The Immunomodulatory Profile of Pseudomonas aeruginosa

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Among the Isopathic/Homeopathic preparations, *Pseudomonas aeruginosa 5X* belongs to the „Haptene“ active group, which is to say that, in this case, rather than using entire bacterial cells or larger structures such as cell walls as active ingredients, a very specific extract is used, enriched primarily with cellwall polysaccharides.

Based an current knowledge, therapy using „Haptenes“ should be particularly well suited to absorb pathogen toxins and antigens or circulating immune complexes and to dissipate the resulting reaction blockades (CORNELIUS, *Nosoden und Begleittherapie* [Nosodes and Adjuvant Therapy] 1990).

Immunological experiments were performed in order to identify more precisely the action mechanisms of *Pseudomonas aeruginosa 5X* on the body's immune system. These investigations were immensely helpful in expanding our understanding of the clinical effect of *Pseudomonas aeruginosa 5X*. Clinical observation does not by itself permit any conclusions regarding the distinctive immunomodulatory characteristics of the active substance; for this, one needs to carry out the appropriate investigations in test tubes. Specific immunological reactions were experimentally simulated which proceed (somewhat) the same in the organism, but which, of course, are not measurable in the organism or the blood. Nearly all modern immunomodulatorily active substances are investigated, tested and characterized as to their effects by this and similar methods.

1. The **target cells** of the active substance in the cell population of human venous blood (leukocytes) should be determined; these determinations should yield information concerning the kind of modulation of the cascade of endogenous immune reactions.
2. The **effect on phagocytosis performance** of monocytes and granulocytes, as the primary reaction of the immunocompetent cells, was investigated.
3. The **direction of modulation** of the immune system should be recognized: dominance of cellular or humoral immunity by investigating cytokine induction in dormant and active peripheral mononuclear leukocytes.
4. The **immune-complex-binding** properties were analyzed.

1. Identification of the Immunological Target Cells for *Pseudomonas aeruginosa 5X*

Bacterial antigens bind to various kinds of immunological structures, among them, as humoral components, the complement proteins and, as cellular structures, the surface molecules of cells generally and of leukocytes in particular. In part, the interaction between cell and bacterial antigen is based an the preceding reaction with the humoral factors; this is known as „opsonization“. There are known specific receptors for bacterial endotoxins (lipopolysaccharide = LPS). One of them, CD14, is found on monocytes/macrophages. The cell is activated via these receptors, inducing the „oxidative burst“ and/or stimulating cytokine synthesis. Binding of endotoxin to free soluble CD14 then neutralizes LPS.

Moreover, there are other cell surface molecules that can react with bacterial structures after opsonization, including complement and immunoglobulin receptors. Therefore, identifying immunomodulatory properties is important for an understanding of the clinical effect of bacterial antigens. This includes characterization of binding and phagocytosis of the bacterial active substance *Pseudomonas aeruginosa 5X* on leukocytes.

To this end, the active ingredient of *Pseudomonas aeruginosa 5X* was coupled to a fluorescing dye (fluorescein isothiocyanate = FITC) and, after incubating Pseu-FITC with freshly-isolated human blood leukocytes, the binding - or the relative amount of bound Pseu-FITC - on the cell surface was measured by means of analytical flow cytometry.

Results

Pseu-FITC binds with roughly equal intensity to the surface of all three leukocyte populations (monocytes, granulocytes, lymphocytes). Preferential or selective binding to one cell type has not been detected. A specific receptor or binding site for binding Pseu-FITC has not been able to be identified.

Pseu-FITC binding to the surface of cells is probably nonspecific. Buildup of Pseu-FITC on the cell surface cannot be reduced by using non-marked Pseu-FITC. This would be expected if there were a specific receptor as binding partner for Pseu on the cell surface.

The idea of a nonspecific binding capability for Pseu-FITC on the surface of various cell subpopulations is supported by the experimentally demonstrated binding of Pseu-FITC on intact yeast cells, which exhibit structures on their surface that probably bind Pseudomonas. Receptors of this sort (e.g. the so-called mannose receptor) are ubiquitous.

