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Effects of a herbal compound containing bupleurum on human lymphocytes

含柴胡的草藥對人類淋巴細胞的影響

Bupleurum-containing compounds, such as KY88 Liver Livo are thought to have immunomodulatory effects. This study investigated the effects of KY88 Liver Livo on the mitogenic induction of lymphocytes in vitro. Fifteen healthy human adult volunteers, aged between 20 and 50 years, provided peripheral blood samples from which lymphocytes were obtained by Ficoll-Hypaque centrifugation. The separated lymphocytes were stimulated by phytohaemagglutinin and KY88 Liver Livo in varying concentrations for 72 hours, with greater cluster and colony formation evident compared with lymphocytes in a control preparation. KY88 Liver Livo was also found to induce the secretion of granulocyte-macrophage colony-stimulating factor in a dose-dependent fashion. These preliminary in vitro studies suggest that KY88 Liver Livo may have potential clinical value in the treatment of chronic viral infection and in the management of immunocompromised patients.

含柴胡成分的合成物，如KY88 Liver Livo被認為有免疫調節的作用。本研究調查了KY88 Liver Livo對試管中淋巴細胞促進有絲分裂的影響。15名年齡介乎20至50歲的健康志願人士提供了血樣本，從這些血樣本中利用Ficoll-Hypaque離心法取得淋巴細胞。利用不同濃度的植物血細胞凝集素和KY88 Liver Livo刺激分隔了的淋巴細胞72小時，結果發現形成的團束和群體明顯比對照中的淋巴細胞大。此外，還發現在不同劑量的情況下，KY88 Liver Livo可以促進粒細胞-巨噬細胞群刺激素的分泌。這些初步的試管研究顯示，KY88 Liver Livo對治療慢性病毒感染和缺乏免疫力的患者可能有潛在的臨床價值。

Key words:

Drugs, Chinese herbal;
 Granulocyte-macrophage colony-stimulating factor;
 Herbs;
 Lymphocytes

關鍵詞：

中草藥；
 粒細胞-巨噬細胞群-刺激素；
 藥草；
 淋巴細胞

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Introduction

Traditional herbal medicine has been used in China since the first century BC. Thereafter, traditional herbal products have been improved on an individual basis. Although still mainly used by Chinese, traditional herbal medicine is gaining acceptance among patients outside China.^{1,2} The search for effective drugs has also prompted researchers to investigate the efficacy of natural products as medicines. The medicinal properties of the *Camptotheca acuminata* and the bark of the pacific yew tree (*Taxus*), have been known to the Chinese for a lengthy period. However, it was only in the 1960s that these substances were discovered by the US National Cancer Institute to have anticancer properties. Despite such findings, herbal medicine is not mainstream therapy and is often categorised as a form of complementary or alternative medicine.³

Currently, most herbal medicine is made into tablets and ingested as a form of drug therapy. Almost all herbal medicine is heterogeneous in nature, comprised of a complex mixture of extracts in a given ratio. The final products, therefore, cannot be characterised easily, or their active substances well defined. The quality and safety of the products is dependent on the processing/ collection of source materials and the manufacturing process.

Traditional herbal medicine uses a compound formulary approach—rather than a simple arithmetic sum of the effect of each individual ingredient—which is considered essential to the medicine's action. This is described in 'Shen Nong Ben Cao Jin', one of the earliest Chinese pharmacopoeia known today, which is thought to have been compiled during the Eastern Han Dynasty, based on the written records of herbalists in earlier centuries. Within the therapeutic herbal compound, some herbs treat the disease, some reduce the side-effects, and the remaining act to 'fuse' and deliver the medicine to targets within the body. This approach makes use of the synergistic effects of herbs to act at different molecular channels and hence achieve the intended function. Clinical experience in China indicates that such an approach is effective.

Chinese herbal medicine is used regularly by practitioners for 'modulating immunity'. Experiments on *Imperata cylindrica* (Beauv) and other purified polysaccharides have demonstrated that these substances are excellent immunomodulating agents.

Polysaccharides or saponins have been shown to stimulate macrophages, promote antibody formation, activate complement, and increase T-lymphocyte proliferation.⁴ Other herbs, such as *Tripterygium wilfordii*, *Aconitum*, and *Artemisiae* spp have apparent immunosuppressive effects. Some of these herbal medicines are now used clinically for the treatment of rheumatoid arthritis, chronic nephritis, systemic lupus erythematosus, and various skin disorders.

