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AUTOSPECIFICITY IN *ESCHERICHIA COLI* AUTOVACCINES: EFFECTS ON THE HUMAN IMMUNE SYSTEM *IN VITRO*

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SUMMARY

The Autovaccine Herborn (AV) consists of a lipid fraction (Lipid A like structures) of the outer membranes of *E. coli* rough strains isolated from the stools of those patients that are to be treated with this individualised preparation. According to previous results the major pharmacological effects of this particular type of autovaccine seem to be mediated through the activation of cells of the immune system (leukocytes).

To evaluate the degree of individuality in the immuno-modulatory effects of the AV, 5 different autovaccines were prepared from the stools of 5 healthy subjects by a particular manufacturing process developed by the Institute for Microecology, Herborn. Each of these autovaccines was tested in whole-blood cultures together with the blood of each of the five stool-donors. For this purpose the cells were co-activated in separated cultures with stimuli specific for a) phagocytes (Zymosan), b) T-lymphocytes (antibodies to CD3 and CD28) or c) B-lymphocytes (Pokeweed mitogen). As readouts for AV activities the production of a series of cytokines was chosen.

A rather homogeneous type of response was observed when the AVs and the blood for the cultures came from different donors. Roughly, this response resembles a Th1 type reaction (i.e., inducing TNF-α, IL-6 and IFN-γ but suppressing IL-5 as a Th2 cytokine). In addition a pronounced increase in the secretion of IL-10 was observed, regardless of whether Zymosan or PWM was used as stimulant.

In contrast, when the five autovaccines were tested together with the blood of the same donor the stool sample was obtained from ("autologous" situation), much more heterogeneity in the production of the different cytokines was to be seen. The basic pattern of a Th1 response still was present, yet the ratios sometimes changed dramatically. For example, in some instances even the synthesis of TNF-α, a mediator usually produced in high amounts in response to Lipid A, did not show any influence by the autologous AV.

Our results provide first clear evidence for the postulated individuality in the reactions of the human immune system to the special, lipid-type of autovaccine (Autovaccine Herborn) tested in the study presented below.

INTRODUCTION

During the fifties of the past century the name "autovaccine" has been introduced to characterise bacterial preparations, for example derived from dermal flora, that were meant to specifically immunise the host against facultative pathogenic bacteria that can cause recurrent local inflammations (*Müller*, 1950). A few years later the Autovaccine Herborn (AV) was created and introduced to human medicine (*Kolb*, 1959). This was long before some decades later the concept of antigen-specific cellular reactions of the immune system was developed and such reactions could clearly be differentiated from those of non-specifically acting cells (the so-called natural immune system).

Today it is quite clear that the antigen-specific cells predominantly respond to polymers of amino-acids and/or sugars. Only rarely they recognise lipid structures specifically enough to elicit a selective antibody or T-cell response (*Porcelli*, 1998). The AV on the other hand consists of a highly purified fraction of lipids isolated from intestinal bacteria. This was shown by a thorough chemical characterisation during the past years which demonstrated the biologically active molecules of AV to be lipid structures that resemble very closely the Lipid A of the outer membrane of Gram-negative bacteria (*Thies*, 2001). Therefore, the postulated concept of the AV inducing an individual-specific immunomodulation is questionable.

The present study was conducted to address this issue. Five different autovaccines, prepared from the faeces of healthy volunteers were tested for immunomodulatory activities in cell cultures prepared from the whole blood of each of the stool donors.

MATERIALS AND METHODS

Preparation of the autovaccines

Stool samples were collected from 5 healthy volunteers who at the time of stool collection had to be clinically free of any symptoms of acute or chronic inflammatory diseases, gastro-intestinal complaints or any other type of immune activating disorders. No drug intake or vaccination was allowed within the last four weeks before stool. All samples were sent to the Institute of Microecology at the day of collection. In this laboratory the AVs were prepared from rough strains of *E. coli* cultivated on selective agars. The stock solutions of the AVs from all 5 donors were then prepared by a standardised procedure from a suspension of 10^9 germs/ml and shipped back to EDI (Experimental & Diagnostic Immunology) GmbH.

Whole blood cultures

Cultures of whole-blood samples of each of the donors from which the AVs were performed as described elsewhere (*Schmolz* et al., 2001). Briefly, whole-blood (anti-coagulated with heparin) was suspended in RPMI 1640 together with the stimulants, which were either Pokeweed Mitogen (PWM, Sigma, Munich), an antibody mixture to CD3 (R&D Systems, Wiesbaden) and CD28 (DKFZ, Heidelberg) or Zymosan (Sigma, Munich) that was opsonised with human AB plasma. RPMI 1640 medium served as control in non-stimulated cultures.

The cultures were incubated (37°C, humidified atmosphere with 5% CO_2) for 24 or 48 hrs, depending upon the type of stimulation and the mediator to

be tested (Zymosan-activated cultures: 24 h; PWM and antibody stimulations: 48 h). Thereafter, all plates were centrifuged and the supernatants were harvested and stored at -20°C until testing for mediator synthesis in standard ELISA assays.

Test for immunomodulatory activities of AVs

Serial dilutions (1:3) were prepared from these lipid suspensions by adding Hanks' Balanced Salt Solution (HBSS). These dilutions were tested in cultures of freshly isolated whole blood (see above). The immunomodulatory effects of the different autovaccines were determined by measuring cytokine synthesis (standard enzyme immunoassays): Activities on monocytes were detected in Zymosan stimulated cultures by measuring the mediators TNF-α, IL-6, IL-10 ("IL-10 Zy") and IL-12; the effects on T lymphocytes were tested in anti-CD3/antiCD28 stimulated cultures by quantifying IFN-γ (Th1 cells) and IL-5 (Th2 cells). Finally, influences of AVs on B cell activation were derived from PWM supernatants in which also IL-10 ("IL-10 PWM") was determined.

