8,9-oxide, 如果與DNA或RNA上Guanine 7N 位置結合則形成 DNA adduct 而傷害正常之細胞, 或此AFB₁-8,9-oxide會與albumin上之lysine residue 結合而影響正常細胞蛋白質之結構⁽¹³⁾。Lin氏等人⁽¹⁴⁾發現在活體外DNA以Guanine與AFB₁結合而可得到guanine-AFB₁之共價物,若將活體外或活體內之DNA-AFB₁共價物在各種不同條件下以酸水解則可產生四種不同產物,而AFB₁-8,9-oxide即為主要攻擊DNA而形成致癌性之主要形式,也為AFB₁之終極致癌物⁽¹⁵⁾。

四、去除黃麴毒素毒性之途徑及相關酵素

AFB₁之終極致癌物AFB₁-8,9-oxide與DNA,或RNA,甚至protein結合之後則對細胞造成傷害或致癌;除了與

這些巨分子結合之路徑外，AFB₁-8,9-oxide可能被非酵
素性質之水解方式而形成AFB₁-8,9-oxide(此物質仍具有
毒性)⁽¹⁶⁾；AFB₁-8,9-oxide 如果與 glutathione(GSH)
conjugation, 經由glutathione S-transferase的作用
則可代謝為8,9-dihydro-8-(S-glutathionyl)-9-hydro-
xyaflatoxin產物而被排除於膽汁中^(17,18)。(Fig.3)

Glutathione S-transferases(GSTs) 為一群 iso-
zymes, 廣泛存在於肝臟, 能夠催化glutathion(GSH)與
親核物質結合, 而使此親核性物質易於排出體外⁽¹⁹⁾。
許多抗氧化劑(BHA, BHT, Oltipraz)即能使老鼠肝臟中
GSTs活性增加而抑制AFB₁終極代謝物之產生⁽²⁰⁾。

五、Gamma-glutamyl transpeptidase (γ -GTase) 及 γ -GTase foci之表現

1. γ -GTase之功能及出現時期

Gamma-glutamyl transpeptidase(γ -GTase;GGT)之
酵素命名為(EC 2.3.2.2) 能催化 γ -glutamyl group 轉
移至其他 peptide 或 L-amino acid上, 而後形成新的
 γ -glutamyl peptides⁽²¹⁾。由 histochemical 及 bio-
chemical 方法可以發現 γ -GTase 在老鼠出生時期(fetal)
的liver parenchyma中大量出現, 而在正常的老鼠
肝中則幾乎不出現⁽²²⁻²⁴⁾。

2. γ -GTase異常表現時期

Fiala等人證明, 經由一些hepatocarcinogen之作用
可 induce mice及rat 肝臟中GTase之活性, 而且在pre-

cancer時期或 carcinoma時期均會發生^(25,26)。因此 γ -GTase 可被用來作為組織是否產生 hepatocellular carcinoma 之早期評估指標⁽²⁷⁻²⁹⁾，並可作為細胞培養時是否變形 (transformation) 之評估⁽³⁰⁻³²⁾。

3. Histochemistry 上 γ -GTase foci 出現之意義

根據 Mbowa MR. 等人 (1975) 研究發現將 AFB₁ 50 μ g / 0.1ml DMSO 以 gastric intubation 方式給予 Wistar rat (1星期2次) 四星期之後，在 histochemistry 觀察發現在肝臟 hyperplastic foci 位置之細胞有很強的 γ -GTase activity⁽³³⁻³⁶⁾。其他學者研究以多種致癌物質如 B(a)P, 7, 12-MBA 等之 polycyclic aromatic hydrocarbons，或 N-Butyl-N-nitrosourea 之 N-nitroso compounds 等均會使肝細胞中出現很多數目的 γ -GT positive foci⁽³⁷⁾。而在 liver cell 中強烈出現這種 γ -GTase foci 的位置即顯示受到致癌物質之毒性的表現。

4. γ -GTase foci 的抑制

Thomas W. 等人 (1987, 1992) 以臨床上用來作為抗霍亂及抗氧化所添加的藥物 1,2-dithiole-3-thione 研究發現此藥物可以 induce 出維持 GSH pools 的 enzymes (如 NAD(P)H : quinoin reductase; epoxide hydrase; glutathione S-transferase 等)；而且可以降低肝細胞被 AFB₁ 誘導產生之 γ -GTase foci 的數目及大小^(38,39)。因此有學者想自 chemopreventor 之藥物中找尋是否具有抑制 γ -GTase positive foci 之效用，以抑制癌症早期之擴展。

第一章 戊乙醯去羥梔子甘對黃麴毒素 急性肝損傷之研究

研究目的

本實驗利用天然物梔子 (*Gardenia jasminoids* Ellis) 之果實粗萃取物乙醯化所得之戊乙醯去羥梔子甘 (AC)₅GP 以胃管方式預先短期處理老鼠，之後再以腹腔注射方法處理 AFB₁ 於老鼠，藉以探討老鼠在受到 AFB₁ 急性肝毒性作用之下，(AC)₅GP 對此毒性之影響。

由於梔子中許多主要成份，如 Crocetin, Crocin 及 Geniposide 均被證實可以抑制因 AFB₁ 所引起之肝毒性，因此本研乃以活體 (*in vivo*) 方式探討 (AC)₅GP 對 AFB₁ 之影響，並檢測老鼠之肝臟功能酵素及 Glutathione 之含量及 Glutathione S-transferase 活性變化。

材料與方法

1. 動物來源

Wistar strain 雄性大白鼠 (體重約150-180g體重) 購自台大醫院動物中心, 以Purina Lab Chow 為飼料, 蒸餾水不限制飲用, 隨意分組方式, 每組別6隻, 均需秤重以備藥物處理。

2. 化學試劑

AFB, GSH, HPO_3 , EDTA, OPT(o-Phthadialdehyde), Tris, Sucrose, GSH, CDNB(1-Chloro-2,3-dinitrobenzene) 均購自Sigma 美國公司。肝功能分析試劑購自德國Merck 公司, Penta-acetyl geniposide((AC)₅GP) 為本實驗室自梔子植物果實中抽取製備而得⁽⁴⁰⁾。

3. 動物處理

(1) 戊乙醯去羥梔子甘前處理3小時部分

戊乙醯去羥梔子甘((AC)₅GP) 及AFB₁均溶解於少量DMSO, 再以二次水稀釋, DMSO 最終濃度為2%, AFB₁ 濃度為2mg/kg Body Weight, (AC)₅GP 濃度為(2mg/kg, 5mg/Kg

及10mg/kg B.W.)。DMSO及AFB₁給予方式為腹腔注射(i.p.)，(AC)₅GP為胃管餵食方式。實驗老鼠共分七組，處理方式如下：Group I 為normal，不作任何處理方式；Group II 老鼠於第4小時i.p. DMSO(2%)0.03ml；Group III 於第4小時i.p. AFB₁ (2mg/kg B.W.) 0.03ml；Group IV 胃管餵食(AC)₅GP (10mg/kg B.W.) 27小時後犧牲；Group V 以(AC)₅GP (2mg/kg B.W.) 胃管餵食，3小時後(即第4小時)i.p. AFB₁(2mg/kg B.W.)；Group VI 胃管餵食(AC)₅GP (5mg/Kg B.W.)，第4小時i.p. AFB₁(2mg/Kg B.W.)，所有老鼠在腹腔注射DMSO或AFB₁ 24小時之後同時犧牲。(處理流程Fig.4)。

(2) 戊乙醯去羥梔子甘前處理3天部分

(AC)₅GP 及 AFB₁均溶於少量DMSO，在以二次水稀釋，DMSO最終濃度為2%。老鼠共分六組，處理如下：Group I 為Normal，不作任何前處理；Group II 於第3天i.p. DMSO(2%)；Group III 於第3天 i.p. AFB₁(2mg/Kg B.W.)；Group IV 於第1、2、3天分別以胃管餵食(AC)₅GP (10mg/Kg B.W.)；Group V 於第1、2、3天胃管餵食(AC)₅GP之後，於第三天i.p. AFB₁(2mg/Kg B.W.)；Group VI 於第1、2、3天分別以胃管餵食(AC)₅GP (10mg/Kg B.W.)，並在第3天同時i.p. AFB₁(2mg/Kg B.W.)，所有老鼠在第4天犧牲(處理流程見C Fig.5)。

4. 分析項目

所有老鼠以斷頭方式犧牲，取得血液分離血清之後分析肝功能診斷酵素，另一方面取肝臟分析GSH含量及GST酵素活性。

(1) 肝毒性分析

以肝功能診斷酵素包括 Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), ALP, γ -Glutamyl transpeptidase (GGT) 作為急性肝毒性之分析指標。

(2) 肝臟GSH含量之定量

檢測方法參考Hissin等人之方法⁽⁴¹⁾, 稱取肝臟180mg, 加入 0.67ml 25% HPO_3 及2.53ml phosphate-EDTA buffer 均質器均質後, 於100,000 $\times g$ 重力離心 30分鐘 (4°C), 取得懸浮液稀釋10倍後, 取100 μ l OPT反應15分鐘 (Room Temp.) 需避光, 之後以Hitachi Fluorescence Spectrophotometer (F2000) 於激發波長 λ_{ex} 350nm, 放射波長 λ_{em} 420nm 測其吸光。(流程請見Fig.6)

(3) 肝臟GST活性之測定

GST (Glutathione S-transferase) 活性之測定係根據Habig等人之方法⁽⁴²⁾, 取肝臟0.8g加入3.2 ml Tris-Sucrose buffer (pH7.5)以均質器均質後於 105,000 $\times g$ 重力下離心30分鐘 (4°C), 取得懸浮液20 μ l 加入1.5ml之2mM CDNB混合均勻後 (以CDNB作為GST之受質), 於340nm波長下檢測 0-1 min, 1-2min 之間GST每分鐘反應之濃度; 另外取同樣量之懸浮液 (20 μ l), 先稀釋50倍之後, 取1ml

稀釋液加入 Protein-Assay Reagent(Bio-Rad Comp.) 4ml反應15分鐘,以280nm波長檢測蛋白質之含量。GST 活性之表示法則以 n mole/min/mg protein)表示。(流程請見Fig.7)。

結 果

經由血清中肝損傷指標酵素如 Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), γ -glutamyl transpeptidase (γ -GT) 及 ALP 之測量, 發現當以單一劑量之 AFB₁ 2mg/Kg 腹腔注射處理 Wistar 品系大白鼠 24小時後, 血清中肝功能酵素指標急遽升高 (Table 1, Table 4), 顯示肝臟機能受到傷害而將肝中酵素釋放至血清中, 造成血清中 ALT, AST, ALP 及 γ -GT 含量急遽升高。而以戊乙醯甈梔子甘 ((AC)₅GP) 不論預先處理 3 小時或 3 天之老鼠, 則血清中 ALT, AST, 及 ALP 被黃麴毒素誘發肝毒性所造成之升高毒性更為顯著 (Table 1, Table 4), 尤其是 (AC)₅GP 預先處理 3 小時組, 添加高劑量之 (AC)₅GP (10mg/Kg) 組則更為顯著 ($p < 0.001$) (Table 1)。而在溶劑控制組 (DMSO) 及 (AC)₅GP 高劑量單獨控制組均沒有影響, 顯示所選擇之劑量對老鼠不具肝毒性。

進一步分析肝臟中 (AC)₅GP 對 AFB₁ 影響之酵素作用, 以 10mg/Kg 之 (AC)₅GP 不論預先處理 3 小時 (Table 2.) 或 3 天 (Table 5.) 均造成鼠肝 GSH 的下降, 但隨著 (AC)₅GP 劑量之升高而升高; 以 (AC)₅GP 高劑量 (10mg/Kg) 單獨處理組則沒有差異。而同時以 (AC)₅GP 前處理 3 小時對肝中 GST 之活性沒有影響之 (Table 3), 而前三天高劑量 (AC)₅GP (10mg/kg) 處理則造成肝中 GST 活性降低之趨向 (Table 6), 顯示以 (AC)₅GP 處理之老鼠對於 AFB₁ 造成之肝毒性並沒有抑制現象, 反而有增強之效應。

