Research Article

Effects of different *Fasciola hepatica* recombinant proteins on the biochemical and serological responses of experimentally infected sheep

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Abstract: In the DELIVER (Full project title: Design of effective and sustainable control strategies for liver fluke in Europe) project the objective was to analyze 3 recombinant antigens for their capacity to induce a satisfied immune response in sheep, to challenge the animals with metacercariae of *Fasciola hepatica*, and to observe their immune response and protection from the parasitosis. The animals were treated initially with metacercariae and every second week blood was taken for liver enzyme estimation of lactate dehydrogenase (LDH), gamma-glutamyl transferase (γGT), and antibody response. After the 3rd month of infection all the animals developed parasitosis, although to different extents. Differential observations of the blood showed a rise in eosinophils after the 3rd month (typical by parasitoses) and specific reactions of the other white blood cells. The LDH and γGT liver enzymes showed a slight fluctuation in comparison with the initial values. The antibody (immunoglobulin G1) formations against the different antigens (made by enzyme-linked immunosorbent assay) were typical as well, but not strong enough to protect the animals from the disease. The immune response was mostly pronounced against the cathepsin L1 antigen, with the highest values after 5–6 months. It turned out that the chosen adjuvant was successful too, because of the high response to it alone. The differential count of the blood cells showed a rise in the eosinophils. The liver enzymes were not typical for this disease. The results gave strong suggestions of how to operate further and what kind of corrections should be made.

Key words: *Fasciola hepatica*, TPx and CL1 antibodies, blood smears, LDH, γGT

1. Introduction

There is no doubt about the great importance of finding a vaccine to combat *Fasciola hepatica* parasitosis. Much effort has been invested in this direction but without achieving great success. The aim of the DELIVER (Full project title: Design of effective and sustainable control strategies for liver fluke in Europe) project was to try new recombinant antigens during this infection in sheep, cattle, and goats (in Bulgaria, lambs) and to measure some parameters in the living organism. Presented are the hematological, biochemical, and immunological examinations of lambs infected with *F. hepatica* as well as in control groups, so as to estimate the impact of these new antigens in preventing animals from contracting this disease.

2. Materials and methods

2.1. Experimental design

Experimental animals (male sheep < 5 months old, i.e. lambs) were immunized with recombinant *F. hepatica* cathepsin L1 (CL1), recombinant *F. hepatica* thioredoxin peroxidase (TPx), recombinant *Schistosoma mansoni* (fatty acid binding protein-(FABP) Sm14), or with a

combination of the 3. Each of the agents was mixed with an adjuvant and injected subcutaneously; one control group (first control) received only the adjuvant (Quil A adjuvant, 1 mL, supplied by Dr JP Dalton, University of Technology Sydney, Australia) and a second control group (double control) was not treated at all. At week 10 after the last immunization (2 occasional immunizations in 1 month) the animals received viable *F. hepatica* metacercariae. Blood samples had been taken from all animals at weeks 1, 4, 6, 8, 10, 12, 14, 16, 18, 20, and 22 and a part of them was stored for antibody analysis; once monthly the samples were used for total and differential cell counts, and simultaneously both liver enzymes lactate dehydrogenase (LDH) and gamma-glutamyl transferase (γGT) were measured. Fecal samples from each animal were taken at weeks 18, 20, and 21 for presence of *F. hepatica* eggs. At the 22nd week the animals were killed. The fecal testing and morphological examinations were the subject of another publication and will not be discussed here.

2.2. Animals

Male, 5 month old Bulgarian Marishka lambs (7 in each group) were used, sourced from fluke-free areas and

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were verified as being free of any infection by serology, fecal examination, and liver enzyme assays. The lambs were housed indoors under controlled conditions for the duration of the trial.

2.3. Antigens

CL1 and TPx were supplied by Dr JP Dalton (University of Technology Sydney, Australia) under sub-contract. FABP (Sm-14) was supplied by Dr M Tendler (Head of the Laboratory of Eco-epidemiology and Control of Schistosomiasis and Soil-transmitted Helminthiases, Oswaldo Cruz Institute, Rio de Janeiro, Brazil).

2.4. Immunizations

Each animal was immunized on 2 occasions (week 1 and week 4 after the start of the study) with 100 µg of each recombinant antigen in 1 mL of adjuvant Quil A. The first control group received the adjuvant alone. The second control group received only the antigens and was not challenged. The animals were inoculated subcutaneously in the neck region.

2.5. Challenge

Each animal from the experimental groups was challenged with 200 viable *F. hepatica* metacercariae within a gelatin bolus, which were administered by a dosing gun 10 weeks after the last immunization (i.e. 14 weeks after the start of the study).

2.6. Antibody estimations by enzyme-linked immunosorbent assay

The standard Liverpool School of Tropical Medicine (LSTM) enzyme-linked immunosorbent assay (ELISA) technique of Salimi-Bejestani et al. (1) was used. The blood samples were centrifuged at 3000 × *g*/min without an anticoagulant and the sera were used for the immunoglobulin G1 (IgG1) estimation. The FABP antigen lost its protein properties during storage and could not be used further in the ELISA estimations.

2.7. Sampling

Blood samples (in 2 options for plasma: with anticoagulant and for sera, or without anticoagulant) were taken from each animal every second week after the challenge and kept for antibody analysis, liver enzyme analysis, erythrocyte, and lymphocyte numbers in the blood (number per liter, manually counted in Burker chambers) were taken into consideration after the 8th week.

