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Influence of Homeopathic Drug Preparations on the Phagocytosis Capability of Granulocytes

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Summary:

Four homeopathic combined preparations with extract attenuations between 1X and 30X (expression for homeopathic level of attenuation) and some additives (minerals and a/toxins) were investigated using two in vitro and one in vivo phagocytosis models. All preparations led to a significant increase in phagocytosis activity in all three immune models. Two preparations (C and D) and a placebo were administered IV on 5 successive days to 12 and 14 preparation group and 13 placebo group male subjects respectively. The phagocytosis indices of peripheral granulocytes were determined over an 11-day period using the microscopic smear test. In both controlled single-blind studies, a clear increase of phagocytosis activity was observed even after the first injection and maximum activity was reached between the 4th and 5th injection days. After the 4th or 5th (next to last or last) injection, a rapid decrease of activity occurred which reached normal values on the 11th day. Other laboratory parameters investigated were not influenced.

Keywords: Homeopathic plant preparations, immunostimulatory activity, in vitro tests, phagocytosis.

1. Introduction

In the German Physician's Desk Reference (22), over 150 preparations are listed under the headings of immunostimulants, stimulation therapy, lym-

phatic agents, irritation therapy preparations, and adjuvant cancer therapy or flu and common cold remedies. In accordance with the indication information, a nonspecific influence on cellular or humoral defensive systems is expected. To the extent that plant-derived preparations are involved, the orally and parenterally administered preparations contain extract combinations with attenuations from 1X to 30X with attenuations from 1X to 6X predominating. They should therefore be classified as homeopathic substances. The expressions 1X to 30X stand for various attenuation levels (potencies). These are produced in accordance with the specifications contained in the HAB, the German Homeopathic Pharmacopoeia. Decimal attenuations are labeled with the letter X. The number preceding the X refers to the number of potentizing steps (attenuations) carried out. The composition of most of the preparation include the addition of minerals (e.g. phosphorus, siliceous compounds, iron salts) or animal toxins (e.g. formic acid, Lachesis, apisin). The extracts which are used in the preparations come from no more than 20 plants. Among these, Echinacea, Eupatorium, Calendula, Thuja, and Baptisia are particularly common (1). The few studies which have been carried out concerning the effects and efficacy of this group of preparations indicate that the phagocytic system of immunodefense seems to be particularly influenced. In previous in vitro studies with a combined preparation

(2-5), Kluthe and colleagues (6) could show in 2 separate double-blind studies with healthy subjects using a daily 0.9 mg PO administration of aristo-lochia acid that increased phagocytosis activity occurred after only 3 days. A further study on human subjects carried out by Mose (7) with an intramuscularly applied Echinacea preparation, led to the same result. Further studies using the previously mentioned preparation with bacterial and viral infections respectively (8, 9) also showed an increase of phagocytosis activity in addition to a subjective improvement in the course of the disease.

The aim of the present investigation was to use in vitro and in vivo studies to explore the influence of some selected example homeopathic drugs with completely different compositions on the phagocytosis activity of human neutrophil granulocytes or macrophages. The in vitro studies should serve above all to identify the most suitable test method and dosage for the experiments with human subjects. For the in vitro studies, a modification of the granulocyte smear test by Brandt (10) and the chemoluminescence test described by Allen (11) were used. For the in vivo evidence of phagocytosis stimulation, the carbon clearance test on the mouse from Biozzi and colleagues (12) was used. For the measurement of phagocytosis in the single-blind study, we used the granulocyte smear test because of its good reproducibility. In addition, immunoglobulin (Ig) serum concentrations, leuko-

cyte concentrations, and the value of the erythrocyte sedimentation rate (ESR) were determined.

