Archivum Immunologiae et Therapiae Experimentalis, 2000, **48**, 31–37
PL ISSN 0004-069X

Effective Phage Therapy is Associated with Normalization of Cytokine Production by Blood Cell Cultures

BEATA WEBER-DĄBROWSKA[1]*, MICHAŁ ZIMECKI[2] and MARIAN MULCZYK[1]

[1] Laboratory of Bacteriophages, [2] Laboratory of Immunobiology, Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Weigla 12, 53-114 Wrocław, Poland

Abstract. The aim of this study was to investigate the effect of phagotherapy on tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6) serum levels and the ability of blood cells to produce these cytokines in culture. Fifty one patients with long-term, suppurative infections of various tissues and organs were enrolled. The ability of cells to secrete cytokines was tested using whole blood cell cultures, unstimulated or stimulated with lipopolysaccharide (LPS) from *E. coli*. In addition, cytokine serum levels were determined. Measurement of cytokine activity was performed using bioassays. We showed that TNF-α, but not IL-6 serum levels, were regulated upon division of patients into categories exhibiting initial: low, moderate and high cytokine levels. The low spontaneous production of IL-6 by blood cell cultures was elevated significantly on day 21 of phage therapy, whereas high release of this cytokine was inhibited. No such correlation was observed with LPS-induced IL-6 production in cell cultures when cells from low-, moderately- or highly-reactive patients were studied. Phage therapy modified TNF release according to the initial ability to produce that cytokine: it reduced TNF production in high responders and increased it in low responders. Patients infected only with Gram-positive bacteria demonstrated analogous changes in the spontaneous and LPS-induced TNF-α production as in the whole studied group. A similar kind of regulation was observed in TNF-α and LPS-induced production, i.e. low production was significantly elevated, high strongly inhibited, and moderate only slightly affected. In summary, we demonstrated for the first time that effective phage therapy can normalize TNF-α serum levels and the production of TNF-α and IL-6 by blood cell cultures.

Key words: phage therapy; TNF-α; IL-6; blood cell cultures.

Introduction

Specific phage therapy has been proven highly effective in combating bacterial infections caused both by Gram-positive and Gram-negative microorganisms[7, 19–25, 27]. The current status of that therapy has recently been reviewed[6]. This therapy is particularly applied in the treatment of infections induced by multi-drug resistant bacteria in which antibiotic therapy is ineffective. In many cases following phage therapy, a long-term state of increased protection against bacterial and viral infections has been observed (unpublished data). Therefore,

* Correspondence to: Dr Beata Weber-Dąbrowska, Laboratory of Bacteriophages, Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Weigla 12, 53-114 Wrocław, Poland, tel. +48 71 373 22 74, fax: +48 71 373 25 87, e-mail: secret@immuno.iitd.pan.wroc.pl

the question arises whether the phage therapeutic effect (disappearance of clinical symptoms and negative bacteriologic tests) is solely a result of the destruction of bacterial cells in the infection sites, or perhaps it is also a consequence of bacteriophage interaction with the immune system. It is known that bacterial infections are associated with the influence of bacterial products on immune system cells leading to their activation as expressed by cytokine release, increase of locomotion and phagocytosis[3, 8]. A prolonged interaction of microorganisms and their products on cells of the immune system may lead to impairment of the defence reactions in patients[14].

In monitoring the immune status of infected or post-surgical patients, two cytokines appeared to be particularly relevant: interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α) [4, 13]. IL-6 is released early in inflammation and its major role is to initiate the immune response by inducing acute phase proteins[2, 17] and to control the activities of other inflammatory cytokines such as IL-1 and TNF-α[1]. TNF-α, on the other hand, is responsible for the development of an adequate course of inflammatory processes and for the immune response to pathogens[4, 12, 15]. High and persistent serum levels of both cytokines are, however, harmful or even fatal[5, 16]. On the other hand, states of hyporeactivity in the production of these cytokines by peripheral blood cells may also lead to increased mortality[1].