綜合以上結果,顯示預先處理3小時或3天之(AC)₅GP對於AFB₁誘發急性肝損傷可能有促進之效應,且對AFB₁解毒之phase II enzyme有下降之趨向,但(AC)₅GP本身對於肝臟並沒有毒性產生。

討 論

本研究將老鼠預先予3小時或3天前胃管餵食戊乙醯化去羥梔子甘((AC)₅GP)再注射AFB₁,則沒有降低黃麴毒素所造成之肝毒性,反而有促進肝毒性之現象。因此以(AC)₅GP餵食及注射AFB₁予老鼠後,血清中ALT,AST,ALP活性明顯上升。當以單一劑量的AFB₁處理老鼠後,會使肝臟中GSH代償性增加,許多研究也証實包括AFB₁之多種致癌物質能促進肝中GSH含量上升⁽⁴³⁾。但本實驗結果發現,以(AC)₅GP處理時GSH有些微下降,可能本身對GSH有消耗作用,這結果可能與梔子中一主要成份-Geniposide本身會消耗GSH之作用相同⁽⁴⁴⁾。但預先胃管餵食(AC)₅GP再注射AFB₁後GSH含量雖然比單獨注射AFB₁組為低,但隨(AC)₅GP之劑量升高而GSH含量升高,表示肝臟並沒有繼續消耗GSH含量(Table 2.,Table 5)由於AFB₁主要的解毒作用是藉由GST之作用與GSH結合,因此在預先處理(AC)₅GP高劑量再注射AFB₁後因為GST活性降低,故無法促進AFB₁的代謝物與GSH結合來減低AFB₁對肝臟之毒性,所以肝中之GSH會隨(AC)₅GP劑量之升高而有升高之趨向。

由於(AC)₅GP結構中在配醣體位置帶有五個乙醯基(acetyl group),因此在經過代謝後可能提供許多acetyl group,而由體內Coenzyme A之結合加速脂肪酸之合成(Fig. 8);另外,AFB₁的代謝產物除了會被GST催化而與GSH共價結合(Conjugation)排出體外,也會與體內蛋白質如albumin等之Lysine residue結合而影響體

內蛋白質之功能⁽⁴⁵⁾,並且蛋白質之合成路徑也被抑制⁽⁴⁶⁾,因此更加使體內脂肪酸堆積在肝臟之機率變大,最後造成肝臟之急性中毒現象。推測此現象可能與酒精因經Aldehyde dehydrogenase 代謝後提供 Acetyl group而使Fatty acid堆積造成急性肝中毒原因相同。但本研究未作病理組織方面觀察,故需進一步探討以確實瞭解(Ac)_sGP經過代謝後之產物及其他作用。根據以前研究結果(Ac)_sGP對AFB₁-induced hepatocyte 的DNA damage (by UDS method)有抑制之作用,為什麼在in vivo,與 in vitro 之結果相異,有待進一步深入的探討。目前我們已知(Ac)_sGP對於活化AFB₁為AFB₁-8,9-epoxide之Cyt P-450 III A(以erythromycin N-demethylase為marker^(47,48))沒有影響(未發表之數據)。

第二章 戊乙醯去羥梔子甘對黃麴毒素 慢性致癌性之研究

研 究 目 的

本實驗同上一章部份以 (AC)₅GP 胃管餵食老鼠，及腹腔注射 AFB₁，但為長期處理八週之後再正常飼養至四十週，探討 (AC)₅GP 對 AFB₁ 引起慢性致癌性之影響。

本章研究方向有檢測肝臟功能酵素；肝臟中 Glutathione 含量，Glutathione S-transferase 活性變化情形；並配合肝組織切片 H.E. stain 方法觀察細胞之變化及以冷凍切片偵測肝細胞中 γ -GTase Foci 的表現程度，藉此瞭解梔子果實中 (AC)₅GP 成份在老鼠活體中長期作用後是否對 AFB₁ 之致癌性有抑制之作用。

材料與方法

1. 動物來源

Wistar R. Strain 雄性大白鼠(體重約150-180g)購自台大醫院動物中心,以Purina Lab Chow 為飼料,蒸餾水不限制飲用,隨樣分組方式每組分為6隻,均需稱重以備藥物處理。

2. 化學試劑

所需之試劑均如同AFB₁急性損傷實驗部份所述;另外 γ -GMNA(γ -glutamyl acid-4-methoxy- β -Naphthyl-amind)購自Bachem.California Comp.;glycylglycine購自fluka公司;Fast Blue BB salt購自Sigma公司;aqueous mounting medium 購自Polyscience Comp.

3. 動物處理

AFB₁及(AC)₅GP先以少量DMSO溶解,再以二次水稀釋,DMSO最終濃度為2%,AFB₁為2mg/Kg Body Weight,(AC)₅GP為1mg/Kg,及2mg/Kg。實驗分為六組,Group I 為正常組,不作任何處理;Group II 為DMSO處理組,第一週至第八週期間每週五次腹腔注射;Group III 為AFB₁處理組,方式同Group II;Group IV 為(AC)₅GP(2mg/Kg)胃管餵食組,胃管處理3天後再連續處理八週(5次/週);Group V 以(AC)₅GP(1mg/Kg)

胃管餵食3天後,同時胃管(AC)₅GP及腹腔注射AFB₁(2mg/Kg)(每週5次,連續8週);GroupVI處理方式同上組,但(AC)₅GP以高劑量2mg/Kg。第九週開始所有老鼠均恢復為正常處理,不作任何藥物處理,第41週全部斷頭犧牲。(流程見Fig.9)

4. 分析項目

所有老鼠斷頭犧牲後取得血液分離血清部份檢測肝功能診斷酵素;肝臟部份一部份分析GSH含量及GST酵素活性,另一部份取右葉約1cm x 1cm block浸於Cold Acetone加乾冰之中急速冷凍後儲存於-80°C冰箱中(可放置2-3個月酵素活性不變),以備作冷凍切片檢測 γ -GTase foci,第三部份肝臟取得約2cm x 2cm 以福馬林固定,以備作H.E.stain,觀察肝臟組織變化。

(1) 肝毒性分析

血液離心(2,000-3,000rpm;15min.)取得之血清部份檢測ALT,AST,ALP, γ -GT 及Total Bilirubin。

(2) 肝臟GSH含量及GST活性之測定

分析方法如第一章材料與方法中分析項目之(2),(3)部份。

(3) Hematoxylin and Eosin stains (H.E stain)

自老鼠肝臟切取約1cm x 1cm,以15%formaldehyde solution固定組織後,以蠟包埋,其後步驟如下:

- ① 脫蠟後，以蘇紫木 (Hematoxylin) 溶液染 2-15 分鐘，(時間可長可短，視蘇木紫溶液之不同而各異)。
- ② 以水浸洗。
- ③ 以 0.5% 鹽酸溶液行分辨染色色度，約 1-5 秒。
- ④ 浸入氨水 (水 1000ml 中滴入濃氨水 2 滴) 至組織呈現藍色，約需數秒。
- ⑤ 於流水中沖洗，至少需 15 分鐘。
- ⑥ 染以 0.5% 伊紅 Y 溶液，數秒至 2 分鐘。
- ⑦ 依次通過 80%，90%，95% 酒精。
- ⑧ 移入 100% 酒精，二甲苯 - 100% (1:1)，二甲苯 - 木餾油 (4:1)，二甲苯 (I)，二甲苯 (II) 各半分鐘。
- ⑨ 以二甲苯使之透明。
- ⑩ 封蓋。

(4) γ -GTase foci 檢測方法

檢測方法根據 1969 年 Alexander 等⁽⁴⁹⁾之方法。取出預先急速冷凍之肝組織，於冷凍切片機中 (溫度控制 20-25°C) 切取 8 μ m 之 section，每一隻 liver 切約 4-5 sections，mount 於 albumin-coating 之玻片上，待稍乾之後滴上新鮮配置之 γ -GMNA Solution (即 substrate solution，內含受質 γ -GMNA, Tris-buffer, Sline, γ -glutamyl group 之接受者，及 Fast Blue BBN salt 等 (配法見 Fig.10) 於室溫下反應 15 分鐘後，置於 0.85% Saline solution 染缸中浸洗 2 分鐘，再移至 0.1M CuSO₄ 之染缸中 2 分鐘 (使呈色穩定)，再於 0.85% Saline solution 中浸洗 2 分鐘，最後於蒸餾水中浸洗 2 分鐘。待乾後以 Aqueous mount medium 滴上蓋以蓋玻片，於顯微鏡下分別以 40x, 100x, 及 200x 觀察，並以顯微尺標 (0.01mm 為一單位) 量取 γ -GTase foci。所有切片均照

相來計算,每一組老鼠均觀察 100mm^2 以上之切片,記錄 0.1mm - 0.2mm , 0.3 - 0.4mm 平均直徑之 γ -GTase foci數目。每一組切片均另外以不含 γ -GMNA受質之substrate solution依相同方式染切片以作對照,另外部份切片作完以上染色後繼續以hematoxilin作counter stain以觀察細胞核之位置。

細胞中 γ -GTase可催化GMNA轉移 γ -glutamyl group至Glycylglycine而與Cu離子形成穩定之紅色呈色物質。
(Fig.11)

結 果

(一) 肝功能酵素及GSH含量, GST活性測定結果

經由血清中肝功能指標酵素ALT, AST, ALP, Total Bilirubin, 及 γ -GT之檢測發現AFB₁(2mg/Kg)連續注射八週(Group IV)之組並沒有變化產生, 但單獨處理(Ac)₅GP組(Group III)及(Ac)₅GP高劑量與AFB₁同時處理組(Group VI)有差異(Table 7), 但此差異是在停止AFB₁及(Ac)₅GP處理之後32週所測定, 故此差異現象並不能表示是由藥物處理所直接造成的。而肝中GSH含量之測定結果顯示所有組別均與正常組無差異(Table 8)。GST 酵素活性方面, AFB₁及(Ac)₅GP 單獨處理組有上升的差異, 但AFB₁與(Ac)₅GP同時處理組與AFB₁組無顯著差異(Table 9)。

(二) 病理組織 H.E stain 結果

組織切片經由H.E stain結果發現:DMSO注射對照組(Fig.12)及(Ac)₅GP(Fig.13)單獨處理組之肝組織細胞均為正常狀態;而以AFB₁注射八週組則發現有eosinophilic cell foci(Fig.14,15), (其中有一隻老鼠以肉眼可見到nodule之病變), 顯示肝細胞在AFB₁八週處理之下出現pre-neoplastic foci;(Ac)₅GP(不論低劑量或高劑量)與AFB₁同時處理組均發現有膽管擴張增生現象(Fig.16,17), 表示組織發生阻塞而致使膽管位置發生擴張。

(三) γ -GTase foci 檢定結果

以GMNA為受質所染的冷凍切片結果發現,在Normal (Group I) (Fig.19),DMSO(Group II) (Fig.20) 及(AC)₅GP單獨處理組(Group III) (Fig.21)僅有少量 γ -GTase positive之細胞表現,而出現位置如同其它研究結果所示, γ -GTase在正常成年老鼠肝臟中多在portal tract位置^(50,51)。

以AFB₁處理組,則很明顯的於顯微鏡下觀察出強烈的 γ -GTase紅色呈色,且形成大區域之 γ -GTase foci (Fig. 22);出現位置則不限於bile duct portal tract,在肝細胞(hepatocyte)中也強烈出現,顯示此區域之細胞對AFB₁之毒性有所反應而提高 γ -GTase之表現,故形成 γ -GTase foci (Fig.23)。以(AC)₅GP與AFB₁同時處理組(Group V, VI)也可觀察到 γ -GTase foci之表現(Fig.24)。