Blood smears were made for differential cell counts: eosinophils, neutrophils, basophils, and monocytes (as an absolute number).

2.8. Grouping

Group I: animals immunized with CL1
Group II: immunized with TPx
Group III: immunized with FABP
Group IV: immunized with all 3 antigens
Group V: immunized with the antigens and without the adjuvant

Group VI: immunized with the adjuvant alone
Group VII: not treated at all

Blood samples were taken from the animals before starting the trial for the purpose of keeping them as double negative controls for all other investigations (group VII).

2.9. Liver enzyme estimations

LDH and γGT were measured in the time periods mentioned above (Anthos Labtec Instruments, UK).

2.10. Statistical analyses

With the help of the computer statistical program "Origin," results from the ELISA were tested for standard deviations and standard errors and simultaneously used to calculate the end point titer using the formula $\{[x2 - x1] \times (1.5 - y1)] / [y2 - y1]\}$ where:

x1 = 1st dilution   y1 = 1st OD (optical density, 450 nm) (above 1.5)

x2 = 2nd dilution   y2 = 2nd OD (above 1.5)

The calculations with "Origin" were employed for the estimation of the real antibody formations.

2.11. Ethical aspects

The experimental procedures were conducted according to the Veterinary Medical Office in Bulgaria, which follows the National Institutes of Health guidelines of care and use of laboratory animals (Permission with registration No. BG 1113–007, valid to 2012). The experiments with animals cannot be replaced with experiments in vitro because the disease can develop only in living animals.

3. Results

3.1 Antibody responses

The antibody response (IgG) in the experimental (immunized and infected) and control animals (from all control groups) was followed once monthly, and the first measurement was made at the 1st month after the immunizations.