Anticipating that phage therapy may change both spontaneous and lipopolysaccharide (LPS)-induced cytokine production by peripheral blood cells and cytokine serum levels, we monitored these immune parameters in 51 patients: before, on day 7 and on day 21 of phage therapy.

Materials and Methods

Patients. The studies included 51 patients with long-term suppurative infections of various tissues and organs caused by drug-resistant strains of bacteria. In the majority of cases the infections were of staphylococcal origin, in other cases the infections were caused by *Pseudomonas*, *Klebsiella* and *Escherichia.* For the therapy, specific bacteriophages were used, administered *per os* and locally. In 44 cases a complete remission of the disease symptoms was achieved, accompanied by negative bacteriologic tests. In other 4 cases a significant improvement of the clinical state was obtained with positive bacteriological results. Only in 3 cases no improvement was registered, including one death (sepsis).

We investigated the ability of peripheral blood cells for TNF-α and IL-6 production and determined the levels of these cytokine in sera. Blood samples were taken before treatment and on days 7 and 21 of phage therapy. The ability of the cells to secrete cytokines was tested using whole blood cell cultures, unstimulated or stimulated with LPS. We also studied the ability to produce cytokines and serum cytokines in 8 healthy volunteers.

Induction of cytokines. Cytokine induction was performed in 24-well culture plates (Nunc). To the wells, 1 ml of blood 5 × diluted with RPMI-1640 was added. LPS was used at a concentration of 5 μg/ml/well. The cultures were incubated overnight in a cell culture incubator. Supernatants were harvested and kept frozen at –70°C until cytokine determination.

Determination of IL-6 activity. The assay was performed according to VAN SNICK et al.[26]. Briefly, 7TD1 indicator cells were washed 3 times with Hanks' medium and resuspended in Iscove's medium supplemented with 10% FCS, HEPES buffer, glutamine and antibiotics in density of 2×10^4 cells/ml. Then the cells were distributed in 100 μl aliquots into 96-well flat-bottom plates containing 100 μl of serially diluted plasma or supernatant in triplicate. After 72 h of culture the proliferation of 7TD1 cells was determined using the MTT colorimetric method[11]. The results of IL-6 activity are presented in pg/ml: such a concentration of IL-6 corresponds to the activity of IL-6 expressed in U/ml[26]. One unit of IL-6 activity was calculated as the inverse dilution of a plasma sample where a half-maximal proliferation of 7TD1 cells was registered.

Determination of TNF-α activity[9]. For the determination of TNF-α activity the indicator clone WEHI 164.13 was used. The cells were washed 3 times with Hanks' solution and resuspended in RPMI 1640, supplemented with 10% FCS, glutamine and antibiotics at a concentration of 2×10^5/ml. The cells were then distributed into 96-well, flat-bottom plates (2×10^4/well). Serially diluted plasma samples were prepared on separate plates and transferred to microtiter plates containing WEHI 164.13 cells. The medium contained in addition 1 μg/ml of actinomycin D to increase the sensitivity of the assay. After an overnight incubation the survival of cells was determined using the MTT colorimetric assay[11]. The results of TNF-α activity are presented in pg/ml, where 10 pg of TNF-α corresponds to 1 U of activity when tested recombinant human TNF-α (kindly delivered by Prof. W. Stec, Department of Bioorganic Chemistry, Centre of Molecular and Macromolecular Studies, Polish Academy of Sciences, Łódź, Poland). One unit of TNF-α was calculated as

the inverse dilution of a given plasma sample where 50% survival of WEHI 164.13 cells took place.

Colorimetric determination of cell proliferation/death. The assay was performed according to HANSEN et al.[11]. Briefly, MTT solution, 5 mg/ml in 0.9% NaCl, was added in a volume of 25 µl/well and incubated for 2–4 h. Then, 100 µl of a lysing buffer was added (20% SDS, 50% DMF, pH 4.7). After an overnight incubation at 37˚C the optical density (OD) was measured using an ELISA reader Dynatech 5000 at a wavelength of 550 nm and a reference wavelength of 630 nm.

