S. Schlecht, M. A. Freudenberg, C. Galanos

Culture and Biological Activity of *Propionibacterium acnes*

Summary: Administration of killed *Propionibacterium acnes* to experimental animals leads to the development of hypersensitivity to the lethal and cytokine-inducing effects of endotoxin. This sensitizing property of *P. acnes* is not always expressed by different bacterial preparations. Its expression depends very much on the conditions employed for the cultivation of this microorganism. The present study investigates which culturing conditions result in *P. acnes* preparations with optimal sensitizing properties. The composition of the medium, the culturing time and temperature as well as the type of cultivation (in minifermentor or stationary culture) were all varied for this purpose. The resulting bacterial preparations were killed at 65°C for 1 h and tested for sensitizing activity. The results show that stationary cultures of *P. acnes* grown at 37°C for 4 to 5 days in the appropriate medium produce biologically active preparations with satisfactory sensitizing activity.

Introduction

Propionibacterium acnes is a normal component of the bacterial flora of the human skin. Occasionally, and recently with increasing frequency, *P. acnes* was found to be the etiological agent in various pathological conditions. Of these, the most interesting from a clinical point of view is acne vulgaris [1]. In recent years *P. acnes* could be identified as the pathogenic agent in endophthalmitis [2], bacterial meningitis [3], osteomyelitis [4] and in a number of other opportunistic infections [5].

One interesting property of live or killed *P. acnes* recognized over three decades ago that has found a wide application in different experimental studies is its ability to sensitize animals to the lethal activity of lipopolysaccharides (LPS). Thus the treatment of mice with heat-killed [6, 7] *P. acnes* leads to the development of hypersensitivity to LPS administered 7 to 10 days after *P. acnes* treatment. This hypersensitivity is characterized by an enhanced production of pro-inflammatory cytokines (TNFa, IL6 etc.) and by a high susceptibility of the animals to the lethal effects of LPS. Moreover, mice treated with *P. acnes* not only produce more TNF upon LPS administration but also exhibit a higher sensitivity to the lethal activity of this mediator [8]. Mice treated with *P. acnes* therefore represent a useful, highly sensitive model for the hyperproduction of cytokines and for evaluating the lethal activity of LPS.

Various more or less complex procedures for culturing *P. acnes* have been described [e.g. 9–12]. Although many of them result in sufficient yields of bacteria, the final preparations are frequently devoid of sensitizing activity, and their administration to mice does not enhance reactivity of the animals to the action of LPS (authors' unpublished results). *P. acnes* is not a strictly anaerobic bacterium. It may best be described as an anaerobic to aerotolerant microorganism. Many strains of *Propionibacterium* grow in deep fluid cultures with no particular precautions against aerobiosis. The optimum culture temperature lies between 30 and 37°C [13]. In the present study we investigated culturing conditions which would both be reproducible and result in bacteria with a satisfactory sensitizing activity.

Materials and Methods

The investigations were carried out on *P. acnes* (ATCC 12930), and the stock culture was kept at −70°C. Tubes containing 10 ml of cooked meat medium (Difco) were inoculated and incubated at 37°C for 5 days. Precultures for batch cultivation were prepared in 20 ml CCM-TT (see below) which had been inoculated with 1 ml of the tube culture and incubated for 3 days at 37°C. Subsequent culturing was carried out a) anaerobically in a minifermentor in volumes of 1 l at pH 7.0 and with N_2 aeration (pO_2 control) and gentle stirring at 30, 35 or 37°C, and b) as a static culture at 37°C in 800-ml quantities in 1-l Erlenmeyer flasks with screw tops. In both cases the incubation time was between 1 and 6 days. The following culture media were used: tryptose tryptone medium (TT) with 1% glucose [14]; cooked meat medium (CMM) (Difco); cooked meat medium filtrate (CMMF, prepared from CMM by filtering off the pellets); a mixture of equal parts of CMM or CMMF and TT with 0.2% glucose (CMM-TT, CMMF-TT). For cultivation in the minifermentor, 0.3 ml polyglycol P2000 (Hedinger, Stuttgart) was added to the medium as an antifoam agent. To harvest the bacteria the cultures in CMM and CMM-TT were first gently agitated and the supernatant containing bacteria decanted from the sedimented pieces of meat. All culture fluids were heated in a water bath at 65°C for 1 h. The bacteria were then obtained by centrifugation and lyophilised.