所有切片利用顯微鏡照相觀察,再以0.01mm為單位之顯微尺標比對之後,計算smaller foci (0.1mm-0.29mm直徑),及larger foci (0.3mm-0.49mm直徑)之數目,每一組切片均至少觀察100mm²,計算每cm²中 γ -GTase positive foci之數目 (Table 10),結果發現Group I, II, III均無 γ -GTase foci出現;Group IV, V, VI三組之Total foci No./cm²相近,但Group IV (AFB₁組)之larger foci No./cm²比率佔82.6%,而Group V (AFB₁+(AC)₅GP 1mg/Kg),Group VI (AFB₁+(AC)₅GP 2mg/Kg)分別為50%及53.8%,顯示出加入(AC)₅GP之後對AFB₁所引起之larger γ -GTase foci有抑制作用。

討 論

血清中肝損傷指標酵素如ALT,AST,ALP及Total Bilirubin, γ -GT活性之測量發現,所有處理組別均與Normal組別差異不大,可能肝臟在經過AFB₁致癌物質的處理8週停止後,再經過32週之正常飼養而有修補之能力;而部份有升高之項目為許多chronic feeding之下可能產生之現象,並不為(AC)₅GP之直接作用。

由組織切片H.E stain 觀察可發現AFB₁ 的注射給予,可能經由代謝活化後產生對肝細胞產生initiation,使肝細胞出現initiated hepatocyte foci⁽⁵⁰⁾(Fig.14,15),如果再繼續受到AFB₁ ultimate product 之刺激則可產生肉眼可辨識的hepatocyte nodules (至少2-3 mm in diameter)之組織變化(本實驗中有一隻AFB₁處理之老鼠肝臟有此發現。在(AC)₅GP與AFB₁同時處理之兩組老鼠均發現有bile duct 擴大增生現象。

由 γ -GTase foci之觀察發現,正常組及DMSO及(AC)₅GP單獨處理組之老鼠肝臟中 γ -GTase之表現位置可能在膽管位置,而且極為少量,此結果符合許多研究所示:正常fetus老鼠之表現在jejunal villi, pancreatic acini, bile ducts, seminal vesicles, proximal convoluted tubules 等之上皮細胞,而在lymphocytes, retina, Choroid plexus中也存在⁽⁵¹⁾;但在成年之後則降為極低,而經過AFB₁處理後之老鼠出現強烈 γ -GTase之表現,而且在某些區域的細胞表現出 γ -GTase foci之出現可作為細胞受到carcinogen toxin而產生反抗的表現。有研究指出

hepatocyte受到 AFB_1 的誘導之後會在Oval cell位置產生增生而且 γ -GTase表現強烈而有hyperplastic nodule的出現⁽³¹⁾。這種在老鼠嬰兒時期肝臟中大量表現的酵素本來在成年期應極少量,但在受到多種致癌物(包括 AFB_1 及其它)的刺激作用之後反而大量表現,而形成 γ -GTase foci或 γ -GTase nodules,由此可以預測此foci之出現與liver carcinoma之前期發展有關⁽⁵⁴⁻⁵⁷⁾。

當以(AC)₅GP與 AFB_1 同時處理之後產生之 γ -GTase foci數目及大小與 AFB_1 組比較(Table 10),發現(AC)₅GP(1mg/Kg, 2mg/Kg)並沒有減少 AFB_1 所產生之 γ -GTase foci總數目,但是larger foci數目則由 AFB_1 組之82.6%降為50%及53.8%,顯示(AC)₅GP可能在 AFB_1 所引起carcinoma之promotion階段(initiated hepatocytes至initiated hepatocyte nodules之階段)(Fig.25,26)。有學者以1,2-dithiol-3-thiones等藥物作用,發現可以降低 AFB_1 所引起之preneoplastic γ -GTase foci之數目及體積,而使 AFB_1 致癌的形成減緩⁽³⁸⁾。因此本篇結果發現長期(AC)₅GP的胃管處理可能使 AFB_1 所誘導產生之larger γ -GTase foci減低為較小之foci,表示(AC)₅GP的處理可使肝細胞受到 AFB_1 損傷的部位減輕,而且 AFB_1 的致癌形成在較為later stage時受到(AC)₅GP抑制。

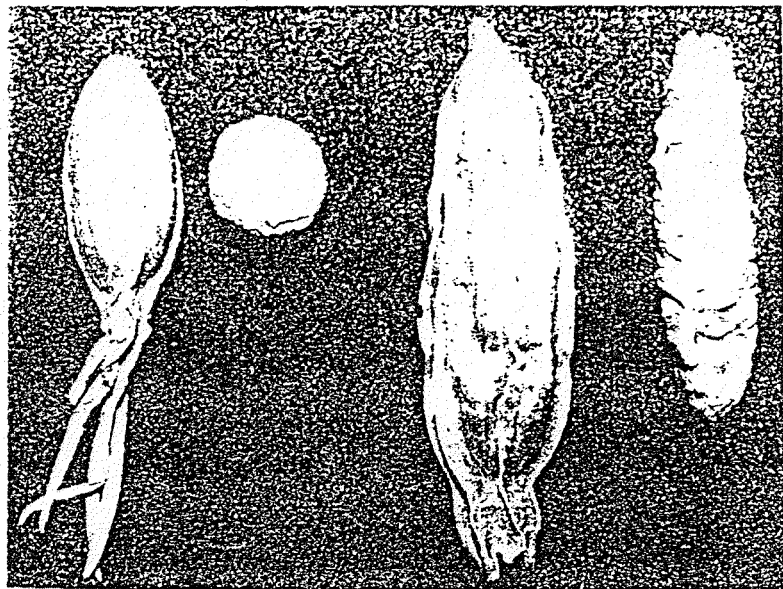
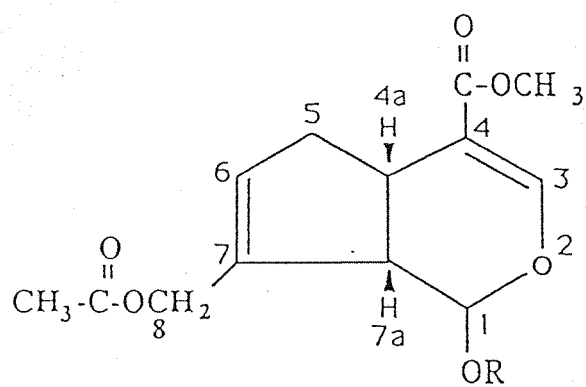


Fig.1 梔子之臺灣市場品×1.5

左起 山梔子・梔子仁(以上為野生品)；

水梔子・梔子仁(以上為栽培品)

山梔子乾燥果實搗碎
 ↓
 以 Ether 浸漬，去脂質
 ↓
 以 Butanol/H₂O 進行 partition
 ↓
 取 Butanol 層進行 acetylation
 ↓
 以 Ethanol recrystallization
 ↓
 所得結晶乾燥即為 (AC)₅GP



R=β-D-2, 3, 4, 6-tetra-acetyl-glucose

Fig.2 自山梔子果實萃取 (AC)₅GP 之流程及 (AC)₅GP 之結構。

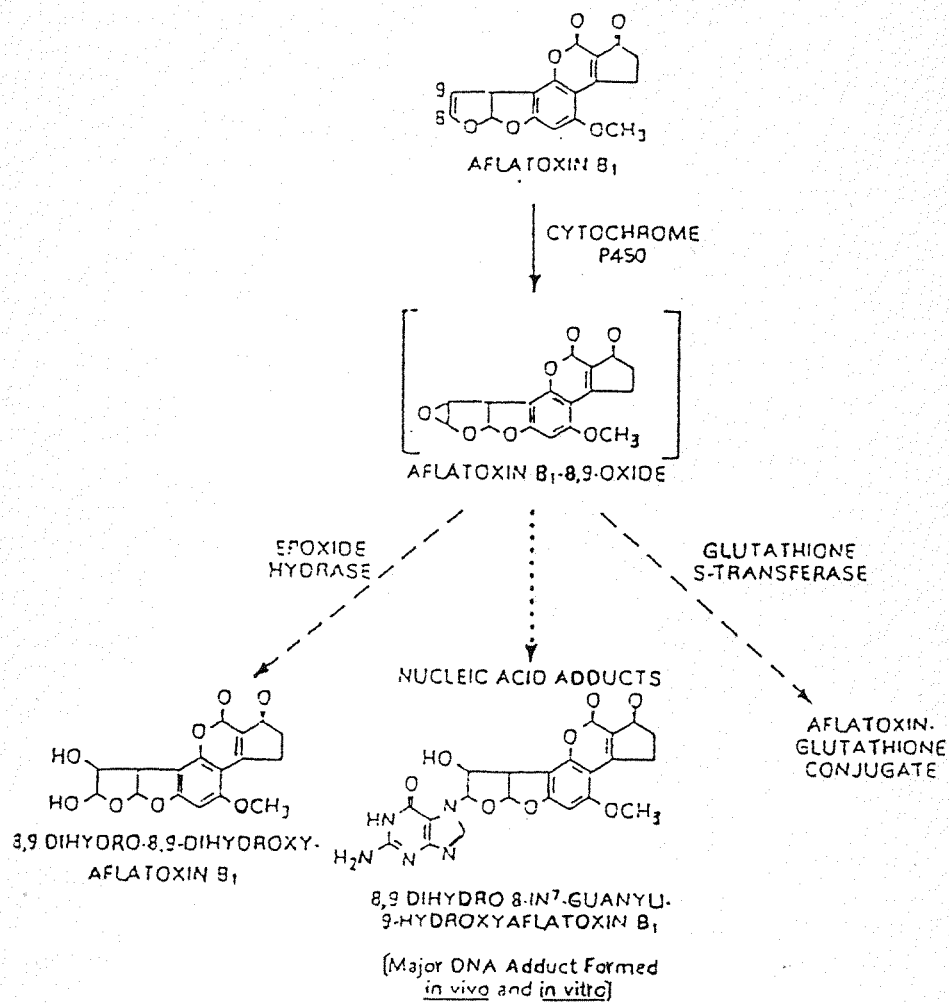


Fig.3 Pathways involved in the metabolic activation of AFB₁.

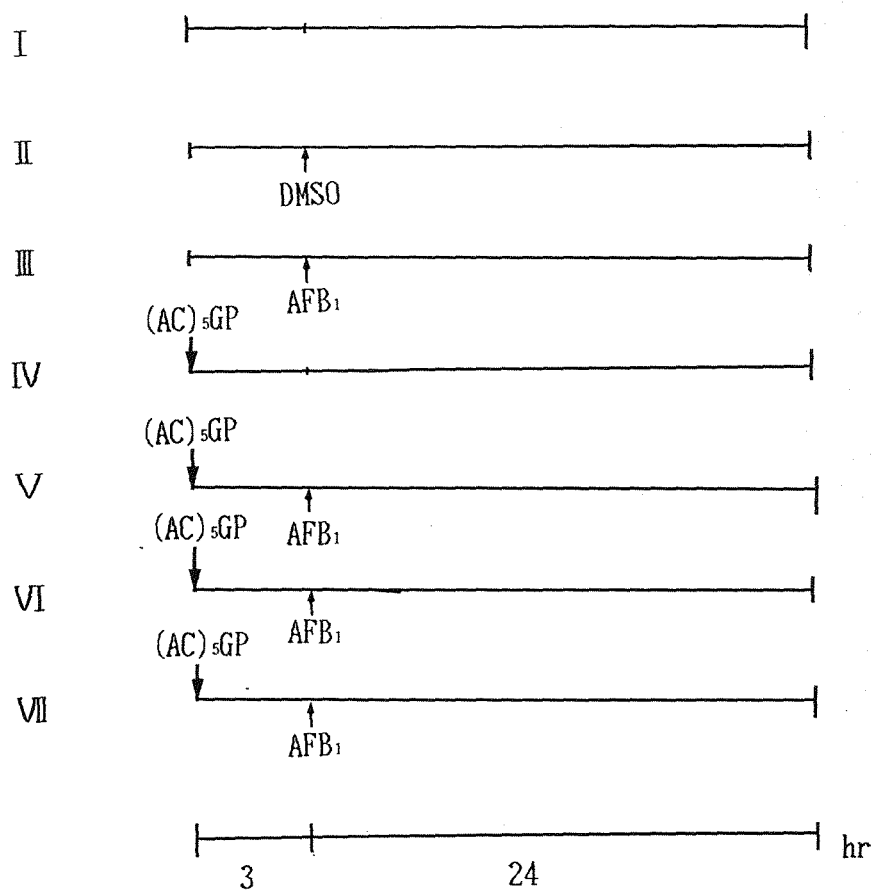


Fig.4 Diagrammatic representation of experimental animal treating. Group I : Normal diet; Group II : DMSO (0.2%) i.p. administration; Group III : AFB₁ (2mg/Kg) i.p. ; Group IV : (AC)₅GP (10mg/Kg) gastric intubation ; Group V : (AC)₅GP (2mg/Kg) g.i. 3hr before AFB₁ (2mg/Kg) i.p. ; Group VI : (AC)₅GP (5mg/Kg) g.i. 3hr before AFB₁ i.p. ; Group VII : (AC)₅GP (10mg/Kg) g.i. 3hr before AFB₁ i.p.. All animals were sacrificed at 24hr after AFB₁ i.p..

