Mitogenic Activity in Skimmed Milk Culture Supernatant of *Aspergillus oryzae* EF-08: Preparation of Mitogenic Fractions and Their Immunopotentiating Effect in Vivo

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A screening test was carried out to detect immunostimulating factor(s) in skimmed milk culture supernatants of various types of food microorganisms using a mitogen assay. High and low molecular weight mitogen-active fractions were successively prepared from skimmed milk culture supernatant of *Aspergillus oryzae* EF-08 using preparative reversed-phase chromatography. The low molecular weight fraction (EF-08ODS) enhanced primary humoral immune response in mice and prolonged the life span of ascites-type tumor-bearing mice when administered intraperitoneally by encapsulation in a liposome. In the tumor suppression assay, it inhibited growth of Meth A fibrosarcoma inoculated subcutaneously in syngenic BALB/c mice. Furthermore, it augmented a delayed-type hypersensitivity (DTH) response in mice in the presence of FIA. EF-08ODS was separated further into two active fractions. These three fractions were mainly composed of amino acid rich substances, whose molecular weights were estimated to be up to 3000.

INTRODUCTION

Aspergillus oryzae have been widely used for the production of traditional Japanese foods, such as soy sauce, miso, and alcoholic beverages. During our studies on immunostimulating substances originating from food microorganisms including lactic acid bacteria, *Bacillus*, and food-grade fungi, we found that *A. oryzae* EF-08, which has been newly isolated from Koji, produced a mitogenic active substance against murine splenocytes in its skimmed milk culture supernatant (Fujiwara et al., 1990). It seems that skimmed milk culture supernatant of edible microorganisms may be a good source of potent immunostimulating substances, especially for use in food.

To date, several papers have been published concerning immunomodulating substances originating from *Aspergillus*, but most of them were concerned with high molecular weight substances, like cell walls (Sakai and Uchida, 1987) and extracellular enzymes (Kusakabe et al., 1974, 1979). Only a few papers (Kato et al., 1969) were published concerning low molecular weight immunostimulating substances originating from *A. oryzae*.

In a previous paper, we reported that the increase of mitogenic activity in *A. oryzae* EF-08 culture supernatant paralleled the degree of proteolysis (Fujiwara et al., 1990). From our results, the presence of a novel mitogenic factor, including peptides, was expected in its supernatant. It is known that some mitogen-active substances, which are of microbial origin (Gotou et al., 1982; Ishizuka et al., 1980a,b; Mine et al., 1983; Yokota et al., 1983), show immunomodulating activities in vivo. To confirm that the mitogenic substance exerts immunostimulating activity, this paper describes fractionation of the mitogenic activity and immunopotentiating activities of resulting fractions in vivo.

MATERIALS AND METHODS

Fungal Strain. A food-grade fungus named EF-08, a potent immunostimulating strain, was screened using a mitogen assay. According to the descriptions by Cohn (1973), the morphological properties of the strain were closely related to those of *A. oryzae*. Therefore, we named strain EF-08 as *A. oryzae* EF-08.

Mitogen Production. Optimal culture conditions for production of mitogenic activity were described in a previous paper (Fujiwara et al., 1990). Briefly, *A. oryzae* EF-08 was grown in 8% skimmed milk containing 0.3% yeast extract and 1% glucose at 28 °C on a rotary shaker at 150 rpm. A 48-h-old culture showed maximum mitogen activity, and the degree of proteolysis reached approximately 75–80%.

Partial Purification of Mitogen-Active Fraction. A skimmed milk culture of *A. oryzae* EF-08 was first filtered with gauze to eliminate the fungal pellet and insoluble materials. The resulting culture supernatant was acidified with HCl to a pH of 4.6 and held for 30 min at 4 °C. After elimination of precipitates by centrifugation at 10000G for 30 min, the supernatant was neutralized with NaOH and filtered with a microfilter (0.45 μm, Falcon 7104, Becton Dickinson and Co., Lincoln Park, NJ). The filtered supernatant (650 mL) was applied to a column packed with preparative C18 (75 μm, 24 mm i.d. × 150 mm long, 70 mL, Millipore Corp., Milford, MA). The eluate was recovered, and the column was washed with 3 column volumes of HPLC grade water (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Both the eluate and the nonretaining fraction were pooled, concentrated, and lyophilized. After a second washing, stepwise elution was performed successively with 50% MeOH (300 mL) and 98% MeOH (300 mL; EF-08ODS). These eluates were collected by each corresponding fraction. The fractions were concentrated and lyophilized to remove water and MeOH. The fraction eluted with 98% MeOH was termed EF-08ODS. To prepare the negative control substance(s), the same fractionation procedure was applied to a freshly prepared skimmed milk medium (without culture) and the fraction eluted with 98% MeOH named SM-ODS.

Further purification was monitored with the mitogen assay and was carried out using a column (30 mm i.d. × 210 mm long, 148 mL) packed with preparative C18 with a linear gradient of MeOH from 50 to 98% in a total volume of 900 mL. Further purified FII2 and FIII fractions were then obtained.

