Effect of cyclophosphamide on

bursal development in the domestic turkey

by

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TABLE OF CONTENTS

INTRODUCTION

The bursa of Fabricius (BF) is a morphologically distinct, primary lymphoid organ of birds which plays a vital role in host resistance to a variety of infectious agents via humoral immunity. An intact, functional BF is required for an optimum immune response. Any environmental, nutritional, or biological factor which adversely affects normal development and function of the BF can increase susceptibility of the bird to infectious diseases.

During investigations on severe, complicated respiratory disease of turkeys, bursal atrophy was frequently found in dead or dying turkeys but not in less affected birds. Newcastle disease virus, Escherichia coli, and Alcaligenes faecalis have been identified as the 3 most important pathogens involved in complicated respiratory disease in Iowa turkeys. It could not be determined whether bursal atrophy or infection occurred first. The following questions were asked: Did bursal atrophy occur and render the bird more susceptible to infection, or did infection with one or more pathogens result in bursal atrophy which further impaired the bird's resistance allowing other infectious agents to complicate the disease process?

These questions remain unanswered. The studies recorded in this thesis were undertaken to develop a model system for producing experimental bursal atrophy in turkeys. The availability of a method to consistently produce bursal atrophy in the turkey would provide a basis for studying the question of what effect a pre-existing atrophied BF would have on a bird's response to infectious agents involved in complicated respiratory disease. The effect of cyclo-phosphamide on the turkey BF was undertaken because this drug has been used to produce bursal atrophy in other avian species and there was a lack of information on its use in turkeys.

LITERATURE REVIEW

Bursa of Fabricius

History

Heironymus Fabricius (1537-1619) first described the avian BF (Hoskins 1977). From accumulated lecture notes left at Fabricius' death in 1619, a manuscript was published in 1621 that included the initial description of the BF (Adelman 1967). Fabricius erroneously believed the BF was found only in the female. He described it as a double sac which communicated with the uterus and acted as a storage place for semen. In honor of Fabricius, the sac-like structure he described has been named bursa of Fabricius (Glick 1977). Later, the BF was found to be single sac present in both sexes that does not directly connect with the uterus or participate in semen storage.

Structure

The BF is a lymphoid organ unique to the class Aves (Warner 1967). It is a blind, round to oval, sac-like diverticulum located dorsal to the cloaca which opens through a bursal duct into the cloaca. The lumen of the organ is filled by about 12 longitudinal folds of the bursal wall mucous

membrane (plicae) which on cross-section resemble villus
projections (Hodges 1974).

The histology of the turkey BF is similar to that of
the chicken BF (Hoskins 1977). Hodges (1974) described the
histology of the chicken BF.

The wall of the BF is composed of 4 basic layers:

(1) An outer thin serosal layer.

(2) A muscle layer formed either of circularly arranged
smooth muscle fibers or an outer longitudinal and an inner
circular layer of smooth muscle.

(3) A mucosa which comprises the greater portion of
the thickness of the wall. It is divided into 2 components;
the lymphoid follicles and a connective tissue framework
surrounding the follicles.

(4) Mucosal epithelia.

Each fold consists of several closely packed polyhedral
follicles separated by a small amount of connective tissue
consisting of a network of fine collagen fibers with
numerous reticulin fibers. Follicles take up the greatest
portion of space within each fold. They have an inner
medulla and outer cortex. Both cortex and medulla possess
a supporting network of reticulo-epithelial cells whose
meshes are filled with lymphoid cells. Separating cortex
from medulla is a distinct basement membrane. Adjacent to
the basement membrane is the outermost layer of the medulla.

This layer is composed of undifferentiated epithelial cells and is continuous with the surface epithelium. Undifferentiated epithelial cells are cuboidal with pale-staining, round nuclei.

The cortex primarily contains closely packed small lymphocytes although lymphoblasts, mitotic figures and macrophages containing remnants of nuclear disintegrations can also be seen (Thorbecke et al. 1957). Fine arterioles, venules or capillaries can occasionally be seen in the cortex but the blood supply is not well developed.

Cells in the medulla are primarily lymphoblasts and medium to small lymphocytes. Blood vessels are rarely (Ackermann and Knouff 1959) or never (Frazier 1974) found in the medulla regardless of the stage of development.

Plasma cells can be found in the connective tissue between follicles and immediately beneath the epithelium (Thorbecke et al. 1957, Frazier 1974).

The ultrastructure of chicken's BF lymphoid follicles has been described by Clawson et al. (1967), Holbrook et al. (1974), Frazier (1974), and Olah et al. (1975). Lymphoid follicles of the turkey BF are ultrastructurally similar to those in the chickens BF (Hoskins 1977).

Both cortex and medulla contain many lymphocytes which vary in nuclear diameter from 3.4 to 7 microns. Small

lymphocytes have only a thin rim of cytoplasm containing few organelles. Nuclei of small lymphocytes contain dense staining clumped chromatin which usually obscures a small nucleolus. Large lymphocytes have a substantial amount of cytoplasm which contains several fairly large open mito-chondria, a Golgi zone with numerous vesicles, a pair of centrioles, and a small amount of rough endoplasmic reticulum with open, vesicular cisternae. Nuclei of large lymphocytes have little chromatin clumping and often contain a prominent nucleolus. The majority of lymphocytes in the bursal follicles fall between these 2 extremes.

Macrophages are fairly frequent in both cortex and medulla. Their content of cytoplasmic organelles is variable. Vacuoles containing phagocytosed material are frequently present. The nucleus is round to oval in shape and often indented by large cytoplasmic vacuoles. Frazier (1974) described large cells, with pale cytoplasm and nucleus in the cortex and rarely in the medulla. These cells have numerous cytoplasmic organelles; their nucleus is round to oval in shape and contains little chromatin.