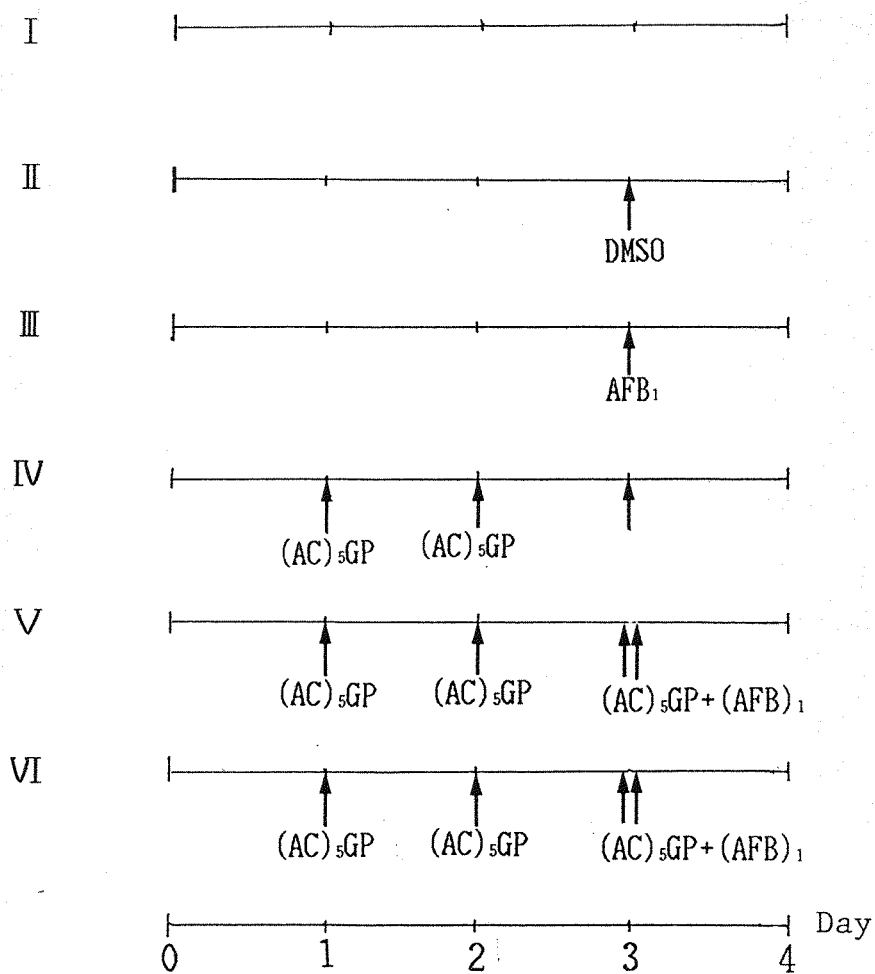


Fig.5 Diagrammatic representation of experimental animal treating. Group I : Normal diet; Group II : DMSO (0.2%) i.p. administration; Group III : AFB₁ (2mg/Kg) i.p.; Group IV : (AC)₅GP (10mg/Kg) gastric intubation at 1st, 2nd and 3rd day; Group V : (AC)₅GP (5mg/Kg) g.i. at 1st, 2nd and 3rd day, then i.p. AFB₁ at 3rd day ; Group VI : (AC)₅GP (10mg/Kg) g.i. at 1st, 2nd and 3rd day , then i.p. AFB₁ at 3rd day. All animals were sacrificed one day after the AFB₁ i.p. administration.

liver Tissue 180mg, adding 0.67ml 25% HPO_3
and 2.5ml phosphate-EDTA

↓

Homogenize, centrifuge at 4°C , 49,000rpm
(100,000xg) for 30min

↓

Take 0.5ml supernatant, adding 4.5ml
phosphate-EDTA for dilution 10times

↓

Take $100\mu\text{l}$ diluted supernatant sol'n,
adding 1.8ml phosphate-EDTA buffer
and $100\mu\text{l}$ OPT solution

↓

Incubate at Room Temp. for 15 min
(must be isolated from light)

↓

Determine fluorescence at 420nm
excitation at 350nm

Fig.6 Protocol for determining the cytosol GSH concentration

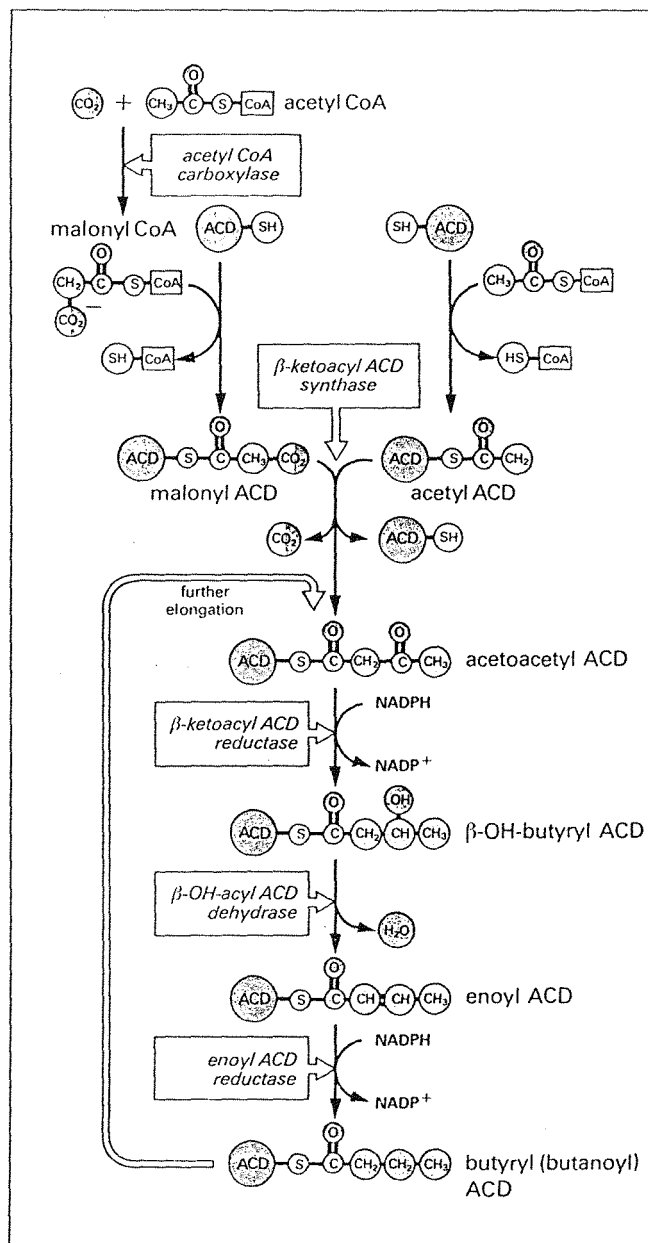


Fig.8 Fatty acid biosynthesis from acetyl-CoA.

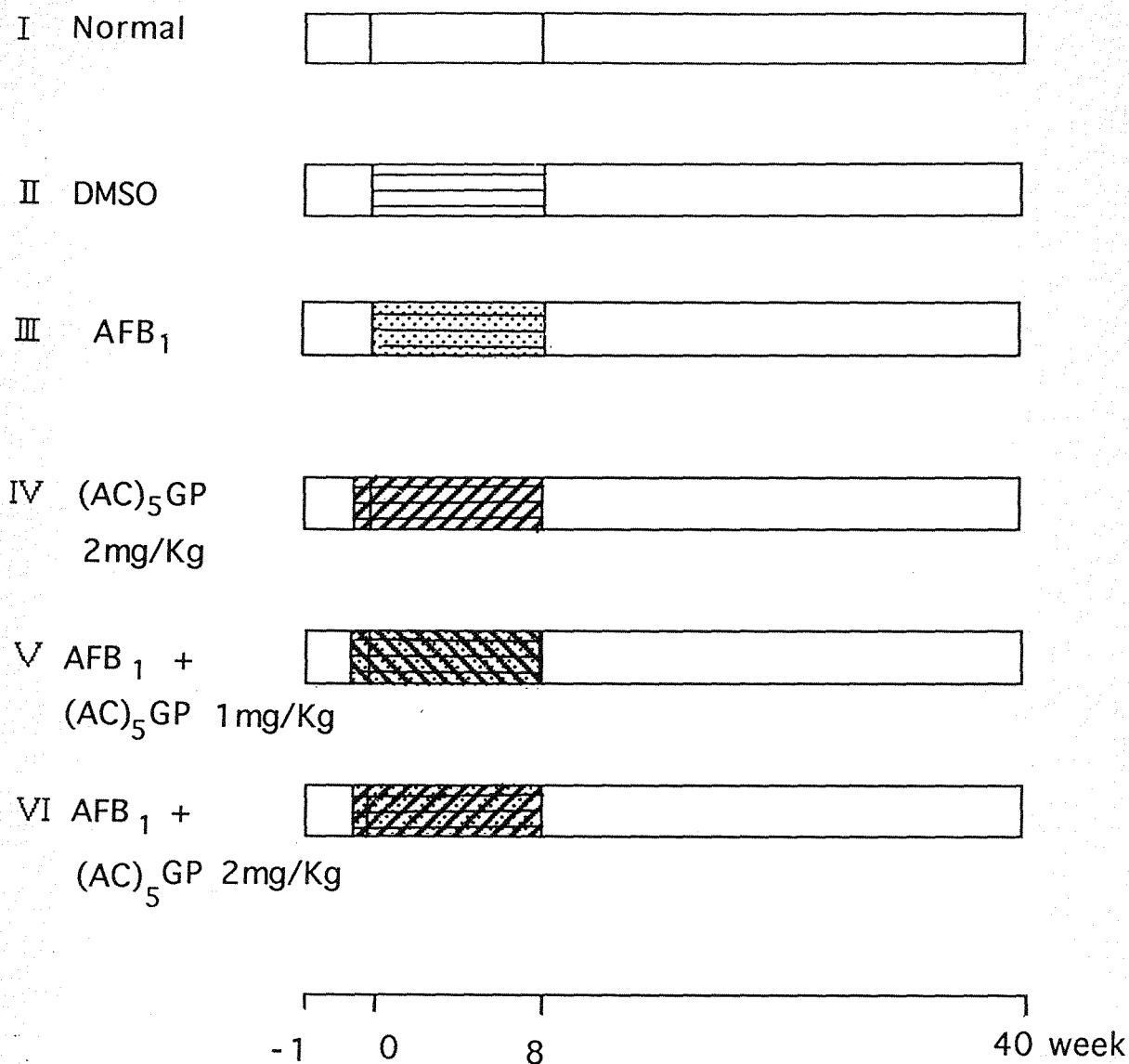


Fig.9 Diagrammatic representation of experimental animal treating. Group I :Normal Group; Group II :DMSO (0.2%) i.p. administration (▨) for 8 wks (5 days/wk); Group III : AFB₁ (2mg/Kg) i.p. (▩) for 8 wks (5 days/wk); Group IV : pretreatment (AC)₅GP (2mg/Kg) (▧) for 3 days, then gastric intubation for successive 8 wks; Group V : pretreatment (AC)₅GP (1mg/Kg) (▧) for 3 days, then g.i. (AC)₅GP and i.p. AFB₁ for successive 8 wks at the same time; Group VI : pretreatment (AC)₅GP (2 mg/Kg) (▧) for 3 days, then g.i. (AC)₅GP and i.p. AFB₁ for successive 8 wks at the same time. All animals were sacrificed 32 wks after the last AFB₁ i.p. administration.