Amino Acid Analysis. After total hydrolysis (5.7 N HCl, 110 °C, under vacuum for 22 h), the amino acid analysis was carried out on an amino acid analyzer (Hitachi Model 835-50).

Antigen, Reference Mitogens, and Immunopotentiator. Crystallized and lyophilized ovalbumin (OVA, type VII) and bovine serum albumin (BSA, fraction V) were purchased from Sigma Chemical Co. (St. Louis, MO). Dinitrophenyl (DNP)-OVA and -BSA were prepared by a coupling reaction with sodium

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dinitrobenzenesulfonate (Eisen et al., 1953). The degrees of substitution (Shiffman et al., 1964), expressed as molar ratio of DNP to protein, were 6.7 for DNP-OVA and 10.9 for DNP-BSA. Concanavalin A (Con A, Vector Laboratories Inc., Burlingame, CA) and lipopolysaccharide (LPS) from *Escherichia coli* 055:B5 (List Biological Laboratories, Inc., Campbell, CA) were used as T- and B-cell mitogens, respectively. Acetylmuramyl-L-alanyl-D-isoglutamine (MDP; Biosys Société Anonyme, Clos des Roses, Campiègne, France) was employed as a reference active compound in the research presented in this paper.

Lipids and Preparation of Liposomes. Dimyristoylphosphatidylcholine (DMPC; Nichiyu Liposome Co., Ltd., Tokyo, Japan) and cholesterol (CHL; Wako) were purchased and stored in chloroform under nitrogen at −20 °C until liposomes were prepared. Multilamellar liposomes (MLV) were prepared with reference to the method of Bangham et al. (1965). MLV liposomes containing encapsulated materials were prepared from a mixture of DMPC and CHL (2.6:1 molar ratio) by mechanical agitation on a vortex mixer. Nonencapsulated solution was removed by washing the liposomes by centrifugation. The internal volume of the MLV was estimated to be approximately 3 μL/μmol of lipid (data not shown). Liposome preparations and reagents were found to contain <0.025 ng/mL endotoxin, as determined by a Limulus amoebocyte lysate assay (McKay and Shapiro, 1958).

Animals. Specific-pathogen-free C3H/HeN male, BALB/c male, CBF_1 female, and ICR male mice were obtained from Charles River Japan, Inc., at 4 weeks of age and maintained under specific-pathogen-free conditions in our laboratory.

Tumors. Sarcoma 180 and Meth A fibrosarcoma in ascites form (from Dr. Takeo Mizutani of the Laboratory Animal Research Center, Institute of Physical and Chemical Research) were serially passed in ascites in allergenic ICR mice and in syngeneic BALB/c mice (Charles River Japan and Atsugi, Kanagawa, Japan), respectively.

Preparation of Spleen Cells. Spleens were removed from C3H/HeN male mice (5–7 weeks of age) and were gently teased through a sterile stainless steel screen (80 gauge, Abe Kagaku, Makuhari, Chiba, Japan) in RPMI 1640 medium (Flow Laboratories Inc., Irvine, Scotland) containing 25 mM HEPES, 0.2% sodium bicarbonate, 100 units/mL penicillin G, and 100 μg/mL streptomycin sulfate (HEPES–RPMI). The cell suspensions were allowed to settle to remove tissue fragments. The supernatant containing single cells was aspirated and centrifuged (260*g* for 5 min at 4 °C). The pelleted cells were resuspended in the same medium, and a dye exclusion test using trypane blue was used to enumerate the viable white blood cell numbers in the suspension (Harlow and Lane, 1988).

In Vitro Proliferation Assay to Mitogens. The mitogen assay was performed by a MTT colorimetric method (Mosman, 1983). Spleen cells were suspended at a cell density of 5×10^6 cells/mL in HEPES–RPMI supplemented with 10% inactivated FCS (HEPES–RPMI–FCS).

One hundred microliters of the cell suspension was plated into 96-well microculture plates (Costar 3596, Data Packaging Corp., Cambridge, MA); 10 μL of mitogen solution (Con A, 10 μg/mL; LPS, 1000 μg/mL; other preparating samples from culture supernatant, 30–2000 μg/mL) dissolved in HEPES–RPMI was added to the wells in quadruplicate. Control wells received the same volume of HEPES–RPMI but without mitogen. The plates were incubated for 48 h at 37 °C in a humidified atmosphere containing 5% CO_2 in air, and then 10 μL of MTT [3-(4,5-dimethyl-2-thyazolyl)-2,5-diphenyl)-2*H*-tetrazolium bromide, 5 mg/mL of PBS] was applied to each well, and plates were further allowed to incubate 2–3 h. The resulting MTT formazan was dissolved by the addition of 150 μL of 2-propanol containing 0.04 N HCl and measured spectrophotometrically by a microplate reader (Model 450, Bio-Rad Laboratories, Richmond, CA) at 595 nm. The results were expressed as means of quadruplicate cultures and as a stimulation index (SI). SI was obtained as the ratio of the amount of absorbed MTT formazan by mitogen-producing cultures over that of control cultures.