Sensitization of mice by Propionibacterium acnes: Mice (C57/BL10) 6 to 8 weeks of age were used. Groups of six animals

Received: 20 September 1996/Revision accepted: 7 April 1997

Dr. *S. Schlecht*, PD Dr. *Marina A. Freudenberg*, Dr. *C. Galanos*, Max-Planck-Institut für Immunobiologie, Stübeweg 51, D-79108 Freiburg, Germany.
Correspondence to: Dr. *C. Galanos*.

Table 1: LPS-sensitizing activity of *Propionibacterium acnes* cultured under different conditions.

Charge	Medium	Cultivation temperature °C	Duration of culture (days)	Sensitizing activity: enhanced TNF production after LPS treatment
MF 1	TT	37	1	–
MF 2	TT	37	3	–
MF 3	TT	37	4	–
MF 4	CMMF-TT	30	4	+
MF 5	CMMF-TT	35	4	+
MF 6	CMMF-TT	35	5	+
MF 7	CMMF-TT	37	5	+
MF 8	CMMF-TT	30	6	–
MF 9	CMMF-TT	35	6	±
ST 1	CMM	37	1	+
ST 2	CMM	37	3	+
ST 3	CMM-TT	37	3	+
ST 4	CMMF-TT	37	4	+
ST 5	CMMF-TT	37	5	+
ST 6	CMMF-TT	37	6	–

Mice received 500 µg *P. acnes* prepared under different culturing conditions. The animals were challenged with LPS 7 days later. Blood was collected and TNF serum levels were determined 1h later.
MF = cultivation in minifermentor: ST = cultivation as standing culture; + = sensitization factor, 50–200; ± sensitization factor, 10–50; – = sensitization factor < 10; TT = tryptose tryptone medium; CMM = cooked meat medium; CMMF = cooked meat medium filtrate.

received 500 mg of lyophilized *P. acnes* suspended in 0.2 ml PBS intravenously. Mice receiving PBS instead of *P. acnes* served as controls. All animals were challenged with LPS 7 days later and the development of hypersensitivity was determined. The presence of hypersensitivity was evaluated by the increase in the levels of serum TNF produced 1 h after the LPS challenge.

TNF-measurement: P. acnes-treated and control mice received 0.2 µg LPS (*Salmonella abortus equi*) intravenously. They were bled 1 h later under ether anaesthesia by cutting the axillary vessels. The blood was allowed to clot and the serum obtained was stored at −80°C until use. TNF in sera was measured in a cytotoxicity test using TNF-sensitive L929 cells (kindly provided by *L. Old*, Memorial Sloan-Kettering Cancer Center, New York, N.Y., USA) as described elsewhere [8].

Results

Results of this series of experiments are listed in Table 1. We had had good results with the TT medium for the cultivation of various gram-positive and -negative bacteria earlier. *P. acnes* underwent significant growth in the medium as well. However, these bacteria revealed only weak activity in exceptional cases, and no activity at all in most cases (MF 1–3 Table 1). Varying the incubation time from 1 to 4 days and the quantity of inoculated material between 20 and 100 ml (2–10% of the culture volume) had no effect on the biological activity (data not shown). It had proven advantageous to use a culture medium with pieces of meat for anaerobic or microaerophilic bacteria [15]. In such a medium (CMM) *P. acnes* grew well and was also active in sensitizing to LPS. A similar result was obtained by using the CMM-TT medium (ST 1–3 in Table 1).

