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Glucan and Tuftsin—Potential Antileishmanial Agents

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Summary of Results

The conditions for optimal glucan mediated suppression of L. major development were examined. Glucan\textsuperscript{p} (0.5 mg) were injected i.v. at 7 day intervals (up to 4 injections) starting 7 days after parasite challenge. Mice receiving only parasites or receiving injections of a glucose solution at concurrent times served as controls. In addition several groups of animals received starch particles in a regime which paralleled the glucan\textsuperscript{p} treatment. Starch was used as a control particulate polysaccharide material since it has a different backbone structure from glucan. When examined at 17, 21 and 28 weeks after parasite injection both the control mice and mice receiving starch particles showed a progressive increase in footpad thickness and developed lesions. Two to four injections of glucan\textsuperscript{p} suppressed lesion development. Four injections gave a more rapid and a higher extent of suppression than 1, 2 or 3 injections. Several of the mice which received glucan\textsuperscript{p} developed relatively big lesions (up to 0.5 mm bigger than the controls) at early stages following parasite injection (i.e. at 9 weeks). However, these lesions healed leaving no visible scar and hair formation resumed normal growth.

Mice were sensitive to the dose of glucan\textsuperscript{p} used for i.v. injection. At a higher dose of particles (1 mg/mouse) many mice died within hours following the i.v. injection.

Injection of glucan\textsuperscript{p} i.p. did not prevent the onset of cutaneous disease.

In a preliminary experiment the alkali soluble glucan (glucan\textsuperscript{as}) was injected two or four times i.p. into two groups of mice. Five hundred μg of glucan\textsuperscript{as} suppressed lesion formation in both groups, a four injection regime resulted in a higher suppressive effect than that of two injections when measured at 12 weeks after challenge.
In another experiment the effects of 4 injections i.p. of 500 μg of glucan\textsuperscript{as}, a gel forming glucan synthesized and secreted by Sclerotium rolfsii, and a low molecular weight glucan solubilized by formic acid treatment were compared. While the glucan\textsuperscript{as} and the gel-forming glucan from Sclerotium rolfsii exerted a suppressive effect on lesion formation, the soluble small molecular weight glucan had no suppressive effect.

The effect of glucan\textsuperscript{as} was further studied, by comparing the effectiveness of 50μg, 200 μg and 400 μg of glucan\textsuperscript{as} given 1 to 4 times by both i.v. and i.p. routes. Glucan\textsuperscript{as} injection i.v. or i.p. blocked lesion development, i.e. footpad thickness did not exceed 3 mm on average (1.5 mm is the thickness of non-treated footpads). In i.v. injected mice (200 μg and 400 μg per mouse) this moderate lesion regressed to almost the control values within 60 to 70 days after promastigote injection. Injection of 50 μg/mouse (i.v.) suppressed lesion formation but a slow continuous increase in footpad thickness was still noticed. Administration of glucan\textsuperscript{as} i.p. also suppressed lesion development and 400 μg/mouse prevented even the initial stages of lesion formation. Seventy days after the promastigote challenge, six mice from the groups receiving 50 μg glucan\textsuperscript{as} with the more pronounced lesions were separated and boosted two more times with glucan\textsuperscript{as} (200 μg i.v. or i.p. respectively, on days 70 and 74). Treatment of these mice with glucan\textsuperscript{as}, even at this stage, resulted in an additional regressive effect on lesion development. Similar treatment of the control mice had no significant effect on lesion progression. At 120-180 days post challenge the feet of these mice were in a state of extensive ulceration so that beneficial effects, even if real, could not be evaluated by footpad measurements of the control mice. It should be noted that mice in the
groups receiving 200 μg and 400 μg glucan<sup>as</sup> maintained a normal footpad thickness for up to 170 days post parasite challenge.

In order to assess whether regression of lesions was correlated with parasite eradication we sacrificed 7 mice from the control group and 7 mice that received glucan<sup>as</sup> treatment (200 μg, 4 times) on day 135 post promastigote challenge. Touch imprints were made from the lesion and from the livers. Many amastigotes could be found within the lesions of control mice. In three out of seven of these mice, parasite metastasis to the liver was also found. In the glucan<sup>as</sup> treated mice no amastigotes could be identified by this technique at either of the sites examined.