The CL1-IgG1 graph in CL1-treated sheep starts with comparatively high levels immediately after the challenge (Figure 1 a) and around the 8th week the antibodies reached maximum levels and remained there until the end of the observation. This already gave an indication that the disease had shown signs of development. We also tested the IgG-response in the lambs injected with the adjuvant and challenged (first controls). The IgG response in the adjuvant treated and challenged animals (Figure 2a) increased earlier than it did in the CL1-treated and challenged animals (although not high), but after the 6th week remained at almost the same value. This showed that the adjuvant itself gave a rather good expressed antibody answer and therefore it might be very appropriate in these experiments as an additive immune inducer. After the calculations by "Origin" (Figures 1c, 1d, 1g, 1h, 1k, 1i, 1o, and 1p) it turned out that the animals treated only
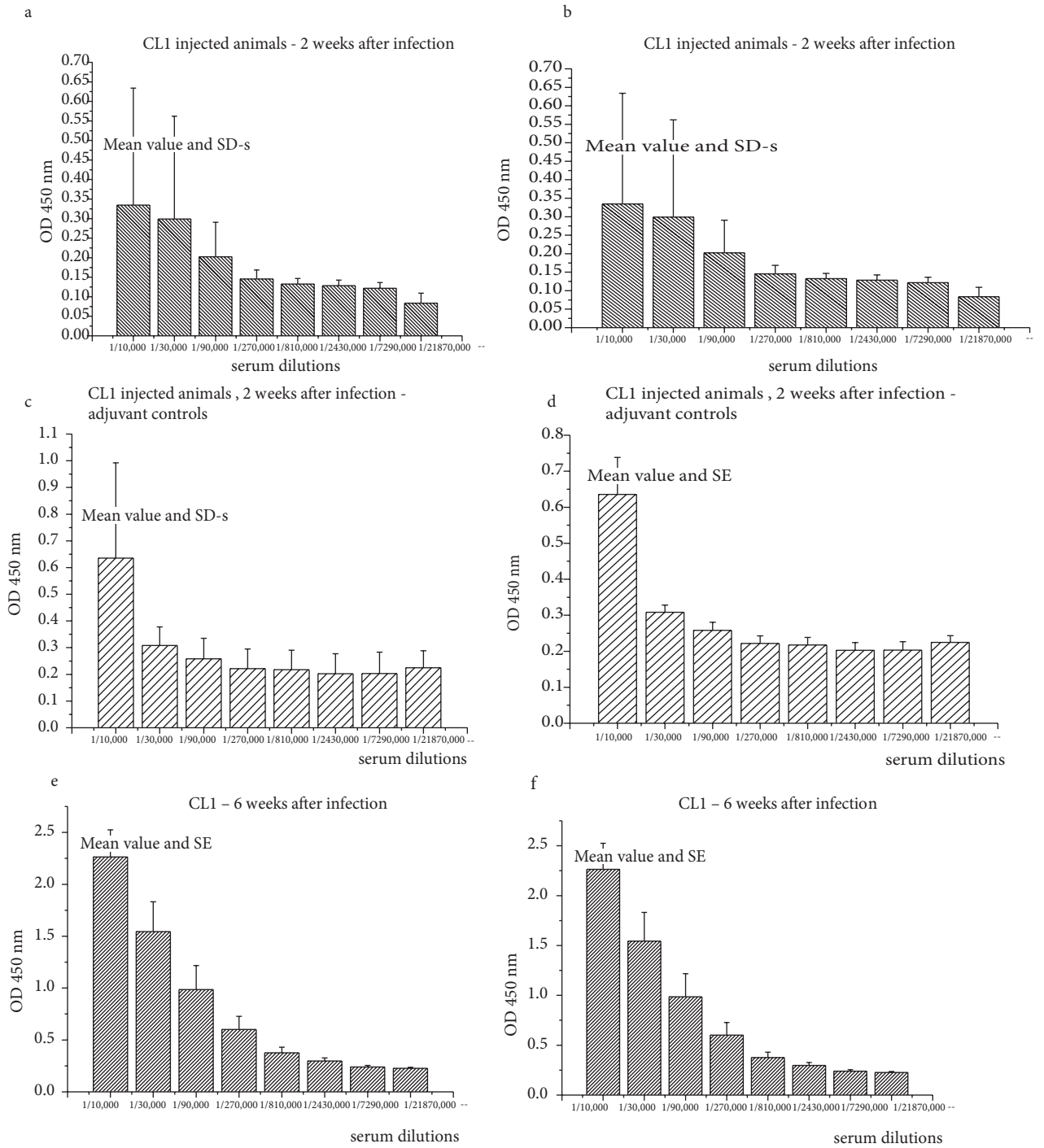
Figure 1 (a–p). Immune response to CL1-antigen (with or without adjuvant) – calculations ("Origin") of SD and SE during the different weeks postinfection.
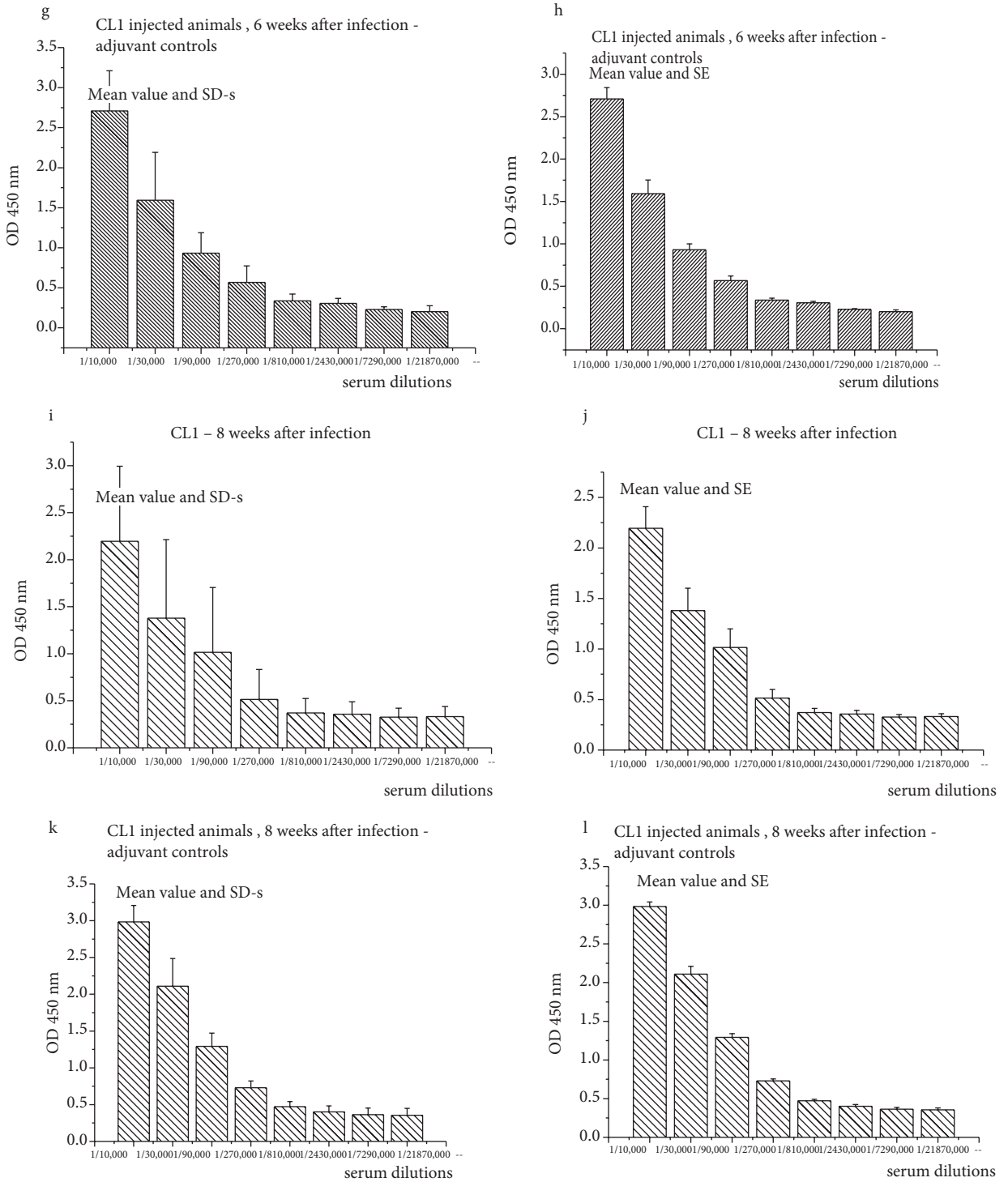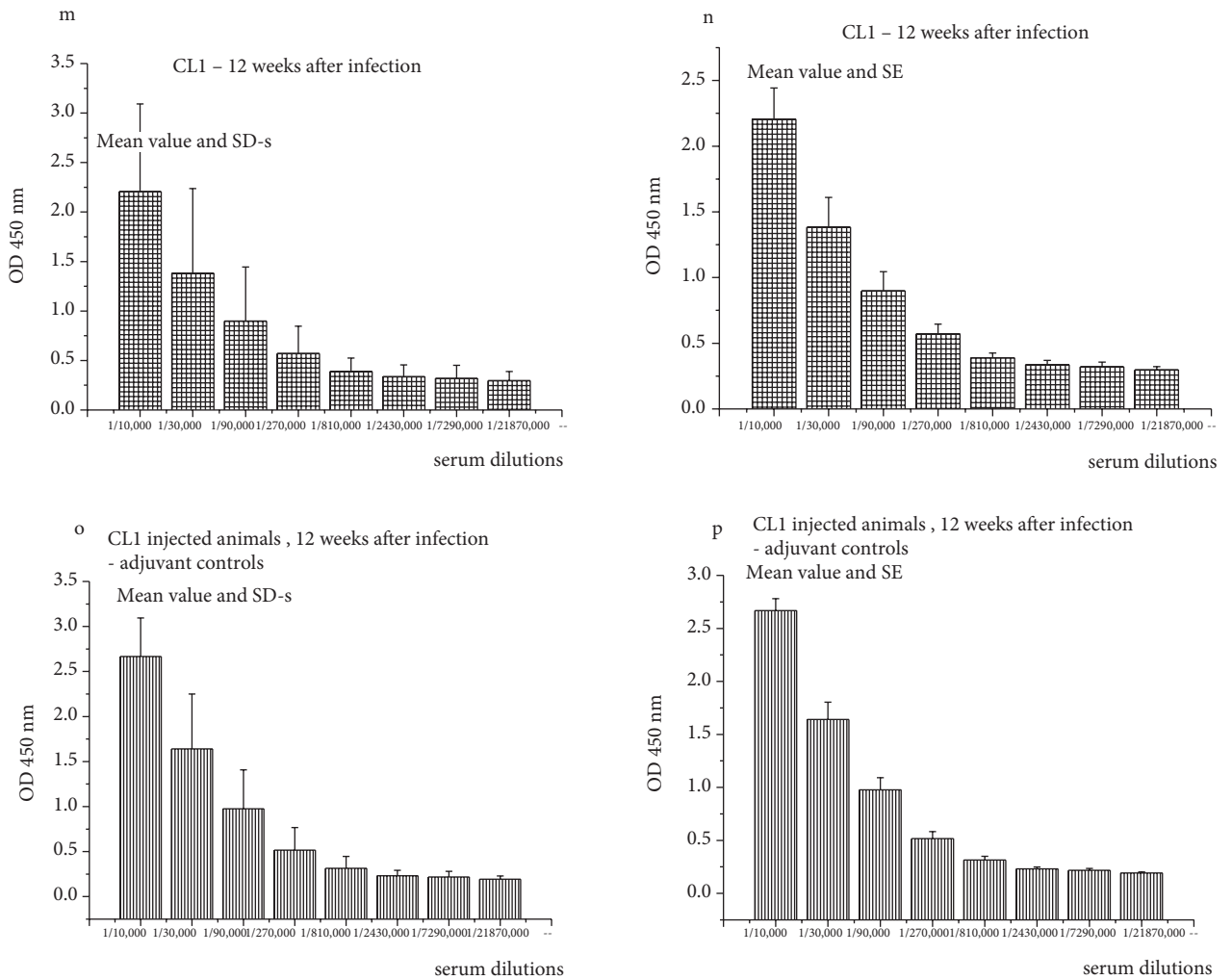
Figure 1. (continued).

