Original Article

CHANGES IN ELASTIN TURNOVER IN PATIENTS WITH DIFFERENT TYPES OF HYDATID DISEASE (ECHINOCOCCOSIS)

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Summary

Echinococcosis is a serious parasitic disease in animals and human beings. *Echinococcus granulosus* grows very slowly and can be localized in different organs, where it damages both specific structure and elastic fibres of the organ concerned. The aim of this study was to assess elastin turnover via changes in levels of specific antielastin antibodies (AEAbs) of different immunoglobulin classes (IgM, IgA, IgG) and IgG subclasses in sera of patients suffering from different types of hydatid disease, and to establish a possible relationship between these levels and the severity of the disease. Anti-human IgM, IgA, IgG peroxidase immunoconjugates and monoclonal IgG subclasses were used to carry out enzyme-linked immunosorbent assay (ELISA). We studied sera from 18 patients with hydatid disease (mean age 42.5 yrs; 16-72yrs; 14 females and 4 males) operated on at Surgery Clinic I, University Hospital, or seen at the parasitology consulting room of the hospital. The diagnoses were confirmed serologically and surgically in all 11 patients with liver echinococcosis. In those with lung and multiple/multiorgan hydatid disease, the diagnoses were additionally confirmed by chest X-ray, computed tomography (CT) and ultrasound imaging. Significant differences with the controls were found for antielastin IgA ($p<0.001$) and IgM ($p<0.01$), most expressed in patients with lung and multiple/multiorgan echinococcosis. Antielastin IgG_1, IgG_2 and IgG_3 levels in the patients investigated did not show significant differences from levels found in controls, except for isotype $AEIgG_4$. This isotype was prevalent in patients with lung and multiple/multiorgan echinococcosis ($p<0.01$). Our results indicate increased elastin turnover that points to a likely relationship between the levels of AEAbs IgA, IgM and IgG_4 and localization and dissemination of E. granulosus, which relationship might be of interest for clinical practice.

Key words: antibodies, IgG subclasses, hydatid disease, elastin

Introduction

Echinococcosis is a serious parasitic disease in animals and humans [1], caused by the larval form of *Echinococcus granulosus*. E. granulosus adult tapewarms are found in nature in the intestines of canines; the larval cyst stage is present in the viscera of herbivores including human beings. Adult tapeworms in the canine intestine produce infective eggs that pass in

feces. When these eggs are ingested by humans, a six-hooked larval stage called an oncosphere, hatches. The oncosphere penetrates the human intestinal wall and enters the circulation to be carried to various tissue sites, primarily the liver and lungs, and to the central nervous system, bones and other sites. It occurs in a number of regions all over the world [1, 2], especially in Mediterranean countries, including Bulgaria [3, 4, 5, 6, 7], where the incidence during the last ten years has increased from 2.36°/$_{0000}$ to 8.14°/$_{0000.}$ The incidence rate among children and adolescents has also increased - the cases registered in these groups account for 22.64% of all registered cases [4], and mortality has reached 96.25% of deaths related to parasitosis [4]. The parasite enlarges very slowly producing tumor-like space-occupying biomass none as hydatid cyst, which damages specific organ structure and elastic fibres. The host is exposed to long-term damage and immune stimulation, as well as the sheer physical consequences of being inhabited by a large foreign body. The most obvious forms of direct damage from the parasite are those resulting from mechanical blockage of internal organs or from the effects of pressure exerted by growing parasite.

The manifestations of the disease are due to the mechanical or chemical tissue damage produced by the parasite, and to the host responses to the presence of the parasite. During a parasitic infection, host cell products such as cytokines and lymphocytes are released from activated cells. Immunopathologic reactions range from anaphylactic reactions to cell-mediated hypersensitivity [8].

Humans can be infected through ingestion, contact implantation, intrabronchially and by aspiration [9]. These facts determine all aspects of interest to the disease.