Medullary reticulo-epithelial cells present a variety of morphological types that have been described as pale, dark or intermediate. Pale cells have a round to oval nucleus that may be slightly indented while dark cells have an

irregular indented nucleus. In all types 1-2 nucleoli
may be visible. Ribosomes, endoplasmic reticulum, mito-
chrondria, and fibrils or tonofilaments are found in the
cytoplasm. They have long cytoplasmic processes which are
frequently joined by well developed desmosomes forming a
meshwork that fills the follicle.

Medullary epithelial cells at the cortico-medullary
border form a continuous layer (undifferentiated epithelium)
which is covered on the cortical side by a basement membrane.
These cells contain an irregularly curved nucleus with loose
chromatin. Scattered in the cytoplasm are elongated mito-
chondria, few rough endoplasmic reticulum cisternae, and
many ribosomes. Beside the nucleus, the Golgi apparatus
and a multivesicular body can be seen. Tonofilaments are
rarely found (Olah et al. 1975).

Olah et al. (1979) have described secretory cells in
the medulla. They usually parallel the cortico-medullary
border, are commonly elongated and possess one long process
containing 3-10 dark membrane-bound granules. In a few
irregularly shaped cells, granules were found gathered near
the nucleus. The secretory cell nucleus is hyperchromatic,
eccentrically located, and contains 1-3 nucleoli. The
cytoplasm is dark and possesses large, round mitochondria,
a small Golgi region, and polyribosomes, but few

ergastoplasmic cisternae are present.

Small dark cells have been observed in small numbers
in the cortex but are absent in the medulla (Frazier 1974).
These cells have spherical to elongated, slightly indented
nuclei. Their cytoplasm contains ribosomes, occasional
mitochondria, vacuoles, and strands of rough endoplasmic
reticulum. A Golgi apparatus and centrioles are occasionally
seen. Associated with the plasma membrane of these dark
cells are collagenous fibers.

Prior to hatching, 3 definitive types of cells have
been described in surface epithelium of the chicken BF
(Ackerman and Knouff 1959). "Type I" cells are oval with faintly
basophilic cytoplasm containing small granules. "Type II"
cells are most numerous and appear as columnar cells with an
oval nucleus which contains a single nucleolus. The cytoplasm
is also slightly basophilic and contain numerous small granules.
"Type III" cells are goblet cells which are more narrow and
contain a hyperchromatic nucleus and secretory material.
No report is available about the presence of these cell types
in post hatching BF.

In the older bird, the mucosal surface is covered by
2 distinct epithelia; follicular epithelium and interfolli-
cular epithelium (Holbrook et al. 1974). Follicular epithelium
is made up of polygonal cells with sharply delineated borders

that possess few, short, regular shaped and evenly dis-
tributed microvilli over their entire lumenal surface. Inter-
follicular epithelial cells are irregularly shaped. They have
pitlike depressions unevenly distributed microbilli on the
surface. They contain smooth membrane vesicles and rest upon
a basement membrane.

Both follicular and interfollicular epithelial cells
have intercellular right junctions. Holbrook et al. (1974)
observed lymphocytes between follicular but not between inter-
follicular epithelial cells in 10-14 day chickens. Hoskins
(1977) identified lymphocytes, heterophils, and degenerating
cells within intercellular spaces of both follicular and
interfollicular epithelium in the turkey BF.

A diffusely infiltrated area of lymphocytes is located
in the dorsal wall of the BF adjacent to the bursal duct
(Odend'hal and Breazile 1979a). This area is believed to
represent a T-cell area of the bursa (Odend'hal and Breazile
1980) and also the source of free lymphocytes that have
been found in the BF lumen (Odend'hal and Breazile 1979b).

Immunological role

Immunofluorescence studies on tissue sections of
chicken BF have revealed that medullary lymphoid cells have
immunoglobulins on their surface almost 1 week before

hatching (Thorbecke et al. 1968). While IgM can be demonstrated on the surface of lymphoid cells in bursal follicles of 14-day-old chicken embryos, IgG is not present prior to the 21st day of incubation. Production of IgA cannot be detected until much later (Cooper et al. 1972, Martin and Leslie 1973). It is of interest that single cells obtained from bursal tissue have been found to exhibit a positive reaction with both anti-IgM and anti-IgG anti-bodies. This finding indicates that various immunoglobulin classes are generated in an orderly intraclonal switch from IgM to IgG to IgA, and that the BF provides a specific induction microenvironment for this sequence of events (Schaffner et al. 1974).

Neither the thymus nor the BF is a major site for antibody production after systemic injection of antigens (Dent and Good 1965, Abramoff and Brien 1968, Choi and Good 1973), although the bursa showed an increased cellular proliferation within 24 hours following intravenous injection of human serum albumin (Back 1973). Intrabursal injection of antigens such as bovine γ-globulin elicited a good antibody response. However, antibody was primarily produced in extrabursal tissues, particularly spleen (Choi and Good 1973). Following intracloacal or intrabursal administration of bovine γ-globulin, bursal cells did not synthesize detectable antibody (Choi and Good 1973).

According to present concepts, the BF is a primary lymphoid organ where the first stage of B lymphocyte differentiation takes place and it functions by offering a microenvironment needed for proper education of these cells (Toivanen and Toivanen 1973). As they mature, they leave the BF and relocate in peripheral lymphoid tissues (lymphoid tissues other than thymus and BF). If lymphocytes in peripheral lymphoid tissues are tested for membrane immunoglobulin, the order in which they appear is found to be similar to that in which they developed within the BF i.e. IgM → IgG → IgA (Sites et al. 1980). Stimulation of bursal lymphocytes by antigen to subsequent proliferation and differentiation into memory B cells or antibody producing plasma cells occurs in peripheral, extrabursal sites (Cooper et al. 1972).