Calculations

When comparing the cytokine syntheses of individual blood donors, a frequently occurring problem is the huge heterogeneity in the capacity of the leukocytes of the single donors in the production of the cytokines. Thus, it was decided to normalise the data by setting the stimulated controls to the level of 1.0 and calculate stimulation indices using the following formula:

$$\text{Stimulation Index (SI)} = \frac{\text{cytokine concentration of sample culture (pg/ml)}}{\text{cytokine concentration of control culture (pg/ml)}}$$

In some cultures a discontinuous dose-response curve was observed. This prevented a determination of half-maximal effective concentrations and thus a direct comparison of the overall activity of the individual AVs. To enable this, the area under the curve (AUC) of each activity curve was determined. For calculating "relative activities" of the homologous AVs compared to the autologous ones, the AUC of the autologous AV was adjusted to 1.0 and the values of all relative activities of the different cytokines were plotted into net diagrams:

$$\text{Relative Activity (RA)} = \frac{\text{cytokine conc. of homologous AV}}{\text{cytokine conc. of autologous AV}}$$

RESULTS

The first series of experiments was performed to give a measure of the variation in activities AVs generate when they are tested on leukocytes of different donors (Figures 1-7). As could be shown, the degree of similarity between the dose-response curves among the AVs when tested on the whole-blood cultures of a single donor was striking (here exemplified by plotting the graphs of blood donor CR). All parameters tested showed clear and characteristic AV-dependent changes.

Instead, when examining the effects of AVs in cultures of blood donors that also gave the stools to prepare the respective AV ("autologous" situation), the picture changed dramatically. In

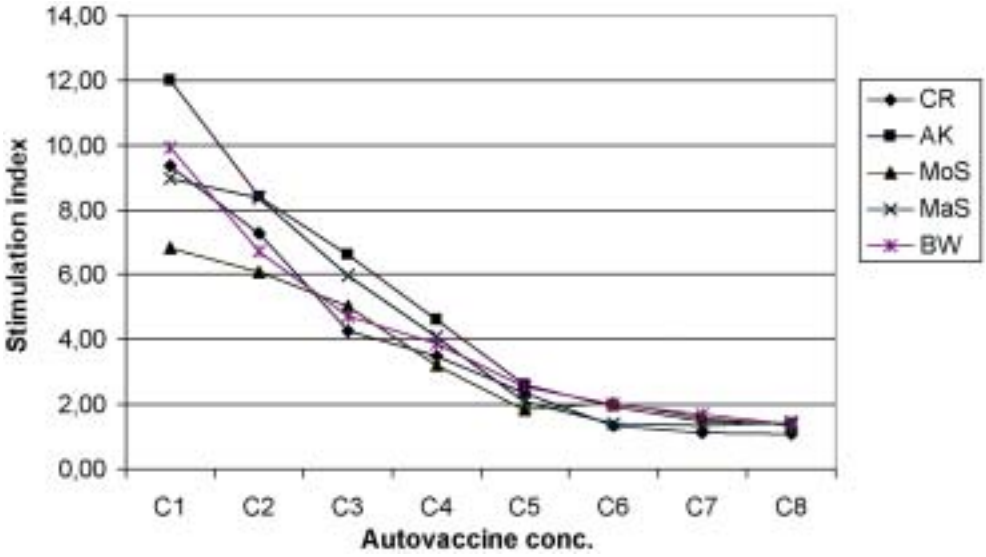

Figure 1: Test of AVs from 5 different donors in whole-blood cultures of one of the 5 donors (CR). The cultures were activated by a combined stimulus using antibodies to the surface antigens CD3 and CD28 (endpoint: release of IFNγ). AVs were tested in 8 different concentrations (C1-C8), starting with 10^9 cells per ml followed by serial dilution (1:3). CR, AK etc. = initials of the donors.

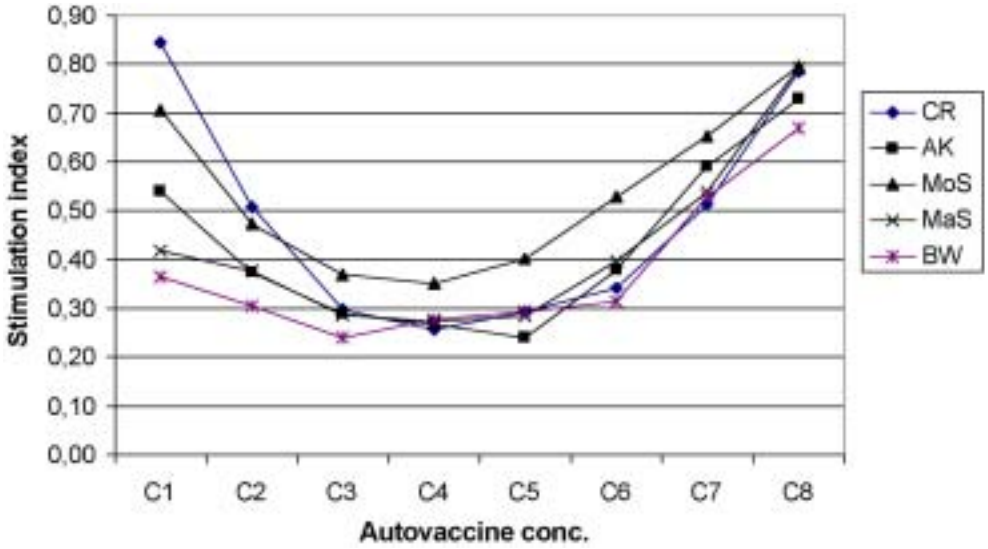

Figure 2: Test of AVs from 5 different donors in whole-blood cultures of one of the 5 donors (CR). The cultures were activated by a combined stimulus using antibodies to the surface antigens CD3 and CD28 (endpoint: release of IL-5). AVs were tested in 8 different concentrations (C1-C8), starting with 10^9 cells per ml followed by serial dilution (1:3). CR, AK etc. = initials of the donors.

