# Experimental and Clinical Results with the *Corynebacterium granulosum*-derived Immunomodulator P40*

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The demonstration that suspensions of heat-killed whole cells of anaerobic corynebacteria are capable of stimulating the natural defenses of an organism has aroused great interest during the two last decades. However, it is possible that the complexity of the intact bacterial cell might obscure the interpretation of experimental data. Furthermore, the high probability that administration of whole bacterial cells to man would elicit untoward reactions could limit their use in human therapy. As a result, many recent investigations have been aimed at isolating fractions from whole bacterial cells which would exhibit either all or only some of the immunostimulating activities of the intact cell. Most work in this field has been concentrated on attempts to obtain water-soluble fractions, as well as fractions which would be possible to synthesise chemically. Considerable achievements have already been attained. In general, myçobacteria have been used for fractionation, while only a few fractionation studies have involved anaerobic corynebacteria. With anaerobic corynebacteria, fractionation has been carried out exclusively on *C. parvum*. For our own investigations, we have used *C. granulosum* for the following reasons.

In the genus "anaerobic *Corynebacterium*" a strain belonging to one particular species may be active, whereas another strain of the same species may be inactive. In addition, an active strain may lose spontaneously its original reticulostimulating activity. A systematic study over a period of several years regarding the immunostimulating capacity of strains belonging to various species of anaerobic corynebacteria showed us[1] that a strain of *C. granulosum* (strain No. 5196) had preserved its reticulostimulating activity, whereas strains of *C. anaerobium* and *C. parvum* had lost practically all of their initial activity. Therefore, strain No. 5196 of *C. granulosum* was chosen for our studies.

This strain of *C. granulosum* was found to stimulate strongly the reticuloendothelial system (RES),[1] to protect mice against infection with *E. coli* 111 B4 and to accelerate the clearance of *E. coli* 111 B4 from the blood stream,[2] and to increase significantly the phagocytic activity of rat circulating leukocytes the phagocytic activity of which had been strongly impaired by extensive thermal injury of the skin surface of the rats.[3] The *C. granulosum* stimulation also resulted in a high increase of the complement activity of sera from thermally injured rats.[4] *C. granulosum* can also substitute for mycobacteria in Freund's complete adjuvant to induce a delayed-type hypersensitivity (DTH) reaction to the simple chemical: azobenzene-arsonate-N-acetyl-tyrosine (ABA-TYR).[5] The production of antibodies directed to sheep red blood cells (SRBC) was also strongly enhanced.[5] Furthermore, *C. granulosum* was found to inhibit the growth of various experimental grafted tumours: Ehrlich tumour,[2] mouse L1210 leukaemia and Friend leukaemia.[5]

Cell walls were isolated from whole cells of *C. granulosum* by using various treatments which resulted in either partly purified cell walls or purified cell walls.[5,6] The cell wall preparations thus obtained were found to exhibit biological activities close to those of the whole cells but, on a weight basis, cell wall preparations were not more active than whole cells. It is also interesting to note that the injection of *C. granulosum* cell walls and SRBCs resulted in two peaks of antibody response to SRBCs, one

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on day 14 and the other on day 28.[5] Thus, it appeared to us that active fractions could be isolated from anaerobic corynebacteria.

Migliore-Samour *et al*[7] reported on the isolation of a water-soluble fraction from *C. parvum* which exhibited strong adjuvant activity. These investigators fractionated whole cells of *C. parvum* by a technique which they had successfully used to fractionate mycobacterial cells. However, when we applied their technique for the fractionation of *C. granulosum* whole cells, we recovered the immunostimulating activities in a particulate fraction which we designated as P40 fraction. Here we report on the preparation of this fraction, its partial chemical characterisation and its main biological activities. We also include the initial results which have been obtained with the P40 fraction in clinical trials. Characteristics of P40 are represented in Table 1.

## Production of large quantities of *C. granulosum* whole cells

*C. granulosum* bacteria were cultivated in fermentors in volumes of 300 or 700 litres. The medium consisted of liver-meat broth supplemented with 4 g of yeast-extract (DIFCO), 2.5 g of NaCl and 10 g of glucose per litre of medium. The pH was adjusted to 7.2-7.3. Cultures were incubated in a nitrogen atmosphere at 37°C and bacteria were harvested in a Sharples centrifuge (Type MV 12-RV 5 P1) after 48-72 hours. The bacteria were washed three times with saline and resuspended in saline before being heated in a water bath at 60°C for one hour. The biological activity of various batches was found to be quite comparable.[8]

## Preparation of P40 fraction

Fractionation of *C. granulosum* bacteria was carried out on the delipidated cells as represented schematically in Figure 1. Delipidation was performed according to the method of Aebi *et al.*[9] The delipidated bacteria were disintegrated mechanically with the aid of an Omni-Mixer and were centrifuged at low speed to remove intact bacteria and large bacterial debris. The supernatant was subsequently fractionated with $(NH_4)_2SO_4$ and the fraction precipitated at 40 per cent saturation was harvested and dialysed exhaustively with distilled water. The dialysate was lyophilised. This fraction was designated as P40 fraction. The P40 fraction batches which resulted from the processing of different lots of *C. granulosum* whole cells exhibited comparable biological activities, indicating that the procedure was reproducible.

