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(54) Title: FRACTION OBTAINED FROM LISTERIA MONOCYTOGENES HAVING THERAPEUTICAL ACTIVITY, PROCESS FOR ITS PREPARATION AND PHARMACEUTICAL COMPOSITIONS CONTAINING IT

(57) Abstract

A fraction isolated from the fermentation product of Listeria monocytogenes strain L 79 is endowed with anti-tumoral, immunomodulating and interferon producing activity. This fraction is prepared by isolation from the fermentation broth of Listeria monocytogenes.

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Fraction obtained from Listeria monocytogenes having therapeutical activity, process for its preparation and pharmaceutical compositions containing it

The present invention relates to a novel therapeutically active fraction, isolated from the fermentation broth of Listeria monocytogenes strain L 79, to the related process of preparation and to the pharmaceutical compositions containing this fraction as active ingredient.

The mononuclear phagocytary system is an important element of the immunitary defences of the host against neoplasiae (D.O. Adams and R. Snyderman, J. Natl. Cancer Inst., 1979, 16, 1341; J.B. Hibbs, H.A. Chapman, and J.B. Weinberg, J. Reticuloendothel., 1978, 24, 549; D.S. Nelson, E.K. Hopper and M. Nelson, Handbook of Cancer Immunology Vol. I, H. Waters ed., p. 107, Garland Publishing Inc. New York, 1979).

The macrophages may exercise a relevant cytotoxic activity against the tumoral cells, in vitro, if an activation takes place, which can be originated from several biological stimuli (R. Keller, Immunobiology of the Macrophage, D.S. Nelson ed., p. 487, Academic press, New York, 1976; L.P. Ruco and M.S. Meltzc, Cell. Immunol., 41, 35, 1978).

Several microorganisms, among which Mycobacterium bovis (R.C. Bast, B. Zbar, T. Borsos and al., N. Engl. J. Med., 290-1413-1420; 1458-1469, 1974), Corynebacterium parvum (G. Mathé, P. Puillart and L. Schwarzenberg., Natl. Cancer Inst. Monogr., 35, 361, 1972; L. Israel and B. Halpern, Nouv. Presse Med., 1, 19, 1972), Corynebacterium granulosum (G. Mathé, ibid) and Listeria monocytogenes (S. Youdim, M. Moser and O. Stutman, J. Natl. Cancer Inst., 52, 193, 1974) show remarkable effects of activation of the normal or depressed macrophages.

Moreover, R.C. et al (J. Natl. Cancer Inst., 54 (3), 749, 1975), described how several microorganisms, among which Listeria monocytogenes, stimulate the immunodefensive response of the host and may inhibit the growth of the tumor.

As a matter of fact, substances of bacterial origin and having immunostimulating properties where already previously isolated from microorganisms of the family of the Corynebacterium, these substances being insoluble in aqueous medium and poorly chemically characterized and being consequently of difficult industrial and therapeutical use.

As an example of the state of the art prior to the present invention there can be cited:

- A.R. Prevot, B.N. Halpern, F. Biozzi, C. Stiffel, D. Mouton, J.C. Morand, Y. Bouthillier and C. Decreusefond (C.R. Acad. Sci., Paris, 1963, 257 series D, 13) described the immunostimulating and anti-tumoral properties of some microorganisms of the Corynebacterium anaerobies species.

A number of subsequent studies where oriented towards the isolation of the active fraction among which, D. Migliore-Samour and P. Jolles (Febs Letters, 1972, 25 (2), 301) disclosed the isolation of a hydrosoluble fraction capable of maintaining the adijuvating properties of the enteric microorganism.

On the other side, P. Lallonette, B. Bizzini and M. Raynaud (N.G. M. 1975, 25, 13) by using the process of Migliore-Samour and Jolles in the fractionating of Corynebacterium granulosum, found that the adjuvating and inmunostimulating activity was associated to an insoluble fraction (bearing the abbreviation P 40) and not to the hydrosoluble fraction (bearing the abbreviation S 70).

