General immunological properties of fat-soluble (Cernitin GBX) and water-soluble (Cernitin T60) pollen extracts

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The immunological properties of fat-soluble (Cernitin GBX) and water-soluble (Cernitin T60) pollen extracts were examined *in vivo* and *in vitro*. For investigations *in vitro* we used the water-soluble pollen extract (T60), and *in vivo* the fat-soluble form (GBX). The aim of the *in vivo* experiments was to evaluate their effect on IgG antibody production, their capability of rosette formation (E-RFC) and cell indicating IgM-plaqueforming cells (PFC) hemolysins. Also observations were made on the effect of the pollen extract on graft *vs* host reaction, the transplantation barrier and delayed hypersensitivity in relation to sheep red blood cells (SRBC). Its assumed effect on phagocytosis and blastic transformation was assessed *in vitro*. In both *in vivo* and *in vitro* systems the influence of T60 and GBX preparations on the population of T- and B-cells was tested. A relationship was revealed between immunological activity and the evaluated Cernitins. Both the Cernitins examined demonstrated moderate immunoregulatory properties, but the immunosuppressive component was predominant.

Keywords: pollen extracts; immunological properties.

INTRODUCTION

Cernitins (pollen extracts obtained from A.B. Cernelle, Vegeholm, Sweden), contain water-soluble (Cernitin T60) and fat-soluble (Cernitin GBX) substances. The aim of the present work was to examine the effect of Cernitins on immunological parameters *in vivo* and *in vitro.* Previously it had been published that Cernitins given orally or intraperitoneally inhibited or counteracted the elevation of aminotransferase activity and the inflammatory process, necrosis and steatosis of liver cells (Wójcicki *et al.,* 1985).

MATERIALS AND METHODS

Effect on the formation of precipitins (IgG). Investigations were carried out on mice of the 129 Ao/Boy strain. The titre of precipitins was detected (every week) by the Ouchterlony (1949) method modified by Wilson and Pringle (1954). Animals were injected subcutaneously with a 1% solution of ovalbumin, 1 mL/kg three times a day every third day. Cernitin GBX was administered 10 mg/kg i.p. from day 3 once a day, for 21 days.

Effect on plaque forming cells (Mishell and Dutton, 1967). A plaque forming cell test was performed according to Jerne and Nordin (1963). Examinations were carried out on mice of 129 Ao/Boy strain. Cernitin GBX was administered i.p. 10 mg/kg, starting from day 1 and afterwards once a day for 4 days. On day 0 a sensitizing dose of 10% sheep erythrocytes was injected i.p. (0.2 mL per

mouse). On the fourth day the test proper was carried out, using the spleen cells.

Rosette E-forming test. The experiment was carried out according to Bach and Dardenne (1972) on mice of the 129 Ao/Boy strain. Cernitin GBX was administered i.p. 10 mg/kg, starting from the day 1 and afterwards once a day for 4 days.

Determination of T- and B-lymphocytes (Boyum, 1976; Gorer and O'Gorman, 1956; Pasternak, 1969). ***In vivo*****.** Mice of 129 Ao/Boy strain received Cernitin GBX for 4 consecutive days (10 mg/kg i.p.). Lymphocytes were isolated by centrifugation (800 revs per min for 5 min at 4˚C) over a Picoll gradient. T-cells were detected by the cytotoxic test. The percentage of B-lymphocytes was determined by direct immunofluorescence. The number of fluorescent cells which were identified as B-lymphocytes is expressed in proportion to 1000 counted cells. *In vitro.* Cernitin T60 was added at 0.6, 3.0 and 15.0 mg/mL final concentration in Hanks' solution. The detailed procedure of T- and B-lymphocyte determination was described by Hoffman and Kunkel (1976).

Skin graft test (Marckman, 1966; Plużańska, 1969). Investigations were carried out on young mice of 129 Ao/Boy strain. Skin grafts of 1 cm^2 in size were transplanted in the allogenic system of mice of C_{57} B1 strain. Mice-recipients were treated with Cernitin GBX (10 mg/kg i.p.) once a day, starting 1 day before being grafted and until complete graft rejection had occurred.

Graft vs host reation (Ford *et al.,* 1970). The spleens of mice of the B_6 strain were removed and rinsed in cold HBS solution. Local graft vs. host (GvH) reaction was induced by injecting 0.05 mL of a suspension of these cells in HBS solution into the right footpad of Balb/CxB_6 mice. The right (test) and left (control) popliteal lymphnodes were removed and weighed 6 days post injection. Cernitin GBX was administered at a dose of 10 mg/kg i.p. daily for 6 days starting one day before the injection in the footpad. Results were expressed by the difference between the mean weights of the left and right popliteal nodes.

