CHAPTER 30

IMMUNOMODULATORY ACTIVITIES OF MUSHROOM MYCELIAL EXTRACTS

W.K. Liu[1], V.E.C. Ooi[2], T.B. Ng[3] and S.T. Chang[2]

Departments of Anatomy[1], Biology[2] and Biochemistry[3]
The Chinese University of Hong Kong, Shatin, N.T., Hong Kong.

1. INTRODUCTION

Mushrooms are a special group of higher fungi which are considered as a delicious health food in Hong Kong because the nutritive value of high-grade mushrooms almost equals that of milk (Chang & Miles, 1989). They also contains high levels of essential amino acids and are particularly rich in lysine and leucine, unsaturated fatty acids, carbohydrates and many vitamins. Their mineral content is higher than that of meat and fish and almost twice that of vegetables. In addition, mushrooms have long been considered to have medicinal value and are devoid of undesirable effects (Sakagami *et al.*, 1991). These special features might be one of the major rationales why the United States National Cancer Institute selects fungi as a source of new drugs. Mushroom extracts have been reported to have hematological, antiviral and antitumor, hypotensive and hepatoprotective effects (Chang & Miles, 1989). In the past two decades, a group of protein-bound polysaccharides have been extracted from an edible mushroom, *Coriolus versicolor*, by scientists in Japan and China, and they are considered by the United States National Cancer Institute to have antitumor activities (Jong & Donovick, 1989). Our previous studies have indicated that the polysaccharopeptide isolated from *Coriolus versicolor* is an immunomodulator which activates the production of antitumor molecules, tumor necrosis factor (TNF), and reactive nitrogen and oxygen intermediates (RNI's, ROI's) by mouse peritoneal macrophages (Liu *et al.*, in press). However, most of these protein-bound polysaccharides are extracted from fruitbodies which might be affected by environmental contamination. In order to avoid probable environmental impact on the quality of the mushroom, submerged culture of the mushroom mycelium is recommended to ensure the production of immunopotentiating agents of a consistent quality. We have selected 11 overseas and local mushroom species for submerged mycelial culture, and the preliminary data indicate that the substances released into the culture media possess no cytotoxicity but some of them can activate the defensive cells such as macrophages and lymphocytes.

2. MATERIALS AND METHODS

2.1. Submerged Culture of Mushroom Mycelium

The mycelia were grown in the laboratory in liquid medium at pH 4.5-6 and at 25-30°C for 7 days and their growth rates were monitored. The media were filtered and the filtrates (MCMs) were lyophilised, weighed and reconstituted in mammalian cell culture media RPMI 1640 (GIBCO, USA) culture medium supplemented with 10% fetal bovine serum and antibiotics before use in bioassays.

2.2. Immunological and Antitumor Bioassays

2.2.1. In vitro assays. The filtrate powders were re-dissolved in RPMI 1640, and their cytotoxicity and antitumor actitivity were tested using a wide range of cell lines, including L929 (mouse fibroblast cell line), H3B (mouse hepatoma, ATCC), E367 (mouse neuroblastoma, a gift from Dr. Stark, University of Duesseldorf, Germany) and JAR (human placenta choriocarcinoma, ATCC HTB 144) in a humidified atmosphere of 5% CO_2 for 24 h. The viable cells were stained with crystal violet and their optical density were measured by a microplate ELISA reader (Liu & Ng, 1991). Mouse peritoneal macrophages were collected from male inbred BALB/c mice and incubated with a range of MCMs for 24 h before they were used to measure the production of reactive nitrogen intermediates.

2.2.2. In vivo assays.

i) Macrophages

Inbred male BALB/c mice were provided with three selected MCMs (#4, #19 and #20) in drinking water for 2 weeks and the macrophages were collected for RNI bioassay (Keller et al., 1990, Liu *et al.*, 1993).

ii) Lymphocytes

Spleens were removed aseptically from the MCM-treated mice and splenocytes obtained by gently pressing of the spleens through a 100-mesh stainless steel sieve to obtain a single cell suspension. The cells were counted and resuspended in culture medium RPMI for proliferative response to the mitogen, concanavalin A (Liu *et al.*, 1989).