Statistical analysis. The Student's *t*-test was used for the statistical evaluation of the results. The results were presented as mean values ± standard errors (SE) and were regarded as significant when $p \leq 0.05$.

Results

Cytokine serum levels during phage therapy

The serum levels of TNF-α and IL-6 in patients subjected to phagotherapy are presented in Fig. 1. For the elaboration of the data we followed a strategy which has proved successful for the assessment of postoperational immune reactivity of patients[28] i.e. we divided patients according to their initial immune status. Otherwise, no immunoregulatory effects of surgery could be demonstrated. Therefore, for calculations and presentation of the regulatory effects of phage therapy described below, we classified patients into categories of low, moderately and highly-reactive individuals. Figure 1A shows IL-6 serum levels during phage therapy in patients exhibiting initially low (0–20 pg/ml) and high (20 pg and more) IL-6 content. IL-6 levels appeared to be constant within the observation period, irrespective of the category of patients. On the other hand (Fig. 1B), TNF-α serum levels underwent alterations depending on the category of patients. Low initial TNF-α levels (0–50 pg/ml) rose more than twice during therapy, moderate ones (50–100 pg/ml) also showed a tendency to increase, but high TNF-α levels (above 100 pg) declined. Serum levels of IL-6 and TNF-α in healthy volunteers were 2 and 44 pg/ml, respectively.

Effect of phage therapy on spontaneous and LPS-induced IL-6 production in whole blood cell cultures

Figure 2 presents the ability of the patients' blood cell cultures to produce IL-6 spontaneously and upon

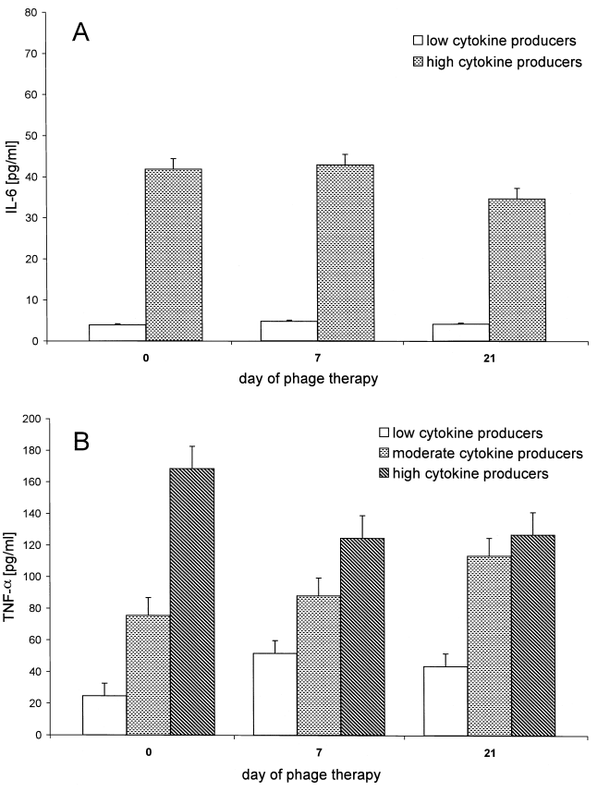

Fig. 1. Interleukin 6 (A) and tumor necrosis factor α (B) serum levels in patients subjected to phage therapy

LPS activation. Patients divided into low (0–50 pg/ml) and high level (above 50 pg/ml) IL-6 – producing groups showed different kinetics in spontaneously released IL-6 by blood cell cultures (Fig. 2A). Low initial IL-6 production was increased very significantly, high initial IL-6 production was, in turn, decreased. Analysis of LPS-induced IL-6 production in 3 categories of patients did not, however, reveal a regulatory effect of phage therapy (Fig. 2B). Low (below 500 pg/ml), moderate (500–1000 pg/ml) and high (above 5000 pg/ml) production of that cytokine showed a slight progressive increase of IL-6 concentrations in blood cell cultures. Spontaneous and LPS-induced IL-6 production were 115 and 1268 pg/ml, respectively in the control individuals.