Delayed hypersensitivity test on sheep erythrocytes (SEC) (Papadimitriou *et al., 1983).* 0.2 mL of a 10% SEC solution was injected intravenously into mice of the 129 Ao/Boy strain on day 0. After 4 days, 0.05 mL of a 50% SEC solution was injected subcutaneously into a hind leg footpad: the thickness of the footpad was measured in mm after 24 h and 48 h. 10 mg/kg Cernitin GBX or 50 mg/kg Cernitin T60 were injected i.p. into the mice on day 1 and on every other day afterwards.

Blastic transformation. Experiments were carried out with human venous blood by the isotope method according to Hersh and Oppenheim (1965), modified by Plużańska (1969). Cernitin T60 was added in a Parker solution to the culture in concentrations of 0.6, 3.0 and 15.0 mg/mL 1 h before the application of 20 µg/mL phytohemagglutinin A (PHA) to the culture. The cultures were incubated for 48 h at 37˚C in a 5% CO_2 atmosphere. ^{14}C-labelled thymidine was added and incubation was continued for another 24 h under the same conditions. Radioactivity was determined by scintillation counting and expressed as counts per min.

Phagocytic activity. The investigations were carried out according to Steuden's (1978) method. The test proper was carried out using 0.1 mL of *Staphylococcus aureus* bacteria (5 x 10^8 per mL) and 0.1 mL of Cernitin T60 in concentrations of 0.6, 3.0 and 15.0 mg/mL + 0.1 mL of granulocyte solution obtained from the peritoneal exudate of a guinea-pig which had received beuillon i.p. The cultures were then centrifuged at 3000 revs per min for 30 min and 37˚C. Radioactivity was measured in 1 mL of supernatant in a Beckman scintillation counter, type 3801. Results were expressed as the percentage of phagocytosed bacteria.

Statistical analysis. Statistical analysis of the data was performed using Student's t-test. In all

comparisons, p <0.05 was considered to be significant.

RESULTS

The effect on the formation of precipitins. It was shown that the titre of precipitins was virtually unchanged in animals receiving Cernitin GBX;

the titre oscillated between 1:128 and 1:512, both in mice treated with Cernitin GBX and in the control group.

The effect on the plaque formed cells (PFC) and on the rosette forming cells (RFC). Cernitin GBX injected i.p. into mice affected the number of cells producing hemolysins (PFC) to some degree and also the ability of lymphocytes to form RFC with sheep erythrocytes. However, the results were contrasting: a marked increase in PFC was demonstrated while the number of RFC was reduced. The differences were statistically significant (p<0.05) in both cases (Table 1).

Table 1. Effective of Cernitin GBX on the plaque forming cells (PFC) and the rosette E forming cell (E-RFC)

Treatment	PFC (2×10^6 cells)	%	RFC (2×10^3 cells)	%
Cernitin GBX (10 mg/kg, $n = 10$)	771[a]	323	3.5[a]	19.4
Control ($n = 10$)	220	100	18.0	100.0

[a] $p < 0.05$.

The effect of Cernitin GBX (*in vivo*) and Cernitin T60 (*in vitro)* on the T- and B-lymphocyte subpopulations. Cernitin GBX (10 mg/kg) and Cernitin T60 (0.6-15.0 mg/mL) did not change significantly (p>0.05) either the number of T- and B-lymphocytes or the lymphocytes of the peripheral blood possessing no receptor (null) (Table 2).

Table 2. Influence of the Cernitin GBX (*in vivo*) and Cernitin T60 (*in vitro*) on the T- and B-lymphocyte subpopulations

Treatment	Lymphocyte subpopulations (%) T	B	Null
Cernitin GBX (10 mg/kg, $n = 10$)	64	24	13
Control ($n = 10$)	64	27	11
Cernitin T60 (mg/mL)			
0.6 ($n = 5$)	58	21	21
3.0 ($n = 5$)	63	19	18
15.0 ($n = 5$)	67	12	21
Control ($n = 5$)	60	22	18

Skin graft test and graft host reaction (GxH). In animals receiving Cernitin GBX, the rejection time of the skin graft was somewhat prolonged (p>0.05). The index of GvH was decreased under the influence of this preparation, but in comparison with the control group there was no statistically significant difference (p>0.05) (Table 3).

Table 3. Influence of Cernitin GBX on the skin graft rejection and graft vs host reaction (GvH)

Treatment	Rejection time of the skin graft (days)	GvH index
Cernitin GBX ($n = 10$)	11.0	1.2
Control ($n = 10$)	10.6	1.8

Delayed hypersensitivity to SEC test. *In vivo* Cernitin GBX and Cernitin T60 did not intensify the response to SEC. The index of the increased in footpad thickness was unchanged after 24 h, while after 48 h it was even slightly diminished (p>0.05) in comparison with the control group (Table 4).