2. Materials and methods

2.1 Injection preparations

Preparation A*): 1.1 ml contains 4.4 µl Aconitum 3X, 1.1 µl Eupatorium perfol. 2X, 0.11 µl Phosphorus 4X, 2.2 µl Lachesis 11X, and 2.2 µl Bryonia 3X. Preparation B*): 1.1 ml contains 6.6@ vincetoxicum 6X, vincetoxicum 10X, vincetoxicum 30X, 1.1@ plant ash from vincetoxicum 30X, 3.3 µl sulfur 4X, sulfur 10X. Preparation C: 2.2 ml (1:1 mixture of preparations A and B). Preparation D: 2 ml contains 0.4 ml Echinacea angustifolia 1X, 0.3 ml Lachesis 10X, 0.2 ml Eupatorium perfol. 3X, 0.2 ml Gelsemium 4X, 0.3 ml Aconitum 4X, and 40 mg ascorbic acid.

*) Prep. A = Gripp-Heel, prep. B = Engystol; Manufacturer: Biologische Heilmittel Heel GmbH, Baden-Baden, Germany.

2.2 Granulocyte test

This was carried out using a modified method from Brandt (10). The granulocytes were extracted as described by Wagner and colleagues (13). The granulocyte suspension was adjusted to 5×10^6 cells/ml with a PBS buffer (phosphate buffered salt). Yeast suspension: Baker's yeast suspended in 0.9% NaCl solution and heated to 100°C was adjusted to $3-5 \times 10^7$ particles/ml.

2.2.1. Test preparation

0.2 ml of pool serum, 0.2 ml granulocyte suspension, 0.2 ml of yeast suspension, 0.2 ml of injection solution; coloring of granulocytes with May-Gruenwald and Giemsa stains. Assessment of 100 granulocytes/slide.

2.2.2. Calculation

Increase of phagocytosis activity = $(\text{phagocytosis index substance} - \text{phag. index control} / \text{phag. index control}) \times 100\%$.

2.3. Chemoluminescence test

The test was carried out in a modified form (13) following Allen (11).

Lumigenous substance: lucigenin (Sigma, Taufkirchen, Germany); zymosan (Sigma). Measurement in the 6-Channel Biolumat LB 9505 (Berthold, Munich, Germany).

2.3.1. Test preparation

645 µl veronal-buffered NaCl solution, 15 µl cell suspension (15,000 PMNL = polymorphonuclear neutrophil leukocytes), 100@ lucigenin 1 (1 mmol/l), and 200@ injection or NaCl solution. The measurements were carried out over a 60 min. time period. The units of measurement are given in cpm.

2.4. Carbon clearance test

The test was carried out following Biozzi and colleagues (12).

2.4.1. Animal material

NMRI mice, female, 30-35 g body weight. Group size $n = 7$ animals/injection solution in comparison to 7 animals pretreated only with NaCl solution. Indian ink suspension (black No. 591017, particle size 20-35 mm; Rotring-Werke, Riepe KG, Hamburg). Experimental procedure according to (14). 24 hours before the beginning of the test, 0.25, 0.5, 0.7, or 1.0 ml of the injection solution/kg body weight was IP injected. On the next day, each mouse was given 0.3 ml Indian ink suspension/30 g body weight IV. Blood was withdrawn from the postorbital venous plexus 3, 6, 9, 12, and 15 min. after the IV injection.

2.5 Prospective single-blind studies

Preparation C (2.2 ml): 27 male test persons aged between 19-30 years, body weights 64-83 kg; 14 subjects in the preparation group, 13 in the placebo group. Preparation D (2 ml): 25 male subjects aged between 27-28 years, body weights 62-85 kg; 12 subjects in the preparation group, 13 in the placebo group. The treatment involved an IV injection of either 2.2 or 2 ml (right side) in the morning between 7:30 and 8:15 a.m. under fasting conditions after taking of blood sample. The placebo group received the same quantity of isotonic saline solution. Blood samples were taken 3 days before the

first injection and on days 1, 2, 3, 4, 5, 8, and 11. The injections were given on days 1, 2, 3, 4, and 5.