Such an insoluble fraction characterized from the chemical and biological point of view by B. Bizzini, B. Maro and J. Lallonette (in Medicine et Maladies infectieuses, 78, n. 9 Vol. VIII, Pages 408-414) revealed a particularly interesting therapeutical activity with respect to the tumor P 815 in the mouse.

Moreover, P. Lallonette, B. Maro, A. Schwartz and B. Bizzini (C. R. Acad. Sci., Paris 1976), still for the insoluble fraction P 40, revealed the capacity of restoring and increasing the immunogenic properties of forming anti-bodies anti-red cells of ram in the depressed mouse by injection of cyclophosphamide.

Later on, in order to have a complete picture of the prior art, T. Metianu and B. Bizzini (French Patent Application No. 8008976, 1980) found a novel fraction extracted from aerobical bacteria and endowed with anti-tumoral, anti-bacterial and interferon production inducing properties,

and the related preparation method.

On the other side, said fraction too, obtained from Corynebacterium catarrhalis, is also insoluble in aqueous medium.

In view of the already achieved knowledge of the possible influence of Listeria Monocytogenes in the immunodefensive processes and on the basis of the hypothesis that the same microorganism may contain or induce the production of active principles capable of activating the immunitary system, the main purpose of the present invention has been just the search and isolation of such an active principle which at the same time would be endowed with well defined chemical and chemico-physical properties and would be reproducible on industriale scale.

Another purpose of the present invention is that of individuating an active principle such as that indicated at preceding paragraph which is also soluble in aqueous medium with the attendant relevant advantages of industrial and therapeutical use.

As already mentioned, the active fraction forming the subject of the present invention, (which is hereinafter indicated for easy use as LM84) has been obtained from a strain of Listeria Monocytogenes L 79 individuated as to the type at the Institute of Microbiology of the University of Messina and corresponding to the following specifications:

Listeria monocytogenes strain L 79 isolated from pathological material and individuated as to the type at the Institute of Microbiology of Messina.

- short rod, Gram-positive, asporigen, aerobical or microaerobical; at 20-25°C, seldom at 37°C, shows a tumultuous rotatory mobility which is fully characteristic;

- katalase	+
- metachromatic granules	-
- haemolysis	+ (beta)
- production of acid from:	
glucose	+
lactose	-

maltose	+
mannitol	-
salicine	+
saccharose	-
threolose	+
xylose	-
dulcitol	-
- UVP	+
- hydrolysis of esculin	+
- nitrate reduction	-
- gelatin liquefaction	-
- urease	-
- hydrolysis of arginine	-
- Simmons citrate	-
- phenylalanine deaminase	-

- carrier of the prophage LM 79.

There is firstly described the method of preparation of the subject active fractions.

Culture a Medium:

Bacto-Nutrient broth dehydrated (Difco)	8 g
distilled water	1000 ml

The pH is adjusted to 7.2 and the medium is sterilized for 15 minutes at 120°C; there is added the 30% (w/v) glucose solution, sterile, at the final concentration of 0.2%.

Culture

The lyophilic strain of Listeria monocytogenes L 79 is taken in 2 ml of culture medium. The bacterial suspension is transferred in 4 tubes containing 20 ml of culture medium (0.5 ml/tube).

Upon the bacteria started the active multiplication, the content of each tube is transferred to an Erlenmeyer flask containing 500 ml of culture medium.

The culture developed in each flask is used for the seeding of a 5 lt big

flask (usually a 15 hours culture is used as inoculum).

The culture is interrupted after 48 hours; at that time the bacteria are deposited at the bottom of the big flask.

The supernatant liquid is removed by siphoning; the bacteria are collected by centrifugation and then suspended again in distilled water, decanted again and washed a second time always with distilled water and subsequently centrifuged.