Effect of phage therapy on spontaneous and LPS-induced TNF-α production in whole blood cell cultures

The results showing the effects of phage therapy on spontaneous and LPS-induced TNF-α production are shown in Fig. 3. The immunoregulatory effect of phage

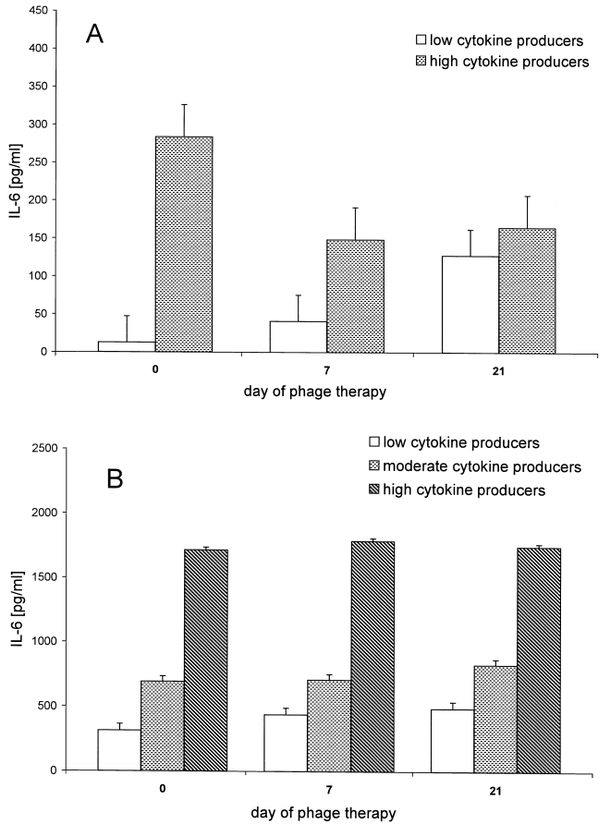

Fig. 2. Spontaneous (A) and LPS-induced (B) interleukin 6 production by blood cell cultures of patients

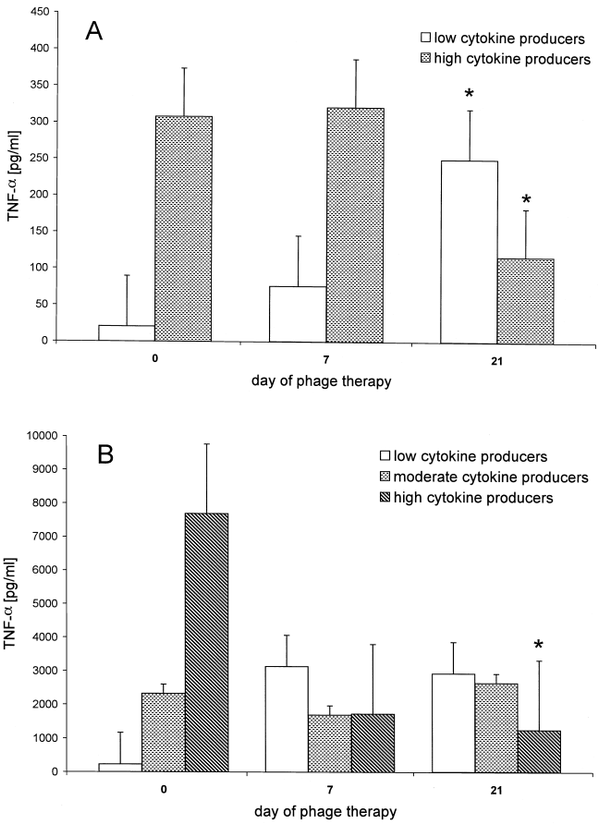

Fig. 3. Spontaneous (A) and LPS-induced (B) tumor necrosis factor α production by blood cell cultures of patients. * Statistically significant inhibition between day 0 and 21