For the granulocyte assessment, two slides were prepared for each subject and test time. For further calculations, the mean of the two values was used.

a) The assessment of the slides (granulocytes) was organized as a double-blind study; i.e. the person making the assessment didn't know from which group the slide came.

b) The subjects were randomly assigned to groups by a third person. The subjects were listed alphabetically, numbered, and then assigned to groups in accordance with the randomization plan.

c) The study was carried out in accordance with the Declaration from Helsinki and Section 40 of the German Federal Drug Act 1976.

The subjects confirmed in writing that they had participated in the study of their own free will and that they were informed by the testing physician concerning the procedure, possible risks, and the possibility that they could terminate their participation in the study at any time without having to state their reasons for doing so.

2.6. Statistical analysis

The statistical analysis was carried out in the Institute for Medical Information Processing, Statistics, and Biomathematics (ISB) of the University of Munich (Director, Prof. K. Überla; Supervisor, Ms. Schubert-Frietschle). The test used was the Friedmann ranks analysis of variance followed by multiple comparisons using the Wilcoxon and Wilcox methods. This test shows significant differences between different sample times within a therapy group.

To compare the therapy and placebo groups, Wilcoxon's U test as described by Mann-Whitney (15) was used. This test determines whether there are significant differences between the independent values obtained at the same sample times for the therapy and placebo groups.

2.7. Analysis of the immunoglobulin (A, G, and M)

The Auto-ICS Laser Nephelometer (Beckmann) was used for the Ig analysis.

2.8. Analysis of hematological parameters

Hemoglobin, the erythrocyte and leukocyte concentrations, and the value of the hematocrit reading were determined using the Coulter Counter Model S 5 (Coulter company).

2.9. Clinical chemical parameters

The analysis of the transaminases of GOT, GPT, μ l-GT, alkaline phosphatase, and total serum protein was carried out using the well-known routine methods. Blood sugar was determined by means of kinetic oxygen measurement. Blood sedimentation was analyzed in the usual way with the Sedifix System (Braun, Melsungen).

3. Results

3.1. Detailed results

The chi2 and U values were taken from the appropriate tables.

3.1.1. Friedmann test

Preparation mixture C

For n = 14 and k = 7, a chi2 = 44.97 was obtained; chi2 = 22.46 required for significance at the 0.1% level.

Preparation D

For n = 12 and k = 7, a chi2 = 54.14 was obtained; chi2 = 22.41 required for significance at the 0.1 % level.

Placebo

For n =13 and k = 7, a chi2 = 15.93 was obtained; chi2 = 12.32 required for significance at the 0.1 % level.

There was a highly significant difference (p= 0.001) between the different sample times within both therapy groups and a significant difference (p = 0.05) within the placebo group.

3.1.2. Multiple comparisons using the Wilcoxon and Wilcox methods

Preparation mixture C

Significance at the 5% level with a rank difference > 33.7; significance at the 1% level with a rank difference >

39.5. Therefore highly significant differences resulted between days 1-5 (51), 1-4 (49), 2-5 (48), 24 (46), 5-11 (45), and 4-11 (43), and significant differences resulted between days 1-3 (37), 5-8 (36), 4-8 (34), and 2-3 (34).

Preparation D

Significance at the 5% level with a rank difference > 31.2; significance at the 1% level with a rank difference > 36.5. Therefore highly significant differences resulted between days 14(55), 1-5 (51),4-11 (48.5), 1-3 (46.5), 5-11 (44.5), 3-11 (40), and 4-8 (37), and significant differences resulted between days 24 (36), 5-8 (33),and 2-5 (32).

Placebo

Significance at the 5% level with a rank difference > 32.5; significance at the 1% level with a rank difference > 38. Therefore significant differences resulted between days 1-5 (33) and 1-6 (38).

3.1.3. Wilcoxon's U test from Mann-Whitney

Preparation mixture C

Significance at the 0.1% level with U values < 23.2. Day 2: 70; day 3: 0; day 4: 1.5; day 5: 0; day 8: 41; day 11:59. Therefore highly significant differences in phagocytosis activity between the therapy and placebo groups resulted on days 3,4, and 5.

