

# Evidences of auto-immunity during avian borreliosis experimentally induced with *Borrelia anserina*

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## SUMMARY

This study on experimentally infected broiler chickens for possibly developing autoimmune diseases and syndromes was motivated by the publications on disseminated intra-vascular blood coagulation, renal immunopathology and immunopathies caused by *Spirochaetaceae* in man.

One-month old broiler chickens (n=130): control (n=10) and intramuscularly infected with avian *Borrelia* BrDSZ strain, Pamukchii serotype (n=120), were used. Ten birds each time were studied 48, 72, 120 hours, 15 days and 1, 2, 3, 4, 5, 6 and 12 months post infection by detecting for lupus erythematosus cells (LE) in blood and bone marrow samples, by the indirect immunofluorescence assay (IIFA) and the direct immunofluorescence assay (DIFA), by histopathologic and electron-microscopic methods.

Nucleolar types of antibodies, including, antinuclear, antimitochondrial, anti-NuMA-2, anti-RNA polymerase I and anti-PM-Scl, were evidenced by IIFA. Moreover, the results from all the methods applied for detecting avian borreliosis were morphologically similar to those observed in human systemic lupus erythematosus (SLE), periarteritis nodosa, dermatomyositis and systemic sclerosis.

Two main phases of the disease were distinguished: an acute with crisis *exitus letalis* and a chronic with autoimmunopathies concluding lethally in result of severe and irreversible visceral deficiency.

**Keywords:** *Borrelia anserina*, broiler chickens, autoimmunity, antinuclear antibodies, immune complexes, systemic lupus erythematosus.

## RÉSUMÉ

### Mise en évidence de mécanismes auto-immuns lors de borréliose aviaire

Cette étude a été motivée par les publications de coagulation intravasculaire disséminée, d'immunopathologie zénales causées par les *Spirochaetaceae* chez l'homme.

120 poulets de chair ont été infectés par voie intramusculaire avec *Borrelia* BrDSZ de sérotype Pamukchii et 10 témoins. 10 oiseaux ont été étudiés 48, 72 et 120 heures, 15 jours, 1, 2, 3, 4, 5, 6 et 12 mois après l'infection par recherche des cellules lufiques (LE) dans le sang et dans la moelle osseuse, par immunofluorescence indirecte (IIFA) et directe (DIFA) et par des méthodes d'histopathologie en microscopie électronique.

Des Ac anti-nucléaires, anti-mitochondrienne, anti-NuMA-2, anti-RNA-polymérase I et anti-PM-Scl ont été mis en évidence.

Les lésions sont semblables à celles mises en évidence chez l'homme atteint de lupus érythémateux.

Deux phases principales de la maladie ont été distinguées : une phase aigüe et une phase chronique.

**Mots clés :** *Borrelia anserina*, poulet de chair, auto-immunité, anticorps anti-nucléaires, complexes immuns, lupus érythémateux systémique.

## Introduction

*Borrelia anserina* [14] causes an endemic disease in birds in several countries from Europe, Asia, Australia, North (USA), Central and South America [3] and it particularly affects the extensively produced chicken flocks in Bulgaria. Production of vaccines and immuno-prophylaxis against avian borreliosis started soon after different serotypes of *B. anserina* were distinguished [4]. The intracellular phase of the causal organism was detected and the concept of its extra-cellular parasitism had to be revised [5]; several years later disseminated intravascular blood coagulation and histopathological evidence of nephro-immunopathology have been found out in hens [10, 11]. It has been determined that some members of the *Spirochaetaceae* family trigger off humoral or cell mediated immune responses against animal and human tissues [2, 6, 8, 9, 12 - 16]. Consequently the aim of the present study was to investigate the occurrence of

immune and auto-immune responses comparative to the responses observed in Lyme borreliosis in man [16] during the development of experimental borreliosis in one-month old broiler chickens.