Adjuvant Activity on Antibody Responses to DNP in Vitro. In vitro antibody responses to DNP were examined by using the method of Mishell and Dutton (1967) with slight modification. Specific-pathogen-free female BALB/c mice (5–7 weeks of age) were immunized with 100 μg of DNP-OVA and 10^9 of *Bordetella pertussis* vaccine (Chiba Serum Institute, Funabashi, Chiba, Japan). Seven days thereafter, spleen cells were prepared and suspended in RPMI 1640 supplemented with 10% inactivated FCS, 0.2% sodium bicarbonate, 100 units/mL penicillin G, 100 μg/mL streptomycin sulfate, and 5×10^{-5} M β-mercaptoethanol (RPMI–FCS–2ME). One hundred microliters of cell suspensions (5×10^6/mL) was placed in a 96-well microculture plate to which 50 μL of EF-08ODS or MDP solutions (dissolved in RPMI–FCS–2ME) and 50 μL of antigen solution (20 ng of DNP-OVA/mL of RPMI-FCS-2ME) were added. Plates were cultured for 7 days and antibodies produced in culture supernatants were determined by ELISA using alkaline phosphatase labeled anti-mouse Ig (IgG + IgM + IgA), IgG, or IgM as a secondary antibody (Zymed Laboratories, Inc., San Francisco, CA). In some cases, antigen solution was replaced by the same volume of RPMI–FCS–2ME.

Adjuvant Activity on Primary Antibody Responses to DNP-OVA in Vivo. Specific-pathogen-free female BALB/c mice (5–7 weeks of age) were immunized intraperitoneally with 100 μg of DNP-OVA in 0.2 mL of an emulsion of Freund's incomplete adjuvant (FIA, a mixture of liquid paraffin and Arlacel A (85:15)) with a saline solution of immunoadjuvant (equal volumes). Seven days later the mice were bled, and the concentrations of serum antibodies were determined by ELISA.

Measurement of Anti-DNP-OVA or Anti-DNP Antibody by Enzyme-Linked Immunosorbent Assay (ELISA). Anti-DNP-OVA antibodies in sera or anti-DNP antibodies in culture supernatants of spleen cells were determined by ELISA. Wells of ELISA plates (Titertek, Flow Laboratories) were coated with 50 μL of DNP-OVA (5 μg/mL, for measurement of anti-DNP-OVA antibodies) or DNP-BSA (5 μg/mL, for measurement of anti-DNP antibodies) dissolved in Tris-buffered saline (TBS, constructed by 20 mM Tris and 500 mM NaCl, pH 7.5). The plates were allowed to stand for 60 min at room temperature and then washed three times with TBS. After unreacted sites on the surface were blocked for 30 min with 150 μL of 1% BSA in TBS, the plates were washed three times with TBS containing 0.05% Tween 20 (TTBS). Fifty microliters of anti-DNP-OVA antiserum or culture supernatant containing anti-DNP antibodies diluted serially with TBS was added to the antigen-coated well in quadruplicate. After 1 h, the plates were washed five times with 50 μL of TTBS, and alkaline phosphatase (AP) conjugated rabbit anti-mouse IgM, IgG, or Ig antibody (Zymed Laboratories) was added to the wells. One hour later the plates were washed thoroughly with TTBS and incubated at 37 °C for 60 min with 50 μL of substrate solution consisting of 1 mg/mL *p*-nitrophenylphosphate–10% diethanolamine buffer, pH 9.6. The reaction was terminated by the addition of 0.5 N NaOH (50 μL/well), and the absorbance of each well was read at 405 nm by microplate reader (Model 450, Bio-Rad Laboratories).

Suppression Assay for Tumor Growth Inoculated Intraperitoneally. On day 0, ICR male mice (6 weeks of age, 16/group) were inoculated intraperitoneally with 5×10^5 cells of sarcoma 180, which was capable of causing ca. 50% mortality within 20 days in the control (saline injected) animals. The fraction tested was dissolved in phosphate-buffered saline (PBS) or entrapped in MLV liposomes (100 μg/2.5 μmol of lipid/head). Animals received intraperitoneal injections on days −4, −1, 1, 2, and 3. They were observed for 40 days following tumor transplantation, mortality rates being recorded daily.

Suppression Assay for Tumor Growth Inoculated Subcutaneously. BALB/c male mice (6 weeks of age, 24/group) were inoculated subcutaneously with 1×10^5 cells of Meth A fibrosarcoma with the fraction to be tested. Seventeen days after tumor transplantation, the animals were sacrificed under CO_2 inhalation. Development of tumor nodules was observed, and the rejection rate was summarized on a contingency table.