Blood was also collected from the sacrificed animals. The antibody titer (in Enzyme-linked immunosorbent assay (ELISA), recorded in absorbance units at 405 nm) for the glucan<sup>as</sup> treated mice (0.25±0.05) was significantly lower (P<0.01) than that of control Leishmania bearing mice (0.48±0.05).

The parasite antigens recognized by the antibodies from both groups of sacrificed mice were analyzed. Reactions using the sera from the infected control group were much stronger, as expected from the titration data. Approximately 14 bands could be seen with molecular weights ranging from <26 to >116 kDa. The broad band at <26 kDa was especially prominent. A much longer exposure (~x25) of the film was required to detect reactions of antigens with the sera from the glucan<sup>as</sup> treated mice. The overall pattern of reactivity of antibody from the glucan<sup>as</sup>-treated mice was very similar to that of control mice. However, no reaction with the prominent broad band (<26 kDa) and with three additional bands (<36 kDa) was observed with sera from glucan<sup>as</sup>-treated mice. The glucan<sup>as</sup>-treated
mice with high titers exhibited antibody binding patterns in Western blots essentially identical to those found with the control infected mice.

In order to better understand the immunological processes that are set into motion by glucanas injection we analyzed the lymphocyte cellular phenotypes in the draining lymph nodes. Lymphocytes expressing the L3T4+ phenotype have been implicated in mediating progressive disease. We used flow cytometry to analyze cell suspensions derived from draining lymph nodes of infected and healing (glucanas injected) Balb/c mice at various stages after infection. T cell expansion in draining lymph nodes occurred early after infection; the most significant observation was the hyperplasia of L3T4+ cells. Before infection the nodes contained 2.2±0.7 x 10^6 cells of which about 60% were L3T4+, after 3 weeks the cellularity increased to 42±1.2x10^6 34% of which were L3T4+, and by 7 weeks the cellularity was 27±1.3x10^6 and 47% of it were L3T4+ cells. The number of Ly2 cells increased proportionately so that the ratio of L3T4+/Ly2 which was 2.5±0.7 before infection was not significantly altered during the 7 weeks of the experiment. The glucanas injected (400 μg glucanas i.p. given 4 times at 4 day intervals starting at 4 days after parasite inoculation) healing mice, showed a significant deviation from this pattern, i.e. their L3T4+/Ly2 cell ratio decreased from the value before infection to 0.9±0.3 and 1.2±0.2 at 3 weeks and 7 weeks after infection, respectively. These findings corroborate the finding that depletion of L3T4+ cells in Balb/c mice by the GK1.5 antibody before infection leads to lesion healing in this susceptible strain.

Several experiments were set to test the potential of tuftsin to suppress lesion formation as a result of L. major injection. Tuftsin at 10, 20
and 40 μg/mouse was injected i.p. or subcutan at different days pre-, concomitant- and post- parasite injection. The experiments suggest a certain suppressive effect but the results were not conclusive. In some experiments it stemmed from a general low level of lesion formation also in the controls and in others from a low response to tuftsin which occasionally occurred in our mouse colony.

Attempts to assess the effect of solubilized glucan and of tuftsin on in vitro infected cultures of macrophages both peritoneal and bone marrow derived were unsuccessful due to the fact that the parasites were spontaneously eradicated by the macrophages during a 72 h to 96 h culture.

A first report appeared in Parasite immunology, 1991, 13, 137-145. The rest will be summarized shortly after completion of the experiments.
Parasites and Glucans

Parasites: Two isolates of L. major were used; Fredlin (World Health Organization designation, MHOM/IL/80/Fredlin) and WR309 (MHOM/IL/79/Perlstein).

Virulent parasites were maintained in BALB/c mice. Fresh isolates were made by needle aspiration and maintained as promastigotes in axenic culture for not more than five to eight passages. The culture medium consisted of Schneider's Drosophila medium containing 200 mM glutamine, 20% heat inactivated fetal calf serum and antibiotics (Penicillin 100 U/ml, streptomycin 100 μg/ml). Stationary phase promastigotes were washed three times in Dulbecco's phosphate-buffered saline and finally resuspended in sterile, nonpyrogenic, 0.9 % NaCl. The promastigotes were injected in 20 to 30 μl, intradermal into one footpad of 6 to 8 week old BALB/c male mice (Olac, England). The basal footpad thickness was 1.5 mm. Glucan and control solutions (nonpyrogenic saline or 5% glucose) were injected i.p. or i.v. at the specified times and amounts. Three hundred μl were injected i.p. and 100 μl were injected i.v. For injection, glucan preparations were suspended or dissolved in the respective solutions (5% glucose or saline), and sterilized by γ-irradiation (2 Mrad, 60Co, Gamma Cell 220, Atomic Energy of Canada). Footpad thickness was measured weekly with a caliper.