In order to obtain a bacterial mass as free as possible from contamination, a particle-free medium is preferred. This is also more suitable for cultivation in a stirred fermentor. Further cultivation was therefore carried out in a cooked meat medium from which the pellets had been removed by filtration (CMMF), as well as in a mixture of this filtrate with the TT medium (CMMF-TT). Besides sufficient growth, an active *P. acnes* preparation was obtained at cultivation temperatures of 30 to 37°C and with an incubation time up to 5 days. Longer cultivation resulted in a decrease of the activity of the bacteria (see Table 1). This is in agreement with previously reported findings of other authors [11, 12]. With some batches of *P. acnes* lethality tests [8] were also performed. The results (not shown) correlate with those of TNF-production.

While cultivation in the fermentor allows large-scale preparation of bacteria, static cultures in the CMMF-TT medium provide a simple and reliable source of biologically active preparations of bacteria when minor quantities of *P. acnes* are required. For this purpose, according to the manufacturer's instructions, the cooked meat medium (Difco) is suspended and mixed in cold distilled water, allowed to stand for 15 min. and then autoclaved for 20 min. at 120°C. After complete cooling, the supernatant is decanted from the meat pellets and passed through a folded filter-paper (e.g. Schleicher and Schüll, No. 595 1/2). Then 400 ml each of the filtrate and TT medium (with 0.2% glucose) are sterilised together in a 1-l Erlenmeyer flask (with screw top) for 20 min. at 120°C. After cooling, the fluid is inoculated with 20 ml of the preculture (3 days at 37°C in

CMM-TT) delivered deep below the surface. Cultivation is carried out without nitrogen aeration and without correction of the pH in an incubator for 4–5 days at 37°C. By the end of incubation, the glucose has been consumed and the pH lies between 6.1 and 6.3. Typical values for the wet weight of the biomasses obtained from 800-ml culture volumes are about 5–6 g.

Discussion

In the present study we investigated the conditions of cultivation of *P. acnes* under which the resulting bacteria express strong sensitizing activity towards the action of LPS. The results make it evident that the property of the final bacterial preparation to express sensitizing activity does not depend only on the efficiency of bacterial growth. Good growth was obtained using TT medium. However, the different batches of bacteria thus prepared expressed only weak or no activity at all. On the other hand, the use of medium containing pieces of meat (CMM or CMM-TT) resulted in satisfactory yields of bacteria with good sensitizing activity.

Under the experimental conditions used, cultivation of *P. acnes* in the fermentor offered no recognisable advantages over the static culture. The fermentor method therefore appears to be a desirable procedure only if a larger volume of culture has to be grown. The addition of an antifoam agent did not have any deleterious effect on the growth or biological activity of *P. acnes*, as could be demonstrated in trials with surface aeration and without adding P2000 (data not shown). The length of cultivation seems to be crucial and according to the present results it should not exceed 5 days. The loss of biological activity of *P. acnes* which has been observed after prolonged cultivation in this study has also been observed previously (based on spleen index) by other authors [11, 12]. Although the precise reasons are not known, it is believed that on longer cultivation the active substance, which is probably a polysaccharide bound to the cell wall, is released into the medium.

The property of *P. acnes* to increase sensitivity to LPS and to prime the organism for an enhanced production of TNF-α and other pro-inflammatory cytokines may be also of clinical interest. Mice treated with *P. acnes* are not only more sensitive to LPS but also more sensitive to the toxic effects of TNF-α. Killed *P. acnes* has also been shown to possess TNF-inducing components and, like LPS or whole killed gram-negative bacteria, it induces lethal shock in mice. The pathogenicity of this microorganism may therefore be due at least in part to its ability to enhance both the levels and the activity of the toxic cytokines produced.

The understanding of the culture conditions under which the sensitizing principle in *P. acnes* is optimally expressed will enable the cultivation of bacteria with reproducible biological properties and will facilitate studies in this field.

Acknowledgements

We are grateful to *G. Backs* and *H. Garbers* and to *Hella Stübig* for their skillful technical assistance during the course of the experiments.