Table 1. LPS-induced TNF-α and IL-6 production by blood cell cultures of patients with partial improvement of the clinical state

Patient	Day of therapy	TNF-α (pg/ml)	IL-6 (pg/ml)
RS	0	1833	165
	7	372	173
	21	55	268
BJ	0	102	1027
	7	211	1557
	21	108	1034
WM	0	21	651
	7	88	89
	21	182	1241
ZD	0	1260	665
	7	2200	703
	21	260	650

Table 2. LPS-induced TNF-α and IL-6 production by blood cell cultures of patients with no improvement of the clinical state

Patient	Day of therapy	TNF-α (pg/ml)	IL-6 (pg/ml)
CZ	0	392	512
	7	95	1181
	21*	–	–
KT	0	216	358
	7	203	571
	21	85	1400
RS	0	1833	165
	7	372	173
	21	55	268

* The patient died.

therapy was evident in both spontaneous as well as in LPS-induced TNF-α production. Low TNF-α production (below 50 pg/ml) was significantly increased (by more than 12-fold) – Fig. 3A. High initial spontaneous responses (above 50 pg/ml), on the other hand, were inhibited. In the case of LPS-induced TNF-α production (0–1000 pg/ml) – Fig. 3B, low initial reactivity was drastically increased (about 20 times), moderate production (1000–5000 pg/ml) not much changed, high reactivity (above 5000 pg/ml), however, was strongly inhibited. It is noteworthy that the mean 3-week values of the cytokine activity in all patient categories were in the optimal range (1000–5000 pg/ml).

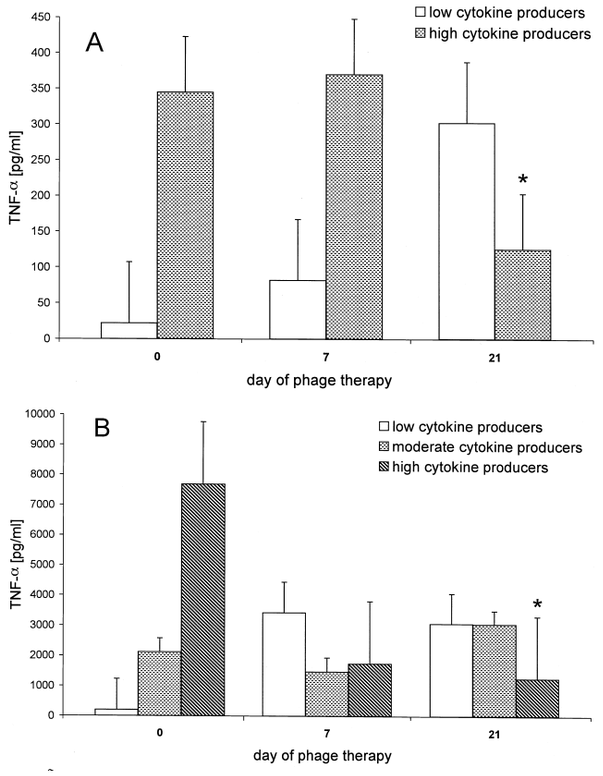

Fig. 4. Spontaneous (A) and LPS-induced (B) tumor necrosis factor α production by blood cell cultures of patients infected with Gram-positive bacteria. * Statistically significant inhibition between day 0 and 21

Figure 4 shows the effect of phage therapy on spontaneous and LPS-induced TNF-α production in 36 patients infected only with Gram-positive bacteria. It appeared that in that specific patient's category the regulatory effects of phage therapy are similar to those of the whole (48 persons) group of patients. Spontaneous and LPS-induced TNF-α production in healthy individuals were 109 and 2750 pg/ml, respectively.

Changes in cytokine production in patients with partially successful phage therapy

Table 1 shows the changes of cytokine production in patients with a partial recovery, whereas Table 2 includes patients in whom no improvement of the clinical condition was registered. In the case of patients with partial recovery, or without any therapeutic effect, progressively decreasing or durably low levels of LPS-induced TNF-α were observed. Also, IL-6 production was in most cases lower than in the healthy controls (mean 1268 pg/ml).