Elastin is a basic extracellular protein that is responsible for elasticity of the skin, blood vessels and all connective tissue. Its biosynthesis and degradation persist throughout human life. In healthy subjects this turnover is slow, and it becomes faster in disease states. Products of elastin turnover reflect to a varying degree the severity of the disease [10]. Apart from other biological activities (chemotactic for fibroblasts and monocytes, modulation of membraneous transport, induced elastase release by monocytes), these products are immunogenic and stimulate the synthesis of antielastin antibodies [11]. The literature reviewed did not reveal data on studies on antielastin antibodies in echinococcosis. This directed our attention to investigating the way *E. granulosus* larvae can change the elastin turnover in infected persons.

The aim of the study was to evaluate the elastin turnover by registering changes in specific antielastin antibodies (AEA_b) of classes IgA, IgM, IgG and IgG subclasses in sera of patients with echinococcosis and clinically healthy subjects, and eventually establish a possible association with dissemination of the disease.

Materials and methods

Subjects investigated

Sera were obtained from 18 patients before surgical intervention (14 females and 4 males) aged 16 to 72 (mean age 42.5±13.3 years) with various localizations of hydatid cysts. The cysts were serologically, surgically, pathohistologically or morphologically confirmed in all the cases. The patients were operated on at the Surgery Clinic I, University Hospital of Pleven or had been referred to the parasitology consulting room at the same hospital. Most of the patients were residents of the Central-North region of Bulgaria. Multiple echinococcosis was found in only four of the patients (Tabl. 1). The sera were stored at a temperature of -20° C before testing.

Sera of 25 healthy controls were investigated,

Table 1. Clinical features of patients with echinococcosis

Localization of Cysts	Number of Cysts	Number of patients
Lung	single	3
Lung	multiple	1
Abdomen and peritoneum	multiple disseminated	1
Liver-lung-brain	multi-organ	1
Liver-lung	multi-organ	1
Liver	single	11

aged 17-65, matched by gender and age with the patients studied. No pathological findings were registered through routine clinical, laboratory and instrumental investigations of the controls.

Antigen

Insoluble elastin was obtained by the method of Starcher and Galione, 1976 [12], which was used to prepare α-elastin after Partridge et al. 1995 [13]. Aminoacid analysis was performed with automated amino acid analyser (Beckman 119 C 1.)

Procedure

Anti-elastin antibodies (IgG, IgM and IgA) and IgG subclasses were detected by enzyme-linked immunosorbent assay (ELISA). ELISA was performed according to the method optimized by Nicoloff et al. 2000 [13].

All reagents, 100 ìl each, were added into wells. The polysterene plates were coated with human aortic á-elastin at a concentration of 10ìg/ml, incubated for 18 hours at room temperature and then washed three times with a phosphate-buffered saline solution (PBS) + Tween 20. After that, sera from the patients and controls (diluted 1:10 in PBS + 0.05 % Tween 20), were added to the wells, incubated for 1 hour at 37ºC and washed fourfold with PBS-Tween. Dilution of human sera at 1:10 was used, following preliminary experiments with serial dilutions of patient sera and immunoglobulins to determine optimal conditions. In the tests for determination of antielastin IgA, IgM and IgG, antihuman immunoglobulin peroxidase conjugates (SIGMA, USA) to the heavy chain of IgG, IgM и IgA, diluted 1:10 000 with PBS containing 0.05% - Tween 20 were added. Incubation for 1 hour at 37ºC was followed by washing and incubation with the substrate solution (0-phenylene diamine 4mg/ml (SIGMA) in 0.05 M citrate buffer, pH 5.0 + 0.01% H_2O_2.

In the systems for IgG subclasses, a second antibody was added (mouse anti-human IgG_1, IgG_2, IgG_3, IgG_4, products of SIGMA), diluted at 1:1000 in 0.05% PBS-Tween 20, incubated for 1 hour at 37ºC, and then washed. Peroxidase conjugate was then added (goat anti-mouse IgG, SIGMA), diluted 1:10 000 in 0.05 % PBS + Tween 20), after which the plates were incubated again for 1 hour at 37ºC and washed with PBS.