DTH Reaction. The effect of EF-08ODS on DTH was studied according to the procedure of Lagrange et al. (1974) as described by Migriore-Samour et al. (1980). Ten-week-old CBF_1 mice (female, 15–16/group) were injected sc in the neck with 10^7 SRBC in 0.2 mL of an emulsion of FIA with a saline solution of the fraction (equal volumes). Six days later, the mice were challenged with 10^8 SRBC (in 0.05 mL of saline) in the left hind foot pad;

Table I. Effect of Medium Composition on the Production of the Mitogenic Factor(s)

media[a]	cultivation[b] cultured MA[c]	cultured DP[d]	not cultured MA	not cultured DP
SM + YE + glucose	1.45	(85.6)	1.00	(16.5)
SM + glucose	1.45	(88.1)	0.96	(16.3)
ISP + YE + glucose	0.93	(98.9)	1.00	(9.8)
ISP + glucose	0.90	(96.7)	0.92	(7.1)
PEP + YE + glucose	1.00	(98.6)	1.00	(100)

[a] Major nitrogen source (SM, ISP, or PEP) was added to the media at the concentration of 8%. YE and glucose were added to the media at the concentrations of 0.3 and 1%, respectively. Abbreviations: SM, skimmed milk; YE, yeast extract; ISP, isolated soya protein; PEP, peptone. [b] *A. oryzae* EF-08 was cultured with various media listed using a rotary shaker (150 rpm) at 28 °C. [c] Optimal mitogenic activities were expressed as mean SI number for quadruplicate cultures. Forty-eight-hour-culture supernatant was diluted serially 2-fold, and 10 μL of each diluted solution was subjected to the MTT assay. Optimal response was observed around 4-fold diluted culture supernatant. [d] Numbers in parentheses show mean value of degree of proteolysis, which was expressed as the ratio of the amount of nonprotein nitrogen to that of total nitrogen in each corresponding supernatant.

the control right foot pad received 0.05 mL of saline. Foot pad swelling was measured 24 h postchallenge.

Statistical Analysis. One-way layout experiments were analyzed by ANOVA or Kruskal-Wallis analysis in variance of ranks. A multiple comparison procedure was then applied. The data summarized in two-way contingency tables were analyzed according to the method described by Hirotsu (1984).

RESULTS

Effect of Compositions in Media on Production of Mitogen Activity. *A. oryzae* EF-08 was cultured in various media with varied culturing time. Table I shows the maximum mitogen activity expression in the culture supernatants of media with various major nitrogen sources. The activity was only shown in the culture supernatant that employed skimmed milk as a major nitrogen source. All of the supernatants, after acid precipitation of these fresh media, showed no mitogenic activity, although the samples were subjected to the assay in a wide concentration range.

Gel Filtration of the Skimmed Milk Culture Supernatant of *A. oryzae* EF-08. For estimating the molecular weight of mitogenic factors, gel filtration on Sephadex G-50 was employed. Figure 1 shows the chromatogram and mitogenic activities of resulting fractions. The activities were observed in fractions 1, 4, and 5. Molecular weights of these mitogenic substances seemed to be over 30 000 (fraction 1) and around 1000 (fractions 4 and 5). As fractions 4 and 5 show relatively high activity compared to that of fraction 1, the mitogenic activity of skimmed milk culture supernatant produced by *A. oryzae* EF-08 is modulated by these low molecular weight mitogens.

Partial Purification of Low Molecular Weight Mitogens. It was revealed that the low molecular weight mitogens were effectively prepared by using preparative reversed-phase chromatography. Table II shows typical results of fractionation using this chromatography employed with batchwise elution. SDS–PAGE (10–20% gradient gel) and urea–SDS–PAGE showed that the fraction eluted with absolute MeOH (EF-08ODS) had a molecular weight distribution similar to that of fractions 4 and 5 chromatographed on Sephadex G-50 (data not shown).

Figure 2 shows further fractionation of EF-08ODS on reversed-phase column with a linear gradient of MeOH from 50 to 98% in a total volume of 900 mL. FII2 and FIII fractions exhibited mitogenic activity.

Amino Acid Analysis of EF-08ODS and Further Fractionated FII2 and FIII. EF-08ODS and further fractionated FII2 and FIII fractions contained large amounts of amino acid containing substances. The amino acid compositions are shown in Table III. All of these active fractions showed relatively high proline (Pro) and glutamate plus glutamine (Glx) contents. It seems that Val and Leu contents in these fractions are relatively rich.

Effect of the Mitogenic Fractions on Blastogenesis of Murine Splenocytes. Figure 3 shows the effect of three mitogenic fractions (EF-08ODS, FII2, and FIII) on the blastogenesis of murine splenocytes. EF-08ODS and FII2 fractions exhibited the maximum mitogenic activity at the concentration of around 50 μg/mL. On the other hand, the optimum concentration of FIII fraction was about 25 μg/mL.

Effect of EF-08ODS on the Production of Antigen-Specific Antibodies in Vitro. When antigen was used for culturing, the addition of MDP to the cultures enhanced their immunoglobulin secretion in a dose range from 2.5 to 50 μg/mL. Supplementation of EF-08ODS showed suppressive effect in a dose range from 25 to 100 μg/mL. Since EF-08ODS failed to enhance the level of antibody secretion in the presence of antigen, we proceeded to examine whether EF-08ODS also suppressed the responses in the absence of antigen. As shown in Table IV, similar response patterns were obtained. EF-08ODS showed the tendency of suppression of the in vitro antibody production in both cases with or without secondary antigen sensitization.