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with the adjuvant showed a very high degree of anti-CL1-response. Two weeks after the infection this response was twice as high as the response to the CL1-injected lambs (OD around 0.6 to 0.3 respectively). During the 6th week (Figures 1e and 1f) the response fell to 0.25 in the adjuvant treated animals, but the values in the OD of the CL1-treated remained the same. Up to the 12th week (end of the trial) the responses in the 8th and 12th weeks remained equal in both cases. The response to TPx was different in the comparison to that of the adjuvant treated animals (Figure 2b), which was strong at the beginning and fluctuating after that with a maximum at the 8th week and decreasing with fluctuations again up to the end of the trial. The statistical analysis of the IgG response as standard deviation and standard errors are given for 2, 6, 8, and 12 weeks after injections and calculated by the given formula and by use of "Origin" (Figures 1a–1p). Two weeks after immunization the CL1-IgG1s had a higher SD. The

SE was within normal boundaries. The adjuvant controls gave higher values (almost twice as high), but the SD was significantly increased as well. It should be noted that all animal groups reacted to the mixture of the 3 antigens in different ways. After the 6th week the CL1-antibodies dropped down slowly, but did not disappear. The adjuvant controls remained with a higher response even after the last (12th) week of the trial (Figures 1o and 1p).

3.2. Liver enzymes
γ-GT was measured before any treatment (first measure, Figure 3, negative control) and each 2nd week after the challenge with the metacercariae. The γ-GT showed 2 slight peaks during the trial, but no special feature was observed. The change in the levels of this enzyme was not indicative of the development of parasitosis. LDH values are given in Figure 4 after 10 measurements for each enzyme. The LDH decreased slowly up to the end of the trial in all the animal groups treated with the different antigens.
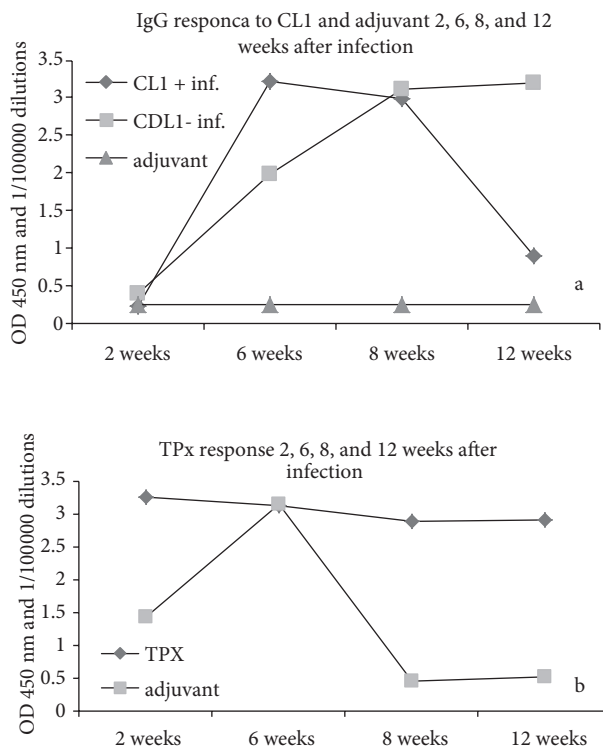
Figure 4. LDH values in the plasma of animals treated with different antigens.

Figure 2. IgG1 response in animals to different antigens during the 2nd, 4th, 6th, 8th, and 12th week after challenge.

3.3 Hematology
During the whole period of the trial the differential blood estimation showed that up to the 2nd month after giving the metacercariae (3rd month after the immunizations) the eosinophils were rising in 4 of the groups (Figure 5). Only in the group injected with CL1 did this rise reach very high values during the 5th month and then it dropped down sharply. The control group (group VI) was taken into account in the comparison.

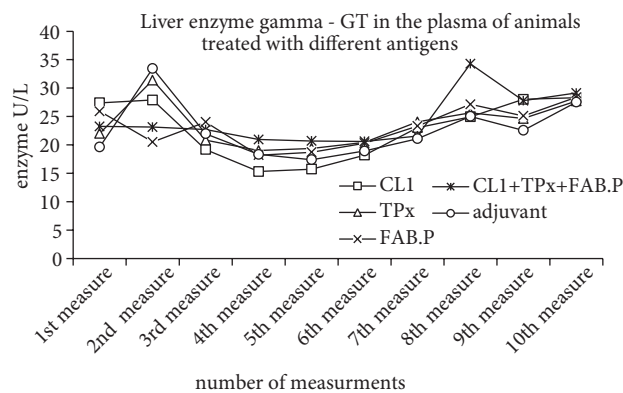The neutrophil number exhibited its own increase (as shown in Figure 6), but a special conformity for these

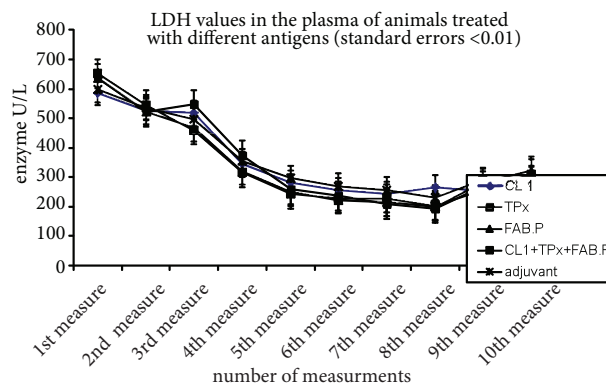**Figure 3.** Liver enzyme γ-GT in the plasma of animals treated with different antigens.

cells during the trial was not observed. Their numbers resembled those of the eosinophils, but with lower values and a special high value in the 5th month for the 1st group.

The number of basophils increased in the 3rd month after the immunizations in the 4th group (treated with the combination of the 3 antigens) (Figure 7). A remarkable rise in the group of adjuvant-treated animals is seen in the 4th month and the highest peak against CL1 is again observed in the 5th month.

The monocytes increased very slowly and a high peak only appeared in the CL1 group in the 5th month and at the end of the trial, when the number of these cells was higher in all groups, their number was quite different in the group of the 3 antigens (Figure 8).

The total leukocyte number (in 1 L) increased during the months 3, 4, and 5 up to 10/L after treating with the mixture of the 3 antigens, followed by the animals treated with FABB.

Only during the last 3 months of the experiment was a slight erythrocytosis recorded, but it can be regarded as negligible.

4. Discussion
These experiments were carried out in the framework of the DELIVERY project and the results should reflect the real situation by using these recombinant antigens. We are obliged to mention that this was not an easy task, because the parasite *F. hepatica* has several steps in its life and it is possible that the immunological answer is not adequate for all the examined phases in the parasite organism.