2. PhagocytosisModulating Properties of *Pseudomonas aeruginosa 5X*

a) The modulatory capacity of phagocytosis performance on monocytes and granulocytes of peripheral blood can be determined by means of a biocatalyst. Phagocytosis, an „archaic" and primary reaction of immunocompetent cells, is an important indicator for finding out the modulatory pathways of immunological feedback control systems.

Both monocytes and granulocytes are capable of phagocytosis of particles and microbial components. With the selected method, the number of phagocytizing cells and the phagocytosis performance of individual cell populations can be determined. Both parameters are important for the characterization of immunocompetent cells in terms of their phagocytotic properties.

Heparinized whole blood was incubated with fluorescent-tagged Pseu (Pseu-FITC) and, after lysis of the erythrocytes, analyzed by means of analytical flow cytometry.

Results

Pseu-FITC is phagocytized both by granulocytes and macrophages. Based on the previously obtained results in identifying the target cells, it seems likely that this is not a case of receptor-mediated phagocytosis. The adhesion potential of Pseu-FITC is in all likelihood considerably reinforced by binding with anti-Pseudomonas antibodies. This immune complex can, via additional receptors - e.g. Fc receptors - react with the surface of phagocytes.

b) Whether *Pseudomonas aeruginosa 5X* impairs or promotes phagocytosis of zymosan was investigated by means of analytical flow cytometry.

Results

We were unable to observe any influence of *Pseudomonas aeruginosa 5X* on the phagocytosis of zymosan-FITC by granulocytes/monocytes (Zymosan is a yeast-cell-wall preparation from *Saccharomyces cerevisiae)*. Pseu itself binds, dosage-dependent, to the surface of yeast cells *(Candida albicans)*. This does not lead to an increase in phagocytosis performance, neither does it influence it negatively, however.

3. Cytokine Induction in Peripheral Mononuclear Blood Cells

Peripheral mononuclear blood cells (monocytes) were isolated from the blood of regular blood donors and incubated with various concentrations of the *Pseudomonas aeruginosa 5X* active factor. The initial reaction was performed first with dormant monocytes and then with active cells. Artificially-produced immune complexes from human IgG were used as stimulus.

Various cytokines were found in the cell culture population, which were synthesized as the monocytes' and lymphocytes' reaction to contact with *Pseudomonas aeruginosa 5X:*

IL-4 could not be determined in the cell culture population; IFN-g was clearly detectable only in 1 of 3 blood donors (the same was true of IL-2 in very low concentration). The other cytokines were released in easily-detectable concentrations in all 6 donors: *Pseudomonas aeruginosa 5X* significantly increased - dosage-dependent - the synthesis of **TNF-á, IL-1ß, IL-6, IL-10 and GM-CSF** compared to the control with no test substance. The following results were particularly remarkable:

- In the case of **TNF-á** and **IL-10**, a significant increase was detectable even at an active factor concentration of 10 ng/ml (=8X).
- In the presence of the immune complexes, the cytokine production of **TNF-á** and **GM-CSF** increased significantly even more.
- **GM-CSF** exhibited weck induction under the sole influence of *Pseudomonas aeruginosa 5X*, but very strong induction as a result of synergy between *Pseudomonas aeruginosa 5X* and immune complexes (cf. *Figs.3-7)*

Monocytes and B lymphocytes are presumably stimulated via the immune-complexes; the site of the immune-complex interaction is probably the Fc-receptors of the blood cells (immunoglobulin's Fc component is responsible for

Final Results

• TNF-α	=	Tumor Necrosis Factor α
• IL-1β, -2, -4, -6, -10	=	Interleukin 1β, -2, -4, -6, -10
• IFN-γ	=	Interferon γ
• GM-CSF	=	Granulocyte/Monocyte Colony-Stimulating Factor

antibody complement and receptor binding).

Concerning the Effect of Cytokines

Cytokines are biologically highly-active polypeptides and glycoproteins (size: 15,000 – 30,000D) which play a significant role in many tissues in intercellular signal transmission, in phenotype and the cytoskeletal structure modulation, and in regulation of the proliferation rate or apoptosis. They are synthesized by more than one kind of cell and exhibit a wide spectrum of overlapping functions. Numerous investigations to date have demonstrated, in vitro and in vivo, both the effects of individual cytokines on particular bone-marrow cells as well as a number of additive and synergistic effects in the area of hematopoesis and the maintenance of immune system defensive preparedness. The labyrinthine interactions among these mediators led to the concept of a functional cytokine network, which is an important element in adapting blood cell production to the organism's current needs.