Substrate Sol'n for γ -Glutamyl transpeptidase
(γ -Glutamyl-4-methoxy-2-naphthylamide; γ -GMNA Sol'n)

(1)	γ -GMNA (2.5 mg/ml)	1.0ml
(2)	Tris-buffer (0.M,pH7.4)	5.0ml
(3)	Saline (0.85%)	14.0ml
(4)	Glycylglycine	10.0mg
(5)	Fast Blue BBN salt	10.0mg

Total 20.0ml

p.s. Stock GMNA Sol'n : 25mg GNNNA dissolved in 0.5ml DMSO and 0.5ml 1N NaOH, then adding 9 ml dist.H₂O ,this agent is stable for 3 days at 4°C, but the substrate Sol'n must be freshly prepared.

Fig.10 Substrate solution for γ -Glutamyl transpeptidase

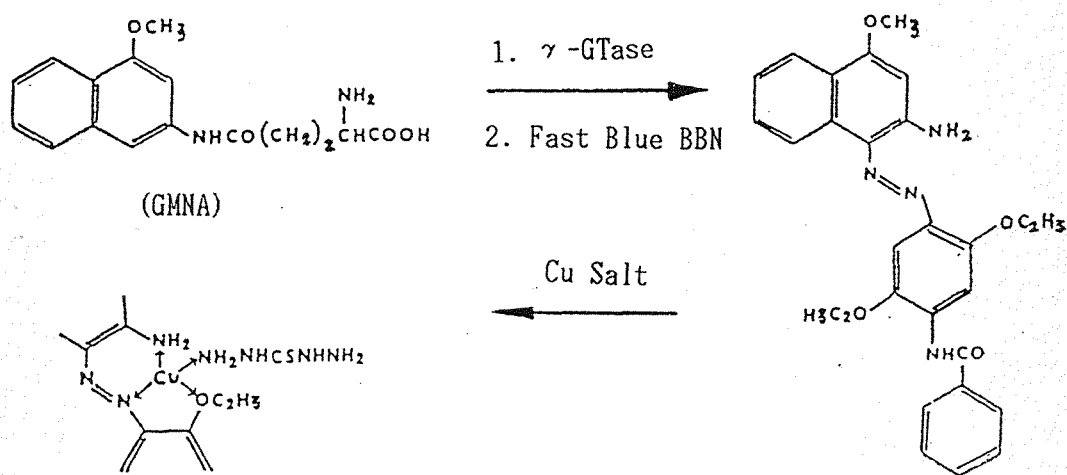


Fig.11 γ -Glutamyl-4-methoxy-2-naphthylamide (GMNA) was transferred the γ -Glutamyl group by γ -Glutamyl transpeptidase (γ -GTase) to glycylglycine, then coupled promptly with the diazonium salt (Fast Blue BBN) to form a red-orange insoluble azo dye which was then chelated with cupric ion to yield an evenmore insoluble intense red dye. (Chelation with Cu ion increased the affinity of the pigment for tissue protein.)

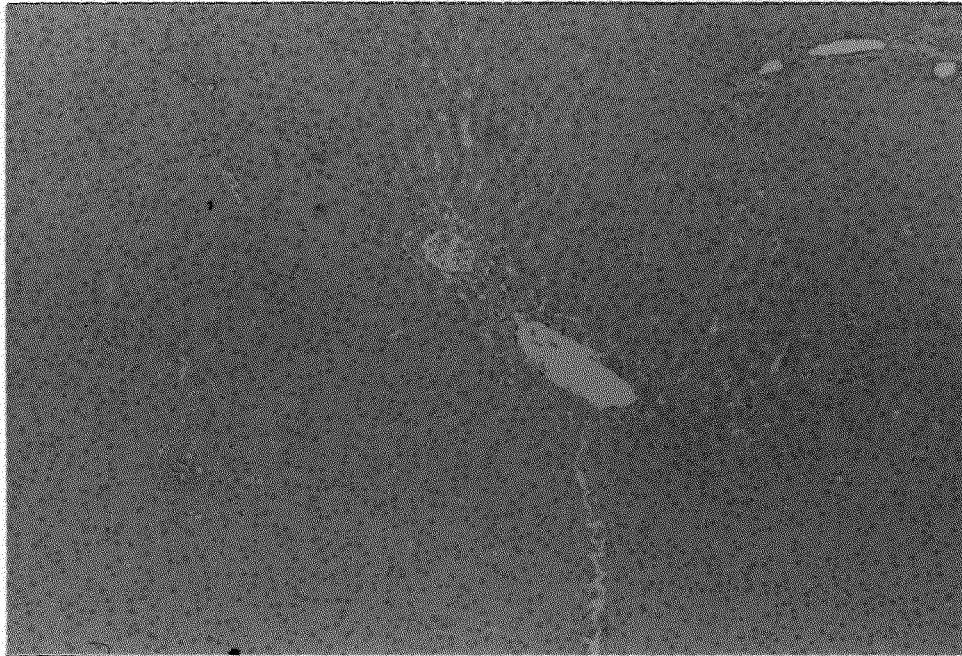


Fig.12 Rat liver examined at 40 weeks. After 8 weeks DMSO i.p. administration (5 days/week), then removed from the i.p. administration for 32 weeks (Group II). H & E. x100.

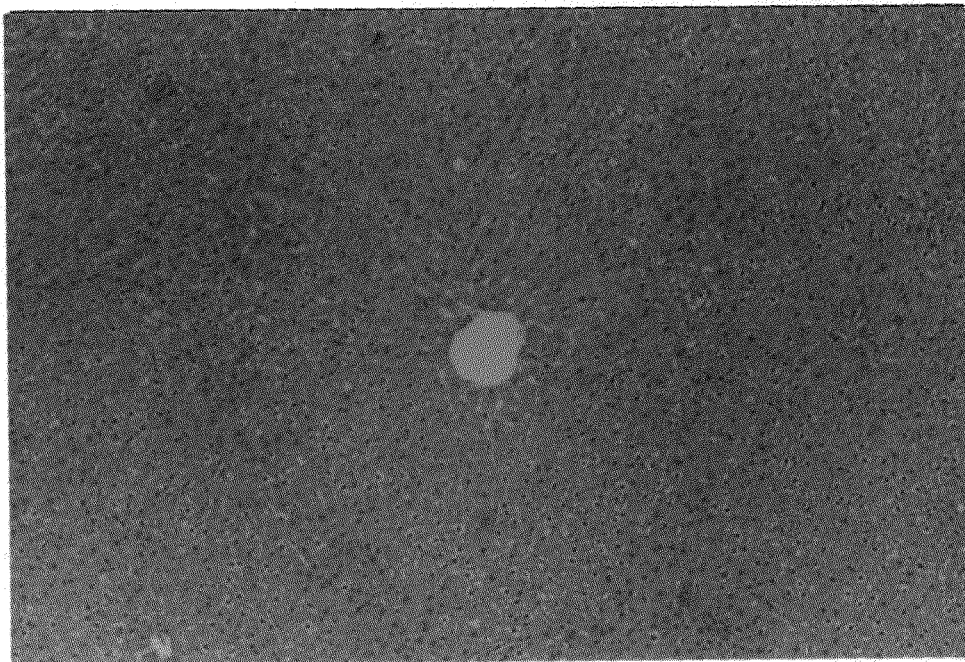


Fig.13 Rat liver examined at 40 weeks. After 8 weeks (AC)5GP (2mg.Kg) gastric intubation (5 days/week), then removed from the gastric intubation for 32 weeks (Group III). H & E. x100.

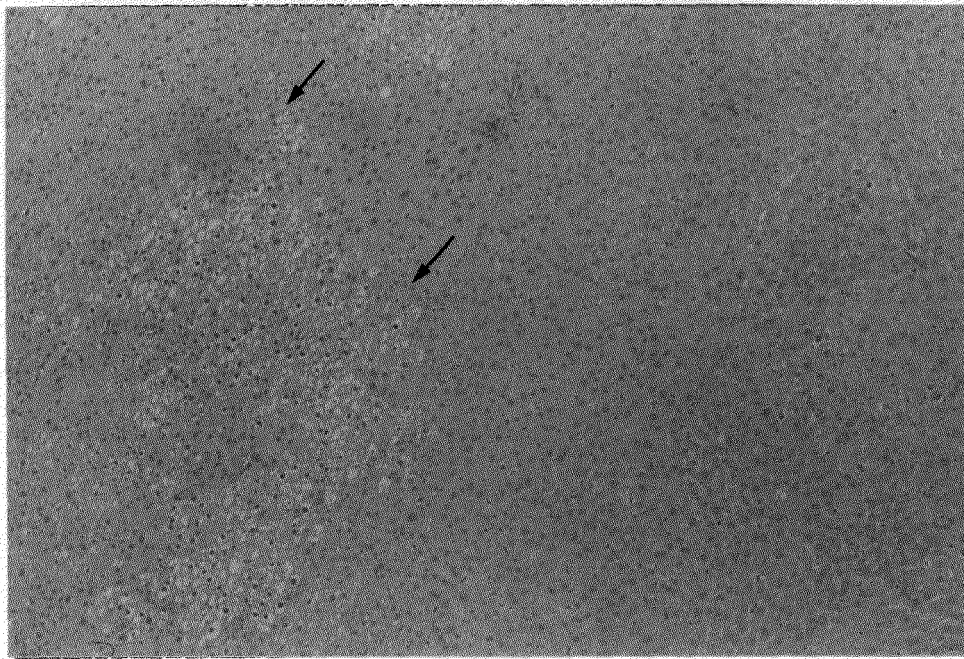


Fig.14 Clear cell foci and eosinophilic cell foci (arrows) in rat livers killed at 40 weeks. Rats were treated with AFB1 i.p. administration for 8 weeks (5 days/week), and then removed from AFB1 i.p. for 32 week (Group IV). H & E. x 100.

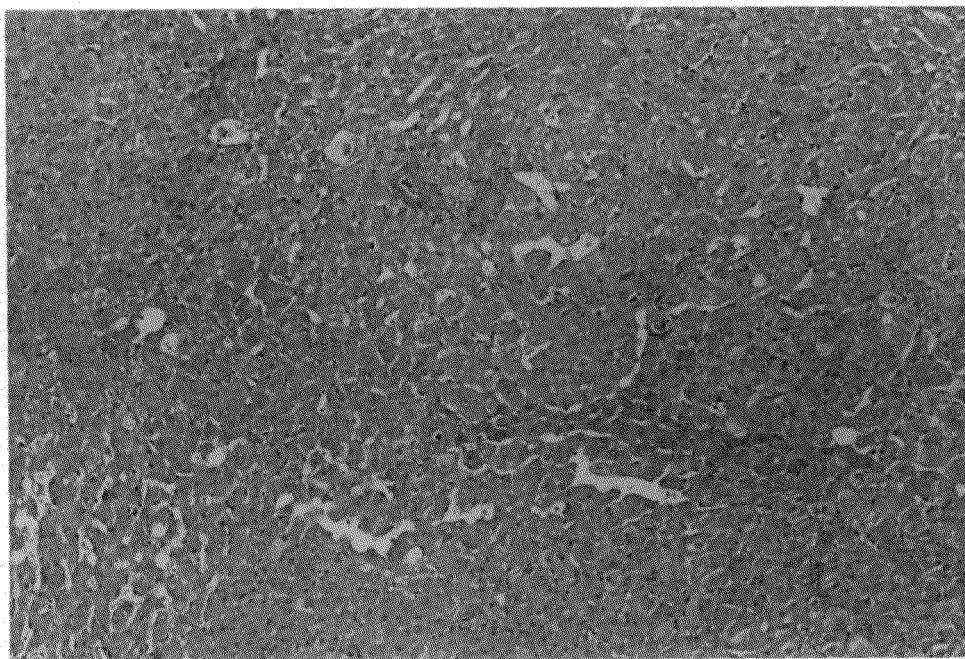


Fig.15 The same section as Fig.14 H & E. x200.