Preparation of the fraction LM 84

On the washed bacteria (about 1.9 g/lt of culture) a delipidation is carried out with a Soxhlet equipment by carrying out alternatively the following treatments:

a) 12 hours with ether-ethanol (1:1), then for 12 hours with chloroform and then for further 12 hours always with chloroform mixed with methanol (2:2).

b) three subsequent treatments with acetone, each having a duration of 12 hours.

The delipidated bacteria are disintegrated in a mechanical Potter homogeneizer (10 times for 1 minute at maximum speed; cooling in ice).

The suspension of disintegrated bacteria is maintained under constant stirring for 5 hours at room temperature before being centrifuged at 2000 x g in order to eliminate the intact bacteria.

The supernatant is heated to 80°C before being adjusted at the concentration of 40% saturation by addition of saturated solution of ammonium sulphate (or, alternatively, with mono- and di-potassium phosphate buffer 1.25 M).

After one night at 4°C, the thus formed precipitated is collected by centrifugation at 10000 g.

The precipitate is suspended again in distilled water and dialized against distilled water until the presence of ammonium sulphate (or alternatively of potassium phosphate) is no longer detectable.

The obtained fraction (about 6 mg/g of delipidated bacteria) corresponding to a fraction insoluble in aqueous medium, is lyophilized.

After lyophilization it is solubilized in the presence of urea at the concentration 6M (or alternatively in the presence of sodium hydrogen carbonate).

In the case in which the solubilization is carried out with urea, a subsequent dialysis against distilled water is carried out until any traces of urea are totally eliminated.

The fraction remains soluble in aqueous medium even after elimination of the solubilizing medium.

Then the purification of the active substance is carried out by chromatography in a column against Tris buffer on Sepharose 6 B or alternatively on Ultragel Ac A 34.

The results of the analysis as thereinafter reported represent the average values obtained on a total number of 10 batches obtained with the process of the extraction and purification as above described.

Protein content

The dosage, carried out according to the method of Lowry, gave a protein content of 0.75 $\pm$ 0.1 mg/mg of active fraction LM 84.

Moisture content

up to 10%

Ashes

from 0.05 to 0.08 mg of ashes/mg of LM 84 (dried weight).

Neutral sugars (anthrone method)

from 0.05 to 2% of neutral sugars/mg of LM 84.

Hexosamines (Elson Morgan method)

from 0.6 to 1.8% of hexosamines/mg of LM 84.

Molecular weight

The molecular weight has been determined by column chromatography on Ultragel AcA 34 set with the following reference substances: IgG, bovine sieroalbumin, egg albumin, myoglobine (kit of Pharmacia).

The fraction is eluted in form of a unique peak slightly asymmetrical in the descending side.

The molecular weight is between 290.000 and 330.000 Daltons (average

value: 300.000 Daltons)

Determination of the aminoacidic composition by aminoacid analyzer

Amminoacid	µg	%	numer of AA residues per molecule
Asp	57.76	6.49	155
Thr	21.32	2.40	64
Ser	22.49	2.53	77
Glu	193.45	21.73	470
Pro	36.25	4.07	113
Gly	18.69	2.10	89
Ala	58.35	6.56	234
Val	26.59	2.99	81
Cys+	-	-	-
Met	5.97	0.67	14
Ile	29.78	3.35	81
Leu	63.60	7.15	174
Tyr	17.21	1.93	34
Phe	12.06	1.36	26
Lys	93.20	10.47	228
His	11.15	1.25	26
Arg	39.25	4.41	81
DAP	153.00	17.19	288
Total	860.12	96.65	2237

Cys+ has been determined in form a CM-Cys.

The determination of the aminoacid composition has been carried out in three fold way by means of an automatic analyzer Beckman model Multichrom B 4255 according to the method of Spackman (Anal. Chem. 1958, 30, 1190) on an acid hydrolisate of 24 hours.

The methionine has been determined according to the method of Moore (J. Biol. Chem; 1963, 238, 235).

Tryptophan has been determined according to the colorimetric method of Gaitonde and Dovey (Biochem. J., 1970, 117, 907).

