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

N. NIKOLOV

Regional diagnostic veterinary institute, Slavianski 58, 6000 Stara Zagora, BULGARIA.

*Corresponding author: nikolov_sz@yahoo.com, rdvi_sz@abv.bg

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, 120 hours, 15 days and 1, 2, 3, 4, 5, 6 and 12 months post infection whereas the 10 remaining birds served as healthy (not infected) controls and were examined on day 0.
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 24th hour after
clotting (2h) and refraction (3h). 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, gland-
dular stomach, liver, spleen, pancreas and brain. From the 6th
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 immu-
nofluorescence 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 para-
sitic diseases, Sofia, Bulgaria) and slides were examined
with a fluorescent microscope ML -2BU42 (LOMO - USSR)
with HBO 200 F 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 pres-
ence of autoanti-NuMA-2.

The presence of ANA deposited within tissues was inves-
tigated 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-
 thickness 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 dehydra-
tion 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 sec-
tions, 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 peri-
vascular oedema, granular disruption of the sarcoplasm,
Zenker dystrophy and necrosis and mononuclear cell prolif-eration in the femoral muscles. Fibrin thromboses, focal
segmental glomerulosclerosis and double contoured basal
membranes of the “loop” or “tail” type were often observed
in blood vessels from oesophagus, glandular stomach, lungs,
liver, heart, spleen, pancreas and brain after 72 hours post-
infection (Figure 1). Degeneration accompanied by homoge-

nization of the media, adventitia and hyperelastosis of tunica
elastica externa, activated and vaculated vascular endothelium
and then vaculization 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
destroyed Borrelia were usually found out in the macrophages
from the 6th hour to the 120h hour post-infection (Figure 3)
whereas in the thrombocyte cytoplasm (Figure 4) and some-
times in fibroblasts (Figure 5) Borrelia, obviously not morpho-
logically damaged, were lying freely.

From 3 to 12 months post-infection, lympho-histocyte pro-
liferation 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 experi-
mentally 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 anti-
bodies 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
120h hour.
Between the 120th – 168th 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 endomysium, in some skeletal muscle bundles, in myocardium and in renal glomerules (granular and sub-endothelial) (Figure 8).

**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 destructed *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 destructed *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.

**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 with the clinical presentation of *Borrelia* infection.
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.

Acknowledgement

The author greatly thanks Prof. Dr. T. Kirev for helping during electron-microscopy studies and Dr Sava Savov for helping with the illustrations.

References

561