Glucan: Baker's yeast (Saccharomyces cerevisiae) were digested sequentially with boiling NaOH and HCl, and extracted with isopropyl alcohol. The glucan particles (glucanp) were further treated to obtain nonparticulate material. Incubating glucanp with 1 M KOH for 20 min at 60 °C resulted in the solubilization of 50-70 % of the glucan (estimated on
the basis of glucose determination). The alkali soluble glucan (glucan\textsuperscript{as}) was precipitated by cold (4°C) 70% ethanol. The precipitate was collected by centrifugation, resuspended in water and alcohol precipitation repeated. The pellet was resuspended in warm water and gave homogeneous turbid solutions. To obtain low molecular weight glucan polymers, glucan\textsuperscript{P} was degraded by treatment with 90% formic acid at 95°C for 20 min. A fraction containing polymers with average chain length of 25 monomers was used as soluble glucan in the study. A gel forming glucan secreted from Sclerotium rolfsii was a gift of Dr. I Chet. Particulate rice starch (starch\textsuperscript{P}) was obtained from BDH (Poole, England).
Work carried out and in progress in Kenya under the supervision of Drs. Kagai and Koech.

The Kenyan group has been concentrating on the establishment of the effect of β-glucan on human lymphocytes from visceral leishmaniasis patients and normal individuals from both endemic and non-endemic regions. The aim of these studies is to assess the potential of β-glucan as an immunomodulator for humans infected with L. donovani.

Specifically, the group has embarked on a program of comparing the stimulatory effect on lymphocytes of β-glucan and other known stimulants such as PHA, concanavalin A (Con A) and PPD as well as of a preparation of L. donovani soluble antigen, in vitro.

Methodology:

β-Glucan:
The polysaccharide is being prepared according to the methods adapted from the literature in the laboratory at the Weizmann Institute.

Lymphocytes:
Three sets of lymphocytes are being analyzed. a- from healthy Kenyans residing in Nairobi, a non-endemic region. b- Kenyans living in Baringo District, an endemic region for kala-azar. c- Kala-azar patients undergoing treatment at the Clinical Research Center, Kemri. Blood samples were taken also before treatment commenced. The people participating in the study filled a questioner and a form of consent. Underage individuals (below 18) require a guardian/parent consent.

Lymphocyte stimulation assay:
The assay is carried out as described by Koech et al. (Am. J. Trop. Med. Hyg.,36(3), 501-503, 1987). Briefly, 20 ml blood collected in heparin (20 u/ml)
are layered in Leuco sep™ tubes (Nyegaard&Co. Oslo, Norway) and centrifuged at 800g for 10 min. The mononuclear cells \((2\times10^6/ml)\) are plated into 96-well microtiter plates and incubated at 37°C in a humidified incubator with 5% CO₂ in air. Plates stimulated with mitogen are incubated for 3 days and with antigen for 6 days.³H-Thymidine is added on the respective last day of culture for 16 h at 0.5 μCi/well.

**Results:**

Several experiments have been carried out. The analysis of the last well defined and controlled study is not complete at this point but is well underway.

Interim conclusions which suffer from several experimental shortcomings are detailed:

β-Glucan at 10 μg/ml was used separately and in combination with PHA, ConA, purified protein derivative (PPD) and L. donovani soluble antigen (SLA) as stimulants.

Mitogens on the whole had the best stimulation. Lymphocytes of 2 out of 8 people of Baringo did not stimulate with 2-3 stimulants. 5 out of 8 from the same group did not stimulate with glucan. Lymphocytes of 1 person of the Nairobi group (10 people) did not stimulate with glucan. Most people (13) whose lymphocytes were stimulated by glucan showed lower indices of stimulation than achieved by mitogens. In 5 persons glucan was equally effective to mitogens. Antigenic stimulation was rather poor in all tested individuals. On the average the Nairobi group had a higher stimulation with mitogens than the Baringo group. The people in the Nairobi group had a better response to glucan+ PPD and PPD as compared to the Baringo group. There was no significant difference between healthy and kala-azar patients (all from Baringo).