Analysis of the pharmacological and biological properties of fraction LM 84

Determination of the toxicity properties

The administration of the substance in the mouse by subcutaneous, intramuscolar and intravenous route, up to dosages of 1 mg/mouse (corresponding to 50 mg/kg of body weight) do not induce any toxic phenomena.

Analysis of the effects on the growth of tumoral mass and inhibiting the metastasis forming

T8 of Guérin, an atypical transplantable epithelioma has been used. The tumor has been transplated in rats, Norvegicus strain, every 20 days in the dorsal subcutaneous area; in a second experiment for the control of the influence of the metastasis formation, the transplantation was carried out in the same area daily.

The fraction LM 84 has been administered in single dose by intravenous route (200 μg/rat) three days after the transplantation of the tumor. As demonstrated by the growth curve of the tumor T8 Guérin in the rat (both treated and non treated), the growth of the tumor T8 is significatively inhibited by a single intravenous administration of the compound.

The development of the metastasis was fully inhibited by the treatment with the compound; the average weight of the metastasis in the non-treated animals 20 days after the transplantation, was 3.196 ± 164 mg.

Phagocytosis and intracellular killing of Candida Albicans

For the test of phagocytosis and of killing of Candida Albicans by the peritoneal macrophages the micromethod of Smith and Rommel was used (J. Immunol. Methods, 1977, 17, 241).

The phagocytic activity of the peritoneal macrophages of the non treated, tumor carrying rats, was remarkably depressed with respect to the control

animals; the intracellular killing of C. Albicans in said animals was unchanged.

On the contrary, in the following table 1, it is demonstrated that the phagocytic activity of the macrophages of tumor carrying rats and treated with the fraction LM 84 is restored to higher values with respect to those of the control macrophages.

In the same table it can be noted that the intracellular killing activity is significatively influenced by the treatment with the fraction LM 84 but at a lower degree of the phagocytic activity ($p < 0.001$).

TABLE 1

Effect of the intravenous administration (only one dose of 200 /ug/rat) of the fraction LM 84 on the phagocytosis and on intracellular killing of Candida Albicans in the tumor carrying rat.

	Phagocytosis %	Killing %
Control rat	60 ± 6[a]	21 ± 4
Tumor carrying rat, non treated	30 ± 5	18 ± 2
Tumor carrying rat, treated with LM 84	94 ± 9[a]	24 ± 2.4[b]

The results are expressed as the average of 5 experiments, ± the standard error;

a= significant difference ($p < 0.0001$) with respect to the tumor carrying rats non treated;

b= significant difference ($p < 0.001$) with respect to the tumor carrying rats non treated.

Effect of the treatment with fraction LM 84 on the chemotactic response of the peritoneal macrophages.

The serum of rat activated with endo-toxin has been prepared by incubation of normal serum with 1 mg/ml of endotoxin from Escherichia coli.

The method used has been that disclosed by Maderazo and Waronick (A modified micropore filter assay of human granulocyte chemotaxis, in: Quie PG,

Gallin JL eds., Leukocyte chemotaxis, Raven Press, New York, 1978, p. 43).

All the experiments have been carried out in three fold manner and the avarage chemiotactic index (CI) and the migration distance have been calculated.

The chemotactic response of peritoneal cells of tumor carrying rats was remarkably depressed with respect with the control macrophages.

Both the chemotatic index and the average distance as /u of migration of cells were significatively reduced in the tumor carrying animals with respect to the control values.

Table 2 demonstrates that the chemotactic response of the peritoneal cells obtained in the animals treated with fraction LM 84 is significatively developed ($p < 0.0001$).

TABLE 2

Effect of the intravenous administration (200 /ug/rat) of fraction LM 84 on the chemotactic response of the peritoneal macrophages in the tumor carrying rats.

	Chemotactic index	Migration front
Control rats	45.25 ± 5.2^{a}	118 ± 9^{b}
Tumor carrying rat, non treated	31.50 ± 2.8	58 ± 6.1
Thumor carrying rat treated with LM 84	50.00 ± 5.0^{b}	122 ± 10.2^{b}

a= significant difference ($p < 0.001$) with respect to the tumor carrying rats non treated;

b= significant difference ($p < 0.0001$) with respect to the tumor carrying rats non treated.