## Materials and Methods

### ANIMALS AND EXPERIMENTAL PROTOCOL

A total of 120 one month old broiler chickens from the Plymothrock x Cornish cross supplied by the Agrarian institute of Stara Zagora were randomly divided into 2 groups: in the experimental group (n = 110), each bird was infected into the pectoral muscles with  $7.10^6$  *Borrelia anserina* (BrDSZ strain, Pamukchii serotype) on day 0 and examined 48, 72, 96 and 120 hours, 15 days, 1 to 6 months and 12 months post infection whereas the 10 remaining birds served as healthy (not infected) controls and were examined on day 0. Blood

was obtained from v.subcutanea ulnaris from the controls on day 0 and from 10 infected birds at each time point. Quantities of 5 cc of blood were poured in 10-cc sterile tubes and left at 20 C. Serum was obtained on the 24<sup>th</sup> hour after clotting (2<sup>nd</sup> h) and refraction (3<sup>rd</sup> h). At each time point after collecting the blood samples, the birds were exsanguinated under ether narcosis and 2 – 3 hours post mortem tissue samples were obtained from the skin, heart, lungs, oesophagus, glandular stomach, liver, spleen, pancreas and brain. From the 6<sup>th</sup> month on all the samples necessary for the histopathological and electron microscopic studies were obtained from birds which had died from the infection.

## METHODS

The presence of ANA (Antinuclear Antibodies) (Figure 7) was researched in sera separated 24 hours after collecting the blood samples diluted at 1:40, 1:80 and 1:160 from infected (n = 10) and healthy (n = 10) chickens by an indirect immunofluorescence assay (IIFA): briefly, immune complexes formed between serum ANA and Hep-2 cells (The Binding site Ltd., UK) were revealed with Fluorescein isothiocyanate (FITC) conjugated monospecific rabbit anti-chicken IgM and IgG fractions antibodies (National Centre of infectious and parasitic diseases, Sofia, Bulgaria) and slides were examined with a fluorescent microscope ML -2BU42 (LOMO - USSR) with HBO 200F mercury lamp (NARVA) [1]. Presence of anti- PM-Scl and anti-RNA polymerase I was evidenced by specific fluorescence of nucleoli while specific fluorescence of NuMA-2 (nuclear mitotic apparatus protein) showed presence of autoanti-NuMA-2.

The presence of ANA deposited within tissues was investigated with a direct immunofluorescence assay (DIFA). For that, 10 µm-thick cryosections were incubated at 20 C for 30 min with FITC conjugated rabbit anti-chicken IgM and IgG antibodies.

Lupus erythematosus cells (LE cells) were investigated in serum containing leucocytes obtained from blood without anticoagulant stored at room temperature for 80 minutes. Identification of LE cells was carried out on swabs, fixed with methanol and stained for 20 minutes by the Giemsa staining method. They contained inclusion bodies with cyto-chemical characteristics of depolymerised DNA. The inclusion bodies were phagocytised by neutrophil cells, whose cytoplasm was not evident, and surrounded by peripheral nuclei [7].

DIF was carried out on 10-µm cryo sections prepared from tissue pieces frozen in liquid nitrogen, stored at -21 C for 24 h, then incubated into a cryostat at -18 C for 2 - 3 h.

Histopathological studies were carried out on 4 - 5 µm-thick paraffin-embedded sections obtained from tissue pieces (6 x 6 mm) fixed in 10% neutral formalin for 24 - 48 h and treated by the routine histological methods for paraffin embedding. The sections were stained with haematoxylin-eosin, Mallory's phosphotungstic acid-haematoxylin (PTAH) [17] and periodic acid Schiff (PAS) [18].

For electron-microscopy studies, small pieces (2 x 2 mm) from bone marrow and kidneys were fixed in 4% glutaralde-

hyde, rinsed twice in PBS for 5 min, fixed again for 2 h in 1% solution of osmium tetroxide by Palade. After dehydration in alcohols (50 – 100%) and processing in propylene oxide and propylene oxide-Durcupan the samples were embedded in Durcupan epoxy resins (Fluka Chemie, Switzerland). Yellow-gold ultra-thin sections 70 – 100 nm thick were obtained on Reichert ultra-microtome. The sections, mounted on 400 mesh copper grids stained with uranyl acetate followed by lead citrate, were studied on JEM 7A electron-microscope and later on JEM 1200 EX (Institute of Experimental Pathology and Parasitology of BAS).