On the level of activated T lymphocytes, and based on the secreted cytokines of the two subgroups of T helper cells T_H1 and T_H2, one can determine in which direction the immune system is being stimulated; thus the T_H1 cells excrete IL-2 and IFN-g, thereby stimulating cellular immune defenses, whereas T_H2 cells excrete IL-4 and IL-10 primarily stimulating humoral defenses (cf. *Fig.1)*. The complex cytokine network represents the basis for regulation of the entire hematopoetic process. *Figure 2* illustrates which cytokines intervene in the differentiation of the pluripotent blood stem cells, as well as the maturation of the precursor cells (cf. *Fig. 2)*.

The cytokines TNF-á, IL-1ß and IL-6 are often called pro-inflammatory cytokines. They are produced particularly by immunocompetent cells. They are of great significance for inflammations and combating tumors. These cytokines are induced by bacterial antigens, for example.

Synthesis of TNF-á is activated by a variety of stimuli, such as interferons, GM-CSF, immune complexes. TNF-á exhibits a broad spectrum of biological activities, including:

- Causing cytolysis or cytostasis of many tumor cell lines in vitro
- Inducing hemmorrhagic necrosis in transplanted tumors
- Strengthening phagocytosis and cytotoxicity of polymorphonuclear granulocytes
- Responsibility for manifold changes in the endothelium