As calculated from the dry weight of delipidated bacterial cells, the P40 fraction was recovered with a yield in the range of 30 per cent.[8]

## Main physico-chemical characteristics of P40 fraction

P40 fraction is insoluble. Examination under the electron microscope shows that its structure resembles pieces of bacterial cell wall (Fig. 2). All attempts to degrade it into a soluble derivative were unsucessful. These attempts included

*Table 1* Immunomodulator P40:

| | |
|---|---|
| 1. Accelerates | CLEARANCE of carbon particles and pathogenic agents |
| 2. Increases | RESISTANCE to infections |
| 3. Enhances | ANTIBODY production |
| 4. Restores | IMMUNE CAPACITY |
| 5. Induces | DELAYED HYPERSENSITIVITY |
| 6. Inhibits or delays | TUMOUR GROWTH |

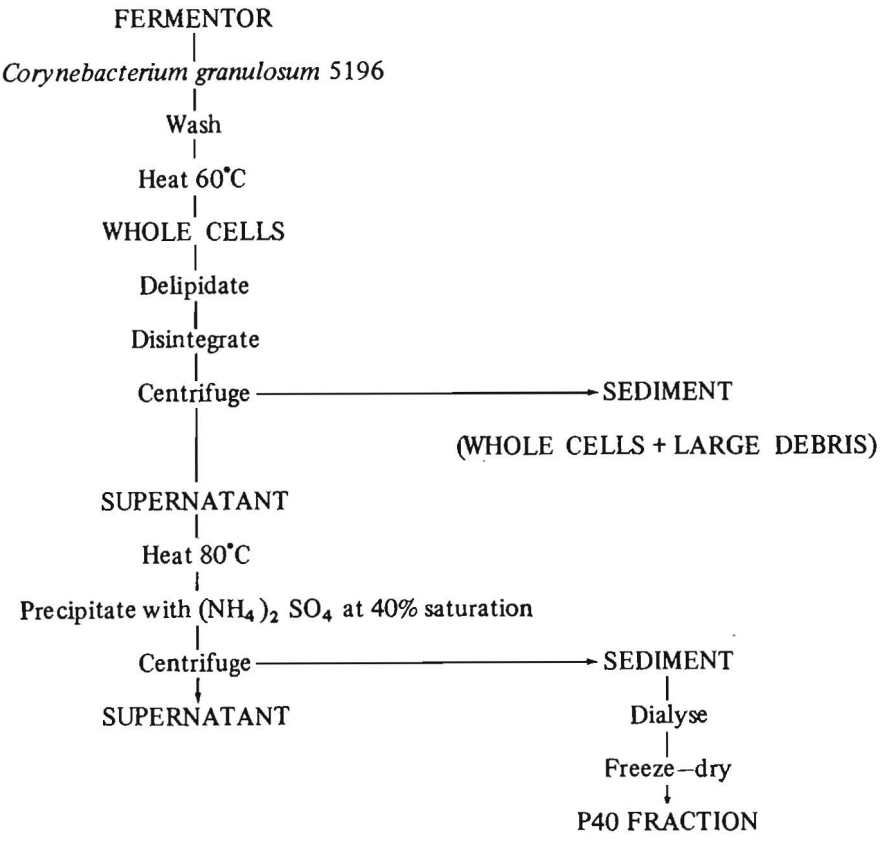



*Fig. 1* Preparation of P40 fraction

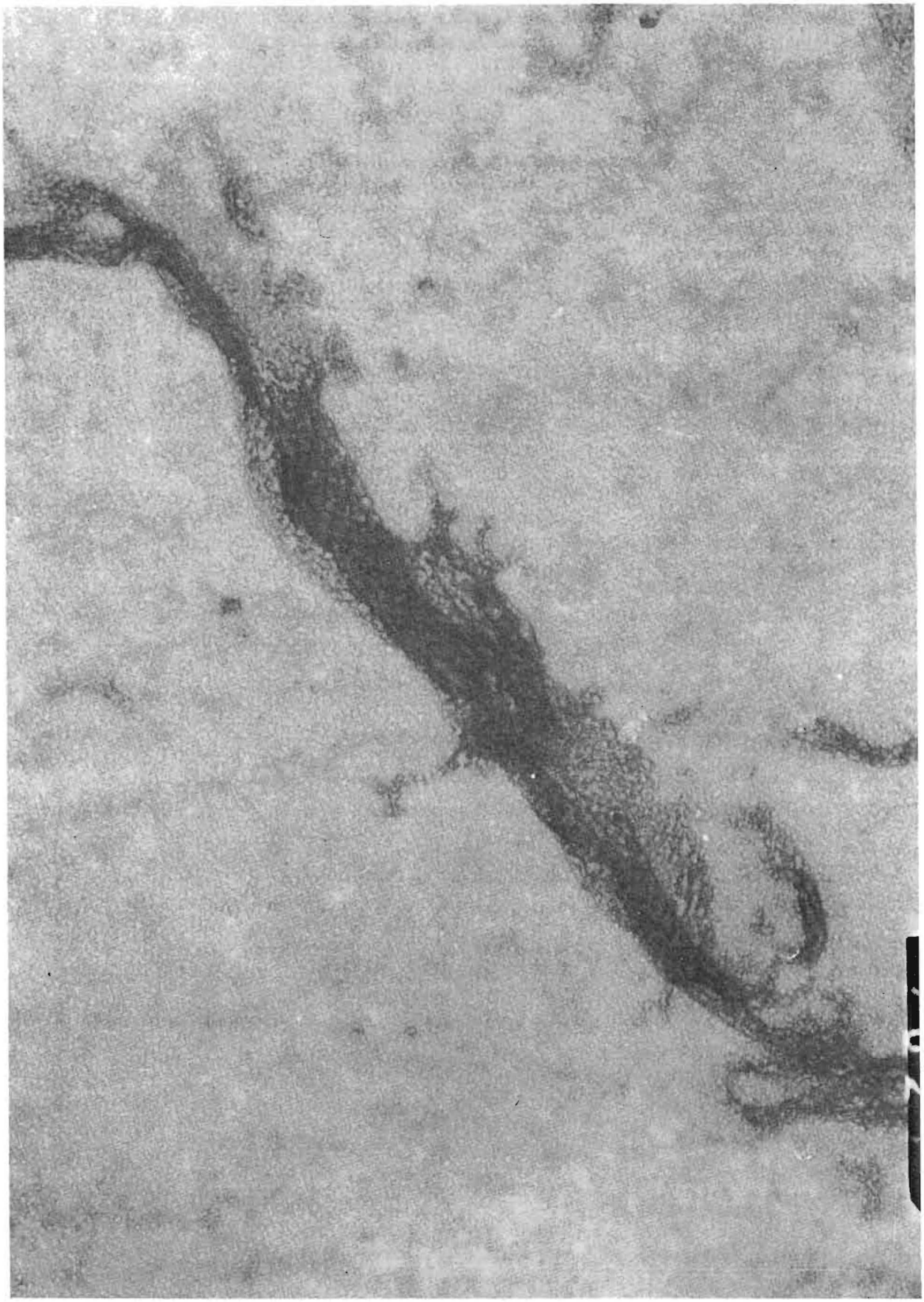


*Fig. 2* Electron microscopic appearance of P40 fraction (magnification x 60,000)

enzymatic degradation with pronase, lysozyme, trypsin, chymotrypsin, RNase and DNase used either separately or sequentially. Chemical reagents such as 0.1 NaOH, 9% HCOOH, or 1% SDS or 6M guanidine in the presence or in the absence of 1% 2-mercaptoethanol were also ineffective in degrading P40 fraction. Therefore, it appears that the P40 fraction has a rigid, compact structure within which the bonds sensitive to breakdown by enzymes and chemical reagents are inaccessible. This also explains why