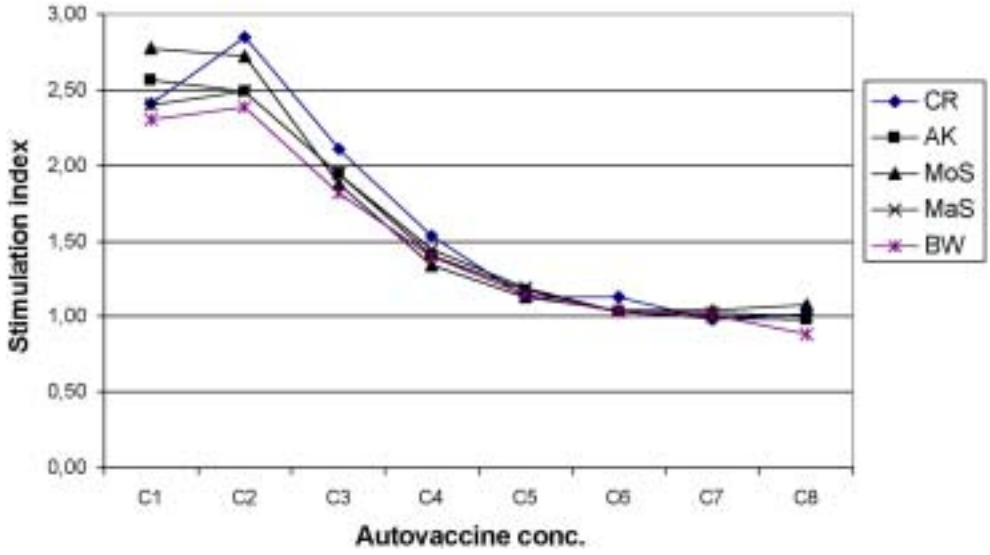

Figure 3: Test of AVs from 5 different donors in whole-blood cultures of one of the 5 donors (CR). The cultures were activated by a mitogen predominantly stimulating B-lymphocytes, PWM (endpoint: release of IL-10 (PWM)). AVs were tested in 8 different concentrations (C1-C8), starting with 10^9 cells per ml followed by serial dilution (1:3). CR, AK etc. = initials of the donors.

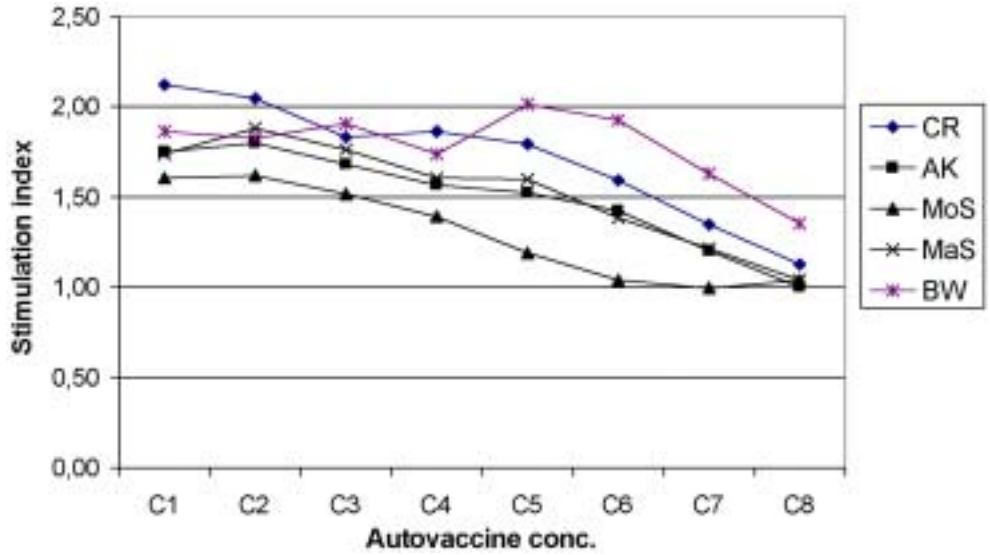

Figure 4: Test of AVs from 5 different donors in whole-blood cultures of one of the 5 donors (CR). The cultures were activated by a particulate phagocytosis stimulus, Zymosan (endpoint: release of TNFα). AVs were tested in 8 different concentrations (C1-C8), starting with 10^9 cells per ml followed by serial dilution (1:3). CR, AK etc. = initials of the donors.

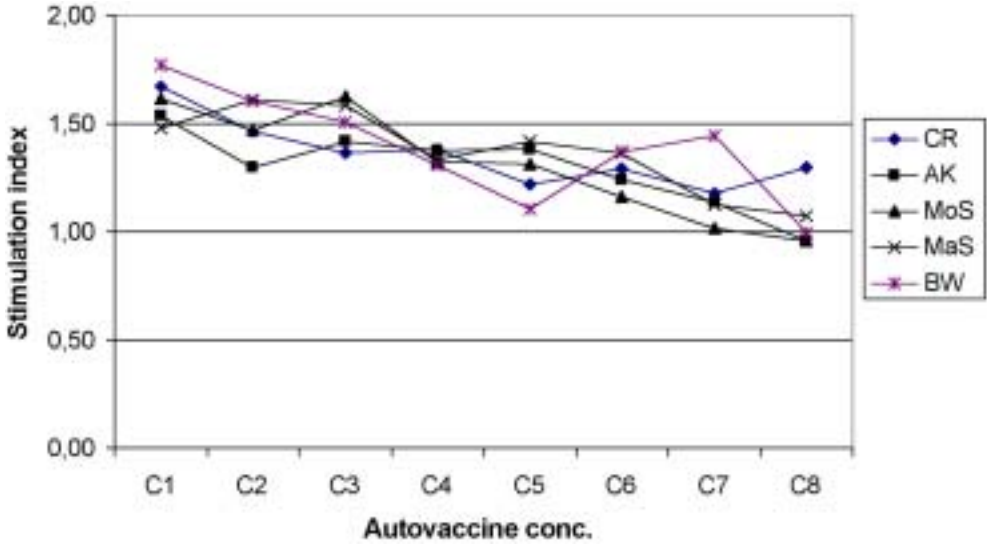

Figure 5: Test of AVs from 5 different donors in whole-blood cultures from one of the 5 donors (CR). The cultures were activated by a particulate phagocytosis stimulus, Zymosan (endpoint: release of IL-6). AVs were tested in 8 different concentrations (C1-C8), starting with 10^9 cells per ml followed by serial dilution (1:3). CR, AK etc. = initials of the donors.