Discussion

In this study we have demonstrated for the first time that effective phage therapy is associated with the normalization of the cytokine production by the patient's blood cells in culture. On the other hand, patients with only partial improvement of the clinical state and those showing no signs of recovery did not follow the general pattern of regulation of the immune parameters studied. To demonstrate the immunoregulatory effects of phage therapy, patients were classified into 2 or 3 categories depending on initial immune reactivity of blood cells or cytokine serum levels. Only such an approach could explain the regulatory effect of surgery in our other studies[28].

Serum levels of inflammatory cytokines such as TNF-α and IL-6 are usually elevated following trauma or infection[18], which is inversely correlated with the ability of cells to produce these cytokines in culture[1]. Such a correlation was not observed in this study. In addition, the initial abilities of blood cells in culture to produce cytokines upon stimulation differed significantly. We suppose that different factors could account for the above mentioned phenomena. The first of these is the high heterogeneity of the studied group of patients. The patients, before subjection to phage therapy, had been in an inflammatory state for different periods. High initial reactivity could, therefore, be associated with a short-term exposure to bacteria, while a distinct hyporeactivity could result from a long-term infection. Secondly, the patients had various histories of disease and antibiotic therapy.

The observed regulatory changes during phage therapy, particularly in the case of TNF-α, seem to be a general phenomenon for all kinds of infections, since in patients infected only with Gram-positive bacteria, both spontaneous as well as LPS-induced cytokine production underwent the same regulatory pattern as for the whole population of patients. Unfortunately, the groups of patients infected with Gram-negative bacteria (6 persons) or with both types of bacteria (7 persons) were to small to demonstrate a similar type of regulation upon further division into categories of low and high responders.

Generally, the effects of phage therapy on IL-6 were less evident, although spontaneous IL-6 production in culture was regulated. We observed a similar phenomenon in other studies on the immunoregulatory action of bovine lactoferrin given *per os* to healthy individuals, where spontaneous production of cytokines (IL-6 and TNF-α) was preferentially regulated in contrast to LPS-induced cytokines[30]. Spontaneous production of cytokines in culture is dependent on some nonspecific[10] and

specific stimuli[29], so that expression of MHC class II antigens and adhesion molecules[29] may be crucial. These peripheral blood cell antigens may be preferentially affected by bacteriophage preparations taken *per os*.

The second major finding of this study was that partial or unsuccessful phage therapy was associated with a decrease in or no increase in the already low ability of cells to produce TNF-α upon LPS induction. Sometimes the LPS-induced IL-6 production was also very low (patients WM, Table 1, and RS, Table 2). During the course of effective phage therapy, low inducible TNF-α concentrations were significantly elevated and moderate ones slightly increased (Fig. 3). This is in contrast to ineffective therapy, where TNF-α was persistently low (patients KT, BJ and CZ – died) or decreasing (WM, ZD, RS).

It is still an open question as to what is actually the cause of immunoregulation during effective phage therapy. There are at least three possibilities. First, the described immunoregulation may be just a result of recovery from infection which is reflected by normalization of cytokine production. Secondly, the bacteriophages themselves may have immunoregulatory properties by interacting with immunocompetent cells. A third possibility is that bacterial debris, present in the phage lysate together with bacteriophages, may account for the described phenomenon. It cannot be excluded however, that a combination of all these factors contributes to the immunoregulatory effect of phage therapy. Studies on mice are underway to establish the key factor responsible for the immunoregulatory influence of phage therapy.

In summary, this is the first report where a direct association between effectiveness of phage therapy and normalization of cytokine production by peripheral blood cells has been demonstrated. Further studies are required to establish the exact mechanism of the described phenomenon.