Effect on the treatment with fraction LM 84 on the intrinsic activity anti-HSV-1 of the peritoneal macrophages.

The effect has been tested with the method described by Bonina at al. (Infect Immun., 1983, 39, 575).

The intrinsic activity anti-HSV-I of the peritoneal macrophages has been evaluated, for several groups of rats, by analyzing the development curves of HSV-I.

The following table III confirms that the production of HSV-I after 24 hours of incubation was higher in the macrophages obtained from tumor carrying rats ($p < 0.002$) than in the macrophaes collected from the control rats or from the tumor carrying rats but treated with the fraction LM 84.

On the contrary no statistical difference exists between the content of HSV-I of the macrophages of tumor carrying rats and treated with LM 84 in comparison with the control macrophages.

TABLE III

Intrinsic activity anti HSV-I of macrophages of tumor carrying rats treated with LM 84 in only one dose by intravenous route (200 µg/rat)

		Production of HSV-I in 24 hours (PFU/ml)
control rats	MOI 1	$2 \times 10^5 \pm 1 \times 10^2$
	MOI 10	$4 \times 10^5 \pm 2 \times 10^2$
Non treated tumor carrying rats	MOI 1	$8 \times 10^6 \pm 3 \times 10^3$
	MOI 10	$2 \times 10^5 \pm 2 \times 10^3$
Tumor carrying rats treated with LM 84	MOI 1	$1 \times 10^5 \pm 2 \times 10^2$ a
	MOI 10	$1 \times 10^5 \pm 1 \times 10^2$ a

The results are expressed as the average of the values of 5 experiments.

a= $p < 0.002$ in comparison with the tumor with the carrying rats non treated.

Effect of the treatment with fraction LM 84 on the extrinsic activity anti-HSV-I of peritoneal macrophages.

By extrinsic activity the mechanism is meant by which the macrophages may inhibit the multipling of virus in permitting neigh bouring cells.

This activity has been tested according to the method disclosed by Wildy et al (Infect Immun. 1982, 37, 40).

The extrinsic activity anti-HSV-I has been expressed in terms of reduction of growth of the virus at the use concentration of arginine (Bonina at al, Virus Res., 1984, 1 501).

As demonstrated by the following table IV the growth of HSV-I as obtained on cells Vero was not statistically different in the case of cells incubated with peritoneal cells of tumor carrying rats or of control rats. On the contrary, the growth of HSV-I on Vero cells cultivated together with different concentrations of peritoneal cells of tumor carrying rats treated with the fraction LM 84 was significatively different ($p < 0.002$; $p < 0.001$) with respect to the values obtained with macrophaes of tumor carrying rats non treated and with macrophages of control rats.

TABLE IV

Extrinsic activity anti-HSV-I of macrophages of tumor carrying rats treated with LM 84

	Ratio macrophages /Vero cells	Extrinsic activity (a)
Control rats	5/1	$6 \times 10^6 \pm 2 \times 10^2$
	10/1	$2 \times 10^6 \pm 2 \times 10^2$
Tumor carrying rats non treated	5/1	$9 \times 10^6 \pm 1 \times 10^2$
	10/1	$6 \times 10^6 \pm 3 \times 10^2$
Tumor carrying rats treated with LM 84	5/1	$1 \times 10^5 \pm 6 \times 10^3$ b
	10/1	$1 \times 10^4 \pm 2 \times 10^2$ c
Vero cells		$2 \times 10^7 \pm 6 \times 10^2$

a= the results are expressed as growth of the virus after 24 hours incubation: average of 4 experiments;

b= significant difference ($p < 0.002$) with respect to non treated carrier

c= significant difference ($p < 0.001$) with respect to the values of non treated carriers.