Finally, O-phenylene-diamine was added to all systems. Incubation in a dark chamber was applied for 30 min at room temperature. The enzyme reaction was stopped by adding 50 ìl of 4N N_2SO_4 into each well. Optic density (OD) was measured at wavelength of 492 nm using Microelisa Reader 210 (Organon Teknika, Belgium). Final extinction value was determined as a mean of three measurements. Relative levels of IgG, IgA, IgM and IgG subclasses were registered as a relation of OD obtained with the respective class- and subclass-specific reagents to OD, obtained with anti-IgG (gamma-specific reagents). Patients were considered positive for AEAb if they had levels of AEAb higher than the mean OD+2SD in healthy individuals, used to establish the confidence interval of the test.

Statistical Analyses

Statistical analyses were performed using EXCEL and STATGRAPHICS* Plus for WINDOWS. The Student's test and ANOVA were used to assess the differences between the groups, and $p < 0.05$ was determined as a statistically significant difference.

Results

The mean extinction values +/-2SD for the levels of basic immunoglobulin classes, obtained by testing of the sera examined are shown in Figure 1. The presence of antielastin antibodies (AEAb) of IgA, IgM, IgG classes (Fig.1), and IgG subclasses were found in the sera of all the subjects studied (Fig.3). The upper extinction borderline for healthy subjects was as follows: 0.325±0.129 for IgA, 0.960±0.259 for IgM and 0.380±0.156 for IgG. All patients with values greater than the highest extinction value of healthy controls (+2SD) were considered positive. A statistically significant difference for AEAb IgA and AEAb IgM (respectively $p<0.001$ and $p<0.05$) was found when the patients were compared to the controls. The levels of AEAb IgG of patients were higher than those of the controls but the difference was not significant. When the patients were classified (Fig.2) according to the number and location of hydatid cysts, a certain tendency were observed. The levels of AEAb IgA were significantly higher ($p<0.001$) in the groups of patients with single lung cysts and multiple/multiorgan echinococcosis (0.721±0.160 and 0.704±0.195, respectively), as compared to the controls

0.333±0.129). For AEAb IgM, however, significant differences ($p<0.01$) were registered in the group with multiple/multi-organ (2.067±0.404; $p<0.01$) and in the group with liver echinococcosis (1.491±0.197; $p<0.05$), as compared to the controls (0.996±0.359). A statistically reliable difference in AEAb IgG levels was found only in the group with lung echinococcosis (0.939±0.391; $p<0.001$).

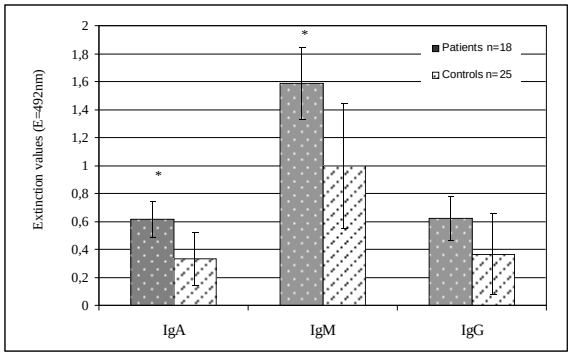

Fig.1. Specific antielastin antibodies (EAb) in sera of Echinococcosis patients obtained by ELISA (* p<00.5 vs. control group)

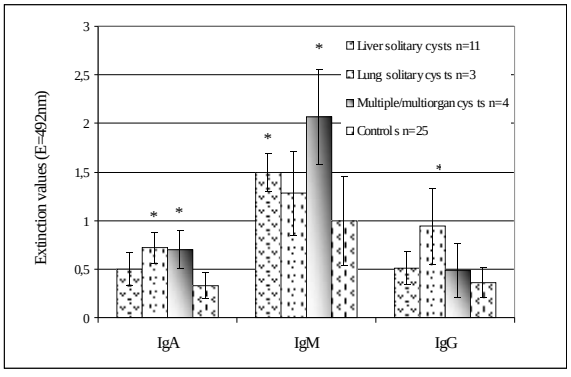

Fig.2 Specific antielastin antibodies (EAb) in sera of Echinococcosis patients with various localization of hydatid cysts obtained by ELISA (* p<00.5 vs. control group)