## Results

### HISTOLOGICAL AND CYTOLOGICAL FINDINGS

The histopathological examination usually evidenced perivascular oedema, granular disruption of the sarcoplasm, Zenker dystrophy and necrosis and mononuclear cell proliferation in the femoral muscles. Fibrin thromboses, focal segmental glomerulosclerosis and double contoured basal membranes of the “loop” or “rail” type were often observed in blood vessels from oesophagus, glandular stomach, lungs, liver, hart, spleen, pancreas and brain after 72 hours post-infection (Figure 1). Degeneration accompanied by homogenization of the media, adventitia and hyperelastosis of *tunica elastica externa*, activated and vacuolated vascular endothelium and then vacuolization of media and destruction of adventitia were also found out. In parallel, plasmatic Mott's cells (ripe plasma cells in active synthesis of antibodies accumulating in the ergastoplasmic sacs and condensed in the form of Russell's bodies known as morulla cells) in peripheral blood and Russell's bodies (big to small confluent hyaline globules) freely lying by the Mott's cells in the duodenum were observed 120 hours post-infection (Figure 2). Phagolysosomes containing destructed *Borrelia* were usually found out in the macrophages from the 6<sup>th</sup> hour to the 120<sup>th</sup> hour post-infection (Figure 3) whereas in the thrombocyte cytoplasm (Figure 4) and sometimes in fibroblasts (Figure 5) *Borrelia*, obviously not morphologically damaged, were lying freely.

From 3 to 12 months post-infection, lympho-histocyte proliferation and growth of fibrous connective tissue in the lungs, kidneys, glandular stomach and duodenum, causing severe fibrosis and irrevocable organ deficiency, were the usual findings.

### AUTO-IMMUNE REACTIONS

LE cells (figure 6) were evidenced in the group of experimentally *Borrelia*-infected birds mainly between 96 hours and 15 days after the inoculation with prevalence ranged from 30% to 50% whereas they were not detected in controls. Anti-nucleolus, anti-mitochondrial, anti-NuMA-2, Anti-RNA polymerase I and anti-PM-Scl anti-nuclear antibodies were detected by IIFA on Hep-2 cells from 1:160 diluted blood sera (figure 7) between 72 to 120 hours post-infection with relative high prevalence (50 – 75%) at the 120<sup>th</sup> hour.

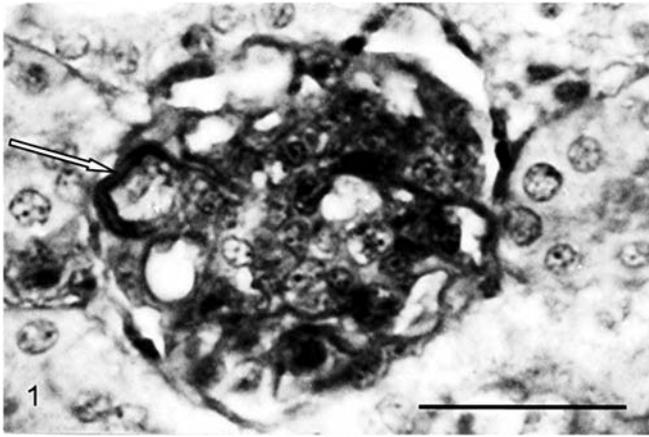


FIGURE 1 : "Rail"-type double contours (arrow) in the basal membranes of a kidney. PAS staining. Bar = 40 µm.