Preparation D

Significance at the 0.1% level with U values < 29. Day 2: 19.5; day 3: 0; day 4: 1; day 5:0; day 8: 15; day 11:54.5. Therefore highly significant differences in phagocytosis activity between the therapy and placebo groups resulted on days 2,3,4,5, and 8.

3.2. Overall results

In the in-vitro granulocyte smear test, all preparations led to a clear increase in phagocytosis (Tables 1 and

Preparation	Ampule solution (ml/test preparation)	Phagocytosis increase compared to control (%)
Preparation A	0.2	30.8 ± 1.0
	0.02	18.1 ± 0.6
	0.002	9.2 ± 4.2
Preparation B	0.2	33.5 ± 2.8
	0.02	27.4 ± 0.8
	0.002	15.5 ± 4.1
Preparation C (1: 1 mixture of preparations A and B)	0.2	16.5 ± 2.1
	0.02	41.0 ± 3.9
	0.002	28.5 ± 3.1
Preparation D	0.2X10 ⁻¹	-63.4 ± 0.9*
	0.2X10 ⁻²	-28.5 ± 0.6*
	0.2X10 ⁻³	-10.4 ± 2.9*
	0.2X10 ⁻⁵	4.3 ± 4.1
	0.2X10 ⁻⁶	12.1 ± 3.2
	0.2X10 ⁻⁷	28.2 ± 2.5
	0.2X10 ⁻⁸	10.0 ± 1.8
* Inhibition of phagocytosis		

Table 1: Phagocytosis stimulation, measured using the granulocytes of the subjects. 0.8 ml of test preparation contained 5 x 10⁶ granulocytes and 3-5 x 10⁷ yeast particles. 100 granulocytes/slide were counted. Phagocytosis index = sum of the incorporated yeast particles/sum of the granulocytes counted Means of triple analyses ± standard deviations.

Preparation	Ampule solution (ml/test preparation)	Chemoluminescence yield (%)
Preparation A	0.2	14.5 ± 1.5
	0.2X10 ⁻¹	12.2 ± 0.8
	0.2X10 ⁻²	10.0 ± 0.6
	0.2X10 ⁻³	7.8 ± 0.5
	0.2X10 ⁻⁴	3.8 ± 2.3
Preparation B	0.2	18.2 ± 1.1
	0.2X10 ⁻¹	9.0 ± 3.5
	0.2X10 ⁻²	7.7 ± 3.4
	0.2X10 ⁻³	5.9 ± 3.5
	0.2X10 ⁻⁴	3.0 ± 1.0
Preparation C (1:1 mixture of preparations A and B)	0.2	27.2 ± 2.0
	0.2X10 ⁻¹	19.4 ± 3.7
	0.2X10 ⁻²	16.0 ± 1.9
	0.2X10 ⁻³	13.5 ± 1.5
	0.2X10 ⁻⁴	7.0 ± 1.7
Preparation D	0.2X10 ⁻¹	-75 ± 1.5*
	0.2X10 ⁻²	-30 ± 0.5*
	0.2X10 ⁻³	-23 ± 2.8*
	0.2X10 ⁻⁴	2 ± 4.1
	0.2X10 ⁻⁵	15 ± 3.2
	0.2X10 ⁻⁶	30 ± 2.5
	0.2X10 ⁻⁷	11 ± 4.8
	0.2X10 ⁻⁸	7 ± 2.1*
*Inhibition of chemoluminescence		

Table 2: Chemoluminescence yields with granulocytic phagocytosis after stimulation with preparation solution; 1 ml of test preparation contained 15,000 granulocytes, 40 µl opsonized zymosan, and 100 µl lucigenin solution. Measurements were taken using the 6-channel Biolumat over a time of 60 min. Means of triple analyses ± standard deviations (see Fig. 1).