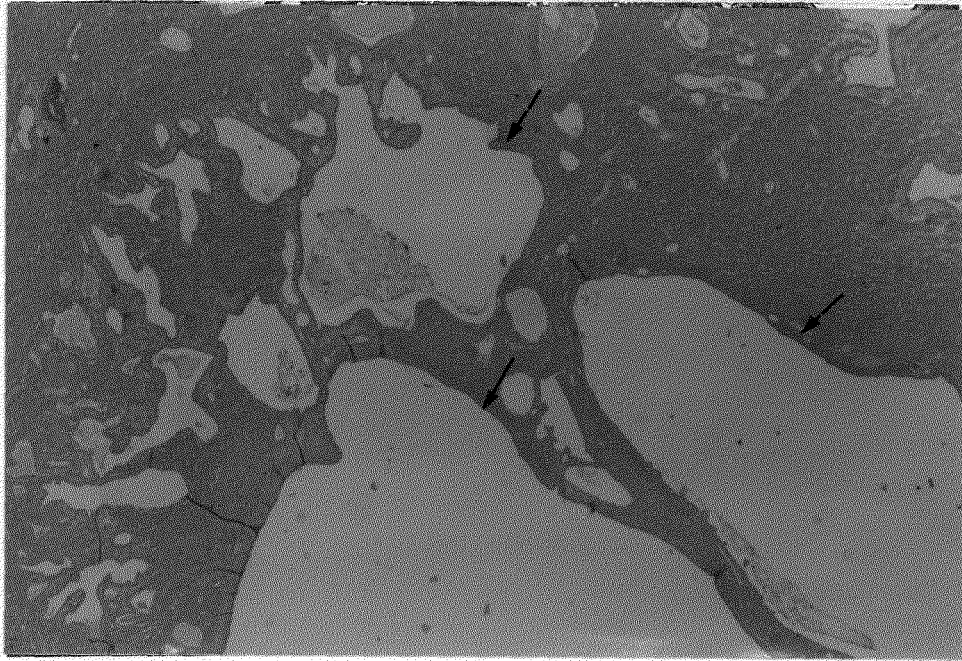


Fig.16 Bile duct dislation in rat livers (arrows) treated with (AC)GP (1 mg/Kg) gastric intubation and AFB_i.p. administration fro 8 weeks(5 days/week) and then removed from thetreating for 32 weeks (Group VI). H & E. x400.

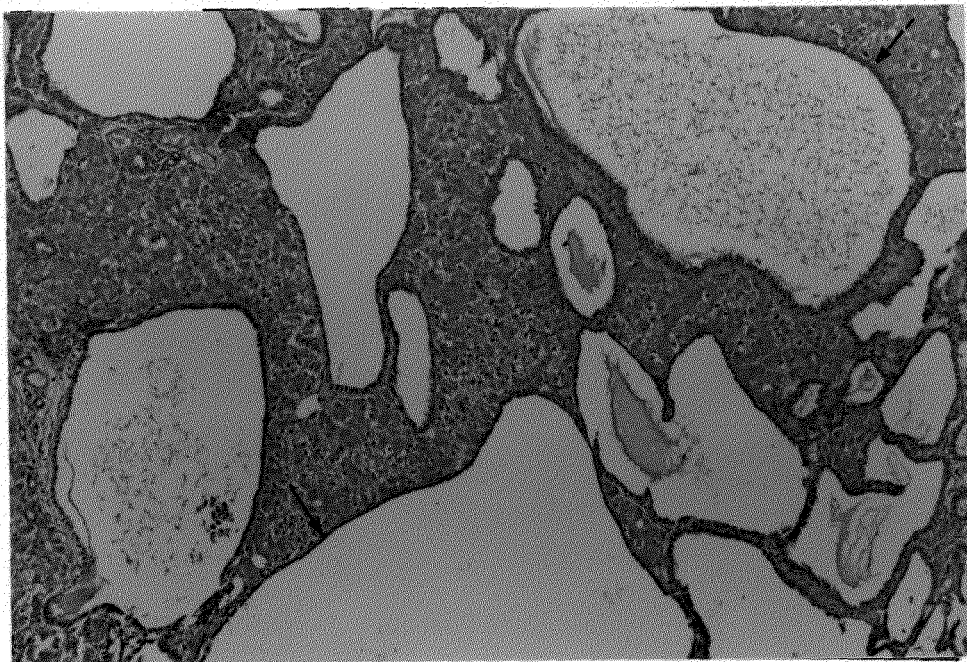


Fig.17 Bile duct dislation in rat livers (arros) wtreated P with (AC)G(2 mg/Kg) gastric intubation and AFB_i. i.p. administration for 8 weeks(5 days/week) and then removed from the treating for 32 weeks (Group VI). H & E. x400.

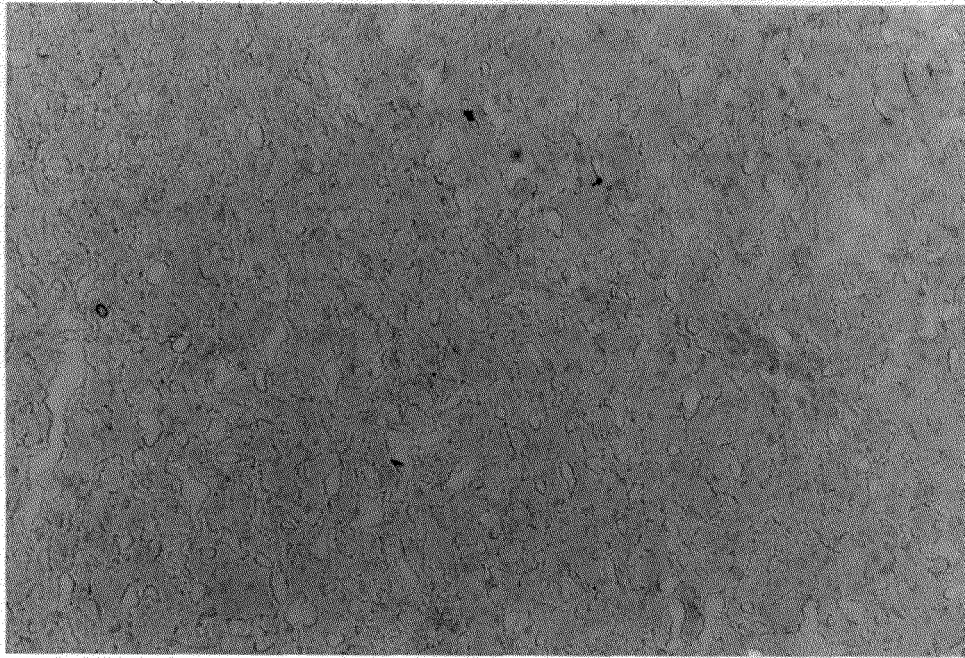


Fig.18 Normal rat liver (Group I). $8\mu\text{m}$ liver section was incubated with substrate sol'n non-containing GMNA. $\times 100$.

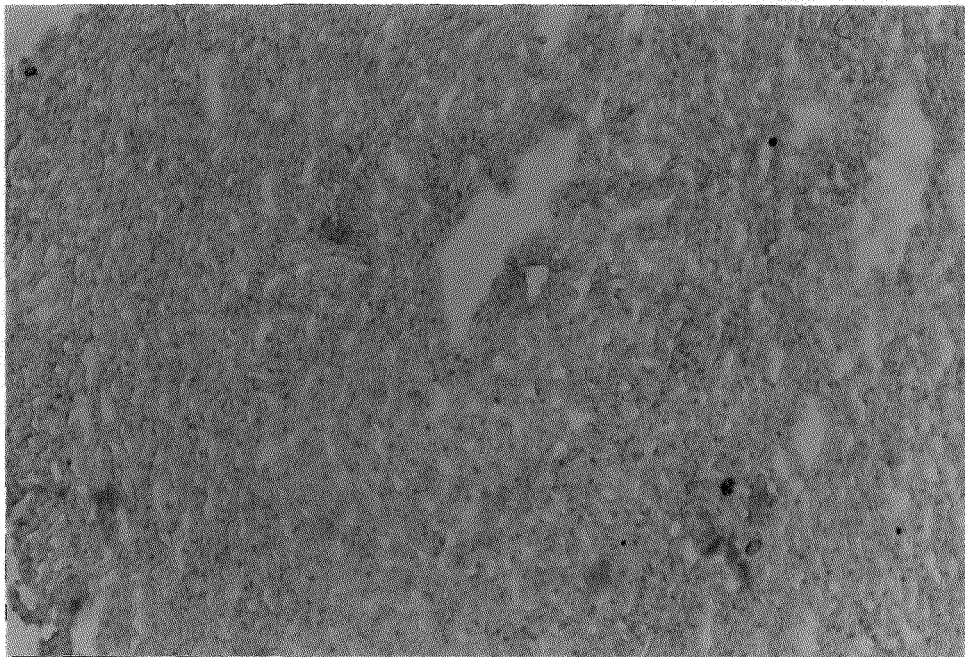


Fig.19 γ -GTase activity in normal rat liver. $8\mu\text{m}$ liver section was incubated with substrate sol'n containing GMNA. $\times 100$.

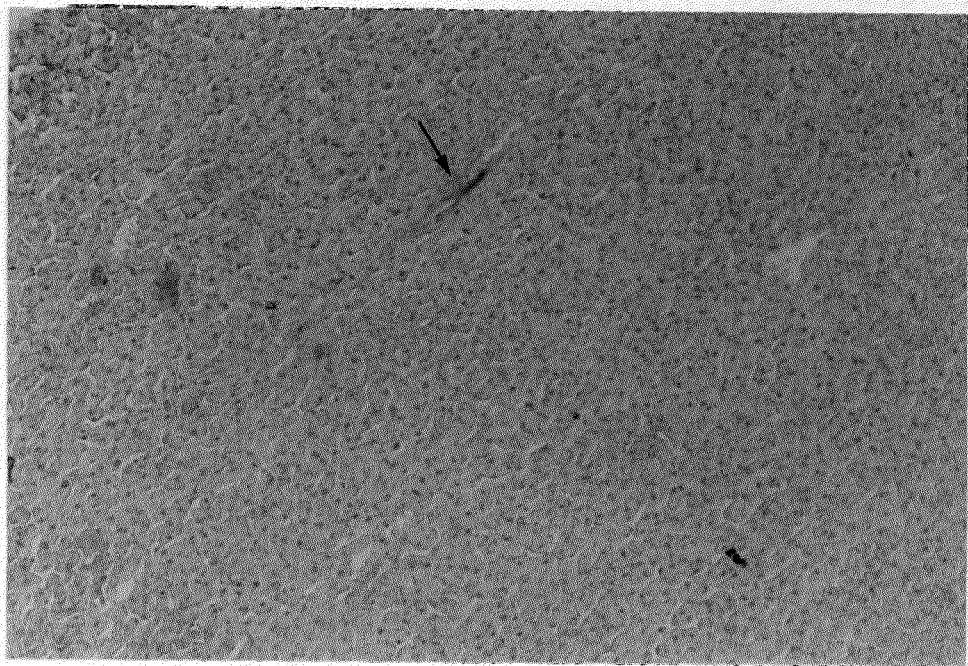


Fig.20 γ -GTase activity in DMSO i.p. administration liver (Group II). Weak γ -GTase activity (arrows) was seen in hepatocytes or bile duct areas. x100.