a water suspension of the P40 fraction can be autoclaved at 120°C for 20 minutes without impairment of its immunostimulating activity. Consequently, suspensions of the P40 fraction can be sterilised by autoclaving, which eliminates the necessity to add formaldehyde which is necessary in whole-cell suspensions.[8]

*Table 2* Chemical composition of P40 fraction

| Amino acids | g/100 g P40 (Dry weight) | |
|---|---|---|
| His | 1.69 | |
| Lys | 3.11 | |
| Arg | 3.09 | |
| Asp | 3.71 | |
| Thr | 1.97 | |
| Ser | 1.37 | |
| Glu | 6.34 | |
| Pro | 1.09 | |
| Ala | 3.38 | |
| Gly | 3.31 | |
| Cys | 0.30 | |
| Val | 2.88 | |
| Met | 1.10 | |
| Ile | 1.79 | |
| Leu | 3.04 | |
| Tyr | 0.91 | |
| Phe | 2.15 | |
| Try | 1.07 | |
| Total | | 42.30 |
| Dap | | 3.23 |
| Neutral sugars | | 13.67 |
| Hexosamines | | 10.09 |
| Muramic acid | | 10.38 |
| Ash | | 0.83 |
| Total | | 80.40 |

As summarised in Table 2, P40 fraction is composed of all the amino acids which are usually present in proteins, as well as diaminopelemic acid, muramic acid, and neutral and amino sugars. On a weight basis, the composition accounts for about 80 per cent of the P40 fraction. The missing 20 per cent could have been lost by destruction of the amino acids or sugar residues, or both during acid hydrolysis of the fraction since the simultaneous presence of amino acids and sugar residues can result in losses of either one by formation of complexes during glycoprotein hydrolysis.