3. RESULTS

The mycelia of 11 overseas and local mushroom species have been selected for submerged culture. The growth of the mycelium depends on a wide range of growth conditions, including the type of culture medium, pH, temperature, cultivation period and the shaking speed. The growth rate of these mycelia were different (Fig. 1). An aliquot of each of these submerged mycelial culture media (MCM) has been subjected to the cytotoxicity test and none of them at concentrations below 1 mg/ml possessed *in vitro* cytotoxic effects on normal fibroblast cell line (L929) or tumor cell lines (Figs. 2 & 3). However, at a dose of 10 µg/ml they augmented the production of antitumor molecules, such as reactive nitrogen intermediates, by mouse peritoneal macrophages (Fig. 4). They also stimulated the proliferative response of mouse splenocytes to concanavalin A (Fig 5). It is of particular interest that the MCM with the most potent biological activity (sample #20) is a local *Tricholoma* species which may be unique to Hong Kong. Its immunostimulatory activity is higher than those of the most widely used species, *Coriolus* (sample #19) and *Ganoderma* (sample #4).

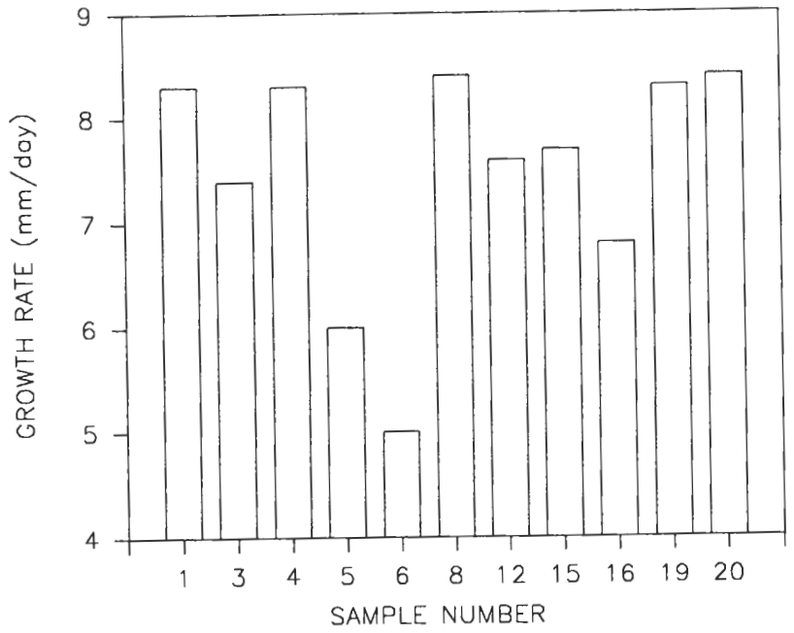

FIGURE 1. Growth rate of selected mushroom mycelia.

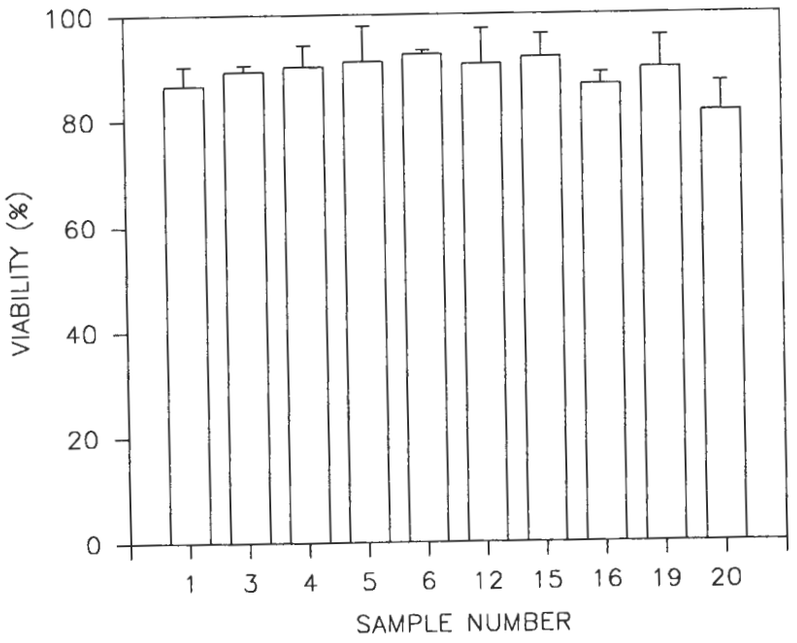

FIGURE 2. Viability of fibroblasts incubated with MCMs (1mg/ml).