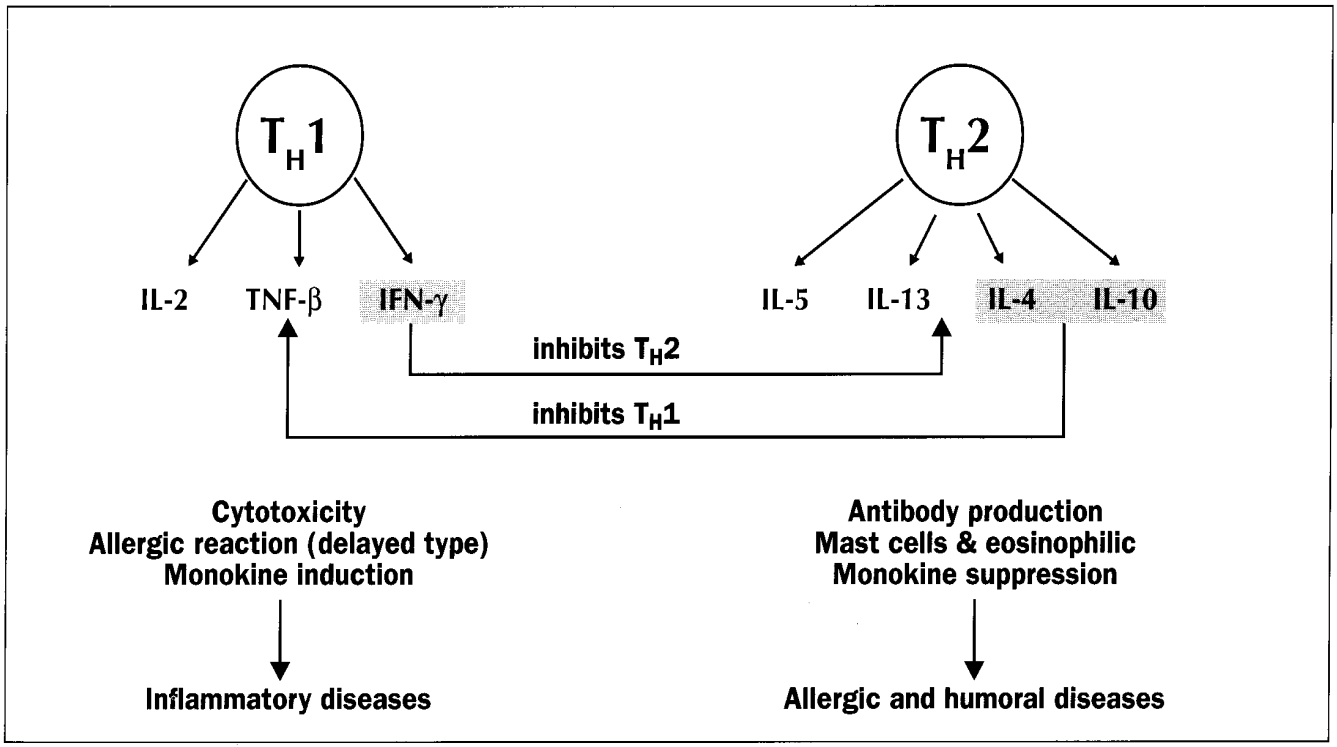


Fig.1: Functional roles and mutual regulation of T_H1 and T_H2 cells.

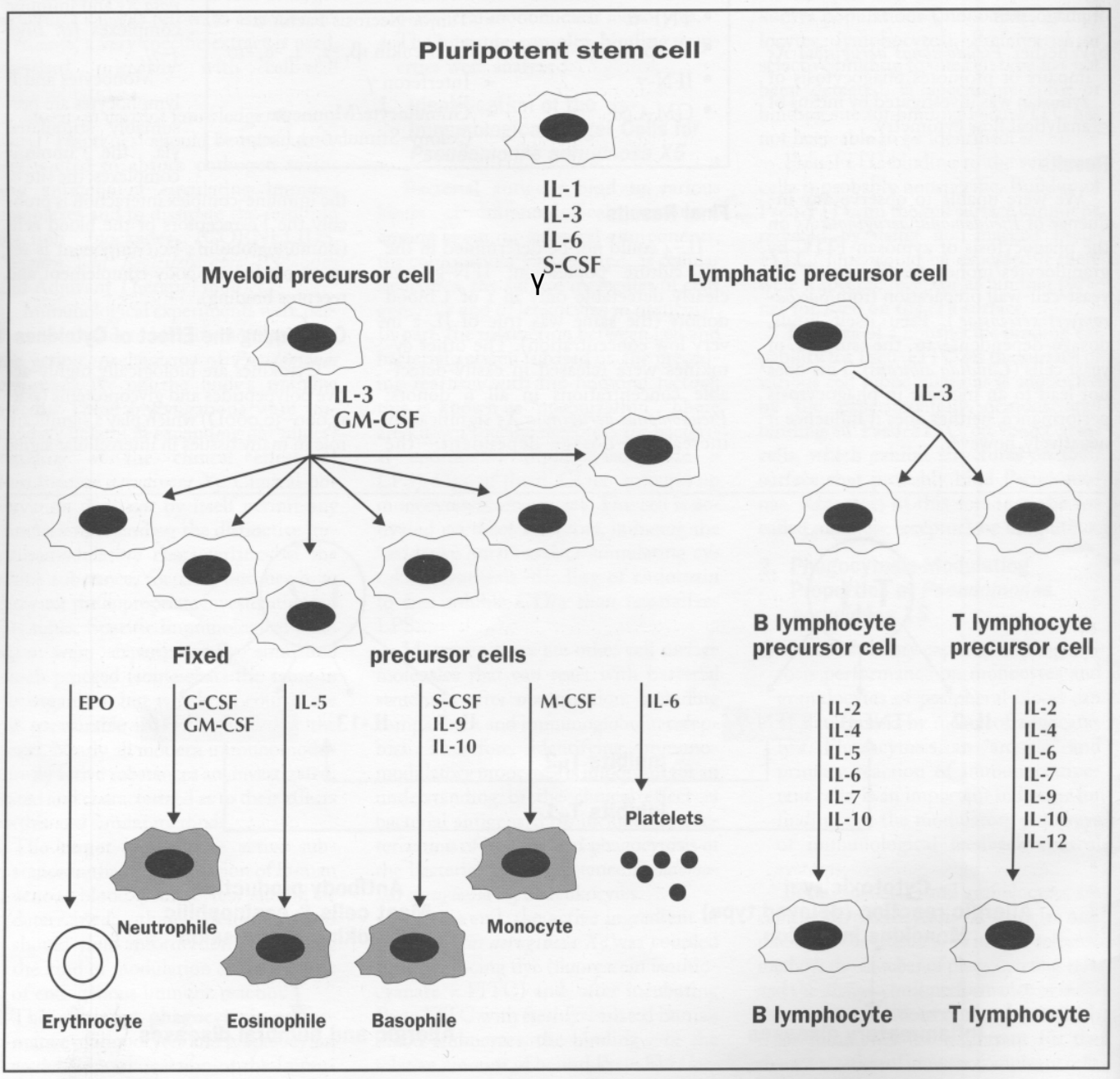
- Reinforcing the proliferation of T & B lymphocytes, as well as differentiation of the latter.