Response of the intravenous treatment of LM 84 (200 µg/kg/die for 7 days) to the administration of ram red cells (GRM)

The haemoagglutinins have been dosed in the Swiss mouse according to the method of Biozzi et al. (Ann. Inst. Pasteur, 1966, 110, suppl. 3, 7-32); the reading has been carried out according to the technique on microplate (Raynaud et al. Ann. Inst. Pasteur, 1972, 122, 695).

The ram red cells have been administered at the dose of 5×10^6 cells.

From the variation of haemoagglutinating concentration as a function of the time it can be noted, in the long run, that the haemoagglutinating concentration is maintained for the control animals on very low values.

On the contrary in the animals treated with the fraction LM 84, the concentration is quickly increased achieving the maximum value at about 1 week from the injection of GRM.

Determination of the interferon inducing activity.

The experiments have been carried out in Swiss mice of the weight of 20g; the product has been administered at dose of 200 µg by intravenous route with a volume of 0.5 ml, whereas the control animals were administered with an equal volume of physiological solution.

The blood abstraction for the determination of the concentration were carried out at 1,2,4,8,24 and 48 hours from the administration of the compound.

The determination of interferon has been carried out according to the method of inhibition of the cytopatic effect by using cells lines L929 and the virus of vescicular stomatities as competitive virus (Metianu et al. Comp. Immun. Microbiol. Infect. Dis, 1981, 4, 24).

The concentration of interferon was adjusted as units with reference to a standard serum.

The results are shown in the following table V:

TABLE V

Interferon inducing activity of intravenous treatment with LM 84

Interferon in international units

	Hours					
	1	2	4	8	24	48
LM 84 (200 ugiv)	100	400	400	300	200	100
carrier	3	3	3	3	3	3

From the above tests and having a specific purpose and validity and which are not generical ones it can be concluded that the fraction abbreviated as LM 84 and forming the subject of the present invention is capable of:

- inhibiting the growth of the tumoral mass and the metastasis formation;
- having immunostimulating effect (stimulation of the phagocytary activity); increase of the killing (activation of the microbicidal activity of the macrophage); restoring of the anti-viral activity of macrophages;
- inducing the interferon production;
- enhacing the anti-body production.

Consequently it can be therapeutically used in the congenic forms, in the infections and generally in all pathological forms involving a compromission of the immunodefending system.

From the experimental condition and from the properties of the substance it is also possible to realize the administration routes (intramuscolar and intravenous) and the dosages (from 50 to 400 γ in only one administration or periodical cycles of 1-3 weeckly administrations according to the specific pathology) to be adopted in the clinical use of the substance itself.

CLAIMS

1. Active fraction, obtained from a culture of Listeria monocytogenes strain L 79, by means of delipidation of the bacteria, their disintegration, precipitation by means of an agent selected among ammonium sulphate and phosphate buffer, dialysis against distilled water, lyophilization of the insoluble fraction, solubilization in the presence of a compound selected among urea and sodium acid carbonate and lastly chromatographic purification.

2. Active fraction according to claim 1, characterized by having the following analitical characteristics:

- protein content according to Lowry: 0.75 ± 0.1 mg/mg of active fraction;
- moisture content: up to 10%;
- ashes: 0.05-0.08 mg/mg of dry weight of fraction;
- neutral sugars (anthrone method): 0.5-2% of neutral sugars/mg of active fraction
- hexosamine (Elson-Morgan method): 0.6-1% of hexosamine/mg of active fraction;
- molecular weight: between 290,000 and 330,000 Daltons, with an average value of 300,000

3. Active fraction according to claim 2, characterized by having the following aminoacidic composition, referred to a 24 hours hydrolisate:

Amminoacid	/ug	%	% number of AA residue per molecule
Asp	57.76	6.49	155
Thr	21.32	2.40	64
Ser	22.49	2.53	77
Glu	193.45	21.73	470
Pro	36.25	4.07	113
Gly	18.69	2.10	89
Ala	68.35	6.56	234
Val	26.59	2.99	81
Cys+	-	-	-
Met	5.97	0.67	14
Ile	29.78	3.35	81
Leu	63.60	7.15	174
Tyr	17.21	1.93	34
Phe	12.06	1.36	26
Lys	93.20	10.47	228
His	11.15	1.25	26
Arg	39.25	4.41	81
DAP	153.00	17.19	288

4. Active fraction according to any of the claims 1 to 3, characterized by being capable of inhibiting the growth of tumoral masses and the forming of metastasis.