Table 2 also shows that glutamic acid, glycine, aspartic acid, and alanine are the major amino acids. Large amounts of neutral sugars and hexosamines were also present. The neutral sugars identified were glucose, galactose, mannose and ribose and the hexosamines were glucosamine and galactosamine, which are present in a molar ratio of 7 to 2. The presence of both diaminopimelic acid and muramic acid indicates that the P40 fraction is derived, at least in part, from the bacterial cell wall.[8]

It is hard to provide a clear interpretation of the analytical results. Because the P40 fraction is insoluble, standard procedures cannot be used to try to resolve its possible complex nature. Therefore, we are not yet in a position to decide whether the P40 fraction comprises only one molecular entity or a mosaic of separate molecular entities, which would be associated in a highly stable structure. However, the presence of diaminopelemic acid and muramic acid is consistent with the existence of a peptidoglycan-like structure in P40. To confirm this assumption, it would have to be shown that diaminopelemic acid is present as its L, L-isomer. In the light of our present knowledge, we may speculate that the P40 fraction is likely to consist of the association of the cell-peptidoglycan and a glycoprotein.

## Biological activities of the P40 fraction

***a) Stimulation of the reticuloendothelial system:***

Like *C. granulosum* whole cells, the P40 fraction was found to be a potent activator of RES. It is capable of increasing both granulopexic capacity (carbon clearance test) and phagocytic activity (enhanced resistance to infections with virulent bacteria). The granulopexic activity of P40 as expressed by the $K/K_O$ index (namely, by the ratio of the activity measured in stimulated mice to that measured in control non-stimulated mice) attained its maximum value about 6-7 days after stimulation with the P40 fraction. A mean value of 4 was found for the $K/K_O$ index of various P40 batches. The increase in granulopexic activity is not correlated with the increase in weight of both spleen and liver; in fact, the spleen and liver continued to grow after the granulopexic capacity had returned to normal values.[2,8]

The P40 fraction is also capable of enhancing the phagocytic and bactericidal activities of macrophages as is evidenced by increased resistance of mice to infection with weakly virulent bacteria (*E. coli* 111 B4, P. Lallouette *et al*)[2] and with strongly virulent bacteria such as *L. monocytogenes* that develops intracellularly (Fig. 3). The increase of the bactericidal activity of macrophages from mice stimulated with P40 is also correlated with the potentiation of their enzymatic activities (K. Masek: personal communication).

The preventive effect of P40 on the experimental *E. coli* infection of the upper and lower urinary tract was investigated in rats.[10] Two-thirds of the rats infected by the lower route were protected against the infection. Under our

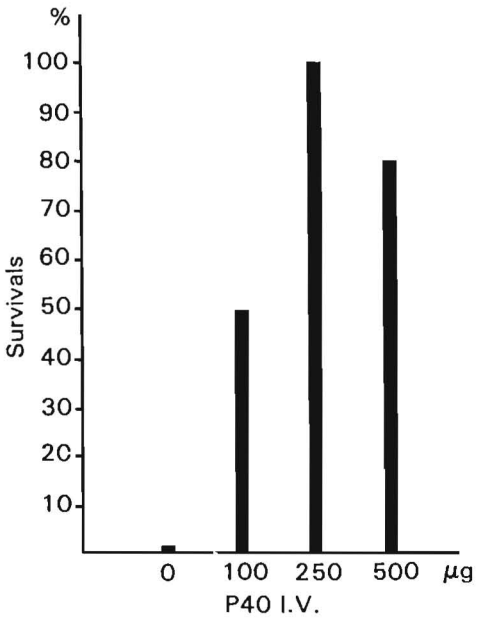



*Fig. 3 L. monocytogenes* (1,6 $LD_{50}$)

conditions, the bacteriological picture of the infection of rats, which were infected by the upper route, was only slightly influenced but the development of nephropathia was significantly decreased.