It is used in cancer therapy as an isolated substance in combination with interferon- to heighten the aggressiveness of lymphokine-activated killer cells.

GM-CSF (Granulocyte/ Macrophage Colony-Stimulating Factor) is secreted primarily by lymphocytes and macrophages. This cytokine is an important factor for growth and differentiation of granulocytes and macrophages. GM-CSF has a strongly chemotactic effect an neutrophil granulocytes and can reinforce the phagocytic activity of granulocytes and macrophages. It stimulates proliferation and differentiation of hematopoetic precursor cells and has a myeloprotective effect. Clinical interest was therefore aroused in the treatment of diseases or bodily conditions involving cytopenia (reduced count in the blood of erythrocytes, granulocytes, monocytes or thrombocytes) or its consequences:

- High-dosage chemotherapy in cancer treatment
- Autologous bone-marrow transplants
- Radiation therapy
- Leukemia
- Agranulocytosis
- Aplastic anemia
- Chronic infections

The cytopenic reaction state can be overcome through the stimulus



that GM-CSF effects in the bone marrow by providing a signal for the differentiation and maturation of blood stem cells.
IL-1ß is produced chiefly by activated macrophages, monocytes and neutrophils. Its production is stimulated by other cytokines, as well as by bacterial antigens, endotoxins, viruses, etc. It strengthens hematopoesis in synergy with other hemato-poetically active cytokines. The effects include:

- Stimulation of T helper cells for the secretion of additional cytokines (e.g. IL-2)
- Promoting the proliferation of B lymphocytes and the production of immunoglobulins
- Promoting the proliferation and activation of natural killer cells
- Anti-proliferative effect on various types of tumor cells
- Responsible for endothelial changes (both alone and in conjunction with TNF-á
- Participating in inflammation reactions by increasing secretion of inflammatory proteins
- Strongly chemotactic effect on leukocytes
- Generating fever as an endogenous pyrogen
- Influencing hormone synthesis via CNS effects
- Synergistic effect on the induction of GM-CSF production and thereby proliferative effect on blood stem cells.

Clinical attention is focused an the use of IL-1ß in cases of T cell defects, in order to accelerate their reconstruction after massive immune suppression or cytostatic treatment. In animal tests, it exhibits a radioprotective effect, promotes wound healing or stimulanes angiogenesis.
IL-6 responds to much the same stimuli as do the other cytokines. It influences antigen-specific immune response and inflammatory reactions. As primary mediator, it induces the so-called „acute phase reaction". Its biological effects include:

- Differentiation factor for B lymphocytes, stimulation of IgG antibody secretion
- Differentiation and activation factor for T lymphocytes
- Thrombopoetic effect as well as promoting the proliferation of blood stem cells (synergistically with IL-3)
- Involved in the pathogenesis of chronic polyarthritis
- Deregulated expression - i.e. excessive overproduction - in various myelomas
- Cellular and biochemical alterations caused by induction of the „acute phase reaction" - which, in the end, aid in local curtailment of inflammatory processes.