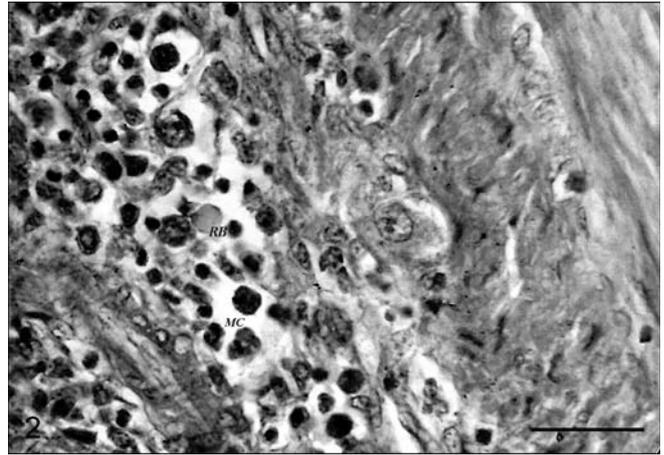


FIGURE 2 : Mott's cells (MC) and Russell's bodies (RB) in a duodenum 120 hours post-infection. Haematoxylin-eosin staining. Bar = 40 µm.

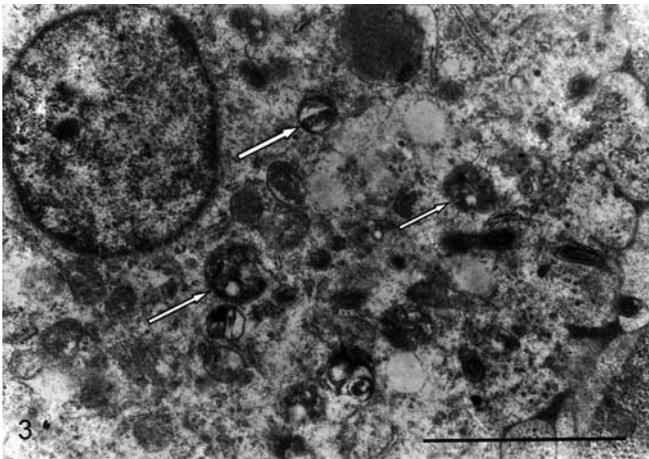


FIGURE 3 : Phagolysosomes (arrow) of a macrophage containing destroyed *Borrelia* 12 hours post-infection. Bar = 1.09 µm.

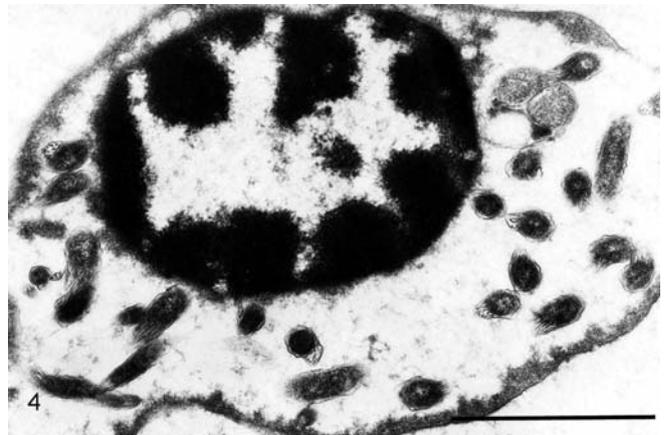


FIGURE 4 : Free *Borrelia* into the cytoplasm of a thrombocyte. Bar = 0.8 µm.

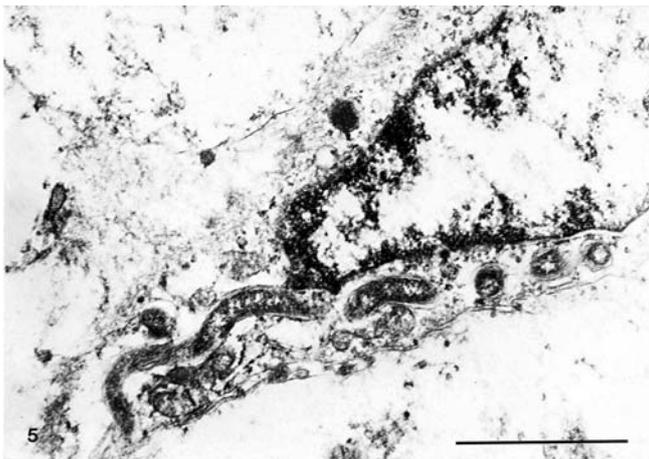


FIGURE 5 : Morphologically not destroyed *Borrelia* free into the cytoplasm of a fibroblast. Bar = 1.04 µm.