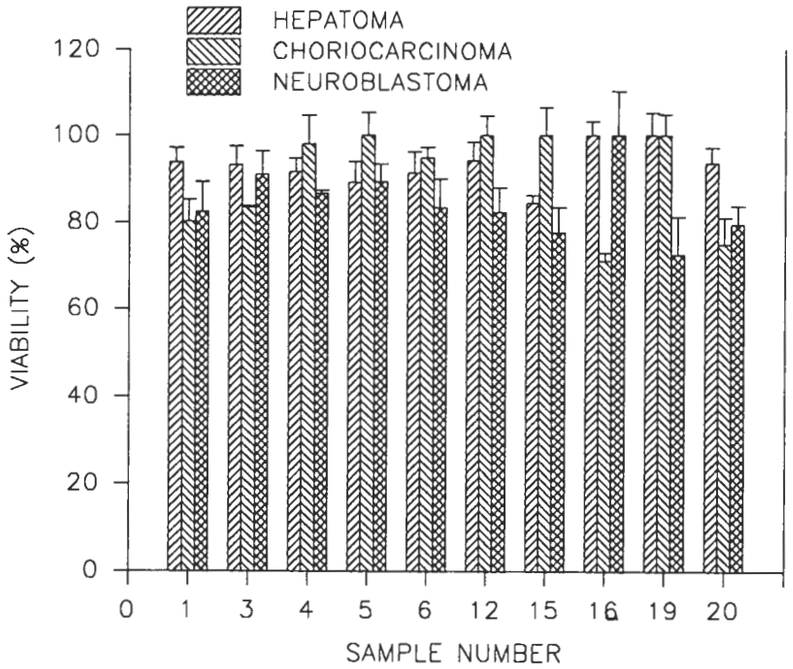

FIGURE 3. Viability of tumor cell lines incubated with MCMs.

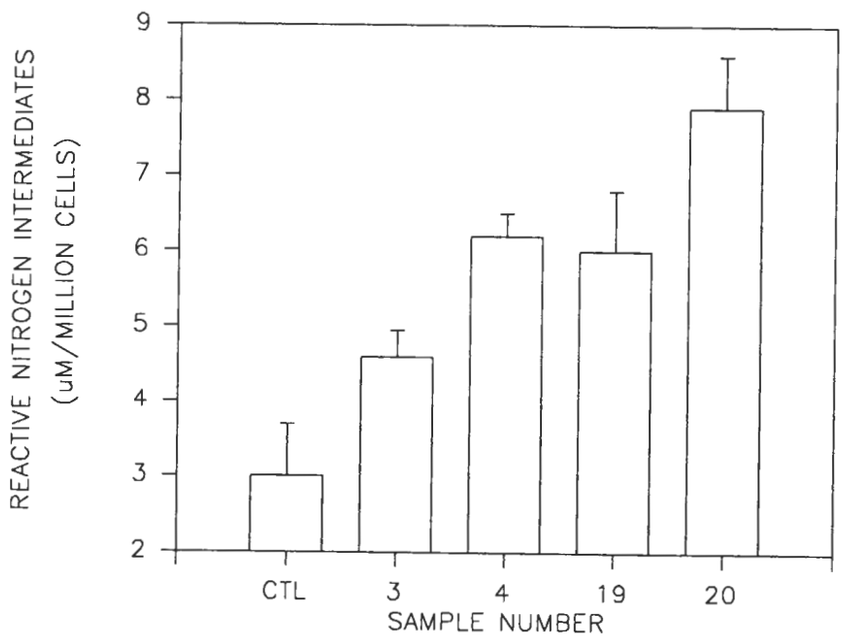

FIGURE 4. Production of reactive nitrogen intermediates by peritoneal macrophages from mice treated with MCMs for 2 weeks.