***b) Effect on antibody production:***

The ability of P40 to enhance the production of antibodies in mice in response to an i.v. injection of sheep red blood cells (SRBC) in saline showed that the adjuvant effect of P40 was maximum at 250 µg per mouse when injected seven days before the SRBC stimulus. Furthermore, the antibody level was at its peak about 6-7 days after the SRBC stimulus was given. For the experiments, a dose of SRBC was chosen that elicited only a negligible antibody response in control, unstimulated mice. Under these conditions the haemagglutinin content remained at a definitely low level in the sera of those mice which were not stimulated with the P40 fraction. In contrast, sera of those mice which were stimulated with the P40 fraction contained high levels of haemagglutinins. The antibody content of these sera decreased slowly; compared with the sera of mice not stimulated with the P40 fraction, antibody content was 11 times higher four weeks later, and three times higher 16 weeks later in the sera of mice stimulated with the P40 fraction.

The effect of the P40 fraction was also investigated with regard to a secondary response of mice boostered 16 weeks after their priming with SRBC. The secondary response in mice which had been stimulated with the P40 fraction seven days before the primary SRBC immunisation was found to be about 4 times higher than that in mice which had not received the P40 injection. Moreover, when the P40 injection was given seven days before giving the mice the SRBC booster, the secondary response was enhanced about 50 times.[8] Other investigators reported an increased production of haemagglutinins in mice on day 5 after a single i.v. injection of 250 µg of P40 given seven days before the i.v. immunisation with SRBC in dose varying from $10^5$ to $10^9$ SRBC per mouse. Increased production of antibodies was verified for all doses of SRBC injected, except for the dose of $10^5$ cells. The major adjuvant activity of P40 concerned the production of IgM- or 2-MER-sensitive antibodies.[11]

Experiments were also performed using tetanus toxoid as a soluble antigen. In this instance, the enhancing effect of P40 on the formation of antibodies was assessed by measuring the amount of toxoid corresponding to one 50 per cent protective dose (1 $PD_{50}$) comparatively in mice stimulated with the P40 fraction and in mice not stimulated. In P40-treated mice, the amount of toxoid corresponding to 1 $PD_{50}$ was found to be decreased with respect to that of unstimulated mice (unpublished results).

***c) Immunorestorative effect:***

It was also verified that the P40 fraction could counteract the immunosuppressive effect of the cyclophosphamide, as measured by the production of haemagglutinins in mice.[12] In fact, the P40 fraction is capable of abolishing not only the immunosuppressive action of cyclophosphamide, but also of enhancing the antibody response of cyclophosphamide-suppressed animals to a primary immunisation with SRBC. However, to counteract the action of cyclophosphamide, the P40 fraction has to be administered before the immunosuppressive drug. Furthermore, cyclophosphamide must not be used at doses which would completely suppress the immune responsiveness of the mice.

These results have been extended by S. Ben-Efraim (Tel Aviv University) who verified in BALB/c mice that the immunosuppressive effect of cyclophosphamide and of methotrexate on contact hypersensitivity to DNFB could be counteracted by the i.v. administration of P40 prior to the injection of the immunosuppressive drug.

A DTH reaction to either ovalbumin or ABA-Tyr was induced when the antigen or the hapten was injected in emulsion in Freund's complete adjuvant in which mycobacteria were replaced by P40 fraction (unpublished results). Recently, an exhaustive study [12,13] of the capacity of P40 fraction injected in saline showed an immunopotentiation of DTH in mice immunised with supraoptimal doses of antigen which would normally depress DTH in control mice. In contrast, when the P40-stimulated mice were immunised with optimal or suboptimal doses of SRBC for induction of DTH in control mice, lower DTH levels were also observed in P40-stimulated mice. This immunoregulatory effect of P40 was ascribed to the accelerated catabolism of the antigen and to the T-cell adjuvant effect of the P40. A significant enhancement of DTH was also recorded when the SRBC were injected s.c. together with the P40 fraction. The strongest effect was observed for a dose of 5 µg of P40 injected along with $10^8$ SRBC. It was further established that the observed

potentiation of DTH did not rely only on a depot phenomenon, as is seen with other adjuvants. Furthermore, the adoptive transfer of DTH indicated that the P40-induced enhancement of DTH could be ascribed to the adjuvant effect of P40 on the lymphoid cells, and not to a non-specific action on accessory cells.

In parallel experiments, the capacity of P40 to induce a state of DTH to certain of its antigens was demonstrated using four different immunological parameters as correlates of cellular immune response.[13] The results obtained suggest that P40 systemic hypersensitivity might be of the first type as opposed to the tuberculin response that is of the second type.

**Anti-tumour effect of the P40 fraction**

The anti-tumour effect of P40 fraction has been investigated using several experimental tumour models.