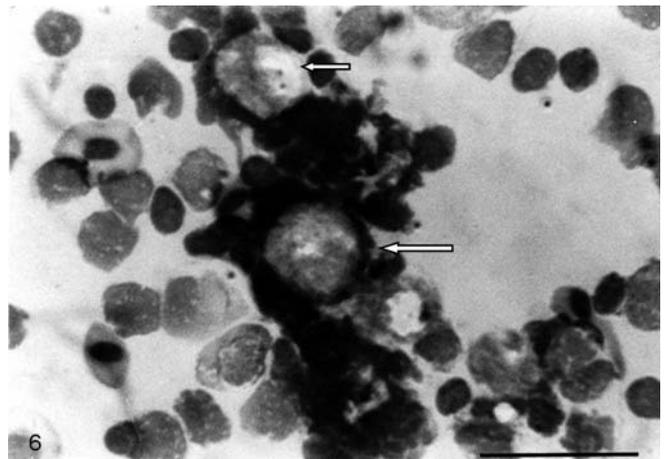


FIGURE 6 : LE cells (arrow) in peripheral blood 120 hours post-infection. Bar = 40 µm.

Between the 120<sup>th</sup> – 168<sup>th</sup> hours granular fluorescence with IgM was observed in the corneal epidermis layer of the comb skin in some of the chickens. Specific for IgM yellow-green fluorescence was observed in the perimysium, the endomygium, in some skeletal muscle bundles, in myocardium and in renal glomerules (granular and sub-endothelial) (Figure 8).

## Discussion

Fibrin thromboses, focal segmental glomerulosclerosis, basal membranes of the "loop" and the double contoured "rail" types observed in this study are similar to the changes seen in people with SLE and these lesions are compatible

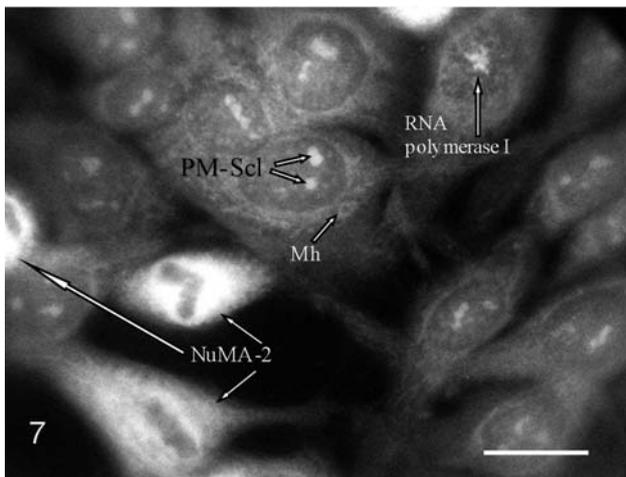


FIGURE 7 : Indirect immunofluorescence assay with Hep-2 and blood serum (dilution 1:160) 120 hours post-infection. Anti-mitochondrial (Mh), anti-NuMA-2, anti-RNA polymerase I, and PM-Scl antibodies Bar = 40 µm.