References

1. ADAMIK M., ZIMECKI M., WŁASZCZYK A. and KÜBLER A. (1997): Immunological status of septic and trauma patients. II. Proliferative response and production of interleukin 6 and tumor necrosis factor α by peripheral blood mononuclear cells form septis survivor, nonsurvivor and trauma patients: a correlation with the survival rate. Arch. Immunol. Ther. Exp., **45**, 277–284.
2. BAUMAN H. and GAULDIE J. (1994): The acute phase response. Immunol. Today, **15**, 74–80.
3. BELLOMO R. (1992): The cytokine network in the critically ill. Anaesth. Intensive Care, **20**, 288–302.
4. BEUTLER B. (1995): TNF, immunity and inflammatory disease: lessons of the past decade. J. Invest. Med., **43**, 227–235.
5. CALANDRA T., GERAIN J., HEUMANN D., BAUMGARTNER J. D. and GLAUSER M. P. C. (1991): High circulating levels of interleukin 6 in patients with septic shock: evolution during sepsis, prognostic value and interplay with other cytokines. Am. J. Med., **91**, 23–29.
6. CARLTON R. M. (1999): Phage therapy: past history and future prospects. Arch. Immunol. Ther. Exp., **47**, 267–274.
7. CISŁO M., DĄBROWSKI M., WEBER-DĄBROWSKA B. and WOJTOŃ A. (1987): Bacteriophage treatment of suppurative skin infection. Arch. Immunol. Ther. Exp., **35**, 175–183.
8. DARVILLE T., GIRROIR R. and JACOBS R. (1993): The systemic inflammatory response syndrom (SIRS). Immunology and potential immunotherapy. Infection, **21**, 279–288.
9. ESPEVIK T. and NISSEN-MAYER J. (1986): A highly sensitive cell line WEHI 164 clone 13, for measuring cytotoxic factor/tumor necrosis factor from human monocytes. J. Immunol. Methods, **95**, 99–105.
10. GERY J. R., DAVIES D., DERR J., KRAFT W. and BARRANGER J. A. (1981): Relationship between production and relevance of lymphocyte-activity factor (interleukin 1) by murine macrophages. Cell. Immunol., **64**, 293–303.
11. HANSEN M. B., NIELSEN S. E. and BERG K. (1989): Reexamination and further development of a precise and rapid dye method for measuring cell growth/kill. J. Immunol. Methods, **119**, 203–210.
12. HAVEL E. A. (1989): Evidence that tumor necrosis factor has an important role in antibacterial resistance. J. Immunol., **143**, 2894–2899.
13. HIRATO T., AKIRA S., TAGA T. and KISHIMOTO T. (1990): Biological and clinical aspects of interleukin 6. Immunol. Today, **11**, 443–449.
14. JIANG W. G. and PUNTIS M. C. A. (1997): Immune dysfunction in patients with obstructive jaundice, mediators and implications for treatments. J. HPB Surgery, **10**, 129–142.
15. LIN Z., SIMPSON J. and CHEERS C. (1995): Interaction of interleukin 6, tumor necrosis factor and interleukin 1 during *Listeria* infection. Immunology, **85**, 562–567.
16. MARANO H. A., FONG Y. and MOLDAWAR L. L. (1990): Serum cachectin/tumor necrosis factor in critically ill patients with burns correlates with infection and mortality. Surg. Gynecol. Obstet., **170**, 32–38.
17. NISHIMOTO N., YOSHIZAKI K., TAGOH H., MONDEN M., KISHIMOTO S., HIRATO T. and KISHIMOTO T. (1989): Elevation of serum interleukin 6 prior to acute phase proteins on the inflammation by surgical operation. Clin. Immunol. Immunopathol., **50**, 399–401.
18. PINSKY M. R., VINCENT J. L., DEVIERE J., ALERGE M., KAHN R. J. and DUPONT E. (1993): Serum cytokine levels in human septic shock. Relation to multiple organ failure and mortality. Chest, **103**, 565–575.
19. ŚLOPEK S., DURLAKOWA I., WEBER-DĄBROWSKA B. and KUCHAREWICZ-KRUKOWSKA A. (1984): Results of bacteriophage treatment of suppurative bacterial infections. III. Detailed evaluation of the results obtained in further 150 cases. Arch. Immunol. Ther. Exp., **32**, 313–335.
20. ŚLOPEK S., DURLAKOWA I., WEBER-DĄBROWSKA B., KUCHAREWICZ-KRUKOWSKA A., DĄBROWSKI M. and BISIKIEWICZ R. (1983): Results of bacteriophage treatment of suppurative bacterial infections. I. General evolution of the results. Arch. Immunol. Ther. Exp., **31**, 267–291.