When mice were stimulated by a single i.p. injection of 10 μg of P40, seven days before the i.p. grafting of $10^5$ Ehrlich tumour cells to mice, inhibition of the tumour growth was achieved in 100% of the animals.[2]

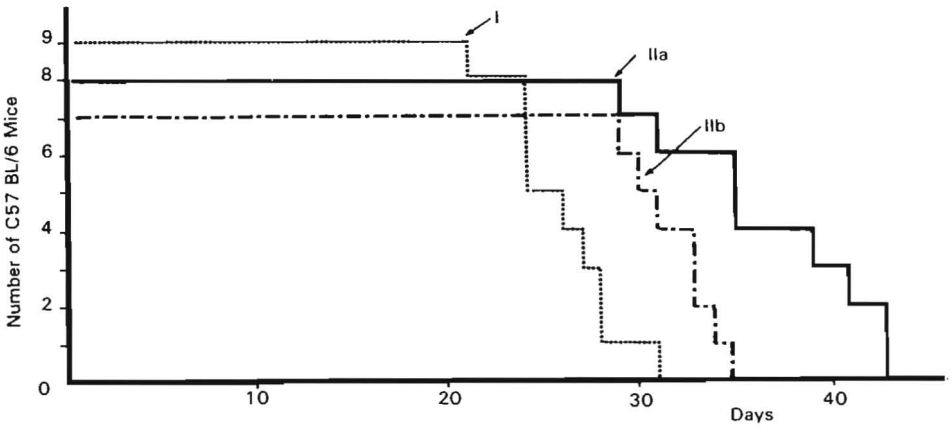
With P815 mastocytoma, the grafting of the tumour in its ascitic form was carried out both in non-isogenic MLTB mice and in isogenic DBA/2 mice. This type of tumour was successfully transplated in MLTB mice by Lalouette and Schwartz and has been serially transplanted in this mouse strain for several years. Although the tumour has lost much of its virulence by transplantation from DBA/2 mice to MLTB mice, an injection of $10^5$ cells will kill all recipients in about 28 days. Spontaneous regressions of established tumours have never been observed. The P40 fraction was injected i.p. at a dose of 100 μg per mouse at various times from one day to 9 nine days after the grafting of P815 mastocytoma to MLTB mice. Under these conditions, 100 per cent tumour rejection was obtained when the P40 fraction was injected up to six days after the tumour grafting. Injected nine days after the tumour challenge, the P40 fraction exercised no effect on tumour growth. The ineffectiveness of the P40 fraction when administered nine days after grafting the tumour is probably due to the fact that the population of tumour cells is too large at this time to be overcome by the immune defenses of the recipient. It was also observed that the P40 fraction was effective only when given i.p. and not when given i.v. In similar experiments in which *C. granulosum* whole cells were used in place of the P40 fraction, tumour growth was only retarded.

When the P815 mastocytoma was grafted to syngeneic DBA/2 mice, the P40 fraction did not influence tumour growth. However, protection could be afforded to a certain extent when the P40 fraction was injected before grafting the tumour.[2]

The Lewis lung carcinoma was investigated as an alternative tumour. It was maintained in its solid form by serial subcutaneous injection in C57B1/6 mice. In the experiments with Lewis carcinoma, the effect of a single i.v. injection of the P40 fraction at a dose of 250 μg per mouse was first studied. This was given either seven or three days before an i.m. challenge with $2x10^4$ tumour-cells, or given on the same day, or three, six or nine days after the tumour challenge. The results showed that the P40 fraction given before or at the same time that the tumour was grafted accelerated the tumour growth as estimated by measuring the variation of the diameter of the leg injected with the adjuvant as a function of time. However, no statistically significant difference was noted with regard to survival time when compared with control animals. However, when the P40 fraction was injected three days or six days after the tumour challenge, the tumour growth was significantly retarded. Given nine days later, the P40 fraction was without effect on tumour development.

Subsequently, we determined the optimum dose of the P40 fraction when injected i.v. four days after the tumour challenge. Under these conditions, the inhibitory effect of P40 on tumour growth was maximum at a dose of 100 μg. At this dose, the prolongation of the survival of the mice was highly significant. At doses of 250, 500 and 1,000 μg, the effect was less marked but still significant (Fig. 4).[8]

Castro Faria and Grynberg[14]




I : Control mice
II : Treated mice : one I.V. injection of a) 100 μg P40 and b) 500 μg P40 4 days after the tumour graft

*Fig. 4* Lewis tumour

have used glycerol-induced vesicles from tumour-cell membranes as a specific tumour antigen. The association of this antigen with P40 after a single i.p. injection resulted in 70-80 per cent-complete regression of a syngeneic methylcholanthrene-induced fibrosarcoma. P40 alone or the vesicles alone were ineffective.

Using L1210 leukaemia as a tumour model in syngeneic (C57B1/6xDBA/2) F1 mice, the effect of P40 alone on the tumour was compared to that of its association with other treatments.[15-19] The i.p. injection of a single dose of 100 $\mu$g of P40 seven days before the grafting of $1x10^5$ L1210 tumour-cells resulted in a significant prolongation of the survival time of the P40-treated mice as compared with the control mice. When glutaraldehyde-inactivated tumour cells (GA-L1210) or glutaraldehyde-inactivated L1210 tumour cells to which tetanus toxoid was coupled (GA-L1210-Tet) were injected seven days before the tumour challenge, tumour growth was not retarded and even facilitated. When two injections were given seven days apart and seven days before the tumour challenge, similar results were recorded. In contrast, the administration of inactivated cells with P40 under the same conditions as above resulted in a highly significant prolongation of the survival time (more marked with the combination of GA-L1210-Tet with P40) as compared with that of the control animals.