Effect of EF-08ODS and Further Fractionated FIII on the Production of Antigen-Specific Antibodies in Vivo. The adjuvant effect of EF-08ODS and FIII fractions on primary antibody response against DNP-OVA was examined by using an ELISA technique. As shown in Figure 4, serum antibody titer (IgG class) in mice immunized with antigen/EF-08ODS/FIA resulted in good anti-DNP-OVA responses as compared to that of mice immunized with antigen only (blank) or with antigen/FIA (control). This enhanced effect was statistically significant as compared with blank and control in doses of 100 and 1000 μg of EF-08ODS/head, whereas no significant difference was observed with a dose of 10 000 μg/head [Kruskal-Wallis test and Tukey's multiple comparison (nonparametric) were applied]. It seemed that an optimal dose of EF-08ODS in the experimental system was around 100 μg. In addition, in a wide dose range from 100 to 10 000 μg/head, SM-ODS did not show any adjuvant activity on primary antibody production.

Suppression Assay. Figure 5 showed the enhanced antitumor activity in ICR mice that were treated with EF-08ODS encapsulated in liposomes intraperitoneally. Groups of ICR mice were inoculated on day 0 with 5×10^5 cells of sarcoma 180 and were utilized to assay for an antitumor effect of intraperitoneally administered unencapsulated or liposome-entrapped EF-08ODS. Only mice in liposome-entrapped EF-08ODS-treated group survived longer than the control mice, while animals in other groups showed no life-span prolongation. At the end of 80 days, 37.5% of the animals in the liposome-entrapped EF-08ODS-treated group were still alive as compared to 0% of the mice in the control group. Although empty liposome treatment seemed slightly effective, only one survivor was finally observed on day 40.

In the subcutaneous tumor suppression assay, the SM-

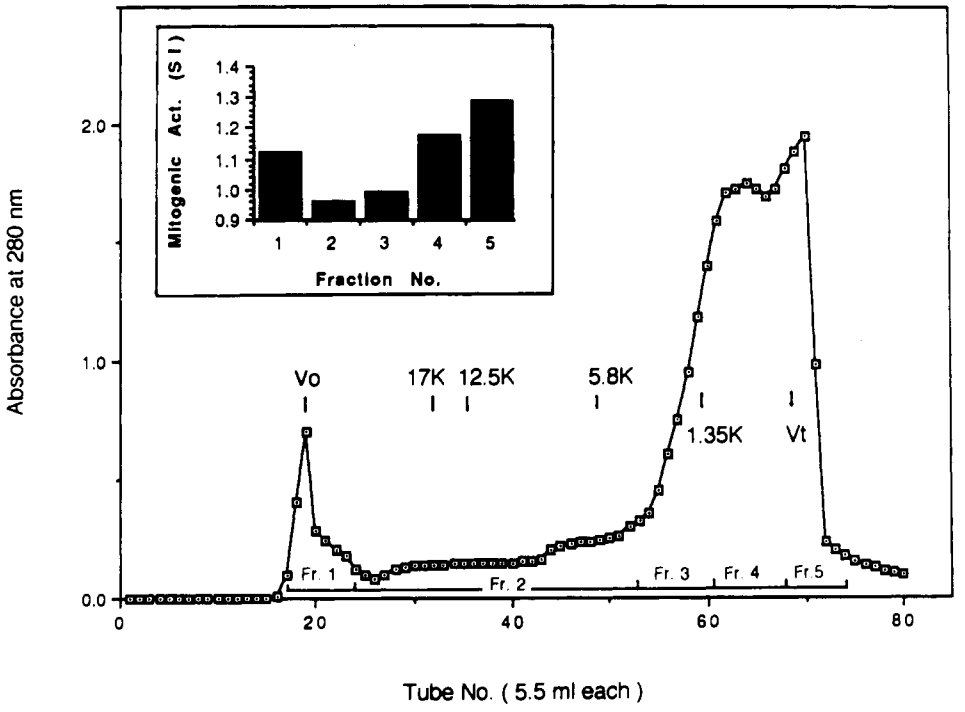

Figure 1. Gel filtration profile of the culture supernatant of *A. oryzae* EF-08 on Sephadex G-50. Each fraction was lyophilized and then subjected to the mitogen assay at a concentration range from 1 to 500 μg/mL. Optimal mitogen response was shown in the inset. Column size: 19.5 × 1000 mm; flow rate, 1 mL/min; mobile phase, water.