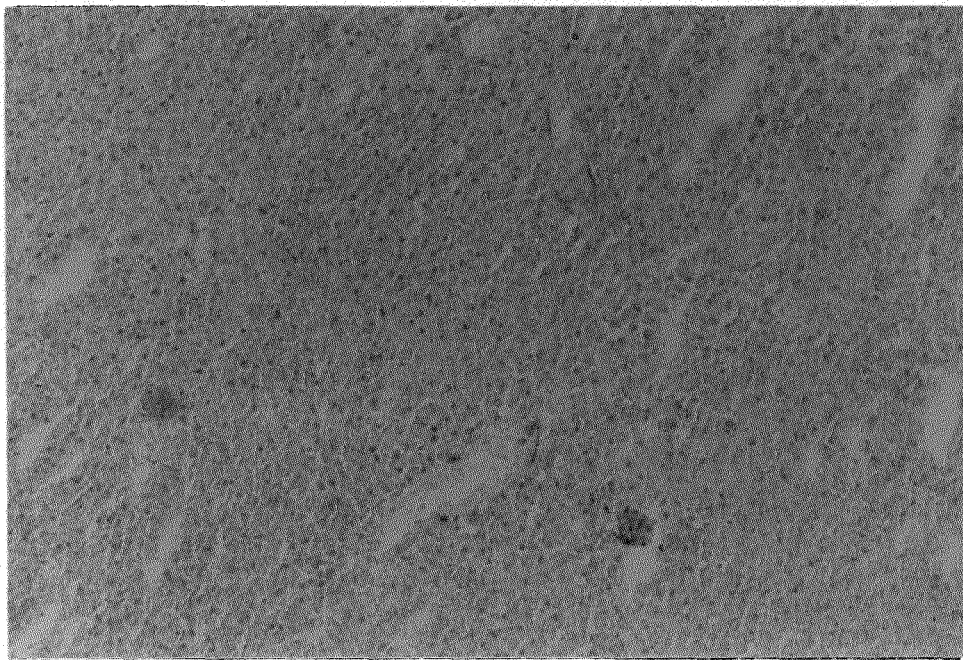


Fig.21 γ -GTase activity in (AC)GP gastric intubation liver (Group III). Weak γ -GTase activity was seen in hepatocytes or bile ducts areas. x100.

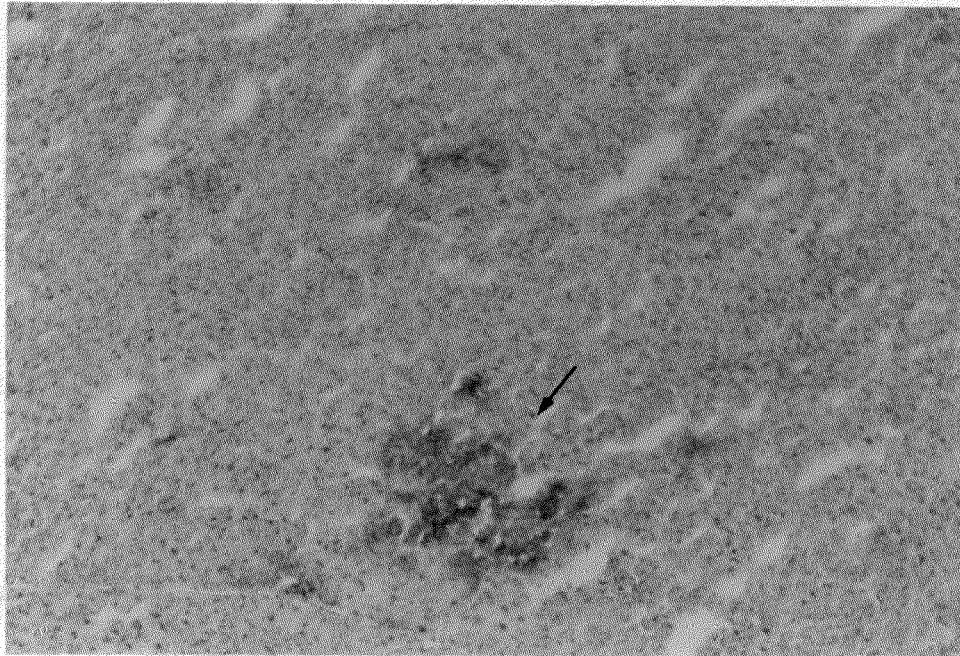


Fig.22 Larger γ -GTase focus in periportal was located (arrow) in AFB₁ i.p. administration (Group IV) hepatocytes. x100.

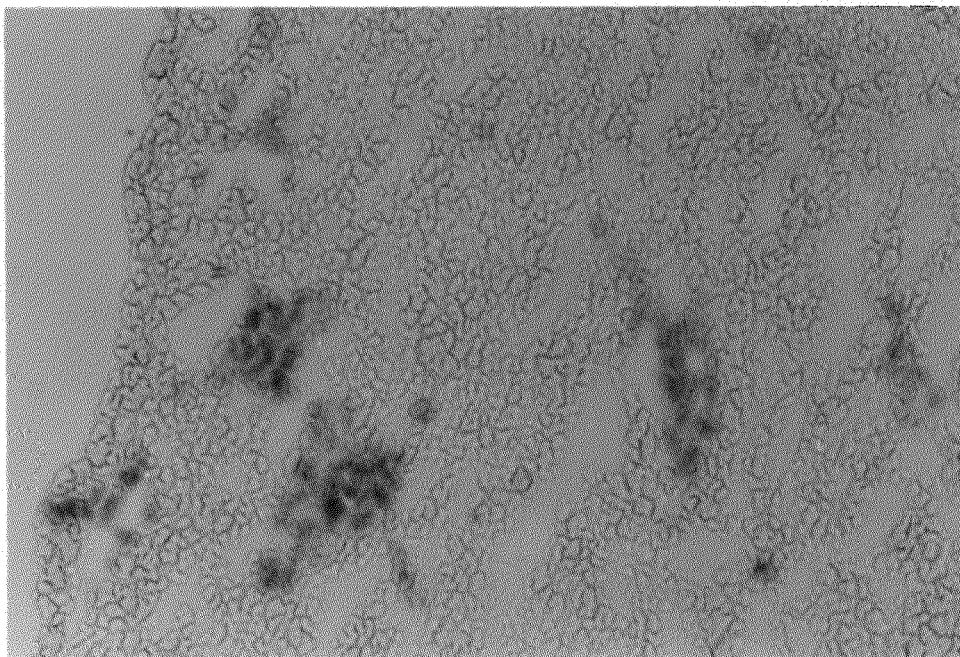


Fig.23 Strong γ -GTase activity was seen in Group IV hepatocytes. x200.

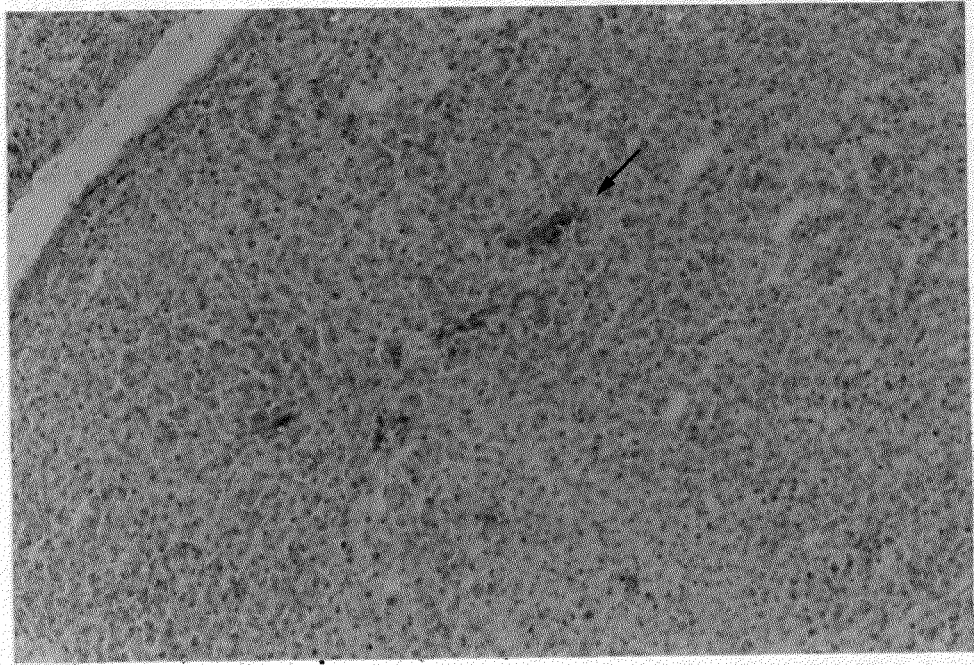


Fig.24 Smaller γ -GTase focus (arrow) in Group V (AFB₁ administration and (AC)GP gastric intubation treated at the same periods). x100.

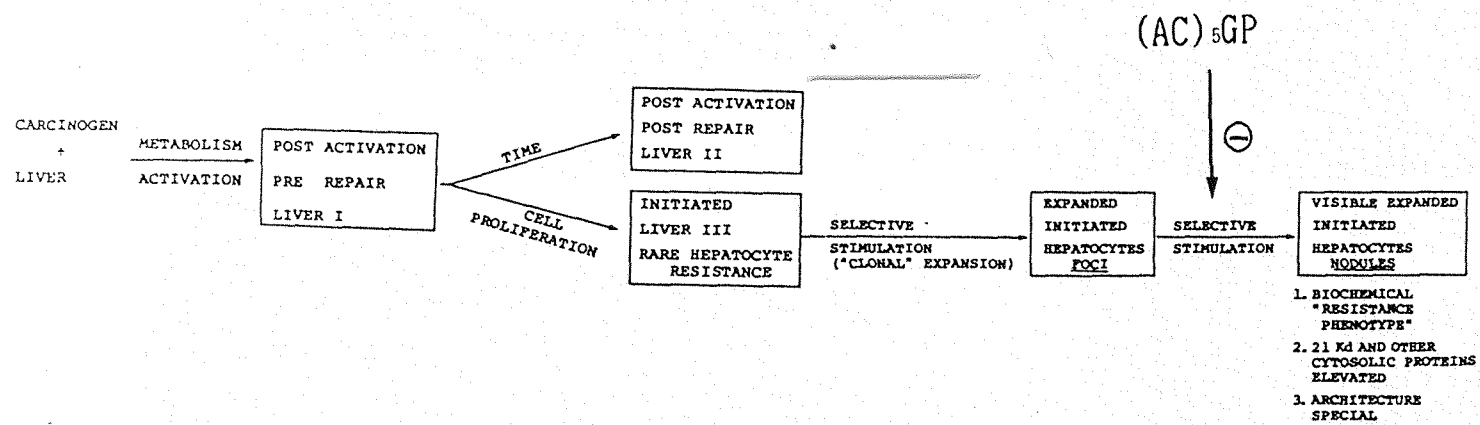


Fig.25 Possible mechanism of (AC)₅GP on inhibitory effect of AFB₁-induced hepatocarcinogenesis.

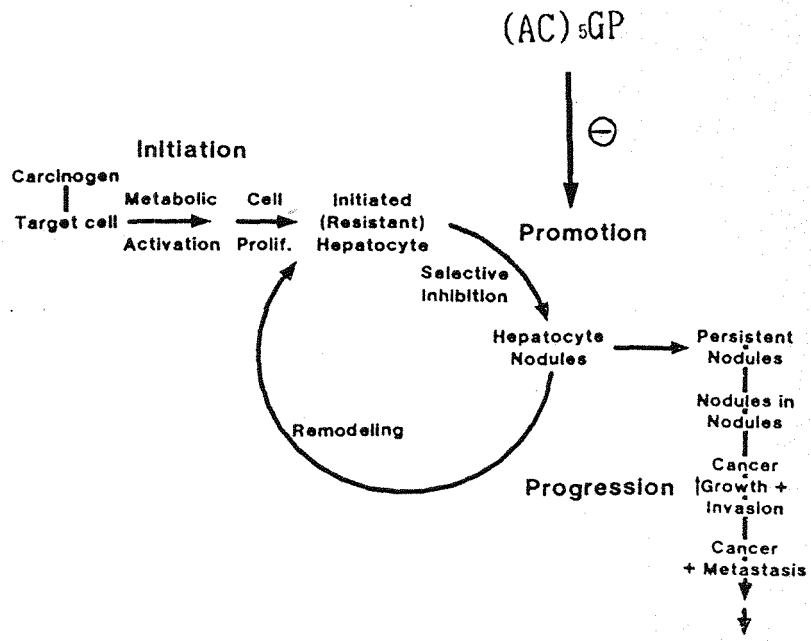


Fig.26 Possible effect of (AC)₅GP on AFB₁-induced hepatocarcinogenesis.