In subsequent studies,[17,18] the effect on L1210 leukaemia of chemotherapy alone, with either daunorubicin or mitomycin, was compared with immunotherapy with P40 alone as was the association of P40 and GA-L1210-Tet with chemotherapy. The treatment consisted of four injections on days -14 and -7 and on days +4 and +11. Chemotherapy alone and P40 treatment alone afforded the same degree of protection. In contrast, the combination of chemotherapy with P40 and GA-L1210 and P40 resulted in a significant increase of survival time in comparison with chemotherapy alone or P40-treatment alone. The survival of a few animals was even recorded under specified conditions. Furthermore, the rechallenge of surviving mice with the same number of L1210 tumour cells showed that about 50 per cent of the animals had developed specific resistance to the tumour.[17,19]

It appears from these results that the combination of chemotherapy with active immunisation and non-specific immunostimulation is more efficacious than single treatments for the control of L1210 leukaemia. Consequently, we are currently carrying out additional experiments in order to specify more precisely optimal conditions for combined treatments.

Experimental DMBA-induced mammary carcinoma was also used in female Sprague Dawley rats as described by Huggins *et al.*[20] The animals fed with DMBA developed mammary tumours beginning the seventh week after administration of the drug. P40 was given either intratumourally (i.t.), or peritumourally (p.t.), or intravenously (i.v.). Two weeks after P40-treatment, all rats underwent surgery for the removal of tumours. A second group of rats received P40-treatment at the time of appearance of the first tumour having an estimated volume greater than 0.6 $cm.^3$ P40 was then injected i.t.

In the first group, no significant difference in the mean survival time was recorded with respect to control rats not receiving P40. In contrast, the volume of tumoural recurrence was highly significantly decreased in the P40-treated rats. Very similar results were obtained in the second group.

## Toxicology

A thorough toxicological examination of the P40 fraction has been carried out in rats and dogs. These experiments showed that the P40 fraction was devoid of any acute and chronic toxicity at doses far beyond the therapeutic doses. The absence of undesirable effects was subsequently confirmed in clinical trials.

## Clinical trials with the P40 fraction

Since the P40 fraction was devoid of toxic effects at a dosage up to 50 times that intended to be used in humans, clinical trials were initiated in patients presenting either of two kinds of pathological conditions: recurring infections and neoplasia.

***Treatment of recurring infections:***

It appears very likely that recurrences of chronic infections may originate from the patient's inability to mount an adequate cell-mediated reaction against the infecting agent. Therefore, it appears to us that stimulation of the patient's natural defenses might substitute for specific immunisation.

To test this hypothesis, 117 patients suffering from recurring infections of the respiratory tract were divided into three groups for treatment (Table 3): Group I received either mono- or poly-microbial preparations (vaccinotherapy); Group II received vaccinotherapy combined with P40, and Group III received P40 alone. Comparison of the effectiveness of the various treatments showed that patients in Groups II and III were significantly improved (on the basis of three criteria) in comparison to Group I patients ($p<0.001$).[21,22]

Subsequently, a trial performed in randomly selected patients with recurring respiratory infections (mostly chronic obstructive bronchitis) confirmed that P40 treatment results in the suppression of symptoms, in a decrease in the number of infectious episodes, and in a decrease in the number of cumulative days of antibiotherapy for the year during which P40 treatment was administered as compared with the year prior to the initiation of P40 treatment.[24]

P40 treatment was also given to patients with recurring herpetic cutaneo-mucous infections. Of 12

*Table 3* Comparative statistical evaluation of the effectiveness of vaccinotherapy alone (group I), vaccinotherapy + P40 (group II) and P40 alone (Group III) in the treatment of patients with relapsing chronic infections of the respiratory tract.

| Treatment | No. of patients | Proportion of improvement according to criteria | % of patients improved |
|---|---|---|---|
| Group I | 79 | 18/79 | 22.78 |
| Group II | 21 | 18/21 | 85.71 |
| Group III | 17 | 14/17 | 82.35 |

Group I – II: $p < 0.001$ (highly significant)
Group I – III: $p < 0.001$ (highly significant)
Group II – III: $0.9 <$ $p > 0.5$ (not significant)

patients who received one or a few injections of P40 0.20 mg/ml during the presence of a herpes blister, nine of them experienced a dramatic shortening of the duration of their blister which faded in 24-48 hours instead of 8-10 days as was the experience of patients in the control group. Of 24 patients who presented frequent recurrences of herpes, 19 did not have recurrent herpes over a period of time ranging from one to four years after 10 injections of P40 spaced one week apart.[21]