Bupleurum-containing compounds are known to possess anti-inflammatory effects. KY88 Liver Livo (Hon Ding International Ltd, Hong Kong, China) is one such compound, used by Chinese medicine practitioners in the treatment of acute and chronic hepatitis B virus (HBV) infection. It is believed to effect elimination of HBV, strengthening the immune system, and stimulating liver cell regeneration. This study aims to evaluate its action on the mitogenic stimulation of lymphocyte proliferation.

Materials and methods

KY88 Liver Livo preparation

The ingredients of KY88 Liver Livo are: *Schizandrae fructus*, *Bupleuri radix*, *Artemisiae capillaris*, *Desmodii herba*, *Poria sclerotium*, *Lithospermi radix*, *Paeoniae radix*, *Phellodendri cortex*, *Scutellariae radix*, and *Trichosanthis radix*.

KY88 Liver Livo (50 g) was extracted with methanol (500 mL x 3). The solid residue of the crude extract was then dissolved in dimethyl sulphoxide to a concentration of 92 mg/mL and stored at 4°C until use. For use, the KY88 Liver Livo was diluted (10 mg/mL) in Hank's Balanced Salt Solution (HBSS) [Invitrogen, Carlsbad, California, US] and filtered using 0.22 Micro Cellulose Acetate (Corning, New York, US) for sterilisation.

Subject selection

Fifteen healthy adults, aged from 20 to 50 years were recruited for the study. Written consent was obtained from each participant and 30 mL of peripheral venous blood subsequently collected from each subject and placed in sterilised tubes containing heparin.

Lymphocyte isolation and culture

The blood samples were centrifuged at 1200 revolutions per minute (rpm) at room temperature for 10 minutes. Cells were collected and mixed thoroughly with ice cold HBSS containing 10 mM Hepes (Sigma, St Louis, US). The samples were carefully layered over Ficoll Hypaque (Pharmacia, Uppsala, Sweden) and centrifuged at 2000 rpm for 20 minutes at 4°C. Peripheral blood mononuclear cells were isolated and the interface was harvested and washed three times with cold Roswell Park Memorial Institute (RPMI) 1640 medium (serum free).

The isolated cells were cultured in RPMI 1640 medium mixed with 10% deactivated foetal bovine serum, 10 mM Hepes, and 1% antibiotics (100 µg/mL penicillin and 100 µg/mL streptomycin) in an incubator at 37°C with 5% carbon dioxide.

After overnight incubation, the separated lymphocytes were stimulated with 10 µg/mL phytohaemagglutinin (PHA) (Roche, Sandhofer, Strasse, Germany), and 0.1 µg/mL, 1 µg/mL, or 10 µg/mL concentrations of KY88 Liver Livo for 72 hours. The optimal concentrations of KY88 Liver Livo were calculated according to the recommended dosage from the supplier. Roswell Park Memorial Institute 1640 medium only was added to one culture as a control.

Proliferation assay for KY88 Liver Livo

Proliferation of lymphocytes was assessed with the cell proliferation reagent WST1 assay (Boehringer Mannheim, Mannheim, Germany). The lymphocytes were cultured at a density of 4000 cells per well into 96-well microtitre plates (Costar, Cambridge, Massachusetts, US) and incubated at 37°C for 24 hours. This cell number was chosen to maintain the cells in an exponential growth phase throughout the experiment, and to allow measurement of optical density at the linear portion of the curves. KY88 Liver Livo was serially diluted with culture medium and different concentrations were added to the plates after 24 hours at a volume of 200 µL per well. The cells were incubated and harvested after 72 hours of culture. At the time of harvest, 10 µL of WST1 was mixed with 100 µL of medium. The mixture was added to each well and the incubation was continued for another 2.5 hours at 37°C. The optical density was read at 540 nm on a spectrophotometer (Bio-Tek Universal Microplate Reader, ELX 800, Winooski, Vermont, US).

Cyto-preparation

After 72-hours' incubation, the cell suspensions were adjusted to 5×10^5 cells/mL in 1 mM phosphate-buffered saline (PBS). Aliquots of 50 µL of the cell suspension were loaded into cuvettes and centrifuged at 2000 rpm for 10 minutes onto a DAKO silanized slide (DAKO, Glostrup, Denmark) in a Cytospin cytocentrifuge (Wescor, Logan, Utah, US). The slides were fixed for 10 minutes in acetone and washed three times with 1 mM PBS. The peripheral blood mononuclear cells were stained with hematoxylin for 3 minutes and eosin for 5 minutes. The finished slides were observed under interference contrast microscope using the Coolsnap Capture system (Roper Scientific Inc., Arizona, US) with 200x magnification. The colonies were measured by an Image Analyser (Metamorph-Winshell, Universal Imaging Corporation, Pennsylvania, US).