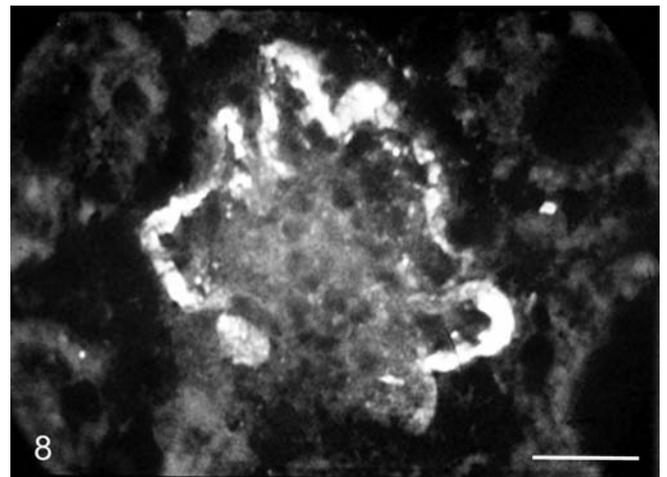


FIGURE 8 : Fluorescent granules and nodules consisting of immune complexes implicating ANA and IgM revealed by the DIFA test on the glomerular basal membrane in kidney 120 hours post-infection. Bar = 40 µm.

with the WHO Mesangial Abnormalities of class II and Focal and Segmental Glomerulonephritis of class III [19]. Fibrinoid degeneration of the walls of the larger blood vessels, occurrence of Mott's cells and Russell's bodies, lympho-histocyte cell proliferation and accumulation of fibrous connective tissue in small intestine and kidneys can be considered similar to those observed in human dermatomyositis. Consequently, these histological and cytological lesions evoked the involvement of the immune system and its activation towards endogenous nuclear antigens. Moreover, the auto-immunity was confirmed by the evidence of LE cells, serum ANAs and deposits of immune complexes in and around basal membranes of kidneys (glomerules), muscles, myocardium and in epidermis. Virtually all cases of systemic lupus erythematosus (SLE) and scleroderma are considered ANA-positive with IIFA titre of 1:40 or more [12, 20]. In the present study, the obtained ANA titre value of 1:160 can be considered as highly positive as in human medicine [1]. The different types of ANAs (anti-nucleolus, anti-mitochondria, anti-NuMA2, anti-RNA polymerase I and anti PM-Scl) observed in *Borrelia* infected chickens are currently identified in autoimmune diseases like SLE, dermatomyositis or scleroderma in man [11, 16, 20]. In the same way, the detection of LE cells confirmed the development of auto-immune reactions against cellular nucleus (figure 6). The granular fluorescent pattern and the localization of the immune complexes along basal membranes, particularly in glomerules, strongly suggest accumulation in such structures of circulating pre-formed immune complexes as observed during SLE [11]. In the present study, such direct and indirect criteria of auto-immunity were detected within the first 120 hours after *Borrelia* infection, suggesting that immune reactions surprisingly rapidly occurred. As *Borrelia* like *B. anserina* and *B. burgdorferi* were engulfed by some cells (macrophages, fibroblasts, thrombocytes and others) [16] very quickly (within the first 120 hours post-infection) and could survive freely in their cytoplasm, it is highly probable that the presence of these micro-organisms into the host cells rapidly initiate immune reactions towards *Borrelia* and also towards endogenous modified cellular

structures. Contrary to the specific Lyme borreliosis in which auto-immune mechanisms were recently explored [19], the time course of immune lesions during *Borrelia* avian infections, i.e. initiation of immune and auto-immune reactions, installation and amplification of tissue injury and transition to a chronic status, has not been investigated for more than 100 years. In this old perspective, it was admitted that survivors to the acute phase of infection were healthy until the present study. The infected broiler chickens still exhibited severe fibrosis in lungs, kidneys and in digestive tract causing organ failure 6 months and even one year after *Borrelia* infection. It can be suggested that the acute phase of *Borrelia* infection is followed by a short or long-lasting interval before a lethal issue, corresponding to the intracellular phase of *B. anserina*, when coagulopathies and autoimmunity are developing. Thus, two alternative courses of avian borreliosis can be distinguished: an acute phase often lethal and a chronic issue dominated by autoimmunity and progressive development of severe and irreversible visceral deficiency fatally leading to death.

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