There is also evidence that the BF functions as a secondary lymphoid organ for taking-up and processing some of the environmental antigens and thereby providing immunity against those antigens (Sorvari et al. 1975, Sorvari and Sorvari 1977).

Role in resistance to infectious diseases

Bacterial diseases
Chang et al. (1959) reported higher mortality in bursectomized chickens compared with

intact controls following experimental <u>Salmonella</u> <u>typhimurium</u> infection. Perek and Drill (1962) found that when birds were challenged with virulent <u>Salmonella</u> <u>typhimurium</u> following immunization with this organism a low mortality rate occurred in the bursectomized group compared with none in the intact group. No difference in mortality was observed between bursectomized and intact turkeys infected with <u>Salmonella</u> <u>gallinarum-pullorum</u> (Awad et al. 1974).

Perek and Drill (1962) found low agglutination titers against <u>Spirochaeta</u> <u>gallinarum</u> in bursectomized chickens experimentally infected with this microorganism. Surgical removal of the BF in chicks at the age of 24 hours lowered their immunohumoral reactivity and increased their suscept- ibility to <u>Treponema</u> <u>anserinum</u> (Soumrov et al. 1967). These studies show that humoral immunity against spirochetosis is of decisive importance.

Chicks bursectomized within 72 hours after hatching and infected with <u>Leptospira</u> <u>icterohaemorrhagica</u> between 2-4 weeks of age had significantly reduced antibody production and infection persisted longer in bursectomized birds causing higher mortality (Kemmes and Pethes 1963).

Chicks bursectomized at 5 days of age and infected with <u>E</u>. <u>coli</u> at 6 or 12 days of age suffered greater mortality than infected nonbursectomized chicks (Sadler and Edgar 1969).

Heller and Perek (1974) noted high mortality in chicks bursectomized at 1 day of age and infected with E. coli strain 078 at 42 and 49 days of age. They did not find differences in mortality between control chicks and chicks bursectomized at 4, 8, 12, 16 days of age.

Neonatal bursectomy did not affect mortality rate or lesion development in Mycobacterium avium infection (Cheville and Richards 1971).

Vardaman et al. (1973) found that resistance to Mycoplasma synoviae was BF dependent because bursectomized chickens had more severe airsacculitis and significant reductions in antibody formation. Kume et al. (1977) showed that B lymphocytes are correlated with resistance to joint lesion development in M. synoviae infection.

Viral diseases   Cho (1963) found similar antibody levels to B-1 strain Newcastle disease virus at 2 and 4 weeks after vaccination in both bursectomized and intact chickens. He concluded that the BF played no role in antibody response to B-1 strain of Newcastle disease virus. The same conclusion was reached by Sadler and Edgar (1968) who found that removal of the BF at 5 days of age did not prevent development of immunity to B-1 strain Newcastle disease virus. Bursectomy at hatching permitted HI antibody formation after exposure to Newcastle disease virus antigen whereas bursectomy

of embryos on the 20th day of incubation resulted in complete suppression of HI antibody formation (Matsuda and Bito 1973). Chickens bursectomized at 1 day of age and vaccinated with B-1 strain resisted intratracheal challenge with a virulent strain of Newcastle disease virus (Ewert and Eidson 1977).

Sadler and Edgar (1968) indicated that the BF was not necessary for development of immunity against fowl pox virus. No difference in mortality was observed between bursectomized and nonbursectomized, nonvaccinated chickens exposed to fowl pox virus.

Biological protection against avian encephalomyelitis is chiefly mediated through the presence of antibody-forming bursal cells (Cheville 1970). Bursectomized chicks developed paralysis and died following inoculation with avian encephalomyelitis virus regardless of age at inoculation, while nonbursectomized chicks developed no clinical signs when inoculated at 28 days of age.

The BF may play a significant role in development of resistance to hemorrhagic viral enteronephritis in goslings because bursectomy increased and prolonged their susceptibility to this disease (Pethes and Bernath 1971).

Robertson (1977) found no difference in morbidity between bursectomized and non-bursectomized chickens challenged with Queensland strain of infectious laryngotracheitis virus. He

concluded that BF-dependent responses are not necessary
for effective immune response development to infectious
laryngotracheitis virus.

Surgical bursectomy of chickens up to 12 weeks of age
has been found to prevent lymphoid leukosis (Peterson et al.
1964, 1966; Cooper et al. 1968). Androgen administered
during embryonic development produced partial to total
atrophy of the BF, with a parallel decrease in lymphoid
leukosis incidence (Burmester 1967, 1969). Mibolerone, a
synthetic androgen, at 1 ug/g of diet during the first 49 days
of age was found to significantly reduce lymphoid leukosis
incidence (Kukuk et al. 1977). These studies emphasize that
BF is an important organ in the pathogenesis of lymphoid
leukosis.

A difference in resistance and tumor spectrum was
observed between bursectomized and control chicks infected
with BAI strain A of avian myeloblastosis virus (Baluda 1967).
Bursectomized chicks had lower resistance, higher incidence
of myeloblastic leukemia and acute anemia, and lower incidence
of visceral lymphomatosis than non-bursectomized chicks.
Baluda indicated that antibody may be responsible for
resistance to tumor formation by avian myeloblastosis virus.

