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7 Prepared by stearoylation or behenoylation of L-Ala-D-Glu(OH)OBzl: stearoyl-L-Ala-D-Glu(OH)OBAl, m.p. 114-6 °C, $[\alpha]_D$ −26.7°(c 0.2, $CHCl_3$); behenoyl-L-Ala-D-Glu(OH)OBzl, m.p. 119-123 °C, $[\alpha]_D$ −14.1°(c 0.4, AcOH).
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Monoclonal antibodies against functionally distinct sites on the delta-endotoxin of *Bacillus thuringiensis* var. *thuringiensis*

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Summary. Monoclonal antibodies against the toxic units of *Bacillus thuringiensis* delta-endotoxin were raised by the hybridoma technique and detected by an indirect enzyme-linked immunosorbent assay (ELISA). Out of 5 positive clones, 1 was found to secrete antibodies which inactivate the toxin.

B. thuringiensis produces a proteinaceous crystal responsible for the insecticidal action of these bacteria towards many larvae belonging to the order Lepidoptera[2]. The crystal, also called protoxin, consists of dimeric subunits of mol.wt 230,000 and can be dissolved under alkaline or reducing conditions[3]. For biological activity a proteolytic activation is required, which occurs under natural conditions in the gut juice of susceptible insect larvae. In vitro digestion with trypsin rapidly leads to a fraction with mol.wt 80,000. It is degraded further to a more stable fragment with mol.wt 60,000 by prolonged exposure to protease. Both units are equally active on a molar basis. Low molecular weight by-products show no toxicity[4]. Little is known about the active site and the molecular mode of action of this toxin. Monoclonal antibodies are considered as a tool to unravel where the toxin acts and which sequences and/or conformation of the polypeptide chain are necessary for its action.

Materials and methods. B. thuringiensis var. *thuringiensis* (serotype H-1, strain LBG B4412) parasporal bodies were purified[5] and dissolved in 0.1 N NaOH for 30 min at 37 °C. The protoxin was digested for 3.5 h with sepharose-bound trypsin in 0.05 M carbonate buffer pH 9.5. The proteolytic fragments were separated on a Sephadex G-200 column using the same buffer. The toxic units, mol.wt 80,000 and 60,000, were eluted in 1 peak, concentrated and used as antigen.

3 female BALB/c mice were immunized twice. First 185 µg active delta-endotoxin in 0.25 ml complete Freund's adjuvant (CFA) were injected s.c., followed 6 weeks later by an i.p. boost with 90 µg of the toxic units in 0.1 ml 0.01 M phosphate buffered saline (PBS) pH 7.4.

A 2nd series of 3 female BALB/c mice was treated 4 times at weekly intervals. For the 1st injection 95 µg of delta-endotoxin in 0.125 ml CFA were given s.c., 2 more injections of 95 µg in 0.125 ml incomplete Freund's adjuvant (iCFA) followed also s.c. The boost of 90 µg in 0.1 ml PBS was given i.p.

4 days after boosting 2 separate fusions were done with spleen cells of mice of both immunization series (47S and 48S). $1.7.10^8$ primed spleen cells were fused with 3.10^7 SP2/O-Ag14 cells[6] with 50% polyethylenglycol[7] according to Köhler and Milstein[8]. Immediately after each fusion cells were diluted in 50 ml HAT medium[9], to which $5 \cdot 10^5$ peritoneal cells were added, and distributed in 96 wells of Costar 3524 trays.

Supernatants from growing hybrids were screened for anti-delta-endotoxin antibodies by the indirect method for microplate ELISA[10]. 200 ng antigen in 0.2 ml coating buffer[10] were incubated overnight in Cooke's Microtiter Plates (Dynatech Microelisa) at 4 °C. In a 2nd step 0.2 ml supernatant in different dilutions were added to the coated plates. Normal mouse serum and culture medium were taken as negative controls. Antibodies were allowed to react with the antigen for 2 h at 37 °C. Alkaline phosphatase was chosen as enzyme label and was coupled to goat antimouse IgG[10]. This conjugate, diluted 1:500, was incubated with the antigen-antibody complex for 2 h at 37 °C. P-nitrophenyl-phosphate (Sigma) was used as substrate. The rate of substrate degradation was examined by a Titertek Multiskan spectrophotometer at 405 nm after 30 min.