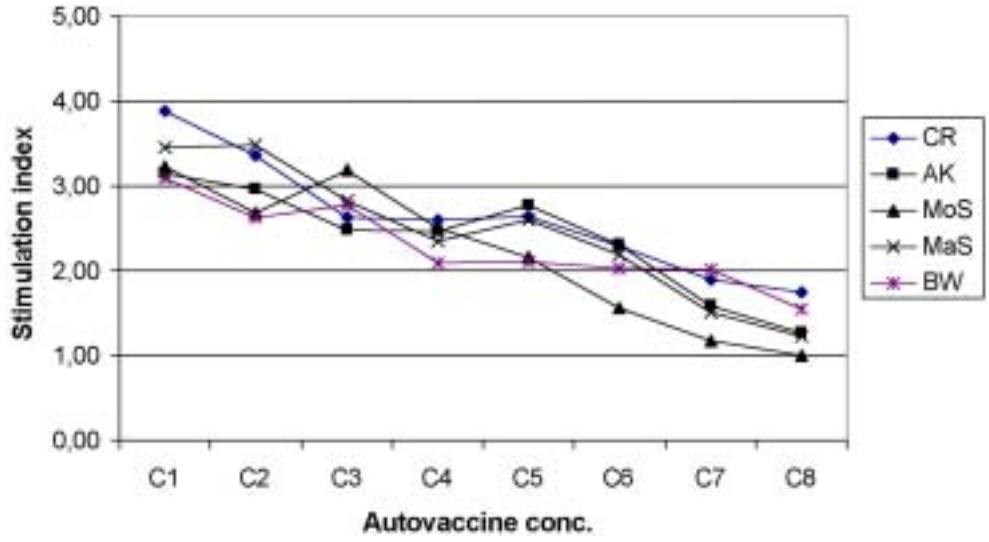

Figure 6: Test of AVs from 5 different donors in whole-blood cultures of one of the 5 donors (CR). The cultures were activated by a particulate phagocytosis stimulus, Zymosan (endpoint: release of IL-10 (Zy)). AVs were tested in 8 different concentrations (C1-C8), starting with 10^9 cells per ml followed by serial dilution (1:3). CR, AK etc. = initials of the donors.

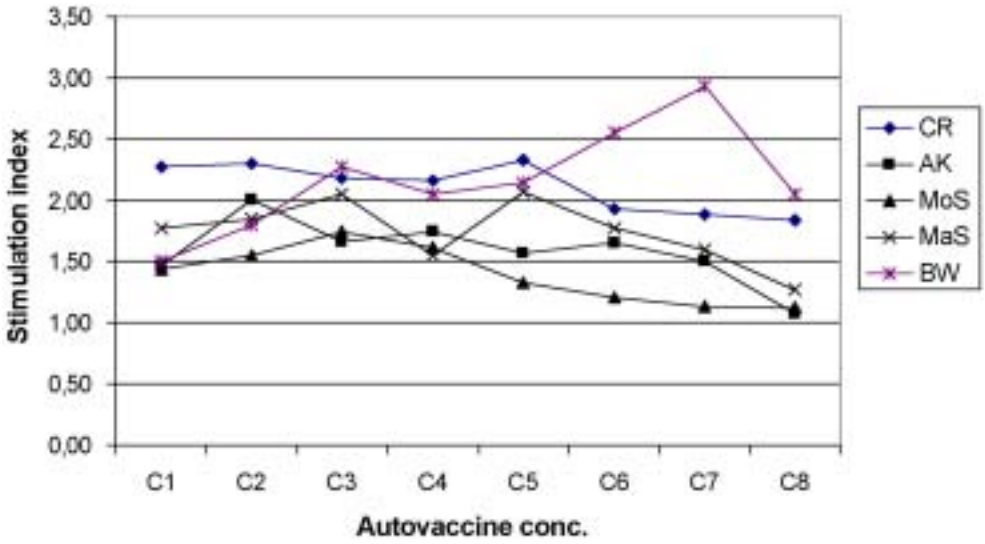

Figure 7: Test of AVs from 5 different donors in whole-blood cultures of one of the 5 donors (CR). The cultures were activated by a particulate phagocytosis stimulus, Zymosan (endpoint: release of IL-12). AVs were tested in 8 different concentrations (C1-C8), starting with 10^9 cells per ml followed by serial dilution (1:3). CR, AK etc. = initials of the donors.

these experiments the effects of the autologous AVs varied over a wide range in the donor group. For instance, the TNF-α synthesis of donor AK obviously did not change at all in the presence of his own AV whereas cultures of CR being incubated with the AV of CR showed a substantial increase in TNF-α production of more than 100% compared to the Zymosan stimulated control (Figure 8). On the other hand, the AV of BW seemed to inhibit the Zymosan triggered release of IL-12 at higher concentrations slightly without exerting any stimulatory effect at any of the concentrations tested. Yet, all other autovaccines at autologous culture conditions induced stimulations to a variable degree (up to 2.5-fold the concentration of the stimulated control, see Figure 11). Similar results could be found with the other monokines (IL-6 and IL-10Zy, Figures 9 and 10).

The same was true for the lymphocytic mediators which reacted with only very little variation when AVs from other donors were applied to the cultures. In contrast, autologous AVs gave rise to a rather broad spectrum of activities, especially regarding the release of IFN-γ and IL-10 (see Figures 12 and 14). IL-5 seemed to react at a much lower degree of variation (Figure 13).

As a last approach to identify individual-specific actions of autologous AVs, we compared the dose-response curves of each of the cytokines and each of the donors with those of the others. Transforming these data into web-like diagrams (see Figures 15-19) it was possible to compare the overall effects of each of the autologous AVs on the complete cytokine pattern to that of the homologous AVs. Compelling activity patterns could be revealed demonstrating a broad heterogeneity generated by the autologous AVs in the immune systems of individual donors, especially when compared to the homologous AVs.