The peptide CL1 was isolated by Cornelissen (2) with the aim of detecting antibodies against *F. hepatica* and to use it in immunodiagnostics. It turned out that the occurrence of common antigens in many helminths remained the main source of the lack of specificity. The authors therefore synthesized species-specific epitopes within CL1 that were specific for *F. hepatica* and demonstrated greater specificity, especially during the first weeks of the fasciolosis.
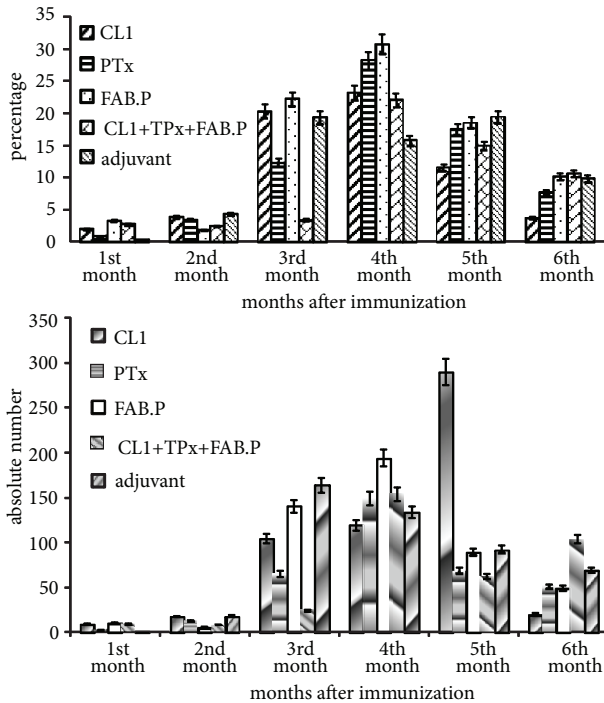
Figure 5. Absolute eosinophil number of animals treated with different antigens.

Figure 6. Absolute neutrophil number of animals treated with different antigens.

It has been proposed that the secreted cathepsin Ls may be involved in the suppression and/or modulation of Th1 immune responses and induction of nonprotective host Th2 responses. An analysis by Dalton et al. (3) of cytokine production by antigen-stimulated spleen cells of *F. hepatica* (adult worms CL1-protease) from infected mice showed that these are predominantly of the Th2 type, i.e. there is production of interleukin IL-4, IL-5, and IL-10, but little or no IFN-γ.

Mulcahy et al. (4) mentioned that the vaccines appeared to target the early migratory stages of the parasite and thus prevented liver damage to the host. In our case the results from the cell differentiation examinations, liver enzyme observations, etc. showed exactly this phenomenon, which will be discussed below. On the other hand, the lack of resistance in sheep has been contrasted to the high resistance in cattle to acute fasciolosis and has led to a general assumption that cattle, but not sheep, have the immunological capability of eliminating liver fluke infections (4).

Harmsen et al. (5) reported some studies on the proenzyme forms of CL and their properties. Vaccinations of rats with these forms showed 64%–76% reduction in fluke load. The authors drew the conclusion that their use might be helpful in a multisubunit vaccine.

In our experiments, more calculations and remodeling were done with the CL1 antigen. The response to CL1 increased quite quickly (the maximal OD is observed
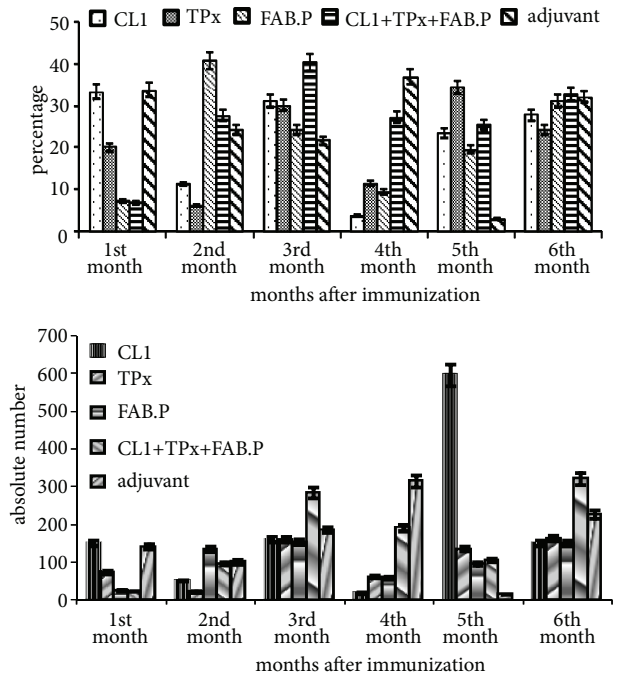
between the 6th and 8th week) and afterwards decreased somewhat and then remained at 1 level (Figure 10 after the "Origin" calculations). This is indicative that this antigen induced a prolonged and more stable IgG1 immune response although not high enough. These results coincide with those published by Dalton et al. (3), who reported this period after 4–5 weeks. Bossaert et al. (6) used a somatic *F. hepatica* antigen and an adult *F. hepatica* ES antigen and infected calves 4 months after immunization with them. They stated that the mean IgG1 titers were significantly higher in the single-dose infected group than in the trickle infected group during the early migratory phase of the infection. IgG2 values were lower than those of IgG1.

Phiri et al. (7) carried out experiments similar to our own with sheep treated with 2 kinds of *F. hepatica* ES and measured the levels of antibody responses after challenge with the parasite. They found IgG1 increases around the 2nd week after challenge, which is in full agreement with our own observations (Figures 1a–1p) after transformation of the expected values by "Origin". It is remarkable that the IgG1 levels in their experiments fluctuated strongly on a week to week basis as was the case in our studies too. The pronounced IgG-answer against CL1 in our experiments is indicative for further success of the present work; there is a possibility to achieve protection after changing some of the parameters in the trial.