5. Active fraction according to any of the claims 1 to 3, characterized by possessing immunomodulating activity deriving from the stimulation of the phagocytary activity, by activating the microbicidal activity of the macrophages and by restoring of the antiviral activity of the macrophages.

6. Active fraction according to any of the claims 1 to 3, characterized by being capable of inducing the production of interferon.

7. Active fraction according to any of the claims 1 to 3, characterized by being capable of potentiating the antibody production.

8. A process for the preparation of the active fraction according to the preeceding claims, characterized by the steps of:

- cultivating Listeria monocytogenes, strain L 79, in a culture medium;
- collecting the resulting bacteria;
- delipidating the bacteria;
- disintegrating the bacteria;
- precipitating an insoluble fraction with an agent selected among ammonium sulphate and phosphate buffer;
- dializing against distilled water;
- lyophilizing the insoluble fraction;
- solubilizing the lyophilisate in the presence of an agent selected among urea and sodium acid carbonate, thus giving a fraction soluble in aqueous medium;
- purifying by chromatography.

9. A process according to claim 8, characterized in that said Listeria monocytogenes strain L 79 has the following characteristics:

- short Gram-positive rod, asporigen, aerobial or microaerobial; at 20 to 25°C, seldom at 37°C, shows a fully peculiar tumultuous rotatory mobility;

catalase	+
metachromatic granules	-
haemolysis	+ (beta)
- acid production from	
glucose	+
Lactose	
maltose	+
mannitol	-
solicine	+
saccharose	-
threolose	+

xylose	-
dulcitol	-
UVP	+
- esculin hydrolysis	+
- nitrate reduction	-
- gelatin liquefaction	-
- urease	-
- Simmons citrate	-
- phenylalanine deaminase	-
- carrier of LM 79 prophage	

10. A process according to claim 8, characterized in that said delipidation is carried out in a Soxhlet apparatus, by alternated treatments with

a) ether-ethanol (1:1) for 12 hours

b) chloroform for 12 hours

c) chloroform in admixture with methanol (2:1) for 12 hours;

d) acetone for three consecutive times, each having a duration of 12 hours.

11. A process according to claim 8, characterized in that said disintegration is carried out in a mechanical homogeneizer with simultaneous cooling.

12. A process according to claim 1, characterized in that the suspension of disintegrated bacteria is centrifugated to remove the intact bacteria.

13. A process according to claim 12, characterized in that the supernatant layer of the centrifuging step is heated to 80°C and adjusted to a 40% saturation concentration by addition of a saturated solution of ammonium sulphate, and is maintained for a night at 4°C, the precipitate being thereafter recovered by centrifugation.

14. A process according to claim 13, characterized in that instead of ammonium sulphate a 1.25 M buffer of mono- and bi-potassium phosphate is used.

15. A process according to the claims 13 or 14, characterized in that the recovered precipitate is suspended in distilled water and depurated from

the ammonium sulphate or phosphate buffer, a fraction insoluble in aqueous medium being obtained.

16. A process according to claim 15, characterized in that said depuration is carried out by dialysis against distilled water.

17. A process according to claim 15, characterized in that said insoluble fraction is lyophilized.

18. A process according to claims 8 and 17, characterized in that said lyophilizate is solubilized in the presence of 6M urea or of sodium acid carbonate, a dialysis against distilled water being thereafter carried out in the case of solubilization in the presence of urea.

19. A process according to claims 8 and 18, characterized in that said solubilized fraction is purified by column chromatography.

20. Pharmaceutical composition characterized by containing, as the active ingredient, the active fraction according to claims 1 to 3.