21. ŚLOPEK S., DURLAKOWA I., WEBER-DĄBROWSKA B., KUCHAREWICZ-KRUKOWSKA A., DĄBROWSKI M. and BISIKIEWICZ R. (1983): Results of bacteriophage treatment of suppurative bacterial infections. II. Detailed evaluation of the results. Arch. Immunol. Ther. Exp., **31**, 293–327.
22. ŚLOPEK S., KUCHAREWICZ-KRUKOWSKA A., WEBER-DĄBROWSKA B. and DĄBROWSKI M. (1985): Results of bacteriophage treatment of suppurative bacterial infections. IV. Evaluation of the results obtained in 370 cases. Arch. Immunol. Ther. Exp., **33**, 219–240.
23. ŚLOPEK S., KUCHAREWICZ-KRUKOWSKA A., WEBER-DĄBROWSKA B. and DĄBROWSKI M. (1985): Results of bacteriophage treatment of suppurative bacterial infections. V. Evaluation of the results obtained in children. Arch. Immunol. Ther. Exp., **33**, 241–260.
24. ŚLOPEK S., KUCHAREWICZ-KRUKOWSKA A., WEBER-DĄBROWSKA B. and DĄBROWSKI M. (1985): Results of bacteriophage treatment of suppurative bacterial infections. VI. Analysis of treatment of suppurative *Staphylococcus* infections. Arch. Immunol. Ther. Exp., **33**, 261–273.
25. ŚLOPEK S., WEBER-DĄBROWSKA B., DĄBROWSKI M. and KUCHAREWICZ-KRUKOWSKA A. (1987): Results of bacteriophage treatment of suppurative bacterial infections in the years 1981–1986. Arch. Immunol. Ther. Exp., **35**, 569–583.
26. VAN SNICK J., CAYPHAS S., VIUK A., HYTTENHOVE C., COULE P. G., RUBIRA M. R. and SIMPSON R. J. (1996): Purification and NH2-terminal amino acid sequence of a T cell-derived lymphokine with growth factor actvity for B cell hybridomas. Proc. Natl. Acad. Sci. USA, **83**, 9679–9683.
27. WEBER-DĄBROWSKA B., KOŹMIŃSKA J., MULCZYK M. and KACZKOWSKI H. (1996): Wykorzystanie bakteriofagów w leczeniu przewlekłego ropnego zapalenia zatok szczękowych. Post. Med. Klin. Dośw., **5**, 291–293.
28. WŁASZCZYK A., ADAMIK B., DUREK G., KÜBLER A. and ZIMECKI M. (1996): Immunological status of patients subjected to cardiac surgery: serum levels of interleukin 6 and tumor necrosis factor α and the ability of peripheral blood mononuclear cells to proliferative and produce these cytokines *in vitro*. Arch. Immunol. Ther. Exp., **44**, 225–234.
29. ZIMECKI M., WIECZOREK Z., KAPP J. and PIERCE C. W. (1989): Structures on T cells and macrophages involved in interleukin 1 (IL-1) secretion by macrophages upon contact with syngeneic thymocytes. Arch. Immunol. Ther. Exp., **37**, 587–592.
30. ZIMECKI M., WŁASZCZYK A., CHENEAU D., BRUNEL A -S., MAZURIER J., SPICK G. and KÜBLER A. (1998): Immunoregulatory effects of a nutritional preparation containing bovine lactoferrin taken orally by healthy individuals. Arch. Immunol. Ther. Exp., **46**, 231–240.

Received in July 1999
Accepted in September 1999