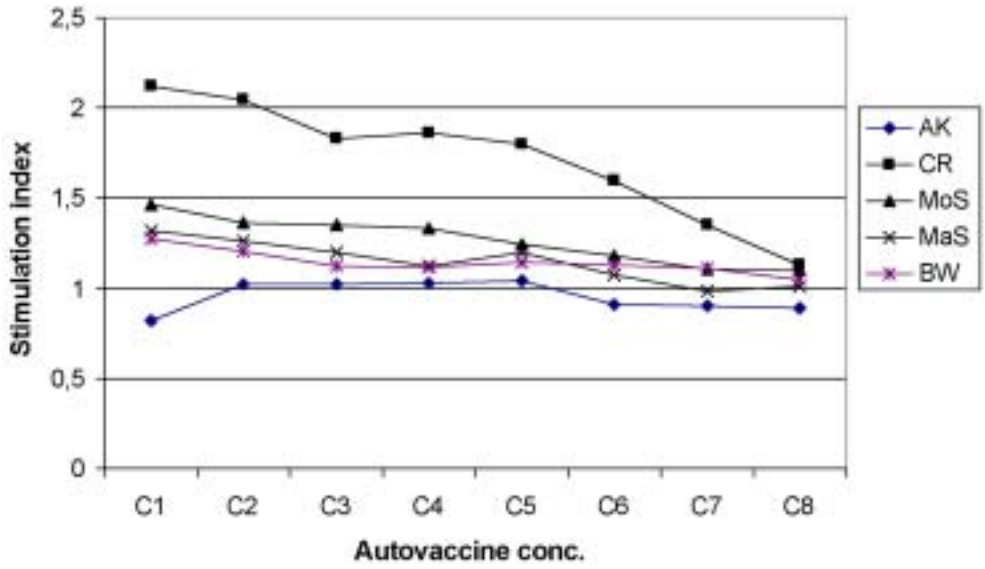

Figure 8: Test of AVs from 5 different donors in whole-blood cultures of those donors the AVs were obtained from (autologous test). The cultures were activated by a particulate phagocytosis stimulus, Zymosan (endpoint: release of TNFα). AVs were tested in 8 different concentrations (C1-C8), starting with 10^9 cells per ml followed by serial dilution (1:3). CR, AK etc. = initials of the donors.

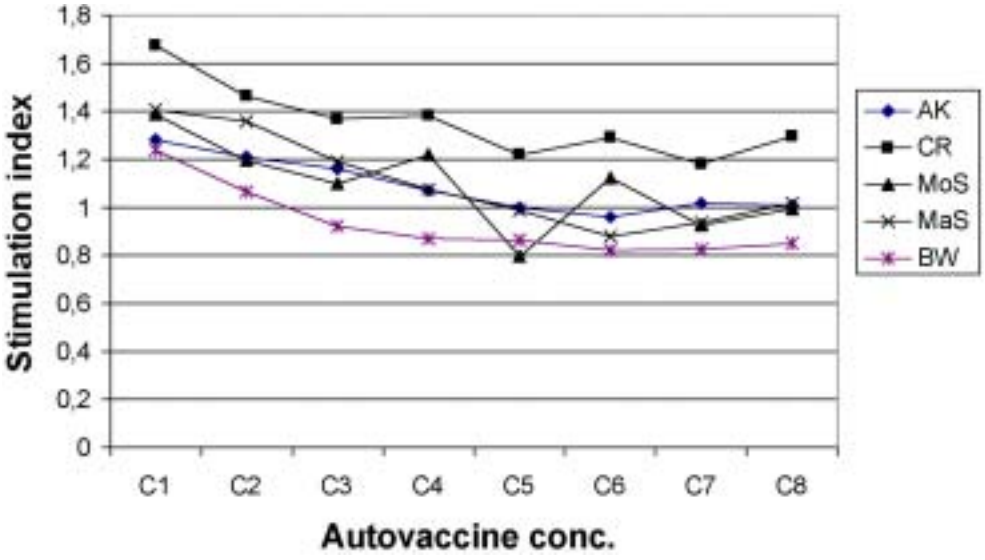

Figure 9: Test of AVs from 5 different donors in whole-blood cultures of those donors the AVs were obtained from (autologous test). The cultures were activated by a particulate phagocytosis stimulus, Zymosan (endpoint: release of IL-6). AVs were tested in 8 different concentrations (C1-C8), starting with 10^9 cells per ml followed by serial dilution (1:3). CR, AK etc. = initials of the donors.

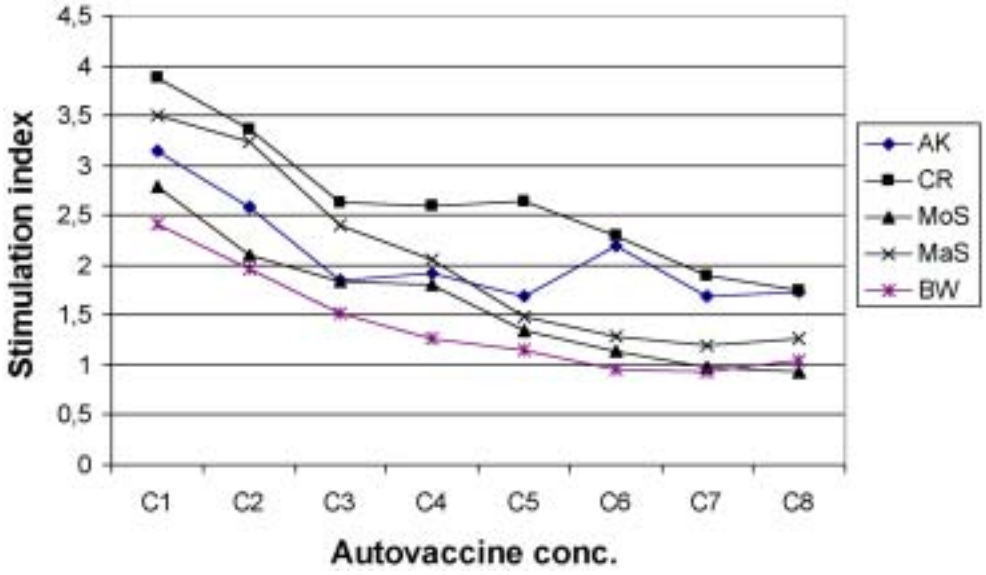

Figure 10: Test of AVs from 5 different donors in whole-blood cultures of those donors the AVs were obtained from (autologous test). The cultures were activated by a particulate phagocytosis stimulus, Zymosan (endpoint: release of IL-10). AVs were tested in 8 different concentrations (C1-C8), starting with 10^9 cells per ml followed by serial dilution (1:3). CR, AK etc. = initials of the donors.