Scoring of lymphocyte cluster and colonies

The scoring of colonies utilised was adapted from the system described by Lange.⁵ In brief, aggregates of 3-20 cells were regarded as clusters and aggregates of over 50 cells considered colonies. The area of each colony was marked out and measured using the accompanying software of the Image Analyser. The mean total area in mm² was used for statistical calculation.

Determinations of granulocyte-macrophage colony-stimulating factor level

Granulocyte-macrophage colony-stimulating factor (GM-CSF) was measured in the supernatant of the

culture system. The lymphocytes were cultured according to the above method. After incubation, the supernatants were harvested and stored at -70°C until analysis. Granulocyte-macrophage colony-stimulating factor concentrations were determined using an enzyme-linked immunosorbent assay (ELISA) commercial kit (Intergen, New York, US). The procedure was performed according to the manufacturer's instructions.

Statistical analysis

All values were expressed as a mean and standard error of mean unless otherwise stated. Parameters were compared using the Statistical Package for Social Sciences (Windows version 6.0; SPSS Inc., Chicago, US). The parameters were evaluated using the paired samples *t*-test. A P value of 0.05 or less was taken as statistically significant.

Results

Lymphocytes were seen to proliferate in response to the stimulation from PHA and KY88 Liver Livo. The proliferation rate as measured by WST1 was greatest when the lymphocytes were stimulated with KY88 Liver Livo at a concentration of 0.1 µg/mL. The rate of proliferation decreased progressively with increasing KY88 Liver Livo concentration (Fig 1). The lymphocyte proliferation rates for KY88 Liver Livo concentrations of 0.1 µg/mL and 1 µg/mL were significantly different from that of the control group ($P < 0.001$ and $P = 0.048$, respectively).

Lymphocytes tended to form clusters and colonies after stimulation. The number of clusters and colonies was determined according to Lange's method.⁵

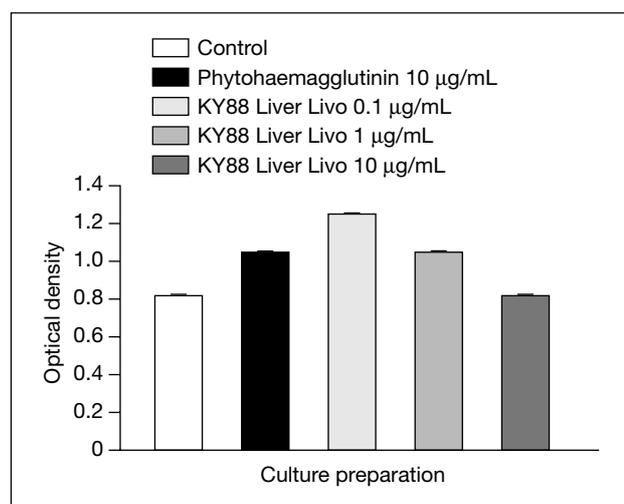


Fig 1. Lymphocyte proliferation after stimulation with phytohaemagglutinin (10 µg/mL) and KY88 Liver Livo in different concentrations