Table 4. Effect of the Cernitin GBX and Cernitin T60 on delayed hypersensitivity against SEC

Treatment	Dose (mg/kg)	Index of the increase of thickness of footpad After 24 h	%	After 48 h	%
Cernitin GBX ($n = 10$)	10	4.5	100	3.0	85.7
Control ($n = 10$)	—	4.5	100	3.5	100.0
Cernitin T60 ($n = 5$)	50	4.5	100	3.0	85.7
Control ($n = 5$)	—	4.5	100	3.5	100.0

Blastic Transformation. Cernitin T60 markedly decreased blastic transformation *in vitro* expressed as the number of impulses for [^{14}C] thymidine per min. Nonspecific induction of blastic transformation with phytohemagglutinin (PHA) confirmed, to some degree, the results obtained. But the interaction between Cernitin

T60 and PHA was irregular and depended on the concentration of the preparation. However, the suppressive component was predominant (Table 5).

Table 5. Effect of Cernitin T60 on the blastic transformation (isotope method)

Preparation	Concentration (mg/mL)	Counts per min
Cernitin T60 ($n = 5$)	0.6	1060[a]
Cernitin T60 ($n = 5$)	3.0	857[a]
Cernitin T60 ($n = 5$)	15.0	170
Control ($n = 5$)		227
PHA ($n = 5$)		7108
PHA + Cernitin T60 ($n = 5$)	0.6	4934
PHA + Cernitin T60 ($n = 5$)	3.0	8934
PHA + Cernitin T60 ($n = 5$)	15.0	1648[a]

[a] $p < 0.05$.

Phagocytic activity. The *in vitro* process of *Staphylococcus aureus* phagocytosis by guinea-pig granulocytes of the peritoneal exudates was completely inhibited by Cernitin T60 in concentrations of 0.6-15.0 mg/mL.

DISCUSSION

The definite anti-inflammatory effect of Cernitin extracts was demonstrated by the *Croton* oil-induced edema test (Itch, 1968). In the cotton pellet test, Cernitin T60 showed an anti-inflammatory activity in rats corresponding to the inflammation-inhibiting effect of phenylbutazone. But T60 was completely devoid of toxicity (Glømme and Rasmussen, 1965). It was also possible to confirm the anti-inflammatory effect of Cernitins on carrageenin-induced edema in rats (Dessi, 1971). Cernitins administered orally to rats demonstrated a marked antiinflammatory effect compared to the very active anti-inflammatory agents injected intraperitoneally as controls. Cernitins also inhibited the inflammatory process induced by galactosamine administration to rats (Wójcicki *et al.,* 1985). The results obtained in this experiment show that Cernitin GBX and Cernitin T60 are able to affect the course of the induced immunological processes. Such an effect is, however, defined and conditioned by the test type used and by the dose applied.

The number of RFC was affected by Cernitin GBX in a quite contrasting way. An increase in the number of PFC was accompanied by a reduction in the RFC. One may argue that the preparation examined plays an essential role in immunological processes due to regulation of the reciprocal relationship between both kinds of cells. Thus, Cernitin GBX may have a quite significant immunomodifying function.
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T- and B-lymphocytes are the morphological basis of the immunologic process. Cernitin GBX (*in vivo*) and Cernitin T60 (*in vitro*) did not alter the reciprocal relationship between the above mentioned subpopulations of lymphocytes. This could mean that another factor modulating reactivity is present or that the changes are not sufficiently marked as to be shown by the quantitative difference between the subpopulations of T- and B-lymphocytes. These results may be due to an ability to produce lymphokins, mainly interleukin 2 (Wybran and Schandene, 1985) rather than differences in morphological element

Cernitin GBX did not influence the barrier of a graft, although slight changes of graft host reaction were noted. Thus, Cernitin GBX did not change essentially either the graft reaction of the graft host reaction. The delayed reaction of hypersensitivity against SEC was not modified by the examined Cernitins. On the other hand, the blastic transformation was affected by Cernitin T60. It was reduced *in vitro* proportionally to the concentration of Cernitin T60. In relation to phytohemagglutinin a two-phase reaction was observed. Cernitin T60 applied in both low concentration and especially in high concentration, diminished the reaction, while the intermediate concentration was not effective. Thus, our observations confirm the results obtained by Kimura and Inoue (1968) demonstrating the lack of allergenic properties of both Cernitins. Our studies showed, however, that there is a relationship between the immunological system and the Cernitins tested. We can conclude, therefore, that the pollen extracts effectively possess an immunotropic/ immunoregulatory component. They show, *in vitro*, a slight immunosuppressive effect (E-

RFC)—B-lymphocyte antagonism in reaction to blastogenic effect of PHA—and occasionally they act as a stimulator (PEC, blastic index). In some experimental systems they are ineffective (GvH, transplantation barrier, SEC test).

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