2). Preparations A, B, and C non-attenuated show the highest phagocytosis rates. After attenuation 1:10 or 1:100, a clear dose-dependent decrease in the phagocytosis index values occurred. Mixing preparations A and B in a proportion of 1:1 resulted at first in a lowering of the phagocytosis rate in comparison to the values of the two single preparations. After a 1:10 attenuation of this mixture, an increased phagocytosis stimulation effect greater than that of the single preparations could again be measured. This superadditive effect is consistent with clinical observations. With preparation D, there was first a suppression of phagocytosis in the non-attenuated state which also then changed into a stimulating effect

at an attenuation of 1:100,000. In every case when single extract components were removed from preparation A, a decrease in the phagocytosis values was observed (Table 3). This indicates that the individual components of the preparation display additive effects. In the chemoluminescence test, which according to our earlier studies is highly correlated with the smear test (12), preparation mixture C also showed a higher luminescence yield than the single preparations A or B (Table 2, Fig. 1). In contrast, preparation D non-attenuated again showed a suppression which like the smear test only changed into a stimulator effect after the appropriate attenuation. In the carbon clearance test on the mouse,

preparations A, B, and C in doses of 0.5 to 1.0 mg/kg led to a dose-dependent increase of the carbon clearance with regression coefficients of 1.20 to 2.02. These correspond to a moderate to good phagocytosis increase (Table 4). With preparation D, the highest regression coefficient (3.22) with an index value of 2 was obtained with the lowest dosage of 0.25 ml/kg. Higher concentrations were almost ineffective (RC tr/RCC = 1.0) or immunosuppressive. When converting the required dosage to one appropriate for human beings, it must be taken into account that the mouse reacts only about 1/4 to 1/5 as well to phagocytosis stimulators as human beings.

In some preliminary experiments carried out with 7 subjects, it soon became clear that the chemoluminescence method was not suitable for a study with test persons. Because the lipofundin test with human beings (16), which is analogous to the carbon clearance method, is too time-consuming to be used here, we chose the granulocyte smear test which has repeatedly proven itself in many previous in vivo studies (17, 18) (Fig. 2).

The single-blind studies carried out on the 25 and 27 subjects with preparation mixture C and preparation D respectively (Fig. 3) showed a clear increase in the phagocytosis rate even after the first injection. This increase reached a maximum phagocytosis rate of 20% after the 3rd or 4th injection on day 4 or 5 respectively (Fig. 2). After discontinuation of preparation mixture C, the phagocytosis activity dropped back down into the normal area within 6 days. Preparation D showed an almost identical stimulation profile (Fig. 3). Here a decrease of phagocytosis activity occurred after the 4th day, which was even before the discontinuation of the injections. After two days of the experiment, the treatment effect exceeded the 5% level of significance value in both studies.

The other control immune parameters (Ig) as well as leukocyte and ESR values remained within physiologically normal limits. No side effects occurred. The tolerance was good in all cases.

4. Discussion

The fact that the completely different homeopathic extract combination preparations used in this investigation resulted in almost identical phagocytosis activity stimulation rates indicates that most of the preparations which are used for this purpose should probably be categorized as paramunity inducers, immunostimulants with non-specific effects (19). In all cases, the phagocytosis increase occurred rapidly. The observation that in the single-blind study with one of the preparations already showed a consistently pronounced decrease after the next to last injection clearly indicates that after a brief stimulation time, a "fatigue or exhaustion phase" occurs. The rapid activity decrease might also be due to an immunosuppressive effect as a consequence of "overstimulation". The results obtained in the in vitro phagocytosis test models clearly show that dose-dependent effect differences exist and that administering a dosage which is too high can sometimes lead to immunosuppressive effects. Whether or not the effect observed with non-attenuated preparation D is related to the fact that here the various extract components were relatively highly dosed (ml) in comparison to preparations A, B, and C, must be more precisely examined. In any case, the result obtained here is in agreement with the in vitro studies we carried out on juices pressed from Echinacea (20) and pure substances (13). When using high concentrations of primary Echinacea extracts (non-attenuated or 1:10 attenuated), we have regularly observed suppressive effects that only disappeared and changed into stimulator effects when the solution was attenuated further. For some pure substances isolated from plants, we also measured the highest immunostimulatory effects in very low concentration areas (10^{-4} to 10^{-8} mg or mol/ml test preparation). That the in vitro results have their counterpart in the in vivo experiments is shown by the dose-dependent positive results of the carbon clearance studies on the mouse. In contrast, with the injection of immunostimulatory preparations in