Table 1. Effects of (AC)₅GP on the hepatic function markers in AFB₁-induced hepatic damage.

Group	Treatment	ALT(EU/L)	AST(EU/L)	ALP(EU/L)	γ-GT(EU/L)
I	Normal	40.8 ± 8.9	191.2 ± 32.8	170.0 ± 44.6	1.0 ± 1.0
II	DMSO(0.2%)	37.6 ± 5.4	183.0 ± 48.4	175.6 ± 51.4	0.6 ± 0.5
III	AFB ₁ (2mg/Kg)	249.5 ± 96.2#	623.0 ± 221.9#	203.4 ± 76.9	3.3 ± 0.9#
IV	(AC) ₅ GP (10mg/Kg)	36.0 ± 3.6	172.7 ± 22.0	160.3 ± 27.2	1.0 ± 0.6
V	AFB ₁ + (AC) ₅ GP (2mg/Kg)	288.7 ± 119.8	655.5 ± 135.1	222.2 ± 36.7	3.5 ± 2.3
VI	AFB ₁ + (AC) ₅ GP (5mg/Kg)	203.0 ± 103.6	437.3 ± 198.0	288.3 ± 100.2	2.8 ± 1.2
VII	AFB ₁ + (AC) ₅ GP (10mg/Kg)	3813.0 ± 593.6*	5251.1 ± 954.9*	315.7 ± 34.0*	4.0 ± 1.5

Experimental rats were pretreated with (AC)₅GP by gastric intubation for 3 hr before the administration of AFB₁. All rats were killed 24hr after the i.p. administration of AFB₁ for the determination of serum ALT,AST,ALP and γ-GT activities. Values are means ± S.D., n=6.

p<0.005, compared with the DMSO control group.

* p<0.005, compared with the AFB₁-treated group.

Table 2. Effects of (AC)₅GP on the levels of GSH in AFB₁-induced hepatic damage.

Group	Treatment	GSH (μ g/g liver)
I	Normal	1993.1 \pm 240.8
II	DMSO (2%)	2051.1 \pm 266.7
III	AFB ₁ (2 mg/Kg)	3050.7 \pm 483.5##
IV	(AC) ₅ GP (10mg/Kg)	1739.7 \pm 275.6
V	AFB ₁ + (AC) ₅ GP (2mg/Kg)	2369.5 \pm 515.7*
VI	AFB ₁ + (AC) ₅ GP (5mg/Kg)	2779.4 \pm 655.8*
VII	AFB ₁ + (AC) ₅ GP (10mg/Kg)	2807.6 \pm 572.9

Exiperimental rats were pretreated with (AC)₅GP by gastric intubtion 3hr before the i.p. administration of AFB₁. All rats were killed 24hr after the i.p. administration of AFB₁. The livers were removed and then homogenized for the determination of cytosol GSH levels. Values are means \pm S.D , n=6.

p<0.05, compared with the DMSO control group.

* p<0.05, compared with the AFB₁-treated group.

Table 3. Effects of (AC)₅GP on the activities of GST in AFB₁-induced hepatic damage.

Group	Treatment	GST (nmol/min mg prot.)
I	Normal	118.7 ± 26.7
II	DMSO (2%)	108.4 ± 15.5
III	AFB ₁ (2mg/Kg)	112.6 ± 34.0
IV	(AC) ₅ GP (10mg/Kg)	93.2 ± 15.6
V	AFB ₁ + (AC) ₅ GP (2mg/Kg)	102.0 ± 10.8
VI	AFB ₁ + (AC) ₅ GP (5mg/Kg)	101.1 ± 9.8
VII	AFB ₁ + (AC) ₅ GP (10mg/Kg)	109.9 ± 4.3

Experimental rats were pretreated with (AC)₅GP by gastric intubation for 3 hr before the administration of AFB₁. All rats were killed 24hr after the i.p. administration of AFB₁ and the livers were minced and homogenized for the determination of enzyme activities. Values are means ± S.D., n=6.

Table 4. Effects of (AC)_sGP on the hepatic function markers in AFB₁-induced hepatic damage.

Group	Treatment	ALT (EU/l)	AST (EU/l)	ALP (EU/l)	γ -GT (EU/L)
I	Normal	40.8 \pm 8.9	191.2 \pm 32.8	170.0 \pm 44.6	1.0 \pm 1.0
II	DMSO (2%)	37.6 \pm 5.4	183.0 \pm 48.4	175.6 \pm 51.4	0.6 \pm 0.5
III	AFB ₁ (2mg/Kg)	249.5 \pm 96.2##	623.0 \pm 221.9##	247.0 \pm 72.3#	3.3 \pm 0.9##
IV	(AC) _s GP (10mg/Kg)	42.0 \pm 6.7	159.3 \pm 47.8	142.2 \pm 26.7	1.0 \pm 0.6
V	AFB ₁ + (AC) _s GP (5mg/Kg)	245.0 \pm 118.1	732.0 \pm 266.5	273.7 \pm 82.9	1.4 \pm 1.1**
VI	AFB ₁ + (AC) _s GP (10mg/Kg)	472.0 \pm 99.9**	941.0 \pm 228.6*	266.3 \pm 70.6	3.0 \pm 1.2

Experimental rats were pretreated with (AC)_sGP by gastric intubation for 3 days. All rats were killed 24hr after the i.p. administration of AFB₁ for the determination of serum ALT, AST, ALP and γ -GT activities. Values are means \pm S.D., n=6.

p<0.05, ## p<0.005, compared with the DMSO control group.

* p<0.05, ** p<0.01, compared with the AFB₁-treated group.

Table 5. Effects of (AC)_sGP on the levels of GSH in AFB₁-induced hepatic damage.

Group	Treatment	GSH (μ g/g liver)
I	Normal	1993.1 \pm 240.8
II	DMSO (0.2%)	2051.1 \pm 266.7
III	AFB ₁ (2mg/Kg)	3050.7 \pm 483.5##
IV	(AC) _s GP (10mg/Kg)	1739.7 \pm 275.6#
V	AFB ₁ + (AC) _s GP (5mg/Kg)	2758.1 \pm 492.0*
VI	AFB ₁ + (AC) _s GP (10mg/Kg)	2849.5 \pm 212.9

Experiment rats were pretreated with (AC)_sGP by gastric intubation for 3 days. All rats were killed 24hr after the i.p administration of AFB₁. The livers were removed and then homogenized for the determination of cytosol GSH levels. Values are means \pm S.D, n=6.

p<0.05,## p<0.005, compared with the DMSO control group.

* p < 0.05, compared with AFB₁-treated group.

Table 6. Effects of (AC)_sGP on the activities of GST in AFB₁-induced hepatic damage.

Group	Treatment	GST(n mole/min mg prot.)
I	Normal	118.7 ± 26.7
II	DMSO (2%)	108.4 ± 15.5
III	AFB ₁ (2mg/Kg)	112.6 ± 34.0
IV	(AC) _s GP (10mg/Kg)	93.2 ± 15.6
V	AFB ₁ + (AC) _s GP (5mg/Kg)	105.9 ± 16.3
VI	AFB ₁ + (AC) _s GP (10mg/Kg)	85.0 ± 5.6*

Experimental rats were pretreated with (AC)_sGP by gastric intubation for 3 days. All rats were killed 24hr after the i.p. administration of AFB₁ and the livers were minced and homogenized for the determination of enzyme activities. Values are means ± S.D., n=6.

* p < 0.01, compared with the AFB₁ -treated group.

Table 7. Effects of (AC)₅GP on hepatic function markers in AFB₁-incuded hepatic damage.

Group	Treatment	ALT(EU/L)	AST(EU/L)	ALP(EU/L)	T.Bili.(mg/dl)	γ-GT(EU/L)
I	Normal	62.7± 8.8	241.0± 34.9	119.6±21.7	0.33±0.05	0
II	DMSO(0.2%)	56.0±11.8	246.7± 46.0	127.0±19.1	0.38±0.09	0
III	AFB ₁ (2mg/Kg)	56.7± 7.0	232.3± 45.7	112.9±19.1	0.33±0.05	0
IV	(AC) ₅ GP 2mg/Kg	63.0±10.8	358.3± 42.3#	176.3±30.9#	0.4 ±0	0
V	AFB ₁ + (AC) ₅ GP 1mg/Kg	54.0±15.6	385.5±120.9	134.7±45.6	0.33±0.06	0
VI	AFB ₁ + (AC) ₅ GP 2mg/Kg	69.6±12.9*	229.4± 31.3	165.2±23.6**	0.32±0.04	0

Experimental rats were pretreated with (AC)₅GP by gastric intubation for 3 days, then successive gastric intubation of (AC)₅GP and i.p. administration of AFB₁ at the wame periods for 8 weeks (5 days/week). Animals were sacrificed for hepatic function markers analysis 32 weeks after removal of rats from (AC)₅GP and AFB₁ treatment.

p<0.005, compared with the DMSO control group.

* p<0.05, **p<0.005, compared with the AFB₁-treated group.

Table 8. Effects of (AC)₅GP on the levels of GSH in AFB₁-induced hepatic damage.

Group	Treatment	GSH μ g/g liver
I	Normal	2012.4 \pm 342.9
II	DMSO (2%)	2349.0 \pm 360.1
III	AFB ₁ (2 mg/Kg)	2349.8 \pm 234.6
IV	(AC) ₅ GP (1mg/Kg)	2311.1 \pm 249.5
V	AFB ₁ + (AC) ₅ GP (1mg/Kg)	2142.8 \pm 481.6
VI	AFB ₁ + (AC) ₅ GP (2mg/Kg)	2293.5 \pm 378.2

Experimental rats were pretreated with (AC)₅GP by gastric intubation for 3 days, then successive gastric intubation of (AC)₅GP and i.p. administration of AFB₁ at the same periods for 8 weeks (5 days/week). Animals were sacrificed for cytosol GSH levels analysis 32 weeks after removal of rats from (AC)₅GP and AFB₁ treatment.

Table 9. Effects of (AC)₅GP on the activities of GST in AFB₁-induced hepatic damage.

Group	Treatment	GST (n mol/min/mg prot.)
I	Normal	173.3 ± 25.7
II	DMSO (2%)	217.0 ± 49.0
III	AFB ₁ (2mg/Kg)	234.3 ± 24.1#
IV	(AC) ₅ GP (2mg/Kg)	253.9 ± 26.0#
V	AFB ₁ + (AC) ₅ GP (1mg/Kg)	211.9 ± 16.4
VI	AFB ₁ + (AC) ₅ GP (2mg/Kg)	233.7 ± 10.6

Experimental rats were pretreated with (AC)₅GP by gastric intubation for 3 days, then successive gastric intubation of (AC)₅GP and i.p. administration of AFB₁ at the same periods for 8 weeks (5 days/week). Animals were sacrificed for cytosol GST activity analysis 32 weeks after removal of rats from (AC)₅GP and AFB₁ treatment.

p<0.005, compared with the DMSO control group.

Table 10. Effects of (AC)_sGP on AFB₁-induced γ -glutamyl transpeptidase positive foci in rat livers.

Group	Treatment	Foci No./ cm ² section		Total Foci No. /cm ² section
		smaller foci No.*	larger foci No.**	
I	Normal	0	0	0
II	DMSO (0.2%)	0	0	0
III	AFB ₁ (2mg/Kg)	1.9 (17.4%)	9.02 (82.6%)	10.92
IV	(AC) _s GP (2mg/Kg)	0	0	0
V	AFB ₁ + (AC) _s GP 1mg/Kg	6.5 (50%)	6.5 (50%)	13.00
VI	AFB ₁ + (AC) _s GP 2mg/Kg	5.19 (46.2%)	6.05 (53.8%)	11.24

* smaller foci : 0.10mm - 0.29mm in average diameter.

** larger foci : 0.30mm - 0.49mm in average diameter.

Rats were treated as described in Fig.9.

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