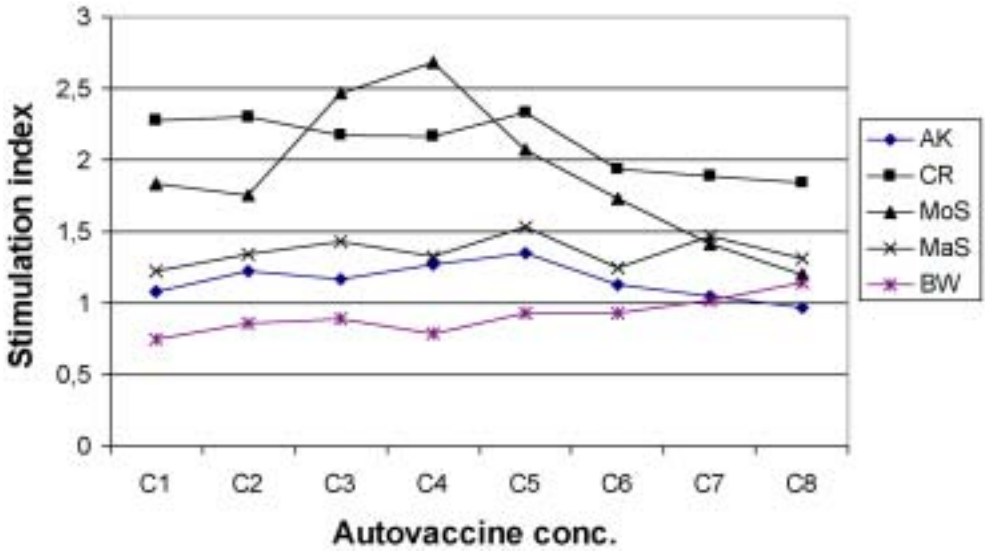

Figure 11: Test of AVs from 5 different donors in whole-blood cultures of those donors the AVs were obtained from (autologous test). The cultures were activated by a particulate phagocytosis stimulus, Zymosan (endpoint: release of IL-12). AVs were tested in 8 different concentrations (C1-C8), starting with 10^9 cells per ml followed by serial dilution (1:3). CR, AK etc. = initials of the donors.

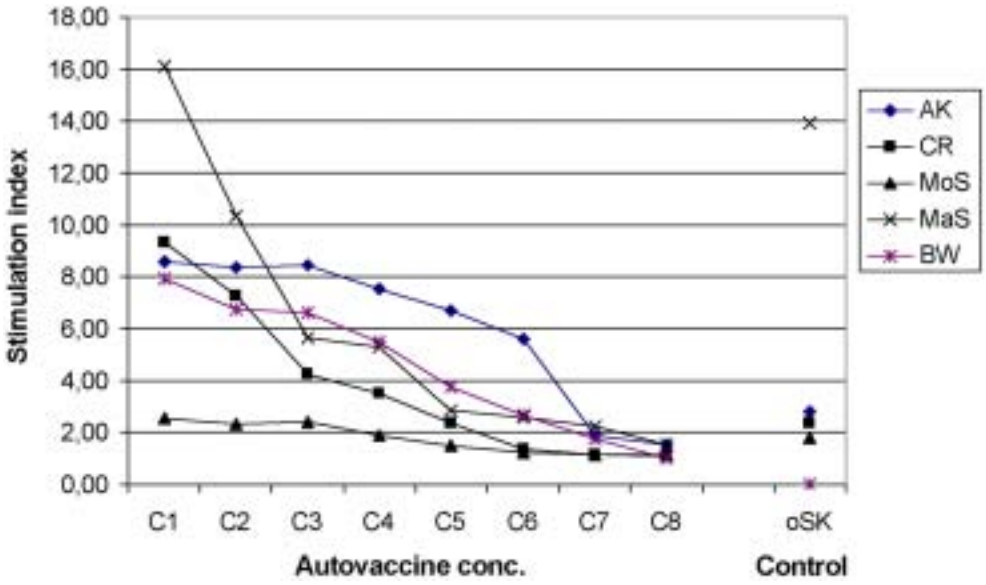

Figure 12: Test of AVs from 5 different donors in whole-blood cultures of those donors the AVs were obtained from (autologous test). The cultures were activated by a combined stimulus using antibodies to the surface antigens CD3 and CD28 (endpoint: release of IFNγ). AVs were tested in 8 different concentrations (C1-C8), starting with 10^9 cells per ml followed by serial dilution (1:3). CR, AK etc. = initials of the donors.

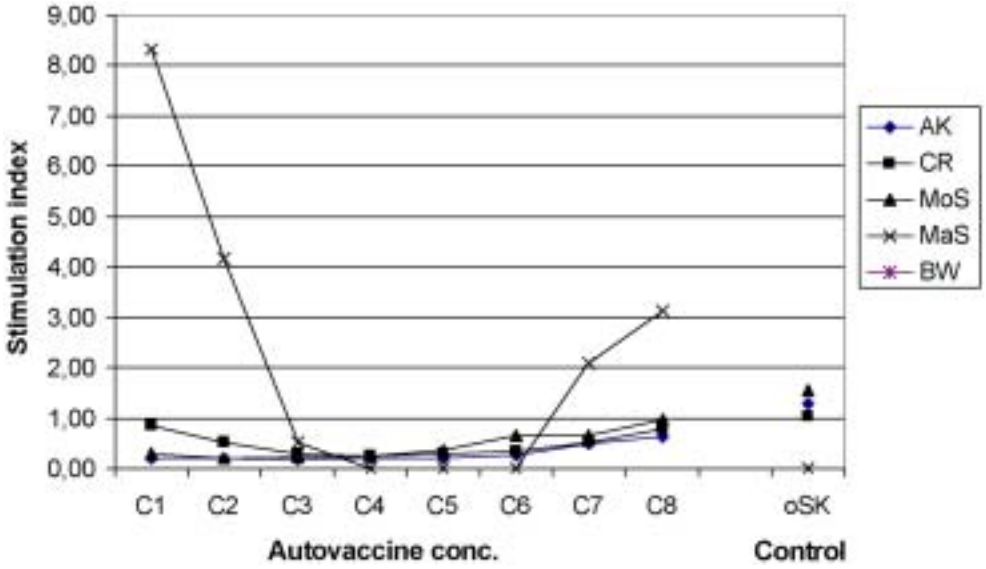

Figure 13: Test of AVs from 5 different donors in whole-blood cultures of those donors the AVs were obtained from (autologous test). The cultures were activated by a combined stimulus using antibodies to the surface antigens CD3 and CD28 (endpoint: release of IL-5). AVs were tested in 8 different concentrations (C1-C8), starting with 10^9 cells per ml followed by serial dilution (1:3). CR, AK etc. = initials of the donors.