Table II. Separation of the Mitogenic Activities in Skimmed Milk Culture Supernatant of *A. oryzae* EF-08 on Waters Preparative C18[a]

fraction	mitogenic activity[b]	optimal dose, μg/mL
eluates	1.26	50–100
washed with water	1.00	none
50% methanol	0.98	none
abs methanol (EF-08ODS fraction)	1.31	25–100
abs ethanol	1.10	50–100

[a] Skimmed milk culture supernatant of *A. oryzae* EF-08 was applied to Waters preparative C18 column (column volume: approximately 70 mL), and batchwise elution was sequentially performed with 200 mL of each solvent. [b] Optimal mitogenic activity was expressed as average SI number for eight wells.

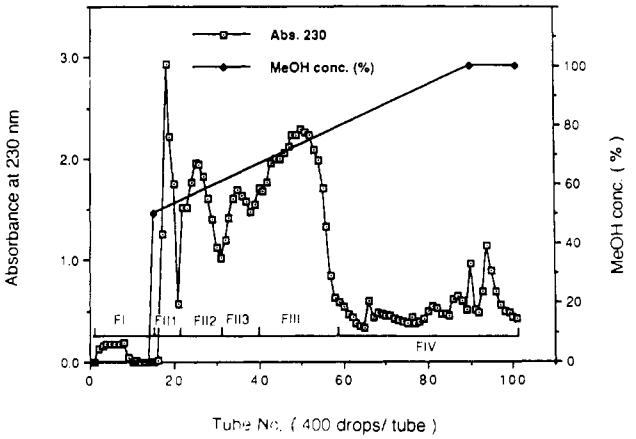

Figure 2. Partition chromatography of EF-08ODS on a Waters preparative C18 column. Each fraction was evaporated under vacuo and then lyophilized to eliminate methanol and subjected to mitogen assay. Resulting FII2 and FIII fractions showed mitogenic activity. Sample size: 400 mg/250 mL of water; column size; 30 × 210 mm; elution, methanol linear gradient (50–98%); flow rate, 3 mL/min.

ODS fraction showed no significant effect on the growth of Meth A fibrosarcoma, while EF-08ODS, at the dose of 500 μg/site, significantly inhibited tumor growth. After 17 days of the tumor transplantation, about 95% of animals were tumor-free in the group (Table V). While direct cytotoxicity of EF-08ODS against the tumor cell was determined by using the MTT assay, no significant cytotoxicity was observed in the concentration range employed in this suppression assay (data not shown).

DTH Reaction. Table VI gives the results of an experiment in which mice were immunized against SRBC in FIA with or without addition of SM-ODS or EF-08ODS, tested 6 days later for DTH by footpad challenge. EF-08ODS stimulated DTH response as compared with that of the control, while SM-ODS showed no stimulation effect.

DISCUSSION

EF-08ODS fraction, which was prepared by using reversed-phase chromatography from skimmed milk culture supernatant of *A. oryzae* EF-08, showed enhancing activity for both host humoral and cellular immunity in experimental animal models. Figure 4 shows a significant adjuvant activity on primary circulating antibody production. Furthermore, fractionated FIII also showed the adjuvant activity, which followed a dose-dependent manner (data not shown). These mitogen-active fractions manifested high adjuvancy on humoral immune response, which is comparable to that of MDP. On the other hand, in vitro, EF-08ODS when applied with or without antigen is similar or less than that of control (Table IV). The reason for this is still unclear, and further investigation is needed to clarify the present phenonena. However, from the results of both EF-08ODS and FIII augmented primary antibody production in vivo, this fact may be related to the mechanisms underlying the activation of antigen-presenting macrophages (Erb and Feldman, 1975). The process relevant to the immunogenic signals that are triggered in the immune systems by these mitogenic fractions is not yet known. Since it was demonstrated class II antigen (Ia) molecule expression and interlukin 1 (IL-1) secretion are necessary for antigen recogition by,

Table III. Amino Acid Compositions of the Mitogen-Active Fractions

amino acid[a]	fraction EF-08ODS	FII2	FIII
Asx (Asp + Asn)	6.13[b]	5.57	5.84
Thr	4.43	5.49	5.60
Ser	3.09	2.85	3.74
Glx (Glu + Gln)	15.14	14.25	15.73
Pro	18.65	20.82	12.08
Gly	1.75	1.65	1.68
Ala	1.36	1.21	0.89
Val	7.64	9.01	7.02
Met	2.55	1.27	1.63
Ile	4.56	4.37	4.26
Leu	7.26	8.64	7.46
Tyr	2.30	1.62	1.47
Phe	3.42	4.92	4.49
Lys	5.84	3.10	3.68
NH_3	1.12	1.56	1.16
His	2.78	1.70	1.90
Arg	1.83	1.40	0.94

[a] Cys and Trp were not analyzed. [b] w/w %.