Foster and Moll (1968) found that hormonal bursectomy
greatly decreased the incidence of Marek's disease in chickens

up to 20 weeks after infection. Morris et al. (1969)
suggested that protection against Marek's disease virus was
provided by the BF and mediated via antibody production.
They recorded higher mortality and gross lesions in surgically
bursectomized chickens than controls. Kenyon et al. (1969)
found surgical bursectomy did not influence mortality from
lymphoblastic leukemia induced by JM-V strain of Marek's
disease virus. Isogai (1978) observed that BF function is not
essential for Marek's disease tumorigenesis. He found that
both bursectomized and control groups had the same frequency
of Marek's disease tumors.

Surgical bursectomy of Japanese quail had no demonstrable
effect on regression of primary tumors induced by SR-Rous
sarcoma virus (Yamanouchi et al. 1971). McArthur et al. (1972)
recorded a similar incidence of Rous sarcomas in control
and bursectomized groups. They concluded that antibody
was not an important factor in growth of Rous sarcoma in
their experiments.

Subcutaneous administration of avian reticuloen-
dotheliosis virus resulted in progressively growing and
ultimately fatal tumors in bursectomized chickens while
tumors regressed in normal chickens (Linna et al. 1974).
Their results indicate that a BF-dependent, antibody
forming system contributes to host defenses preventing

reticuloendotheliosis virus lesion development.

Parasitic diseases   Challey (1962) found significantly
higher mortality among surgically bursectomized chickens than
among intact controls during initial infection with Eimeria
tenella which indicated that BF was important in host re-
sistance to coccidiosis. Other studies using hormonally
bursectomized and intact chickens revealed only minor
differences in the ability of the 2 groups to resist challenge
infection with 50,000 E. tenella oocysts (Long and Pierce
1963, Pierce and Long 1965). They concluded that humoral
antibody was not significant in resistance to E. tenella
infection. Rouse and Burns (1971) found surgical removal of
the BF within 24 hours of hatching did not impair the ability
of chickens to survive E. tenella infection or develop
resistance to reinfection.

Hormonally or surgically bursectomized chickens developed
higher parasitemias following experimental infection with
Plasmodium lophurae (Longenecker et al. 1966, Farmer and
Breitenbach 1968) and Plasmodium gallinaceum (Stutz et al.
1972).

The BF was not important in mediation of immunity to
Syngamus trachea in chickens (Varga 1971).

Johnson et al. (1974) found chicks bursectomized in

ovo, at 3 days or 14 days of age had significantly greater worm burdens and incidence of infection with Ascaridia galli than intact controls. No alteration in the worm burden was observed in chicks bursectomized at 35 days of age. They concluded that early bursectomy resulted in insufficient humoral antibodies to provide protection against A. galli.

Cyclophosphamide

History

Cyclophosphamide (CY) was among a large number of nitrogen mustard derivatives first synthesized by Arnold and Bourseaux (1958). It was subsequently shown to possess an excellent therapeutic index in tumor models (Brock 1967). The approach in CY synthesis was essentially a continuation of earlier attempts to prepare latent alkylating agents which could be activated by acid phosphatase in the hope that toxic products would be formed selectively in tumor cells. Although CY was synthesized to release non-nitrogen mustard after selective activation by enzymes within tumor cells, it soon became apparent that its toxicity was dependent on an earlier activation event which occurred in liver microsomes (Foley et al. 1961). Furthermore, a detailed

analysis of the metabolic pathway of CY revealed that its greater toxicity for tumor cells was attributable to a much less efficient enzymatic detoxification of primary oxidation products in tumor cells than in normal cells due to an absence or lower concentration of appropriate enzymes (Connors 1975).

Clinically, CY has proved to be a relatively safe and effective drug for cancer chemotherapy. Furthermore, following demonstration that CY was immunosuppressive, it has been utilized extensively as an alternative to irradiation to pretreat potential bone marrow transplant recipients (Glucksberg et al. 1974). The immunosuppressive potential of CY has also been applied to the treatment of immunological disorders, notably autoimmune diseases and rheumatoid arthritis but with rather less spectacular results.

In experimental immunological models, 2 attributes of CY have been exploited more fully: (1) its ability to substantially lower the quantity of antigen required to induce immunological tolerance, and (2) the recent finding that suppressor T cells are particularly sensitive to the drug in vivo.

Metabolism and pharmacologic action

Cyclophosphamide is inert in direct contact with bacteria, leukocytes, and most tissue cultures of tumor

cells (Foley et al. 1961, Hample et al. 1966). Activation of CY occurs primarily in the liver generating cytotoxic alkylating substances (Bagley et al. 1973). There is a direct correlation between alkylating activity and cytotoxicity (Bardos et al. 1969). Liver activation depends upon a functional microsomal oxidase system, requiring reduced nicotinamide adenine dinucleotide phosphate (NADPH) and oxygen (Cohen and Jao 1970, Sladek 1971).

In general, it is considered that alkylating agents exert their immunosuppressive activity by inhibiting nuclear DNA replication. Inhibition is mediated through formation of covalent bonds with guanine molecules of chromosomal DNA (Calabresi and Park 1970, Gerald 1974). Covalent bonding results in cross linking 2 DNA strands or linking DNA to protein. Linking results in major disruption of DNA function and subsequent interference with normal cell mitosis and replication. It is accepted that rapidly proliferating cell populations are considerably more sensitive to alkylating agents than those not undergoing active division. In the case of CY, certain metabolites are effective phosphorylating agents and thereby additionally interfere with cellular function (Back et al. 1964).

Effect on lymphocyte populations and immune responses

The relative susceptibility of B and T lymphocytes to CY in vitro remains controversial. The original observation in the chicken (Lerman and Weidanz 1970) that CY was selectively toxic for B cells was later confirmed in the guinea pig utilizing morphological criteria (Turk and Poulter 1972). These experiments implied that B cells were affected to a much greater extent than T cells. This agreed with earlier findings that CY induced a more substantial inhibition of antibody response than allograft rejection (Berenbaum and Brown 1963, Fox 1964, Brody et al. 1965).