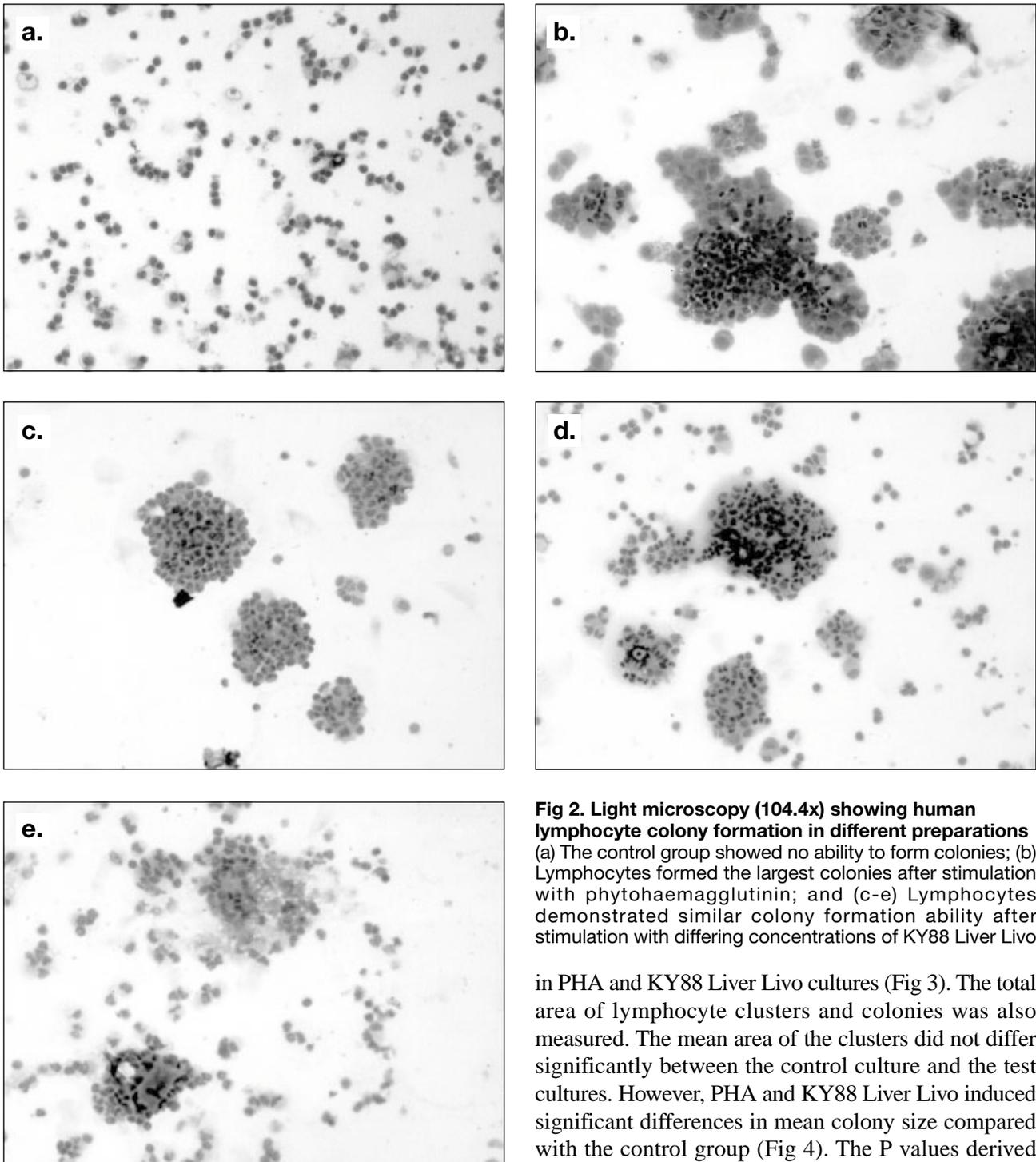


Fig 2. Light microscopy (104.4x) showing human lymphocyte colony formation in different preparations (a) The control group showed no ability to form colonies; (b) Lymphocytes formed the largest colonies after stimulation with phytohaemagglutinin; and (c-e) Lymphocytes demonstrated similar colony formation ability after stimulation with differing concentrations of KY88 Liver Livo

Lymphocytes in the control culture were scattered and demonstrated no ability to form a colony (Fig 2a). However, lymphocyte colony formation was evident after the addition of PHA (Fig 2b) and KY88 Liver Livo (Figs 2c to 2e). Although the number of clusters was similar between the control and test cultures, the number of colonies in the test cultures was significantly greater than the control group. The respective P values were 0.016, 0.044, and 0.037 for KY88 Liver Livo at concentrations of 0.1 $\mu\text{g}/\text{mL}$, 1 $\mu\text{g}/\text{mL}$, and 10 $\mu\text{g}/\text{mL}$ of KY88 Liver Livo. The number of colonies were similar

in PHA and KY88 Liver Livo cultures (Fig 3). The total area of lymphocyte clusters and colonies was also measured. The mean area of the clusters did not differ significantly between the control culture and the test cultures. However, PHA and KY88 Liver Livo induced significant differences in mean colony size compared with the control group (Fig 4). The P values derived were <0.001, 0.002, 0.03, and 0.05 for PHA, and KY88 Liver Livo concentrations of 0.1 $\mu\text{g}/\text{mL}$, 1 $\mu\text{g}/\text{mL}$, and 10 $\mu\text{g}/\text{mL}$, respectively. There were no statistically significant differences seen between the different KY88 Liver Livo concentrations in this regard.