Positive cultures were cloned by limiting dilution according to Herbst and Braun[11]. Supernatants from growing clones were screened for anti-toxin antibodies and the positive clones were grown in DFCS culture medium[12]. Immunoglobulin subclasses were determined by immuno-diffusion of rabbit antiserum to the different mouse classes and subclasses (Bionetics) and 20 times concentrated supernatants of positive clones[11]. SDS-PAGE with ^{14}C-leucine labeled supernatants[13] was carried out according to

Feeding inhibition of *Pieris brassicae* larvae in percentage as a function of toxin concentration and antibody containing supernatants from different clones

Supernatant applied	Toxin (ng/larvae) 0	5	10	20	40
Culture medium	0	100	100	100	100
47S94.2	0	0	0	10	40
48S34.1	0	100	100	100	100
48S44.1	0	100	100	100	100
48S54.1	0	80	100	100	100
48S54.4	0	100	100	100	100

Laemmli[14] under reducing conditions on 10% acrylamide gels. For that purpose, cells of well-growing clones were centrifuged at 200 × g for 15 min, their supernatants were decanted and replaced by 0.2 ml ^{14}C-leucine culture medium (5 µCi/ml). The cells were grown in this medium for 18 h and supernatants containing the secreted ^{14}C-labeled antibodies were harvested.

The bioassay was performed with 5th instar larvae of *Pieris brassicae* according to Lüthy[15]. Each test series consisted of 4 larvae to each of which 5 µl of the examined samples were force fed. Supernatants of 5 clones, concentrated 10 times by ultrafiltration, were tested for toxin inactivation by adding toxin up to concentrations of 1, 2, 4 and 8 ng/µl, respectively. Culture medium, also concentrated 10 times, was injected perorally to the larvae as a negative control. The same medium concentrate, to which toxin was added up to 1, 2, 4 and 8 ng/µl, respectively, was used as a positive control. Antigen and antibodies were allowed to react for 2 h at room temperature before the solutions were fed to the larvae. Feeding inhibition was taken as a measure of toxin activity[15].

Results and discussion. Fusion rates, i.e. percentage of the number of growing hybrids to the number of cultures started, were 84% for the 47S immunization series and 96% for the 48S one. Fusion efficiency, defined as percentage of delta-endotoxin specific antibody producing hybrids related to the number of growing hybrids was 12% and 22%, respectively. Hybrids whose supernatants showed in the ELISA at 405 nm after 30 min absorption values larger than 0.5 were considered as positive. Negative controls never exceeded an absorption value of 0.1. From each fusion series supernatants of 6 hybrids were noteworthy as their ELISA values surpassed 2.0 after 30 min. Therefore, they were chosen for cloning. 5 of these 12 hybrids gave rise to 48 specific clones. 16 arose from one hybrid of the 47S series and the other 32 from 4 hybrids of the 48S series.

All clones secreted antibodies consisting of γ_1 heavy chains and κ light chains. These antibodies did not precipitate the toxin in the immuno double diffusion test, indicating that the antigenic determinant they recognize is not repetitive in the toxin molecule. However, all of these clonal supernatants crossreacted with protoxin in the ELISA. Therefore, their epitopes must already be accessible on the protoxin.