References

1. **Pulverer, G., Roszkowski, W., Beuth, H.J., Ko, H. L., Quie, P.:** Granulocyte activating factor released from *Propionibacterium acnes*. A possible mediator of inflammation in acne vulgaris. Zbl. Bakt. Hyg. A 270 (1988) 246–251.
2. **Huber-Spitzy, V., Arocker-Mettinger, E., Herkner, K., Schiffbänker, M., Georgiew, L., Steinkogler, F. J., Grabner, G.:** Diagnosis and therapy of bacterial endophthalmitis, and serum levels of inflammation markers. Infection 20 (1992) 122–127.
3. **Neu, H. C.:** Microbiology of bacterial meningitis pathogens. Antibiot. Chemother. 45 (1992) 52–67.
4. **Abolnik, I. Z., Eaton, J. V., Sexton, D. J.:** *Propionibacterium acnes* vertebral osteomyelitis following lumbar puncture: case report and review. Clin. Infect. Dis. 21 (1995) 694–695.
5. **Dworniczek, E., Modarska, H., Bizuniak, I., Smogor, W., Szklarz, E.:** Anaerobic propionibacteria in opportunistic infections. Medycyna Doswiadczalna i Mikrobiologia 45 (1993) 219–222.
6. **Halpern, B. N., Prévot, A.-R., Biozzi, G., Stiffel, C., Monton, D., Morard, J.C., Bouthillier, Y., Decreusefond, C.:** Stimulation de l'activité phagocytaire du système réticuloendothélial provoquée par *Corynebacterium parvum*. RES J. Reticuloendothel. Soc. 1 (1964) 77–96.
7. **Green, S., Dobrjansky, M. A., Chiasson, M. A., Carswell, E., Schwartz, M. K., Old, L. J.:** *Corynebacterium parvum* as the priming agent in the production of tumor necrosis factor in the mouse. J. Natl. Cancer Inst. 59 (1977) 1519–1522.
8. **Katschinski, T., Galanos, C., Coumbos, A., Freudenberg, M. A.:** Gamma interferon mediates *Propionibacterium acnes*-induced hypersensitivity to lipopolysaccharide in mice. Infect. Immun. 60 (1992) 1994–2001.
9. **Cove, J. H., Holland, K. T., Cunliffe, W. J.:** Effects of oxygen concentration on biomass production, maximum specific growth rate and extracellular enzyme production by three species of cutaneous propionibacteria grown in continuous culture. J. Gen. Microbiol. 129 (1983) 3327–3334.
10. **Fujimura, S., Nakamura, T., Pulverer, G.:** Purification and properties of a proline iminopeptidase from *Propionibacterium acnes*. Zbl. Bakt. Hyg. A 260 (1985) 175–183.
11. **Koukalova, D., Kod'ousek, R., Hajek, V., Kolar, M.:** Experimental nonspecific immunostimulation by the *Propionibacterium acnes* vaccine. Acta Univ. Palacki, Olomuc, Fac. Med. 133 (1992) 19–23.
12. **Mara, M., Julak, J., Bednar, M., Ocenaskova, J., Mikova, Z., Dohnalova, A.:** The influence of *Propionibacterium acnes* (*Corynebacterium parvum*) fractions on immune response *in vivo*. Zbl. Bakt. 281 (1994) 549–555.
13. **Sneath, P. A., Mair, N. S., Sharpe, M. E., Holt, J. G. (eds.):** Bergey's manual of systematic bacteriology. Vol. 2. Williams and Wilkins, Baltimore 1986, pp. 1346–1353.
14. **Schlecht, S., Galanos, C.:** Influence of the glucose concentration on the yield of biomass and lipopolysaccharide in *Salmonella* cultures. Zbl. Bakt. 281 (1994) 30–37.
15. **Müller, R.:** Medizinische Mikrobiologie. 4th ed. Urban und Schwarzenberg, München 1950, p. 137.