P40 treatment was also successful in treating patients with either vaginal candidiasis or *E. coli* cystitis. Patients improved by the treatment (about 75%) have not experienced relapses over a period of time ranging from one to four years post-treatment.[21,25]

We must emphasise that skin tests for delayed hypersensitivity with recall-antigens (bacteria from the respiratory tract, SK-SD, PPD, candidin, *E. coli*) which were negative before P40 treatment became positive after the treatment in improved patients. In particular, in a series of 10 patients with vaginal candidiasis, who were unresponsive to candidin, eight of them showed improvement and at the same time they became responsive to candidin. In contrast, the two patients who were not improved by the P40 treatment also remained unresponsive to candidin.

*Table 4* Evaluation of the cutaneous response to recall-antigens before and after treatment with P40 fraction (n = 67)

| Score | No. Responsive/total Before P40 | After P40 |
|---|---|---|
| 4+ | 41/67 (61%) | 63/67 (94%) |
| 5+ | 31/67 (46%) | 55/67 (82.%) |
| 6+ | 27/67 (40%) | 45/67 (67%) |
| 7+ | 18/67 (27%) | 32/67 (48%) |

The importance of performing skin tests on patients with recurring infections as a guide for therapeutic decision-making and as a test to evaluate the efficacy of the treatment has previously been emphasised.[26] In this connection, the possibility of modulating skin response to an antigen by P40 fraction has been reported.[27] The P40 fraction would either enhance a poor skin reaction or weaken an exaggerated skin reaction as a function of the ratio of P40 to antigen.

### P40 treatment in breast cancer patients

Skin-testing of breast cancer patients with recall-antigens showed that a large proportion of them were immunosuppressed, since their scores turned out to be much lower than those of a control population of individuals apparently free of cancer.[28,29] In fact, the exploration of the immune status of patients with breast cancer was performed using skin tests with five different recall-antigens to evaluate their DTH responses. Each patient was assigned a composite score based on the intensity of the skin test response to the five antigens (Table 4). The results showed that the frequency of anergy was significantly higher in breast cancer patients than in a control group of individuals apparently free of cancer. Furthermore, for patients with mean DTH scores, a significant difference was found between those who had lymph node involvement and those who had no lymph node involvement. In the former patients, fewer showed mean scores when compared with the latter. A significant difference as a function of age was found only for the highest scores.[20,30]

An attempt to restore the DTH reactivity of the immunosuppressed cancer patients by P40 treatment (1 subcutaneous injection of 0.2 mg three times weekly for three weeks) resulted in a significant decrease in the incidence of anergy. Lymph node involvement had no influence on the issue of P40 treatment. Thus, P40 treatment restored DTH reactivity in about half or more of the patients according to the type of antigen used for the skin tests (Tables 4 and 5).

Evaluation of the effect of P40 treatment on the survival rate of breast cancer patients is currently being carried out.


### Summary

We report the preparation of an immunostimulating fraction from mechanically disrupted, delipidated cells of the anaerobic *Corynebacterium granulosum*. Since this fraction is precipitated by ammonium sulphate at 40 per cent saturation, it has been designated as P40 fraction. P40 is a particulate substance appearing in the electron microscope as pieces of the bacterial cell wall. The chemical composition of P40 is consistent with the assumption that it consists of an association of the cell wall peptidoglycan

*Table 5* Comparison of the results obtained in breast cancer patients before and after treatment with P40 fraction and with controls when skin tested with recall-antigens. Results are expressed as per cent of positive reactions

| Tests | Patients | | Controls |
|---|---|---|---|
| | before P40 | after P40 | |
| PPD | 61 | 83 | 74 |
| Candidine | 39 | 73 | 80 |
| Divasta | 42 | 67 | 76 |
| Streptococci | 34 | 61 | 85 |
| Streptococcal exoproteins | 18 | 40 | 74 |

together with a glycoprotein. P40 fraction can exercise a broad spectrum of activities on the immune system: it can 1) stimulate RES; 2) increase nonspecifically the resistance of animals to various bacterial infections; 3) enhance the production of antibodies to SRBC; 4) counteract the immunosuppressive effect of cyclophosphamide and methotrexate; 5) induce delayed-type hypersensitivity to various antigens and to itself; and 6) inhibit the growth of various experimental tumours.

Clinical trials have indicated that it is effective in the treatment of recurring infection of either the respiratory tract or the uro-genitary tract. In breast cancer patients, P40 was found to be capable of restoring cell-mediated immunity, as measured by skin tests with recall-antigens, in immunosuppressed patients.

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