However, later studies reported that relatively large doses of CY could suppress lymphocyte functions which were mediated exclusively by T cells, e.g. graft-versus-host reaction and delayed hypersensitivity (Turk 1964, Owens and Santos 1968, 1971, Winklestein et al. 1972). Further work established that even small doses of CY which were ineffective in suppressing antibody synthesis, dramatically enhanced a delayed hypersensitivity reaction providing the drug was administered prior to the sensitizing antigen (Askenase et al. 1975, Gill and Liew 1978). The general consensus was that potentiation of delayed hypersensitivity was due to elimination of suppressor T cells. Other inhibitory effects of CY on suppressor T cells have been reported (L'age-Stehr and Diamanstein 1978, Ferguson and Simmons 1978, Stevenson

and Fauci 1980).

In a recent study on human lymphocytes, it was found that B cells were most sensitive to CY, followed in sensitivity by suppressor T cell populations with relative resistance of helper T cells (Stevenson and Fauci 1980). Among different T cell subpopulations, cytotoxic killer cells were found to be more resistant to the action of CY than suppressor T cells. A large dose of CY was required to suppress generation of cytotoxic T cells in mice (Ferguson and Simmons 1978).

It was concluded that CY is cytotoxic for rapidly dividing cells whether B or T lymphocytes (Turk and Parker 1979). CY will suppress any immune response, humoral or cell-mediated, if given during the first week after immunization when there is rapid proliferation of specifically stimulated lymphocytes.

Use of Cyclophosphamide in Avian Species

Lerman and Weidanz (1970) reported that chickens treated with high doses of CY developed deficiencies in both immunoglobulin synthesis and antibody production. Following this report, many immunological studies have been done using CY as an immunosuppressant in chickens, ducks, and pigeons.

Apparently no attempt has been done to study the effect
of CY on the immune system of turkeys. CY has been used
more extensively in chickens than ducks or pigeons. In
this review, information will pertain to chickens unless
otherwise stated.

Dose and route of inoculation

A wide range of CY doses (1-24 mg) has been used.
In most cases, the total dose was divided into small equal
doses injected on different post-hatching days. Three or 4
equal doses injected for 3 or 4 successive days starting on
the day of hatching was the most common procedure (Lerman
and Weidanz 1970, Glick 1971, Linna et al. 1972, Hiraga
et al. 1976, Sharma and Lee 1977, Hoffman-Fezer et al. 1977,
Prasad 1978, Sachs et al. 1979). In some cases, the dose
was calculated on the basis of body weight (Toma et al. 1977,
Olah et al. 1979). The drug was administered intramuscularly
or intraperitoneally. Cyclophosphamide was also injected
intravenously into embryonated eggs for 3 successive days
beginning on day 12, 14 or 16 of incubation (Eskola and
Toivanen 1974).

Ducks were injected with 2.5 mg CY intraperitoneally
for 2 and 4 successive days staring from the hatching day
or day 1 after hatching (Hashimoto and Sugimura 1976, Sugimura

and Hashimoto 1976).

Pigeons were given 10 mg CY/bird intraperitoneally for 4 successive days in 4-week-old birds (Coignoul and Vindevogel 1980).

Effect on host survival

Lerman and Weidanz (1970) found high mortality occurred in chickens that received 18 mg CY. The majority of deaths occurred within 2 weeks following treatment. The lowest mortality occurred in the group that received 12 mg. In ovo treatment with CY induced significant mortality, both in ovo and after hatching (Eskola and Toivanen 1974). Mortality in ovo was found to be dose dependent. High post-hatching mortality occurred in the group inoculated on days 12-14 of incubation and dose-dependent mortality occurred in the group inoculated on days 16-18 of incubation. Rouse and Szenberg (1974) reported 50% mortality in 16 day-old chickens following administration of 10 mg of CY. More than 90% died by 6 weeks of age following a total injection of 10 mg of CY.

Effect on growth

Both slow growth and decreased body weight have been reported in chickens (Glick 1971, Hiraga et al. 1976, Prasad 1978) and ducks (Hashimoto and Sugimura 1976) treated

with CY. Hiraga et al. (1976) reported depressed growth in CY-treated groups from 7 days to 3 weeks but not after 5 weeks of age. In ovo treatment caused a significant dose-related retardation in body weight gain in the post-hatching period (Eskola and Toivanen 1974).

Poor weight gain and decreased body weight have been observed in CY-treated ducks at one week of age (Hashimoto and Sugimura 1976).

Effect on lymphoid organs

The weight of the BF, thymus and spleen in CY-treated chickens (Glick 1971, Hiraga et al. 1976, Toma et al. 1977, Sharma and Lee 1977) and ducks (Sugimura and Hashimoto 1976) was consistently decreased in comparison to controls. Regardless of the dose used, CY had a more prolonged suppressive effect on BF weight than spleen or thymus weights. The time required for lymphoid organs to regain normal weight depended on the dose of CY. Cyclophosphamide suppressed BF weight in CY-treated pigeons (Coignoul and Vindevogel 1980).