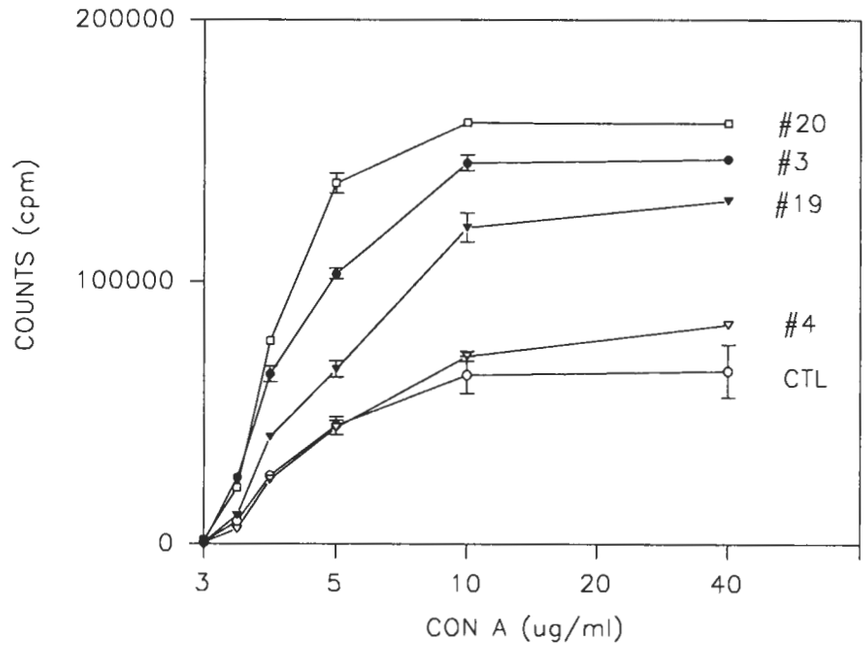

FIGURE 5. Mitogenic responses of splenocytes from mice treated with selected MCMs for 2 weeks.

4. DISCUSSION

It is an impossible task to have a continuous supply of 10-30 fresh mushroom species of various origins maintained for experimental purpose. However, the method of submerged culture of mushroom mycelium can solve the problem with the advantage that a consistent culture condition can be maintained and also environmental contamination can be avoided. The biological activities of the secretions in mushroom mycelial culture medium can therefore be intensively studied using bioassays and modern molecular techniques.

Macrophages and lymphocytes are two major populations of cells in the host defense system which act against invading pathogens. Macrophages are responsible for the non-specific cellular response. They ingest infectious microorganisms and digest them with lysosomal enzymes (Liu & Ng, 1991). Macrophages can also be activated by lymphokines and other cell mediators to kill tumor cells by producing TNF (Keller *et al.*, 1990), ROI's (Fridovich , 1976) and RNI's (Hibb *et al.*, 1988). When non-specific responses fail to check the invasion of pathogenic organisms, specific immune responses are activated whereby the lymphocytes recognize a particular pathogen and generate antibodies to promote destruction of this pathogen. In addition, lymphocytes and macrophages co-operate through the production of cell mediators. Lymphocytes cannot recognize free antigen in the absence of antigen presenting cells, namely macrophages, which produce a cell mediator called interleukin 1 (IL-1) which can stimulate the lymphocytes to produce interleukin 2 (IL-2). IL-2 is a T cell growth factor which promotes the proliferation of T-lymphocytes, increases the cytotoxic capacity of natural killer cells and increases the production of gamma interferon (IFNγ) which is one of the major macrophage activating factors. Our previous studies have demonstrated that a polysaccharide-protein complex (PSPC) isolated from *Coriolus* augmented the production of IL-2

and IFNγ by lymphocytes and also the production of TNF, RNI's and ROI's by peritoneal macrophages in mice. Northern blot analysis of TNF mRNA provided further support that macrophages upon exposure to PSPC had an earlier TNF transcription (Liu *et al.*, in press). The molecular basis for the tumoricidal activity of activated macrophages is not clearly known, but their secretory products, such as TNF, RNI's and ROI's might play an important role in this process (Kilbourn *et al.*, 1984; Ding *et al.*, 1988). RNI's suppress mitochondrial respiration of tumor cells (Nathan & Hibbs, 1991) while ROI's appear to be important components of the oxygen-dependent killing of ingested microbes by phagocytes (Fridovich, 1976). Therefore, it is possible that enhancement of production of reactive intermediates and TNF underlies the tumoricidal activity of the PSPC.

We have also observed increases in the expression of the proto-oncogenes, such as *c-fos* and *c-myc* which code for components of the signal transduction pathway, in macrophages from PSPC-treated mice. Hence, it is possible that PSPC modulates cytokine action and also participates in signal transduction in the defensive cells. Experiments are required to study the mechanism of action of active ingredients in the MCM in the activation of macrophages.

Several species of basidiomycetes were reported to produce antitumor polysaccharide-protein complexes (Jong & Donovick, 1989). Since these substances were plant extracts, they might contain several active ingredients. However, so far only unfractionated preparations have been widely used in almost all experiments (Sakagami *et al.*, 1991). The polysaccharide-bound protein comprises a polypeptide moiety as well as a carbohydrate moiety. It is soluble in hot water, heat-stable and light-stable. Although these polysaccharide-bound proteins are classified as antitumor chemicals by the United States National Cancer Institute (Jong & Donovick, 1989), the molecular basis of their biological activity is largely unknown. Experiments are in progress to identify the active ingredients and the mechanism of action of these antitumor substances.

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