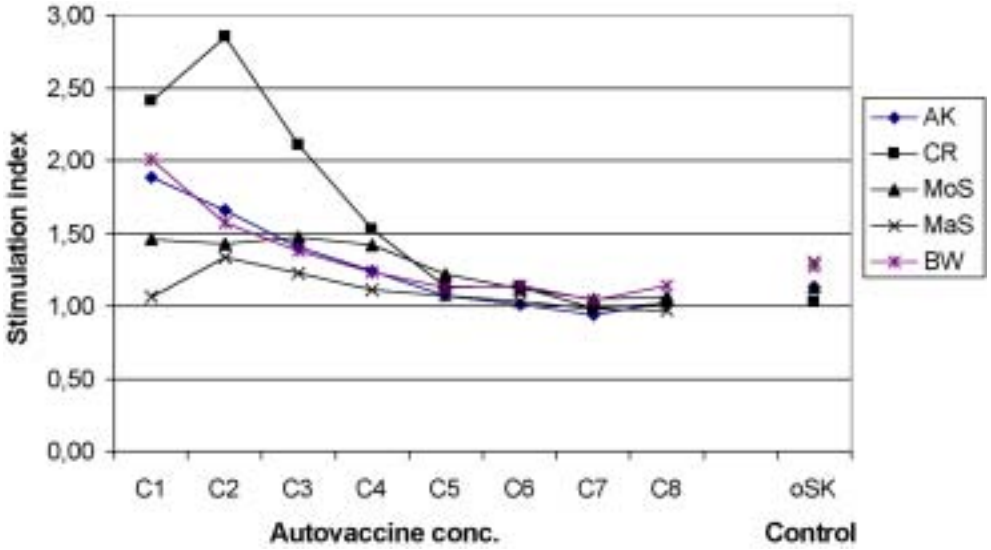

Figure 14: Test of AVs from 5 different donors in whole-blood cultures of those donors the AVs were obtained from (autologous test). The cultures were activated by a mitogen predominantly stimulating B lymphocytes (endpoint: release of IL-10). AVs were tested in 8 different concentrations (C1-C8), starting with 10^9 cells per ml followed by serial dilution (1:3). CR, AK etc. = initials of the donors.

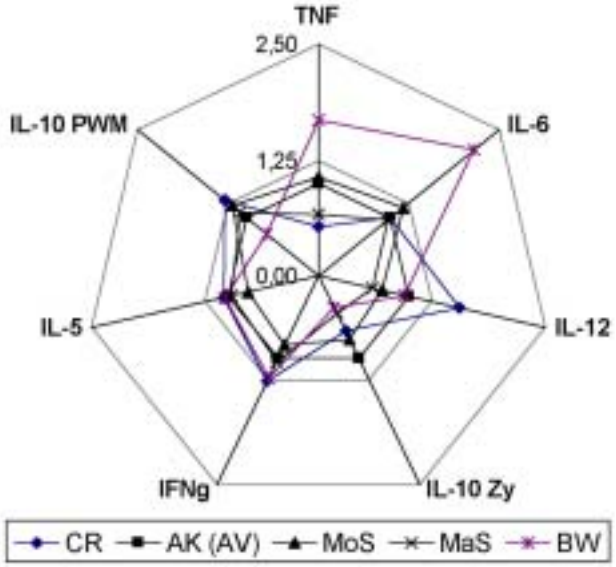

Figure 15: Cytokine response of the blood cells of donor AK to his autologous AV compared to the AVs of four other donors. For each of the cytokines the area under the curve (AUC) was calculated from the dose-response curve. Then the autologous AV was set to 1.0 and a ratio was formed between the homologous and the autologous AVs. CR, AK etc. = initials of AV donors; AK (AV) = donor of blood and AV (autologous situation).

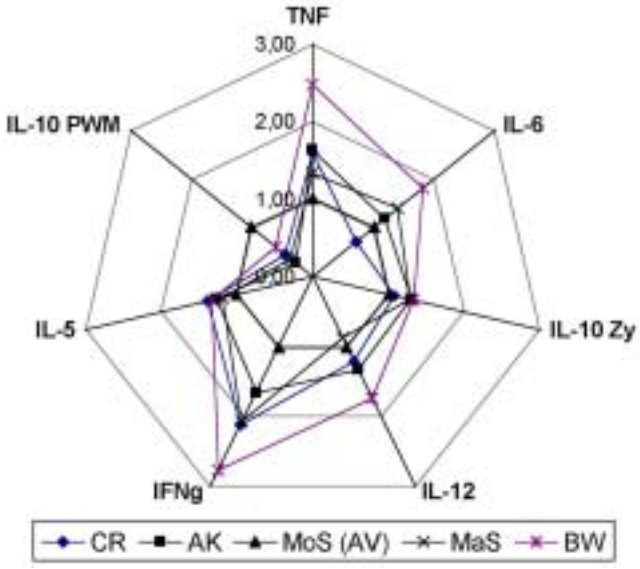

Figure 16: Cytokine response of the blood cells of donor MoS to his autologous AV compared to the AVs of four other donors. For each of the cytokines the area under the curve (AUC) was calculated from the dose-response curve. Then the autologous AV was set to 1.0 and a ratio was formed between the homologous and the autologous AVs. CR, AK etc. = initials of AV donors; MoS (AV) = donor of blood and AV (autologous situation).

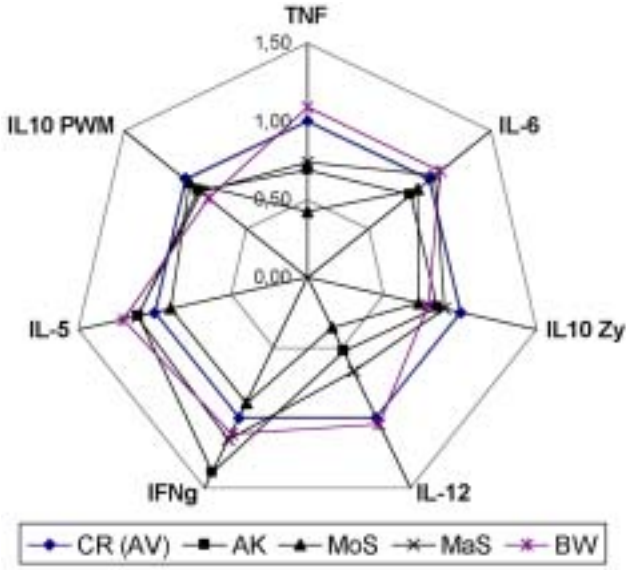

Figure 17: Cytokine response of the blood cells of donor CR to his autologous AV compared to the AVs of four other donors. For each of the cytokines the area under the curve (AUC) was calculated from the dose-response curve. Then the autologous AV was set to 1.0 and a ratio was formed between the homologous and the autologous AVs. CR, AK etc. = initials of AV donors; CR (AV) = donor of blood and AV (autologous situation).