The production of GM-CSF was minimal in the control culture, whereas KY88 Liver Livo and PHA induced significant production of GM-CSF (Fig 5). The levels of GM-CSF show a linear relation with the different concentrations of KY88 Liver Livo used. The levels were six, eight and 12 times higher than that of

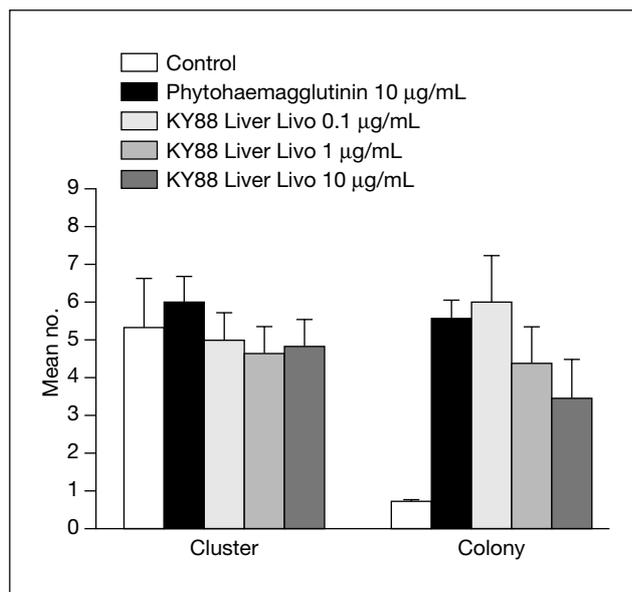


Fig 3. Lymphocyte proliferation: cluster and colony formation in differing preparations after 72 hours
 Significant differences were detected between the mean number of colonies in the phytohaemagglutinin and KY 88 Liver Livo preparations and the control preparation ($P < 0.05$ for all comparisons)

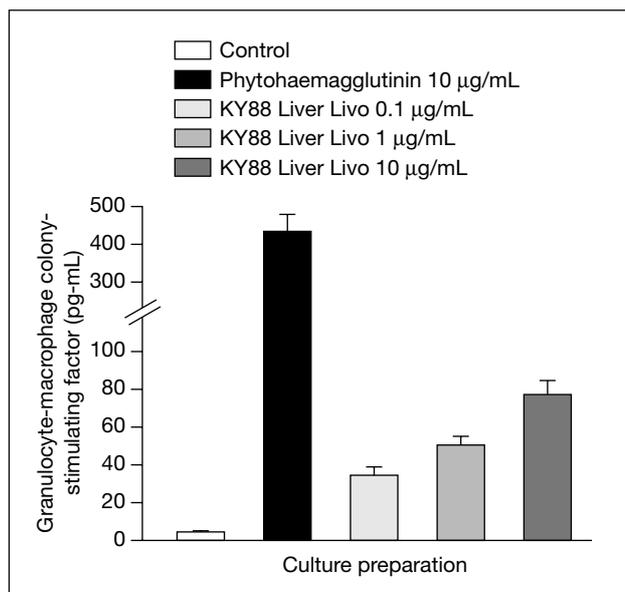


Fig 5. Production of granulocyte-macrophage colony-stimulating factor by human lymphocytes in differing preparations after 72 hours
 Phytohaemagglutinin and KY88 Liver Livo induced increased production of granulocyte-macrophage colony-stimulating factor