^{14}C-leucine labeled monoclonal antibodies originating from the most positive clones were examined by SDS-PAGE. All antibodies derived from 47S94 showed the same banding patterns of heavy and light chains as 47S94.2 (fig. 1). Out of culture 48S54 2 families of clones could be recovered by cloning; these differed slightly in the molecular weight of their light chains like 48S54.1 and 48S54.4. Also 48S34.1

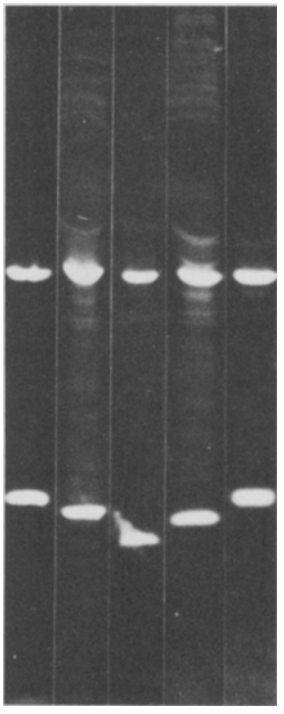

Figure 1. Autoradiograph of SDS-PAGE analyzed ^{14}C-leucine labeled antibodies secreted by the clones 47S94.2, 48S34.1, 48S44.1, 48S54.1 and 48S54.4 (from left to right).

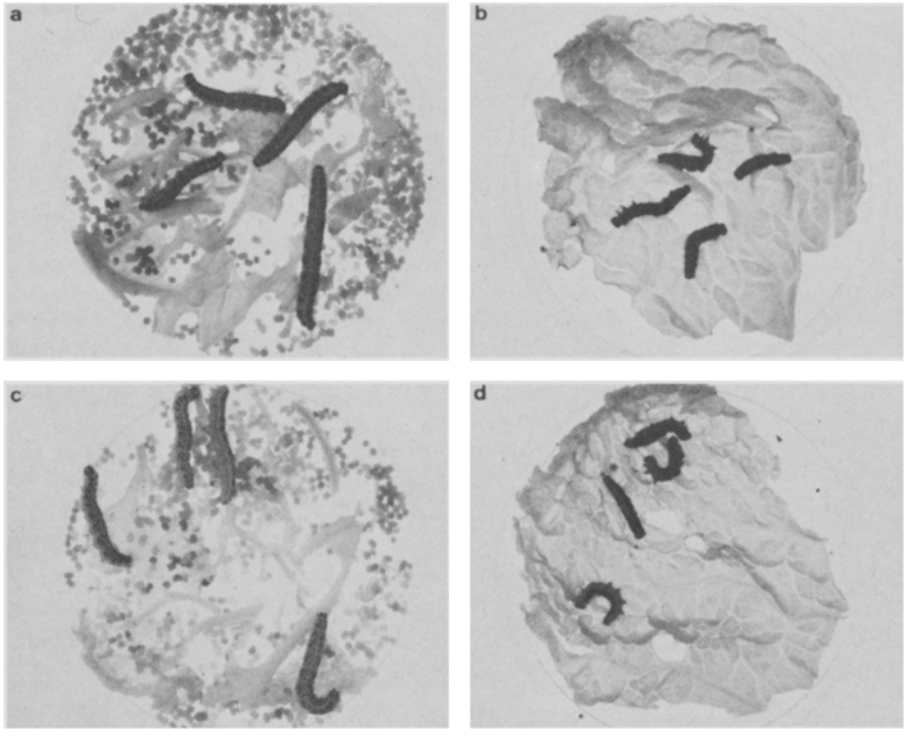

Figure 2. Bioassay with larvae of *Pieris brassicae*: Effect of monoclonal antibodies on the activity of the toxin of *Bacillus thuringiensis*. *a* Negative control with culture medium; *b* positive control with culture medium and 10 ng toxin/larva; *c* supernatant of clon 47S94.2 with 10 ng toxin/larva; *d* supernatant of clon 48S34.1 with 10 ng toxin/larva.

and 48S44.1 differed from one another in this aspect (fig. 1). Those 5 clones were further examined in the bioassay (table). Larvae fed with 10 times concentrated culture medium containing 5, 10, 20 and 40 ng toxin/larva, respectively, all exhibited 100% feeding inhibition (fig. 2, b). The same result was obtained with toxin and concentrated supernatants of clones derived from the 48S series (fig. 2, d). The only exception were the 4 larvae fed with supernatant of clone 48S54.1 and the lowest toxin concentration, showing a 80% feeding inhibition, i.e. a slight reduction in toxin activity.