The levels of specific antielastin IgG subclasses are demonstrated on Fig. 3 and Fig. 4. Though no significant differences were found between the patients and controls (Fig. 3), the levels of specific AEAb of isotype IgG_3 and IgG_4 showed a tendency to increase: upper extinction borderline for AEAb IgG_3 was 0.155+0.063, and for AEAb IgG_4 it was 0.169+0.059. As mentioned above, analyses by groups (Fig.4) regarding basic immunoglobulin classes showed that the differences were significant for AEAb IgG_4 in the groups with lung and multiple/multiorgan echinococcosis (0.224±0.067 and 0.229±0.035, respectively; $p<0.01$). Significant differences for AEAb IgG_3 were found only in patients with a solitary liver cyst (0.209±0.072; $p<0.05$). The rest of the patients presented levels similar to normal ranges detected in the controls (0.155±0.063).

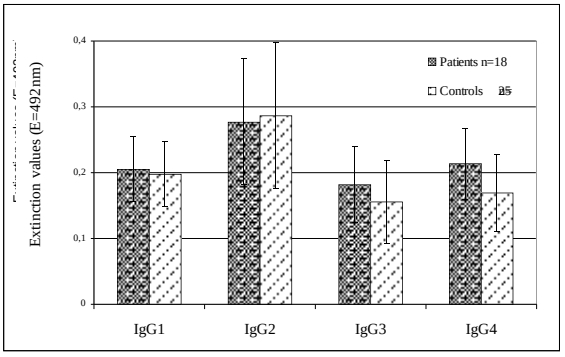

Fig.3. Specific antielastin IgG subclasses in sera of Echinococcus patients obtained by ELISA

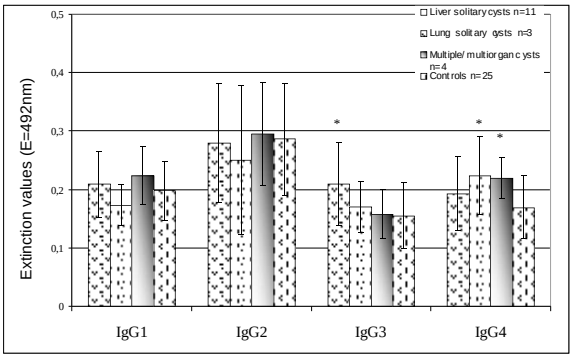

Fig.4. Specific antielastin IgG subclasses in sera of Echinococcosis patients with various localization of hydatid cysts obtained by ELISA (* p<00.5 vs. control group)

Discussion

In patients with echinococcosis, mortality and lethality were most commonly associated with late complications such as relapses, dissemination of cysts, rupture and abscess. In a few cases, sclerosis of a solitary cyst occurred, which occurrence was followed by a spontaneous recovery.

In all cases, however, damage of elastin structures was detected, irrespective of location

and number of hydatid cysts. Stein et al. 1965 [15] were the first to report antigenicity of elastin and circulating antielastin antibodies (AEAb). This was later confirmed by a number of further studies [11, 16, 17, 18]. The presence of AEAb in sera of healthy subjects suggests that these antibodies are not auto-aggressive. Elastin in elastin fibres is covered with fibrillin microfibrils [19], which are probably capable to protect it against the action of antielastin antibodies, thus allowing for the preservation of the nature of its turnover in health.