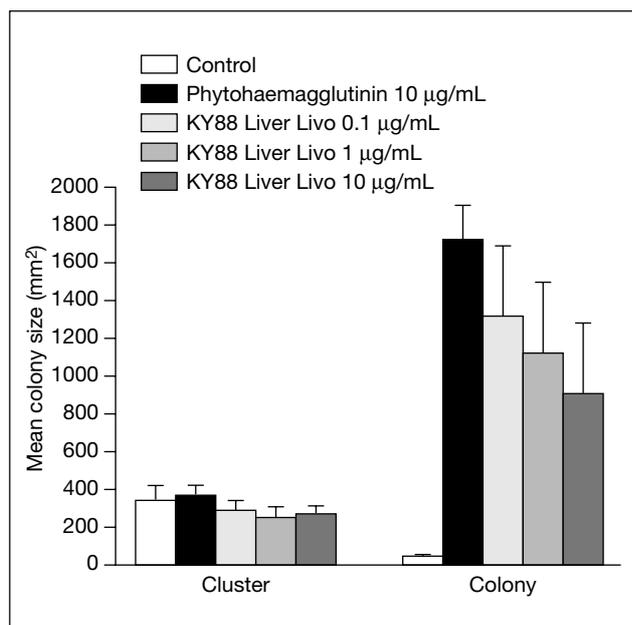


Fig 4. Lymphocyte proliferation: colony size in differing preparations after 72 hours
 Significant differences in colony size were seen in the phytohaemagglutinin and KY 88 Liver Livo preparations compared to the control preparation

the control group when the lymphocytes were stimulated with KY88 Liver Livo at concentrations of 0.1 µg/mL, 1 µg/mL, and 10 µg/mL. The P values derived were 0.166, 0.047, and 0.003, respectively.

Discussion

The herbal compound formulation approach for

chronic hepatitis therapy aims to eliminate the HBV, strengthen the immune system, and induce regeneration of the liver. While this study has not demonstrated that KY88 Liver Livo is able to eliminate the HBV, it does indicate that the compound appears to enhance the immune system, specifically demonstrating that it stimulates lymphocyte proliferation in vitro.

Although the formation of lymphocyte clusters was common, even in the control culture, lymphocyte colonies were seen to form only in response to stimulation. KY88 Liver Livo at the lowest concentration of 0.1 µg/mL was seen to induce a similar number of colonies compared with other concentrations. Size of the colonies varied, and therefore error in interpretation may still occur if only the number of colonies is considered. With respect to the total area of the colonies, PHA was noted to have a greater ability to induce lymphocyte proliferation as determined by the mean total lymphocyte colony area. There was no significant difference between KY88 Liver Livo stimulation at differing concentrations, indicating that the induction of lymphocyte proliferation with KY88 Liver Livo occurs irrespective of the concentration used.

This study has demonstrated that the mitogenic action of KY88 Liver Livo is coupled with the production of GM-CSF by lymphocytes. The level of GM-CSF secretion was related to the concentration of KY88 Liver Livo used. A linear relation between the level of GM-CSF production and the KY88 Liver

Livo concentration was seen, with GM-CSF production 12 times higher than that of the control group when KY88 Liver Livo at a concentration of 10 µg/mL was used.

It is possible that the formation of colonies is related to the release of GM-CSF.⁶ This could be determined by adding neutralisation antibodies against GM-CSF to the culture system. A decrease in colony formation would support this hypothesis.⁶ Other investigators have reported that the secretion of cytokines is time-dependent, with secretion levels significantly increased with a longer period of culture.⁷ These researchers also found that a high concentration of a herbal extract did not produce the same augmentative effect as a low concentration. This study does confirm that a longer reaction time is required for significant secretion of GM-CSF to be noted. However, the secretion of GM-CSF was found to be concentration dependent, implying that different compositions of herbal extracts would tend to produce different effects, even at similar culture conditions.

Granulocyte-macrophage colony-stimulating factor is produced by a number of cells, including lymphocytes and monocytes,⁸⁻¹⁰ and has a broad impact on haematopoiesis. Its production is triggered by exposure to bacterial lipopolysaccharides, activating the host inflammatory response to invading pathogens.¹¹⁻¹³ Granulocyte-macrophage colony-stimulating factor induces the proliferation of monocytes and macrophages, enhances neutrophil survival and phagocytosis,^{14,15} and stimulates committed stem cells to mature and develop. Low doses induce the formation of macrophage colonies, whereas high doses induce the formation of myeloid colonies.¹⁶

This study provides insights into the mechanism of action of KY88 Liver Livo. Knowledge on this subject is limited, although KY88 Liver Livo is believed to be effective against the HBV. While KY88 Liver Livo may act on the virus directly, activation of the host inflammatory response is certainly an important means of combating viral infection. This study suggests potential new areas of research into the treatment of chronic viral infection. It has demonstrated that properly manufactured herbal medicine is effective in inducing lymphocyte proliferation and the secretion of GM-CSF, which is linked to the host inflammatory response against pathogens. Further clinical evaluation of this herbal compound would appear worthwhile.

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