Clinical application is frequently in combination with GM-CSF after highdosage chemotherapy and bone-marrow transplants.
Unlike the above cytokines, **IL-10** is characterized as an **anti-inflammatory** cytokine. We know from in-vitro experiments that IL-10 **down-regulates** the secretion of pro-inflammatory cytokines such as TNF-á, IL-1ß and IL-6. In this context, this cytokine is also ascribed immunosuppressive properties, and clinical applications for IL-10 have been derived from this, for example in treating chronic inflammations, rejection reactions and autoimmune diseases.
The biological effects include:

- Growth and differentiation factor for activated B lymphocytes
- Direct antagonist of TNF-á, which is stimulated by lipopolysaccharides, e.g. in cases of gram-negative sepsis by bacterial endotoxins, meningococcus sepsis
- Anti-inflammatory effect in cases of ulcerative colitis and Crohn's syndrome
- Sharply reduced in cases of alcohol-induced cirrhosis of the liver (whereas TNF-á is overproduced)
- Inhibits blast proliferation (leukocyte precursor) in cases of acute myeloid leukemia.

Summary of results of the effect of *Pseudomonas aeruginosa 5X* on cytokines

Pseudomonas aeruginosa 5X does not seem to be cytokine-inducing in every case on the subclass of the T_H1 cells. Therefore, the immunomodulatory effect of *Pseudomonas aeruginosa 5X is* more strongly seen to lie in the direction of the T_H2 cells - i.e. of humoral immunity, via:

- Induction of the pro-inflammatory cytokines (TNF-á, IL-1ß and IL-6)
- Induction of the hematopoetic cytokine GM-CSF

- Induction of the anti-inflammatory cytokine IL-10
- In part, considerable increase of cytokine production of TNF-á, IL-1ß, IL-6 and particularly GM-CSF in the presence of immune complexes as immune stimulants.

4. Reaction of *Pseudomonas aeruginosa* 5X with Immunoglobulins and Immune Complexes

It has been recognized in recent years that the humoral part of the immune system is more tightly coupled with the cellular part than had previously been thought. The division into two parts turns out actually to be more historical than real. There is a whole series of receptors on the cell surface which can react both with immunoglobulin as well as with other immune-system structures (complement proteins). This network regulates thus via receptors - e.g. antibody production - but also via the induction of certain regulatory cytokines.

An in-vitro test using a microtiterplate-based ELISA (enzyme-linked immunosorbent assay) was developed to investigate binding properties. The microtiter plates were coated with *Pseudomonas aeruginosa 5X*, human Serum, human immunoglobulin subclasses or synthesized immune complexes (cf. *3rd below)*, incubated and the quantity of bound immunoglobulin determined by means of an enzyme reaction.

Results

The test exhibited no relevant binding of the immunoglobulin subclasses IgG1, IgG2 and IgG3 - nor was any expected, since these isolated, so-called „inert“ antibodies in all likelihood exhibit no specificity whatsoever regarding Pseudomonas antigens.

On the other hand, there was a concentration-dependent increase in binding with the use of highly-purified but undefined IgG from human serum (normal donor serum,

Induction of Cytokine Release by *Pseudomonas aeruginosa* 5X In Vitro with Human PBMC

(See the text for illustration details)