21. Pharmaceutical composition according to claim 20, characterized by being in form suitable for the administration by intramuscular and intravenous route.

22. Pharmaceutical composition according to claim 20, characterized by containing 50–100 γ of said active fraction.

23. Pharmaceutical composition according to each of the claims 20 to 23, useful in the treatment of the congenic forms.

24. Pharmaceutical composition according to each of the claims 20 to 23, useful in the treatment of infections pathologic states of microbial and viral origin.

25. Pharmaceutical composition according to each of the claims 20 to 23, useful in the treatment of the pathological states wherein the immunodefensive system is affected.

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 86/00679

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: A 61 K 35/74; A 61 K 39/39; C 07 K 15/00; // C 12 P 21/02

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	A 61 K; C 12 P

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	FR, A, 2293213 (J. HESSE) 2 July 1976 see page 1, lines 10-15; page 3, lines 21-31; claim 1 --	1-25
Y	FR, A, 2480604 (INSTITUT PASTEUR) 23 October 1981 see page 1, lines 1-4; page 5, line 38 - page 6, line 13; page 7, lines 1-28; claims 10,11 cited in the application --	1-25
A	Cancer Immunology Immunotherapy, volume 17, no. 1, 1984, Springer Verlag, D. Iannello et al.: "Inhibition of normal rat macrophage functions by soluble tumor products. Effect of systemic treatment with bacterial immunomodulators", pages 38-41 see the whole article --	1-25
A	Chemical Abstracts, volume 97, no. 23, 6 December 1982, (Columbus, Ohio, US), see page 454, abstract 196828w, ./.	1-5,8-25

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
10th April 1987	29 APR 1987
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	M. VAN MOL

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	& JP, A, 82139019 (Y. YAMAMURA et al.) 27 August 1982	
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A	FR, A, 2164510 (M. FAUVE) 3 August 1973 see page 1, lines 1-8, 23-33, and line 37 - page 2, line 14; page 2, lines 26-32; page 3, line 40 - page 4, line 14; claims 1,2,6,12-14	1-3,5,8-22, 24,25
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A	Can. J. Microbiol., volume 28, no. 12, 1982, National Research Council of Canada, (CA), T.V. Otokunefor et al.: "Immunosuppression, nonspecific B-cell activation, and mitogenic activity associated with a high molecular weight component from Listeria monocytogenes", pages 1373-1381 see the whole article	1-3,5,8-22, 24,25
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A	Infection and Immunity, volume 39, no. 3, March 1983, American Society for Microbiology, (US), N.W. Hether et al.: "Chemical Composition and biological functions of Listeria monocytogenes cell wall preparations", pages 1114-1121 see page 1114, left-hand column, lines 3-15; page 1116, table 2, right-hand column, lines 18-19; page 1117, left-hand column, line 9 - right-hand column, line 5; right-hand column, lines 15-18; page 1118, left-hand column, lines 14-24; right-hand column, lines 21-33; page 1119, table 10; page 1120, left-hand column, lines 23-44	1-5,8-25
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A	Cellular Immunology, volume 88, no. 1, 1984, Academic Press, Inc., A. Nakane et al.: "The significance of alpha/beta interferons and gamma interferon produced in mice infected with Listeria monocytogenes", pages 29-40 see the whole document	1-3,6,8-22

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON

INTERNATIONAL APPLICATION NO. PCT/EP 86/00679 (SA 15671)

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 22/04/87

The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
FR-A- 2293213	02/07/76	None	
FR-A- 2480604	23/10/81	BE-A- 888430	15/10/81
		US-A- 4479935	30/10/84
FR-A- 2164510	03/08/73	NL-A- 7217588	26/06/73
		DE-A,B,C 2262427	12/07/73
		BE-A- 793260	22/06/73
		GB-A- 1419969	31/12/75
		CA-A- 991543	22/06/76
		JP-A- 48068729	19/09/73
		US-A- 4180563	25/12/79