A variety of morphological changes have been described in the BF lymphoid follicles of CY-treated chickens. Lymphocyte depletion was the most consistently described histological change (Glick 1971, Linna et al. 1972, Toivanen et al. 1972b, Eskola and Toivanen 1974, Hiraga et al. 1976,

Prasad 1978, Hoffmann-Fezer et al. 1977, Sachs et al. 1979).
Other changes were follicular fibrosis (Linna et al. 1972,
Toivanen et al. 1972b, Prasad 1978, Sachs et al, 1979) and
increased numbers of macrophages (Hiraga et al. 1976). Olah
et al. (1979) described changes in secretory cells. A spher-
ical nucleus with loose chromatin structure was a characteristic
feature of these cells in the BF of CY-treated chickens.
Histological changes have also been observed in the follicular
and interfollicular epithelium. There was thinning of
follicular epithelium, hyperplasia of interfollicular epithe-
lium, and appearance of cysts in the epithelium lining (Sachs
et al. 1979). Ultrastructurally, dilation of rough endoplasmic
reticulum cisternae and multiple Golgi bodies in the inter-
follicular epithelium were found in CY-treated chickens
(Sachs et al. 1979). Hoffmann-Fezer et al. (1977) gave an
excellent description of the sequential changes and recovery
stages in the BF of CY-treated chickens. They performed
histopathological analysis up to 11 weeks of age.

Sugimura and Hashimoto (1976) characterized histological
changes in the BF of CY-treated ducks. They observed lympho-
cyte depletion, loss of follicular epithelium, and a signifi-
cant reduction in plasma cells.

In pigeons, CY caused depletion of lymphocytes in both
cortex and medulla, changes in the follicular epithelium,

and proliferation of the epithelial layer which separates cortex from medulla (Coignoul and Vindevogel 1980).

In the thymus, CY caused depletion of lymphocytes in both cortex and medulla (Glick 1971, Linna et al. 1972, Rouse and Szenberg 1974, Hiraga et al. 1976, Sharma and Lee 1977, Prasad 1978), occasional necrosis and hemorrhage (Rouse and Szenberg 1974, Prasad 1978), and, increased reticulo-epithelial cells (Hiraga et al. 1976, Sharma and Lee 1977).

Lymphocyte depletion was the only histological change described in the thymus of CY-treated ducks (Hashimoto and Sugimura 1976).

Coignoul and Vindevogel (1980) described degeneration and depletion of lymphocytes and an increase in the size and number of Hassal's corpuscles in the thymus of CY-treated pigeons.

Absence of germinal centers and depletion of plasma cells and lymphocytes were the only morphological changes described in the spleen of CY-treated chickens (Glick 1971, Toivanen et al. 1972b, Rouse and Szenberg 1974, Prasad 1978).

Hashimoto and Sugimura (1976) reported complete absence of lymphocytes and granulocytes in the white and red pulp of the spleen of CY-treated ducks.

Absence of germinal centers (Glick 1971, Linna et al. 1972) and plasma cells (Linna et al. 1972) has been observed

in the cecal tonsils of CY-treated chickens.

Effect on humoral immunity

Various antigens have been used to test immune responsiveness of CY-treated birds including sheep red blood cells (Glick 1971, Eskola and Toivanen 1974, Sharma and Lee 1977, Sachs et al. 1979), horse red blood cells (Rouse and Szenberg 1974), killed Brucella abortus (Linna et al. 1972, Eskola and Toivanen 1974, Sharma and Lee 1977), and killed Salmonella pullorum (Hashimoto and Sugimura 1976). Cyclophosphamide treatment resulted in suppression of primary antibody response and profound deficiency of humoral immuno- logical function (Lerman and Weidanz 1970, Glick 1971, Linna, et al. 1972, Rouse and Szenberg 1974, Hashimoto and Sugimura 1976, Sharma and Lee 1977, Sachs et al. 1979).

Effect on cell-mediated immunity

Three methods have been used to evaluate cell-mediated immunity (CMI) in CY-treated chickens: (1) skin grafting techniques (Linna et al. 1972; Rouse and Szenberg, 1974, Prasad 1978); (2) graft-versus-host (GVH) reactivity (Lerman and Weidanz 1970, Seto 1970, Linna et al. 1972, Rouse and Szenberg 1974); and (3) lymphocyte response to phyto- hemagglutinin (Eskola and Toivanen 1974, Sharma and Lee

1977). Most evidence indicates that CY temporarily suppresses cell-mediated immunity (Seto 1970, Rouse and Szenberg 1974, Sharma and Lee 1977, Prasad 1978).

Effect on immunoglobulin synthesis

Cyclophosphamide treated chickens lack detectable IgG or IgM or have low levels of IgG and IgM (Lerman and Weidanz 1970, Glick 1971, Linna et al. 1972, Rouse and Szenberg 1974). Decreased γ-globulin levels have also been observed in CY-treated ducks (Hashimoto and Sugimura 1976).

Cyclophosphamide-induced immunosuppression in avian disease research

Cyclophosphamide has been used as an immunosuppressant to study the course and pathogenicity of several avian infectious diseases. It has also been used to study the immune response in immunologically impaired vaccinated chickens. Cyclophosphamide was used with avian malaria (Herman and Shiroishi 1973), fowl typhoid (Cameron et al. 1974, Smith et al. 1977), inclusion body hepatitis (Fadly et al. 1976), avian reticuloendotheliosis (Linna et al. 1974), Marek's disease (Purchase and Sharma 1974, Kermani-Arab et al. 1975, Lu et al. 1976, Loliger et al. 1978, Payne et al. 1978), lymphoid leukosis (Purchase and Gilmour 1975), avian sarcomas

(Smida and Smidova 1978, Loliger et al. 1978), and infectious
avian encephalomyelitis (Westbury and Sinkovic 1978).

Cyclophosphamide increased susceptibility of chickens
to the second generation of the preerythrocytic form of
Plasmodium gallinaceum (Herman and Shiroishi 1973).