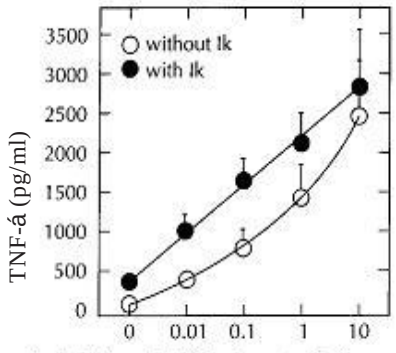


Fig 3: TNF-α

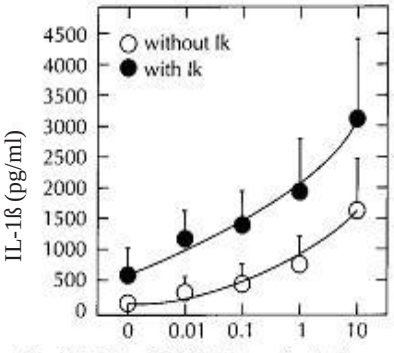


Fig 4: IL-1β

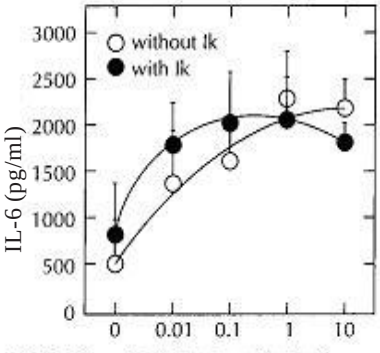


Fig 5: IL-6

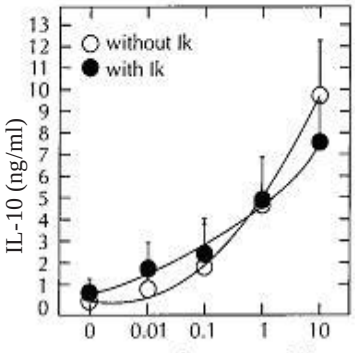


Fig 6: IL-10

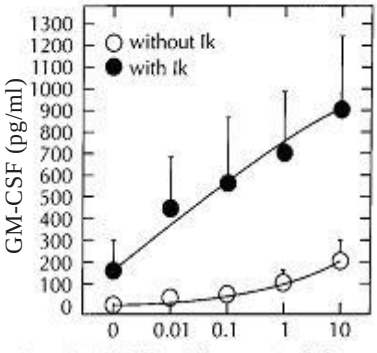


Fig 7: GM-CSF

Zytokin	Zytokin Induction			
	Comparison Contr. vs. San Pseu (without IC stimulation)		Comparison San Pseu vs. San Pseu with IC	
	Test substance concentration		Test substance concentration	
	10ng/ml	10µg/ml	10ng/ml	10µg/ml
TNF-α	⇧	⇧	⇧	⇧
IL-1β	-	⇧	⇧	⇧
IL-6	-	⇧	-	⇩
IL-10	⇧	⇧	-	-
GM-CSF	-	⇧	⇧*	⇧
IFN-γ*	-*	⇧*	⇧	-*
IL-2*	-*	⇧*	-*	⇩*
IL-4	n.p.	n.p.	n.p.	n.p.

* only with one of three cell donors, tendency provable
n.p. not proven
⇧ significant zytokin induction
⇩ reduced zytokin induction
\- no detectable difference

not from patients). This evidently implies that Pseudomonas antibodies are present in human blood, since the human immune system has undergone confrontation with the antigens of the classic commensal *P. aeruginosa*. This result was confirmed with patient sera, in which antibodies were detectable even before administering *Pseudomonas* aeruginosa 5X. Antibodies against the microorganism are evidently a natural part of the immune defense system, which is thus able to control the pathogen.

Summary of the In-Vitro Experiments with *Pseudomonas aeruginosa 5X*

An idea can be derived from these results as to how Pseudomonas aeruginosa 5X might work in vivo. The introduction of highly-antigenic enriched *Pseudomonas* aeruginosa 5X structures into the immune system (subcutaneously or intramuscularly) probably leads rapidly - because of the immunoglobulins present in the body (antibodies) - to an immune complex formation.

This substance probably represents the actual immune modulator. The effect of the complex likely has less to do with induction of antibodies against *Pseudomonas* aeruginosa 5X than with regulation of immunological processes or correction of immunological imbalances, and develops its effect, for example, via induction of cytokines, particularly GM-CSF and IL-10.

The former sends a strong hematopoetic signal to the bone marrow in the form of a pro-inflammatory stimulus, which, after it has had sufficient time to overcome the immune system's reaction blockage, is „reined in" again by the anti-inflammatory effect of IL-10. What is interesting about this activity profile is that, due to the influence of homeopathic dilutions of the *Pseudomonas* aeruginosa 5X active substance, the body's own mechanisms for dealing with immune deficiencies are stimulated, whereas conventional tumor therapy tries to achieve this by administering, for example, isolated pure substances from cytokines, but at the cost of triggering side effects that are difficult to control.

The results permit certain conclusions regarding the areas of indication for *Pseudomonas* aeruginosa 5X: in disease cases in which an immune defect is involved - whether it be the disease itself or caused by immunosuppressive treatment - Pseudomonas aeruginosa 5X could be used with immunological justification:

- With patients undergoing radiation therapy
- With patients undergoing cytostatic therapy
- With patients under long-term immune suppression; i.e. for all disease states associated with leukopenia.

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