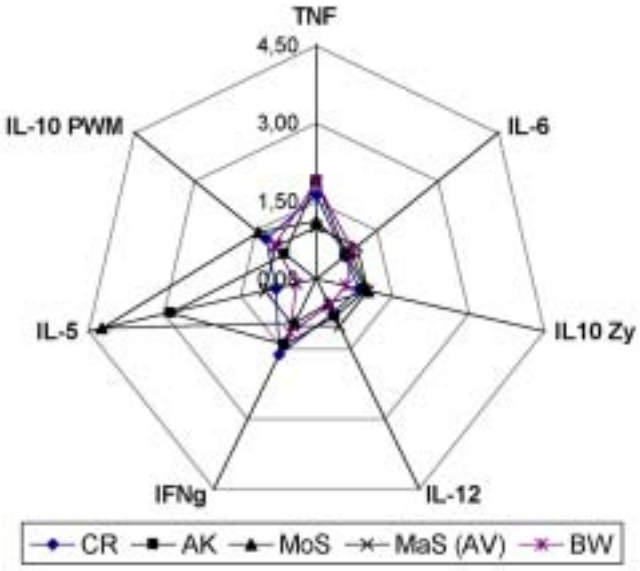

Figure 18: Cytokine response of the blood cells of donor MaS to his autologous AV compared to the AVs of four other donors. For each of the cytokines the area under the curve (AUC) was calculated from the dose-response curve. Then the autologous AV was set to 1.0 and a ratio was formed between the homologous and the autologous AVs. CR, AK etc. = initials of AV donors; MaS (AV) = donor of blood and AV (autologous situation).

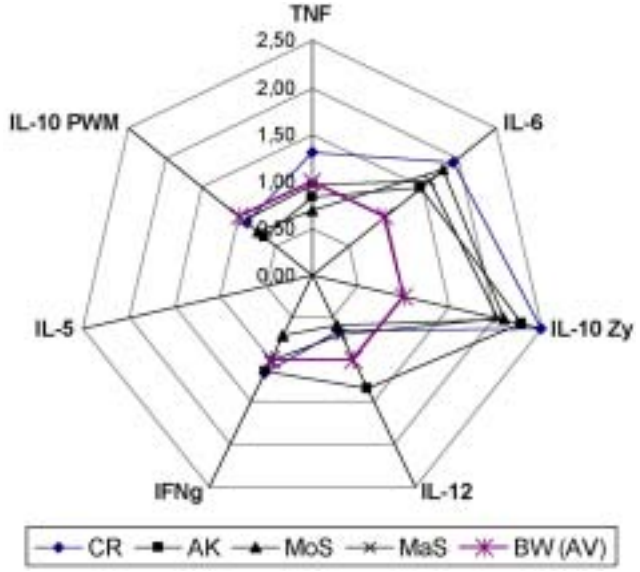

Figure 19: Cytokine response of the blood cells of donor BW to his autologous AV compared to the AVs of four other donors. For each of the cytokines the area under the curve (AUC) was calculated from the dose-response curve. Then the autologous AV was set to 1.0 and a ratio was formed between the homologous and the autologous AVs. CR, AK etc. = initials of AV donors; BW (AV) = donor of blood and AV (autologous situation).
The gap seen with IL-5 is the result of a cellular response that was too low to give reliable results, therefore, the data had to be omitted.

DISCUSSION

The Autovaccine Herborn (AV) is composed of bacterial lipids, the structure of which is very closely related to the well-known Lipid A of Gram-negative bacteria. Lipid A in turn is the central building block of the so-called lipopolysaccharide (LPS), also known as endotoxins. The immune system uses LPS as well Lipid A as common indicators of the presence of bacterial infection and, by means of specific cellular receptors, as trigger signals for antigen non-specific leukocytes, such as granulocytes and macrophages (*Jackson*, 1997; *Ozinsky* et al., 2000). Those receptors, besides displaying more or less selectivity for several such types of stimulating molecules, exhibit only a low degree of fine specificity. Thus, although different types of LPS molecules can bind to TLR4 or the LPS-binding protein, they nevertheless induce the same type of cellular reaction. In contrast to Lipid A, LPS often displays considerable structural variability, although not in its lipid moiety but rather because of differences in the polysaccharide "side-chain". In Lipid A the side-chain usually is truncated down to 1 or 2 sugar rests, leaving only little space for this type of variation (*Rietschel* et al., 1993; *Mueller-Loennies*, 1998). Taken together, these facts reduce the degrees of freedom to produce structural heterogeneity in Lipid A molecules dramatically. The fact that AV consists of a highly purified fraction of Lipid A raises the question if individuality can actually be found in the immunomodulatory activities of AVs, even if one takes into account that each of the patients usually harbours an individual set of *E. coli* rough strains in the gut.

To address this issue, a series of five AVs was prepared from the stools of different healthy volunteers, which later were also employed as blood donors to prepare whole-blood cultures to test their AVs for immuno-pharmacological effects. An extensive cross testing of each AV with the blood of each of the donors ensured a complete overview on the influences the AVs showed on the pattern of immunoregulatory mediators (7 different cytokines). The sets of results were obtained: a) data derived from cultures where AVs were tested in cultures from the same donor who gave the stool sample to prepare this very AV ("autologous" situation) and b) data from tests where AVs were added to cultures of blood of the other donors ("homologous" experiments). This allowed to detect the overall differences in the effects the AVs caused under both test conditions and by using these data characterise the degree of autospecificity AVs are able to produce.

Interestingly, when the different homologous AVs were tested on the blood of a single donor there was considerable homogeneity in the reactions they generated, indicating that generally there is not much individuality in the response of the antigen non-specific leukocytes towards AVs of different origin. The opposite seems to be true when testing AVs in an autologous system: Each test donor reacted to his/her own AV with a cytokine pattern that revealed distinct differences to what was seen in response to the AVs of other donors. In addition, every volunteer's leukocytes demonstrated a particular (set of) cytokine(s) that seemed to be influenced much stronger (or much weaker) by most if not all other AVs compared to the effects of the autologous AV.

Our *in vitro* experiments clearly demonstrated the autologous AVs to exert immuno-pharmacological activities showing pronounced individual-specific effects. Further experiments using either

healthy volunteers or patients who receive autologous or homologous AVs respectively will now have to prove in *ex vivo* experiments if this holds true also for the situation as it is *in vivo*.

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