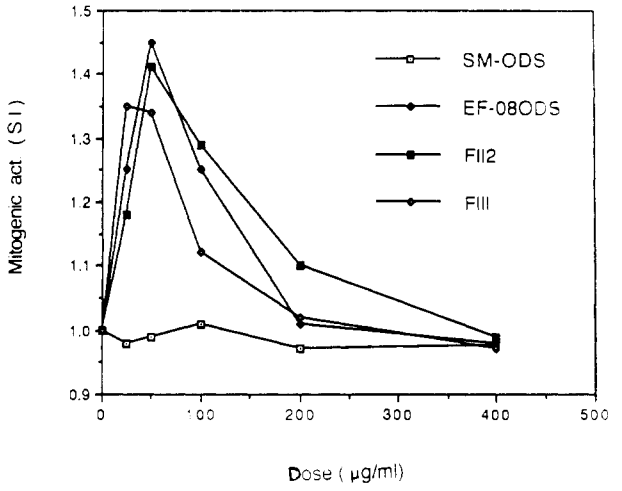

Figure 3. Blastogenic responses by splenocytes from C3H/HeN mice induced by different concentrations of partially purified fractions from skimmed milk culture supernatant of *A. oryzae* EF-08. Each point represents the mean number of SI (eight determinations).

Table IV. Effect of EF-08ODS on Primary and Secondary Anti-DNP-Igs Production in Vitro[a]

adjuvant	dose, μg/mL	antigen[b] without	with
control		0.115 ± 0.033[c]	0.187 ± 0.038
MDP	2.5	0.171 ± 0.038	0.187 ± 0.038
	25	0.220 ± 0.042	0.275 ± 0.060
	50	0.135 ± 0.012	0.221 ± 0.039
EF-08ODS	25	0.105 ± 0.009	0.120 ± 0.035
	50	0.075 ± 0.007	0.085 ± 0.010
	100	0.072 ± 0.006	0.081 ± 0.011

[a] BALB/c female mice were immunized with 100 μg of DNP-OVA and 10^9 of *B. pertussis* vaccine. The sensitized spleen cells were cultured with or without addition of the antigen in the presence or absence of each adjuvant for 7 days. [b] Final concentration of antigen (DNP-OVA) in culture: 5 ng/mL. [c] Anti-DNP-Igs in the culture supernatant were determined by ELISA and the results (A_{405}) are expressed as means ± SD for eight wells.

and for proliferation of, T lymphocytes (Unanue et al., 1984; Mizel et al., 1982), it may be considered that these mitogenic fractions affect the immunological status of macrophages, including their ability to produce cytokines and Ia molecule expression.

In the in vivo tumor suppression assay, intraperitoneal administration of EF-08ODS entrapped in a liposome

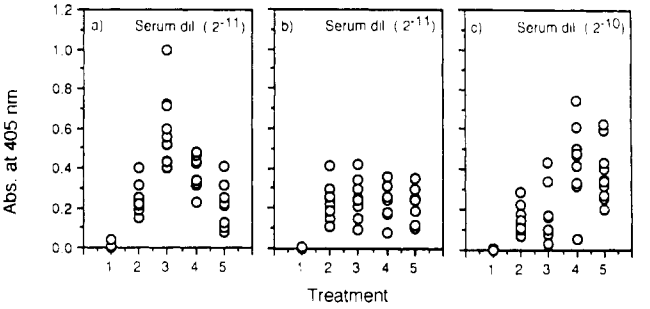

Figure 4. Effects of EF-08ODS and its further fractionated preparations on the primary humoral immune responses in mice. Mice were injected intraperitoneally with 100 μg of DNP-OVA and each adjuvant preparation in emulsion of FIA. Seven days thereafter, anti-DNP-OVA IgG was determined by using ELISA. Serum dilution rate was expressed in each column. (a) Mice (n = 8) immunized with (1) DNP-OVA alone, (2) DNP-OVA/FIA, (3) DNP-OVA/EF-08ODS 100 μg/FIA, (4) DNP-OVA/EF-08ODS 1000 μg/FIA, and (5) DNP-OVA/EF-08ODS 10 000 μg/FIA. Statistical analysis: 3 vs other groups; $p < 0.05$ (Scheffe-type multiple comparison). (b) Mice (n = 10) immunized with (1) DNP-OVA alone, (2) DNP-OVA/FIA, (3) DNP-OVA/SM-ODS 100 μg/FIA, (4) DNP-OVA/SM-ODS 1000 μg/FIA, (5) DNP-OVA/SM-ODS 10 000 μg/FIA. Statistical analysis: No significant difference was observed. (c) Mice (n = 8) immunized with (1) DNP-OVA alone, (2) DNP-OVA/FIA, (3) DNP-OVA/FII1 100 μg/FIA, (4) DNP-OVA/FIII 100 μg/FIA, (5) DNP-OVA/MDP 100 μg/FIA. Statistical analysis: 2 vs 4, 3 vs 4, and 3 vs 5, $p <$ 0.05; 2 vs 5; $p < 0.01$ (Tukey-type multiple comparison). No significant difference was observed.