Antielastin antibodies of various immunoglobulin classes have also been detected in sera of patients with other conditions [20, 21, 22, 23, 24, 25]. However, results reported by authors are controversial. Bako et al., 1987 [26], using DOT technique and ÷- elastin from bovine ligamentum nuchae as antigen, detected antielasin antibodies in 90% of the patients with obliterating endarteriitis, in 67% of patients with ischaemic heart disease and in 60% of hypertensive patients. In young and middle-aged subjects, they did not detect antielastin antibodies. Using the same technique and antigen, Gminski et al., 1992 [27], studied the levels of AEAb in sera of patients with various forms of lung cancer and sera from healthy controls. In all patients, the authors found high levels of AEAb of IgG and IgM classes, but the ratio between the two classes in the respective patient groups was different. They detected AEAb in only 5% of the healthy controls.

This study is the first in Bulgaria to report data from investigations of serum specific AEAb of IgM, IgA, IgG classes, and IgG subclasses in patients with echinococcosis. The results we obtained regarding levels of AEAb IgM and IgG in sera of patients with multiple/multiorgan echinococcosis agree to a great extent with the results reported by Gminski et al. 1992 [27]. Presumably, on the site where the process of growing occurs, elastases or elastase-like enzymes are released by cells such as macrophages and polynuclear granulocytes, attempting to limit the growth of the tumor or cyst [28]. Usually, the proteolytic activity of elastase-like enzymes is controlled by physiological inhibitors, mostly á-1-proteinase inhibitor, which is present in body fluids. In some diseases (echinococcosis, carcinoma), these inhibitors are probably unable to provide full protection, and more intensive degradation occurs in elastin structures on the site affected. Nicoloff et Baydanoff, 1997 [29] have reported significantly higher levels of elastin peptides in sera of patients with Diabetes Type I without vascular complications and abnormal findings on physical examination decreased pressure and impaired blood supply established through Doppler ultrasound. Probably, higher levels of elastin peptides in the circulation act as a pathological stimulus for increased synthesis of antielastin antibodies.

Although Siracusano et al., 2008 [30] recently reported that the major *E. granulosus* antigen B, isolated from hydatid fluid inhibits elastase activity and neutrophil chemotaxis, higher levels of serum AEAb IgM in patients with multiple/multiorgan and liver echinococcosis could be the first signs of the onset of pathologically activated elastin degradation and progress of the disease. On the other hand, a significant increase of AEAb IgA in patients with a single lung cyst and such with multiple/multiorgan echinococcosis is probably "overlooked" by phagocytes that mostly possess receptors for Fc-fragments of IgG and IgM. In this way, the elimination from circulation of IgG, bound to the specific antigen would be faster as compared to that of IgA [31] during the phase of enlarged echinococcus cyst or a larger number of cysts. Since the cells, responsible for the cell-mediated immune response (phagocytes, T-killers, NK-cells, etc.) also possess more receptors for Fc-fragments of IgG and IgM, they would predominantly interact with immobilized and soluble immune complexes, formed with AEAb IgG and IgM.

The detection of specific AEAb of all IgG subclasses suggests that they participate in the turnover of elastin in both health and disease [27, 32, 33]. The prevalence of some specific IgG subclasses ($AEIgG_4$) in the sera of 11 of the patients investigated is in favor of the antigen that could unlock immune response. It seems that some of the circulating elastin peptides express epitopies, which specifically stimulate synthesis of AEAb IgG_4. This assumption is in agreement with previous studies of ours on patients with sclerodermia, in which we registered a significant increase of AEAb to human á-elastin of both IgG and IgE classes [32], and that IgG_4 possesses biological activities similar to those of IgE.

Conclusions

In conclusion, our results demonstrate an important association between serum levels of AEAb IgA, IgM and IgG_4 and the development of lung and multiple/multiorgan echinococcosis. To asses if the AEAbs are really the result of the echinococcus infection or due to another reason, a long-term investigation of their serum levels in patients who have had the cyst removed might be necessary. On the other hand, though the role of antielastin antibodies in metabolism of elastin in echinococcosis needs to be studied further and in a larger number of patients with different forms of the disease are necessary, investigations on these antibodies could contribute with indirect information about the increased turnover of elastin, which could reveal the progress and dissemination of echinococcosis.

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