In contrast, antibodies of clone 47S94.2 completely inactivated the toxin at toxin concentrations of 5 and 10 ng/larva (fig. 2, c). Increasing the toxin concentration to 20 and 40 ng/larva caused a weak feeding inhibition of 10 and 40%, respectively. Culture medium as well as all concentrated supernatants fed without toxin had no effect at all on the larvae (fig. 2, a).

We conclude that the antibodies described here detect non-repetitive, functionally different sites on the delta-endotoxin. 47S94.2 blocks the toxin activity. It is not yet clear whether inactivation of the toxin is due to a direct interference with the active site, an indirect effect as e.g. a conformational change of the antigen, or a blocking of its possible binding site.

Further studies on the characterization of the monoclonal antibodies and their mass-production in mice are in progress.

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Decrease of mast cells in regional lymph nodes in response to allogeneic antigens and syngeneic tumor antigens

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Summary. The absolute number of mast cells in regional lymph nodes decreases on the 5th day after stimulation by allogeneic lymphocytes and semisyngeneic leukaemic cells, despite the enlargement of stimulated lymph nodes. It is postulated that the reaction of lymphatic mast cells could be a sensitive test for tissue incompatibility, and probably also for the presence of tumor associated antigens.

Basophilic granulocytes and mast cells (MC) play an active role in a variety of immune responses[2-6] (for review see Włodarski et al.[7]) including antitumor reactions[8-11], but in spite of numerous experimental studies the mechanism and the significance of their participation remains to be elucidated[12].

MC rapidly respond by increase in number in murine lymph nodes regional to the site of a localized primary heterologous (BSA, sheep erythrocytes) antigenic stimulus[3,4], but within h this reaction ceases, and between 24 and 48 h the level of MC declines below the normal values[4]. According to the previous publication the early increase (within h) of the number of MC developing after injection of antigenic material, as well as of syngeneic cells seems not to be related to the antigenicity of the injected material[4]. In the present study an attempt was undertaken to find out what is the relation between the decline in the number of MC, following the early increase, and the antigenicity of the injected cells.

Material and methods. 4-5-month-old Balb/c × DBA/2W F_1 mice of both sexes, raised in our laboratory, were injected in the right footpad with 5×10^6 semisyngeneic leukaemic L1210 cells, passaged on DBA/2W mice; with allogeneic splenocytes of strain 129; with semisyngeneic splenocytes and thymocytes of DBA/2W; or with Parker medium. Contralateral left footpads were injected with 0.05 ml of Parker medium without serum. All cells were irradiated in vitro with 11000 R, 3–4 h prior to the inoculation.

The animals were killed 5 days after injection and the popliteal lymph nodes were excised, fixed in Bouin solution and embedded in paraffin. The entire nodes were sectioned serially at 7 µm thickness. Sections were stained with 0.1% aqueous solution of toluidine blue. Every 5th section was carefully examined and the content of MC scored under the microscope at a magnification of × 120. From the number of sections obtained from a given lymph node and from the number of MC scored in every 5th section, the total content of MC per lymph node was calculated.

The size of a given lymph node, as expressed as its mean diameter, was calculated from the number of sections obtained multiplied by 7 µm. Arithmetical mean values for each group were calculated. The differences between mean values were analyzed by Student's t-test.

Results and discussion. The results obtained are presented in the figure. The MC number in the regional popliteal lymph node decreases after the injection of allogeneic splenocytes or semisyngeneic leukaemic cells, as compared to non-stimulated contralateral and intact control nodes. This difference is statistically significant as determined by Students's t-test at $p < 0.05$.