Preparation	Ampule solution (ml/test preparation)	Phagocytosis increase compared to control (%)
Preparation A	0.2	30.8 ± 1.0
Preparation A without Aconitum 3X	0.2	26.5 ± 2.1
Preparation A without Bryonia 3X	0.2	23.0 ± 2.1
Preparation A without Lachesis 11X	0.2	20.5 ± 3.6
Preparation A without Eupatorium perfol. 2X	0.2	19.8 ± 1.9

Table 3: Phagocytosis stimulation by means of Preparation A and preparations with altered compositions, measured using human granulocytes. See Table 1 for measurement conditions.

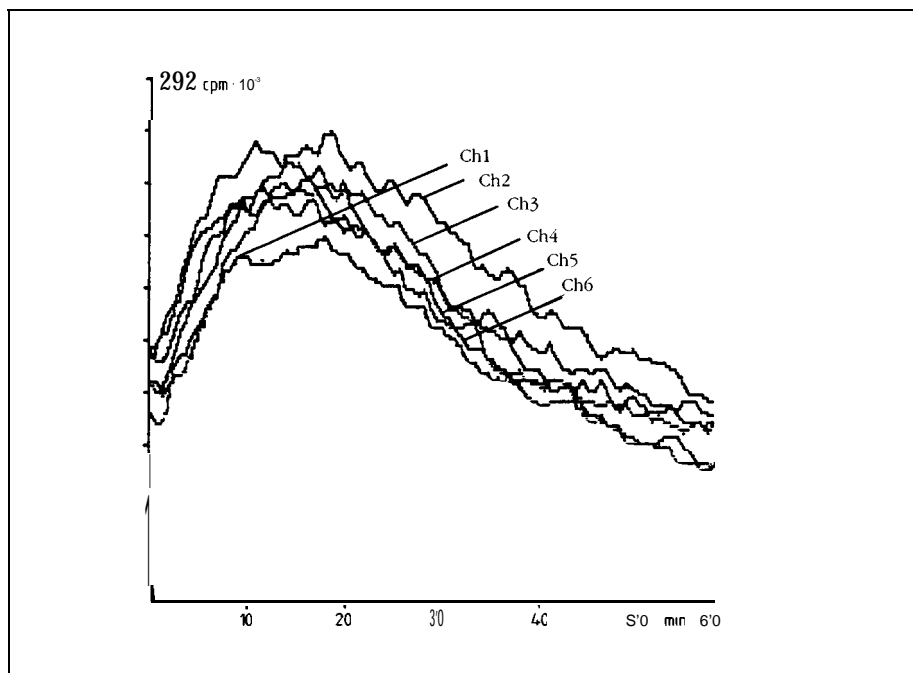


Fig. 1: Chemoluminescence yield with granulocytic phagocytosis after stimulation with Preparation C. For concentrations and test design see Table 2 and the methodology section. Channel (Ch) 1 = control; Ch 2 (0.2 ml) 29.3%; Ch 3 (0.2×10^{-1}) = 25.3; Ch 4 (0.2×10^{-2}) = 16.6; Ch 5 (0.2×10^{-3}) = 15.1; Ch 6 (0.2×10^{-4}) = 9.7.

human beings, dosage differences within certain limits seem to be less important. The studies with test persons also showed that the plant-derived immunostimulants, in contrast, for example, to levamisol, can also induce a stimulating effect within the normal immunodefense situation (21).

The studies do not permit a statement to be made concerning whether

these drug preparations exert their effects directly by stimulating phagocytizing leukocytes or indirectly by means of the stimulation of T-cell subpopulations or via a release of certain mediators. It is also not possible to draw conclusions from these results concerning the efficacy on certain immunodeficiencies or infectious diseases.