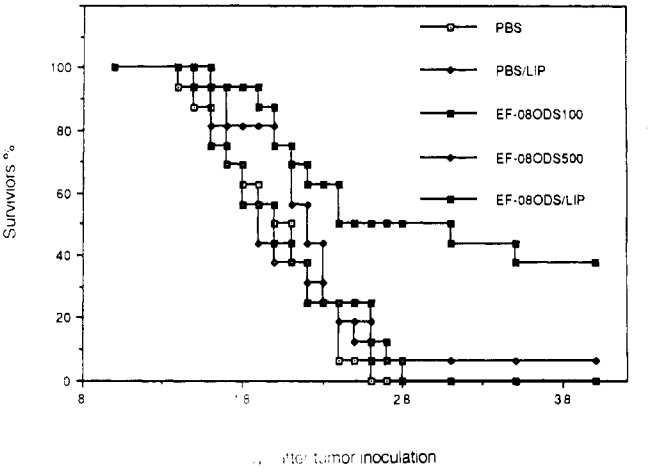

Figure 5. Effect of MLV liposome-entrapped EF-08ODS fraction on survival of ICR male mice bearing ascite-type tumor. Groups were treated intraperitoneally with encapsulated EF-08ODS (100 μg, EF-08ODS/LIP), empty liposome (PBS/LIP), 100 and 500 μg of free EF-08ODS (EF-08ODS (EF-08ODS100 and EF-08ODS500, respectively), or PBS. Encapsulated EF-08ODS recipients survived longer than mice in other treatment groups. Schedule of treatment was described under Materials and Methods.

exerted antitumor activity. However, free EF-08ODS showed no significant effect. Recently much attention has been focused on the use of liposomes as carriers of macrophage-activating agents to cells of the reticuloendotherial system in vivo. In fact, Fidler et al. (1980, 1981a,b) have demonstrated that synthetic MDP or lymphokines encapsulated in liposomes are far more effective than the free compounds in rendering murine alveolar macrophage tumoricidal and in the treatment of pulmonary micrometastasis in mice. The present findings that EF-08ODS encapsulated in MLV liposomes could render the antitumor activity of tumor-bearing mice much more effectively than the free EF-08ODS may be closely associated with these observations. It is conceivable that

Table V. Effects of EF-08ODS and SM-ODS on Suppression of Syngenic Tumor Growth[a]

fraction tested	dose, μg/site		
	10	100	500
control 2/24			
SM-ODS	0/24[b]	1/24	2/24
EF-08ODS	0/24	2/24	23/24[d]
MDP	ND[c]	0/24	ND

[a] Direct cytotoxicity was not found in this dose range (in vitro MTT assay). Meth A fibrosarcoma (1×10^5) was inoculated with or without addition of effector subcutaneously to BALB/c mice. Seventeen days after the transplantation, tumor-rejected mice were counted. [b] Number of tumor-rejected mice over total tested. [c] Not detected. [d] Significantly different from control and SM-ODS(500 μg)-treated groups.

Table VI. Activities of EF-08ODS and SM-ODS on DTH Reactions in Mice Immunized against SRBC in the Presence of FIA[a]

fraction	no. of animals	dose, μg/head	DTH reactions: foot pad swelling (mean ± SD), mm	DTH reactions: % increase	P
control	15		0.91 ± 0.24	0	
SM-ODS	16	100	0.85 ± 0.21	−0.76	NS[b]
EF-08ODS	16	100	1.09 ± 0.18	21	<0.05

[a] Statistical analyses were performed by one-way analysis of variance and Tukey's multiple comparison procedure. Probabilities (*P*) were expressed against control group. [b] Not significant against control.

immunostimulatory effects of these mitogenic fractions are achieved through activation of macrophage which contribute antigen presentation to helper T cells (Shevach et al., 1973; Erb and Feldman, 1975) and expression of their own cytotoxicity against tumor cells (Pace and Rusell, 1981). However, further detailed examinations are needed to clarify the mode of action of these mitogenic fractions.

We have not examined whether or not immunopotentiating activities of these mitogenic fractions are even expressed following their oral administration. Banks et al. (1988) reported tuftsin administered orally exerted a significant immunopotentiating effect in tumor-bearing C57BL/6 mice, so that it caused prolongation of their average life span. In the course of studies of Bestatin, Ishizuka et al. (1980a) also reported that it affected host immune system in an animal model system by its oral administration. One of our interests is focused on studying the effects of these mitogenic fractions on the host immunological status following their oral administration.

This work strengthens the possibility that *A. oryzae* EF-08 processes milk constituents to form immunoactive substances during its cultivation, including peptides (Nishioka et al., 1973; Starosik et al., 1983; Jollès et al., 1982; Parker et al., 1984; Gattegno et al., 1988), and the immunoactive substances are capable of augmenting both humoral and cellular immunities of the host following systemic administration. However, purification, characterization, and structural studies remain to be performed and are now in progress.

ABBREVIATIONS USED

DTH, delayed-type hypersensitivity; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; TTBS, Tris-buffered saline containing 0.05% Tween 20; Ig, immunoglobulin; FCS, fetal calf serum; 2ME, 2-mercaptoethanol; MDP, muramyl dipeptide; BSA, bovine serum albumin; DNP, dinitrophenyl; FIA, Freund's incomplete adjuvant.

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