Cameron et al. (1974) found that immunity to fowl typhoid
was not impaired in CY-treated chickens vaccinated with
Salmonella gallinarum although there was a severe depression
in humoral immune response. He concluded that immunity to
Salmonella gallinarum is not dependent on a humoral immune
mechanism. No difference in mortality has been observed
between CY-treated and untreated 1-day-old chickens infected
with Salmonella gallinarum (Smith et al. 1977).

Interference with the BF by CY enhances pathogenicity
of inclusion body hepatitis virus and renders chickens more
susceptible (Fadly et al. 1976). They stated that biological
protection against inclusion body hepatitis is chiefly mediated
through bursal cells.

Protection against Marek's disease afforded by vaccination
with turkey herpesvirus is initially impaired by treatment
with CY (Purchase and Sharma 1974, Payne et al. 1978).
Cyclophosphamide was found to reduce Marek's disease virus
viremia (Kermani-Arab et al. 1975) and to delay development
and reduce frequency and intensity of Marek's disease lesions
(Kermani-Arab et al. 1975, Lu et al. 1976). Cyclophosphamide

does not affect the transplantation rate metastasis in Marek's disease (Loliger et al. 1978).

Chemical bursectomy with CY prevents lymphoid leukosis development (Purchase and Gilmour 1975). Smida and Smidova (1978) reported progressive growth of sarcomas and high mortality in CY-treated chickens experimentally infected with avian sarcoma virus B77. Restriction of transplantation rate and metastasis rate of lymphosarcoma in CY-treated chickens has been observed by Loliger et al. (1978).

Twenty-day-old or older chickens immunologically suppressed with CY developed clinical infectious avian encephalomyelitis following oral exposure to NSW-1 strain of infectious avian encephalomyelitis virus (Westbury and Sinkovic 1978). It was concluded that the humoral immune system plays a significant role in pathogenesis of infectious avian encephalomylitis and development of immunity to this disease.

EXPERIMENT I. EFFECT OF CYCLOPHOSPHAMIDE ON BURSAL

MORPHOLOGY AND HUMORAL IMMUNITY OF TURKEYS

MATERIALS AND METHODS

Turkeys

Fertile eggs were purchased from a commercial hatchery and incubated at the Veterinary Medical Research Institute. After hatching, 50 poults were weighed, identified with wing tags, divided randomly into 5 groups and transferred to a wire-floor electrically heated battery unit[1] in a temperature controlled room. Each group was kept in a separate identified unit. Poults were fed a commercial starter ration[2] and given tap water ad libitum.

Experimental Design

Three groups were treated with either 2, 4, or 6 mg CY/poult/day for 3 successive days, one group was treated with 2 mg/CY/poult/day for 6 successive days. The remaining group served as untreated controls. Cyclophosphamide treatment of all groups was started on the day of hatching. Parameters used to assess the effect of CY were: (1) daily clinical signs, (2) mortality, (3) weekly body weight,

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[1]Oakes 801A Brooder, Oakes Manufacturing Co., Inc. Tipton, Indiana.

[2]Agri Group, Domain Industries, New Richmond, Wisconsin.

(4) bursa weight expressed as a percentage of total body weight, (5) antibody response to sheep red blood cells (SRBC), (6) total serum protein, (7) serum protein electrophoresis and (8) gross and microscopic lesion.

Preparation and Use of Cyclophosphamide

Cyclophosphamide (Cytoxan[R])[1] was obtained in vials containing 100 mg of active ingredient. The drug was kept in the refrigerator at 5°C. Two different aqueous solutions were prepared by dissolving 100 mg CY in 25 ml (solution A, 4 mg/ml) or 12.5 ml (solution B, 8 mg/ml) sterile distilled water. Solutions were freshly prepared and administered intraperitoneally. Control birds were injected intra-peritoneally with sterile distilled water for 3 successive days.

Sheep Red Blood Cell Preparation and Turkey Immunization

Blood from the jugular vein of a ram was drawn into a heparinized syringe fitted with a 1½ inch 16 gauge needle. Heparinized blood was centrifuged at 3000 xG for 10 minutes, plasma discarded, and sedimented SRBC resuspended in sterile normal saline. Washing and centrifugation were done 4-5

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[1]Mead Johnson & Company, Evansville, Indiana.

times until a clear supernatant was obtained. After the final centrifugation, supernatant fluid was discarded and the test tube, containing only sedimented SRBC was refrigerated at 5°C. Fresh SRBC were prepared each time before use for immunization.

At 1 and 2 weeks of age, each poult was injected intramuscularly with 0.5 ml SRBC.

Necropsy

All poults that were found moribund or dead during the experiment were necropsied. Surviving poults were killed and examined at 4 weeks of age. At necropsy, body weight was recorded and a blood sample for serum was collected from the heart. Poults were killed by cervical dislocation, BF was removed through an incision dorsal to the vent and weighed, and the carcass opened and examined for gross lesions. Portions of BF and any organs with gross lesions were placed in 10 percent neutral buffered formalin. Tissue sections were prepared by conventional paraffin-embedding procedures and stained with hematoxylin and eosin.

Hemagglutination Inhibition Test

Clotted blood, collected at necropsy, was centrifuged at 3000 xG for 10 minutes. Serum was transferred with a

Pasteur pipette into 2 ml screw-capped vials and kept frozen
until tested.

Hemagglutinin levels were determined by microtiter
technique. Two-fold serial dilutions of serum in saline
were made in conical-bottom microtiter plates using 50 ul
volumes. To each dilution, 50 ul of a 0.25% SRBC suspension
were added. Plates were incubated at 37°C and read after
2 and 12 hours. Titers were recorded as the reciprocal of
the highest dilution showing complete agglutination of SRBC.