The calculations gave us the opportunity to compare the IgG1 profiles at the same dilutions. This part of the investigation was not entirely satisfactory because the
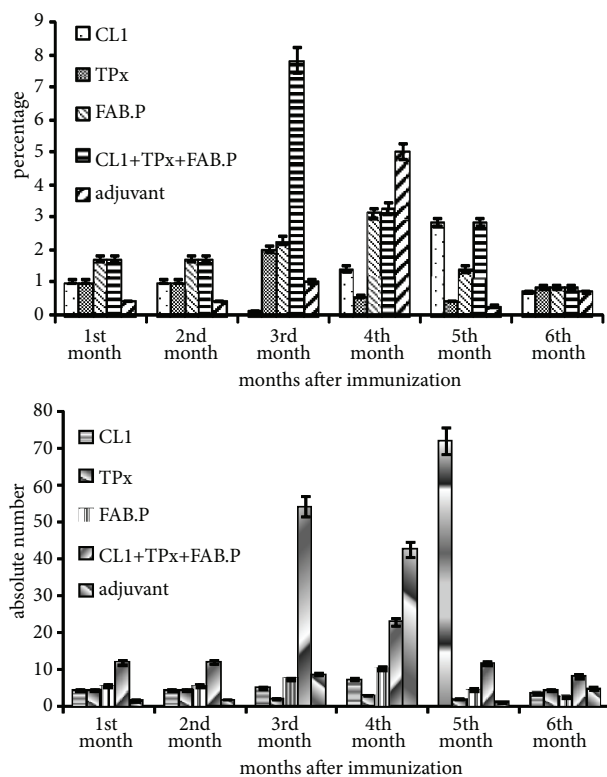
Figure 7. Absolute basophil number of animals treated with different antigens.

Figure 8. Absolute monocyte number of animals treated with different antigens.

immunological responses were not strong enough. The poor IgG1 response in all treated groups is well explained in the work by Wolstenholme et al. (8), who discussed in great detail the resistance of the organism to a variety of parasites, the parasite genetics, and biology and the use of antihelminthic compounds. The authors came to the conclusion that further detailed research is needed to discover the strategy for effective treatment. This was why the goal of our project was to investigate all circumstances necessary for achieving positive results against *F. hepatica* as well.

The IgG1 responses in the adjuvant Quil A-treated animals remained comparatively high. Figure 2a and Figures 1c, 1d, 1g, 1h, 1k, 1i, 1o, and 1p show that these responses are even higher at the start of the trial, and after falling in the 8th week they remained equal to the CL1- response at the end of the observation. This adjuvant, initially refined by Dalsgaard (9) and containing triterpene glycosides, was estimated by Kensil et al. (10) and has subsequently been subjected to intensive investigations. Its efficacy was followed in lambs with *F. hepatica* parasitosis by Haçarız et al. (11), who established a maximum response during the 8–10-week period after a sharp start. Our observations in this respect generally coincide with those of these authors, but the maximum value is somewhat earlier than 6–8 weeks.
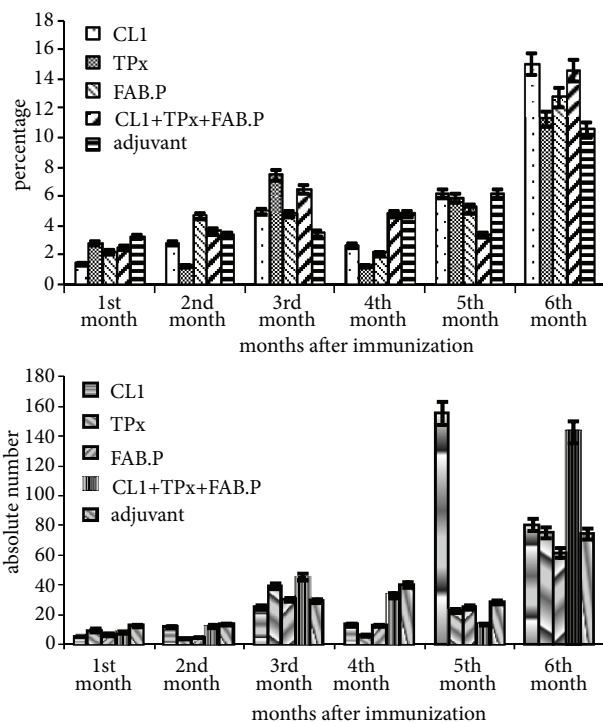
The fact that the antibody response to the adjuvant alone was stimulated to a great extent shows that the adjuvant was well chosen. Haçarız et al. (11) was investigating 3 adjuvants and came to the conclusion that the Quil A adjuvant could be useful in anti-*Fasciola hepatica* vaccines, because it induces a strong elevation of the immune response after such an infection. It can be seen in Figures 1c and 1d of our results that in the same week after challenge (2nd week) the response to CL1 in adjuvant-treated animals is even higher than that in the group without the adjuvant.

The additive effect after the simultaneous application of the 3 antigens (group IV) is not surprising, because each separate antigen gave its own positive response and this was completed by the adjuvant. Donnelly et al. (12) made a recombinant antioxidant thioredoxin peroxidase (TPx) (bearing in mind that it is present in the *F. hepatica* parasite excretory-secretory/ES/products) and showed that TPx not only increases the number of peritoneal macrophages, but directly converts them to RAW 264.7 phenotype, characterized by production of high levels of IL-10, prostaglandin E_2. In our experiment animals treated with TPx (group II) showed a better increase in the monocytes only during the 3rd and 6th months. In Figures 1a–1p and 2 one can see that in almost every case the application of the 3 antigens simultaneously leads to an increase in the immunocompetent cells (eosinophils, monocytes, etc).