In our opinion, the immunostim-

Test series	Preparation	Concentration (ml/kg)	Regression coefficient RC_{tr}	$\frac{RC_{tr}^a)}{RC_c}$	Index ^{a),b)}
I	Preparation A	0.7	-0.041	1.36	1
	Preparation B	0.7	-0.046	1.53	2
	Preparation C	0.7	-0.059	1.96	2
II	Preparation A	0.5	-0.0385	1.20	1
		1.0	-0.051	1.60	2
	Preparation B	0.5	-0.0423	1.32	1
		1.0	-0.0647	2.02	2
	Preparation D	1.0	-0.028	0.875	0
		0.5	-0.0382	1.2	1
III	Preparation D	0.25	-0.087	3.22	2

a) Control values: 0.3 ml 0.9% NaCl solution/30 g mouse. Regression coefficient for test series I:-0.0307; II:-0.0320; III:-0.027.

b) $\frac{RC_{tr}}{RC_c}$: 1<1 (index 0 = ineffective); <1.5 (index 1 = effective); > 1.5 (index 2 = very effective).

Table 4: Analysis of the carbon clearance in mice after preparation injections. Injections 1 P of 0.25, 0.5, 0.7, or 1.0 ml ampule solution 24 h IV injection of 0.3 ml Indian ink suspension/30 g mouse. Spectrophotometric analysis of the clearance at 650 nm after 3, 6, 9, 12 and 15 min.

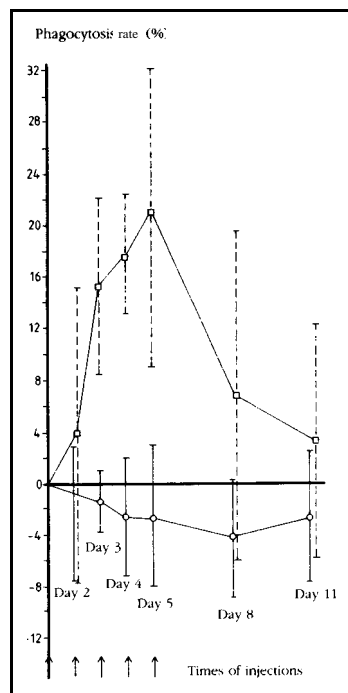


Fig. 2: 11-day single-blind study with Preparation C (□—□). IV injections of 1 ml ampule solution or 0.9% NaCl solution on days 2, 3, 4 and 5. 14 subjects in the therapy group, 13 in the placebo group (○—○). Measurement of the phagocytosis index 3 days before the 1st injection and on days 2, 3, 4, 5, 8, and 11.

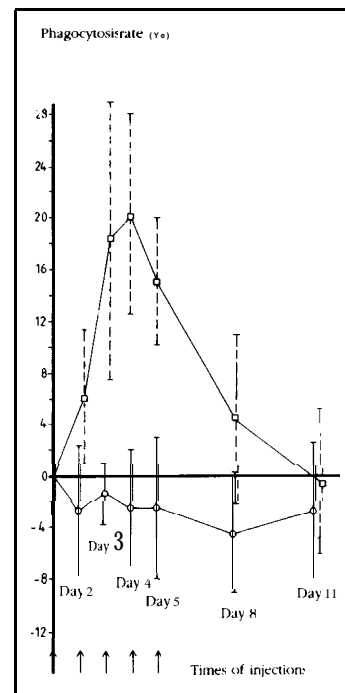


Fig 3: 11-day single-blind study with Preparation D (□—□). IV injections of 2 ml ampule solution or 0.9% NaCl solution on days 1, 2, 3, 4, and 5. 12 subjects in the therapy group, 23 in the placebo group (○—○). Measurement of the phagocytosis index 3 days before the 1st injection and on days 2, 3, 4, 5, 8 and 11.

ulation measured is an effect of higher attenuations, as is already known for lipopolysaccharides or phorbol ester.

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