The liver enzymes (LDH and γGT) had their own shape in these experiments. Their values in our trial are different; the γGT rose only between the 8th and 9th weeks and the LDH values fell continuously until the end of the trial (not characteristic for liver damage). During the same parasitosis Haçarız et al. (11) established an increase in this enzyme between the 13th and 23rd weeks, with a maximum in the 19th week, which is different from our data (Figure 4). The activity of γGT in the adjuvant-treated animals in our experiments did not change too much in comparison to the value of the controls (Figure 3, the first measurement with an exception in the 2nd measurement), but this evaluation cannot be explained by an adoptive or antiparasitic reaction. The values of γGT are not as high as in the experiment reported by Bossaert et al. (6). According to them, the immunization with 2 different *F. hepatica* ES-proteins 4 months prior to infection of calves with *F. hepatica* led to mean serum levels of LDH and γGT around 12–14 weeks after primary infection. Their activities were higher in the single-dose infected groups but only around the peak values. They did not find a correlation between the fluke number and antibody titers and suggested that IgG1 production has little protective effect against *F. hepatica* infection. It is possible that the antigen used, CL1, prevented the liver damage, but not the infection of sheep. Yang et al. (13) estimated the values of LDH and γGT after fluke infection and stated that plasma LDH was significantly elevated from 6 to 21 weeks postinfection. Significant increases in plasma γGT occurred from 8 to 26 weeks postinfection, reaching maximum values at the 15th week. They were working with other animals, and so their results are different from ours.

The LDH values in all experimental groups had the same shape and the highest values were observed during the first week of the parasitosis. The lambs injected with the adjuvant did not show different features, which was not the case in the experiments conducted by Haçarız et al. (11), who established a maximal increase in this enzyme during the 13th week.

The results concerning the differential blood count are in agreement with the work by Pulendran and Ahmed (14), who pointed out that the helminths induce differentiation of Th2 cells, whose cytokines (IL-4, IL-5, and IL-10) induce IgE and eosinophil mediated destruction of the pathogens. Serradell et al. (15) performed experiments showing eosinophil apoptosis during *F. hepatica* infection in rats and concluded that diminution in Eo survival in early infection could be a parasite strategy in order to prevent a host immune response. Unfortunately, they did not present any data about the changed eosinophil levels in the blood during different periods of the parasitosis. As seen from our Figure 5, the levels of the eosinophils increased in the experimental animals (independent from the injected antigen) between the 3rd and 4th months after infection, but a high peak only in the CL10 group appeared in the 5th month. Subsequently, a decrease was noticed—the question is why? The answer might be found in the work of Serradell et al. (15) and it would be worth following up. The eosinophil's number is unstable in our experiments although it increased after the 3rd month. It should be noted that the eosinophils in the adjuvant-treated examples remained at higher and almost equal values up to the 6th (last) month (Figure 5). Whether this happens because of some protective effect should be examined.

Zhang et al. (16) presented some data about the cellular response in sheep, only they infected the animals with 2 kinds of parasite, *Fasciola hepatica* and *Fasciola gigantica*, and ascribed the answer to parasitic excretory-secretory products (ESPs). Eosinophil numbers in their experiments increased more quickly and strongly in *F. gigantica*-infected sheep than in *F. hepatica*-infected ones. In both groups, peripheral blood mononuclear cell (PBMC) proliferation in response to the ESPs showed similar kinetics. The kinetics of the eosinophil count in their examinations was biphasic with peaks at the 4th week postinfection (WPI) and 9–10 WPI for the group with *F. hepatica* and the 4th WPI and 11–13 WPI for the group with *F. gigantica*. The second peak of the eosinophil count for the group with *F. hepatica* coincides completely with our investigations and the decrease in this count after this maximum peak is the same. These authors give data about the count of other cells; the PBMC received a proliferation of these cells during the first 8 weeks postinfection with *F. hepatica*. In our experiments the mononuclears increased slowly with an exception during the 5th month in CL1 and during the 6th month in Ag-combination–treated animals (Figure 8). Furthermore, we studied the number of the neutrophils (Figure 6) and basophils (Figure 7) during the parasitosis with the different antigens. The neutrophils did not show a specific response, but again increased in the 5th month among the CL1-group. In contrast, the basophils remarkably only increased in the 3rd month postinfection in antigen-mixture treated animals; during the 4th month an increase in the same cells was observed in the adjuvant group and during the 5th month in the CL1-group (Figure 7). For now we cannot give any reasonable explanation for this phenomenon.

The use of new *F. hepatica* antigens with the aim of reaching some protection from this parasitosis gave discrepant results. The immune response was mostly pronounced against CL1, with highest values after 5–6 months, but with the decrease thereafter we could not achieve full protection results. It turned out that the chosen adjuvant is very successful because the animals reacted to it with a comparatively high response. The

differential count of the blood cells did not show any special features; the eosinophils rose very significantly in each group after the initial 3–4 months and showed in this way that development of the parasitosis had already started. Nevertheless, the liver enzymes were not typical for this disease.

Our conclusion after finishing the trials of the project is that several aspects have to be changed to achieve

successful results. As we assumed, it might be useful to try performing the same experiments in all the directions, firstly in small animals, and to take all the above mentioned untried ideas into account. Secondly, it would be good to think about a 2-step vaccine (antigens), which might be different in each period of the examinations.

The problem is of great importance especially for farms and the expectations are even greater.

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