<div align="center">

Total Serum Protein Determination and

Serum Protein Electrophoresis

</div>

Total serum protein was determined with a Goldberg
refractometer.[1] Serum proteins were quantitatively separated
by cellulose acetate membrane electrophoresis using Gelman
serum protein electrophoresis system.[2] The system is composed
of Tris-Barbitol-Sodium Barbitol (pH 8.8) high resolution
buffer, Sepratek[TM] electrophoresis chamber, power supplier,
staining tray, Sepratek sample applicator, and ACD-15
automatic computing densitometer. Electric current of

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[1]TS meter, American Optical Company, Instrument
Division, Buffalo, New York.

[2]Gelman Instrument Company, Ann Arbor, Michigan.

225 V and 5 milliamps was provided by the power supplier. After separation of protein fractions, the cellulose acetate membrane was put into a clear plastic strip holder and read in the densitometer.

Statistical Analysis

Duncan's new multiple range test was used for testing significant differences between individual pairs of means.

RESULTS

Clinical Signs and Mortality

Poults in CY-treated groups remained active with good appetite. Cyclophosphamide had a significant dose-related suppressive effect on growth of poults up to 4 weeks of age. Body weight of poults in 6 mg x 3 and 4 mg x 3 groups was significantly different from body weight of control poults at 1, 2, 3, and 4 weeks of age. Body weight of poults in the 2 mg x 6 group was significantly different from body weight of control poults at 2, 3, and 4 weeks of age. Body weight of poults in the 2 mg x 3 group was not significantly different from body weight of poults in the control group at any age (Table 1).

Mortality in the 2 mg x 6 group occurred during the first week and in the other CY-treated groups during the third week (Table 2). The cause of mortality was determined to be acute septicemia, probably caused by Escherichia coli, characterized by hemorrhagic pneumonia and purulent meningitis.

Development of Bursa of Fabricius

The BF from CY-treated poults were obviously smaller with poorly developed folds compared to those from untreated poults. There was a significant difference in relative

Table 1. Mean body weight of CY-treated and control groups (gm).[a]

Age	Control	2 mg x 3	2 mg x 6	4 mg x 3	6 mg x 3
1 day	60.73±1.49[b](10)[c]	59.41±1.50(10)	60.83±1.41(10)	61.30±1.29(10)	60.82±1.6 (10)
1 week	108.07±3.85(10)	103.25±4.83(10)	98.84±5.08(9)	85.25±1.92(10)	70.24±4.10(10)
2 weeks	257.35±0.01(10)	226.62±18.78(10)	208.13±11.99(9)	171.18±14.43(10)	115.10±16.60(10)
3 weeks	486.80±16.11(10)	473.33±18.24(9)	402.22±22.77	376.87±16.27(8)	292.67±21.80(6)
4 weeks	757.20±29.76(10)	750.00±32.84(9)	615.44±32.86(9)	571.00±24.35(8)	470.17±26.56(6)

[a]Underlined groups are not significantly different from each other (p>0.05).

[b]Mean, ± standard error of the mean.

[c]Number of poults.

Table 2. Mortality in CY-treated and control groups.

Group	No. of deaths/total
Control	0/10
2 mg x 3	1/10
2 mg x 6	1/10
4 mg x 3	2/10
6 mg x 3	4/10

bursa weight (expressed as a percentage of total body weight) between CY-treated and control poults. However, BF weight was not significantly different among poults treated with different doses of CY (Table 3).

Antibody Response to Sheep Red Blood Cells

No SRBC antibody titer was detected in any CY-treated poult. Response in the control group was low: 2 poults had 1:4 titers and 4 poults had 1:2 titers.

Serum Proteins

Cyclophosphamide had a marked suppressive effect on -γ globulin synthesis. A significant difference in γ-globulin level was found between CY-treated and control groups. There was a direct relationship between CY dose and severity of γ-globulin deficiency although the difference was not significant. There were no significant differences among other globulin fractions (α, β), albumin, or total serum proteins (Table 4).

Gross Pathology

The only gross lesions observed were in the lungs of 5 CY-treated poults: 2 poults in 2 mg x 6 group and 1 poult in each of the other CY-treated groups. The lung lesion

Table 3. Mean bursa percent in CY-treated and control groups[a]

Control	2 mg x 3	2 mg x 6	4 mg x 3	6 mg x 3
0.173±0.010[b] (10)[c]	0.057±0.005(9)	0.054±0.003(9)	0.048±0.003(8)	0.051±0.004(6)

[a]Underlined groups are not significantly different from each other (p>0.05).

[b]Mean, ± standard error of the mean.

[c]Number of bursa.

Table 4. Mean serum protein levels in CY-treated and control groups (g/dl)[a]

	Control	2 mg x 3	2 mg x 6	4 mg x 3	6 mg x 3
Albumin	1.434 ± 0.142[b]	1.642 ± 0.093	1.504 ± 0.087	1.670 ± 0.065	1.650 ± 0.130
α - globulin	0.360 ± 0.063	0.194 ± 0.020	0.377 ± 0.028	0.336 ± 0.017	0.360 ± 0.026
β - globulin	0.500 ± 0.042	0.462 ± 0.034	0.508 ± 0.029	0.452 ± 0.021	0.478 ± 0.029
γ - globulin	0.178 ± 0.020	0.121 ± 0.010	0.099 ± 0.033	0.095 ± 0.023	0.072 ± 0.013
Total protein (gm)	2.470 ± 0.212	2.494 ± 0.132	2.459 ± 0.142	2.529 ± 0.010	2.534 ± 0.190
	(10)[c]	(9)	(9)	(8)	(6)

[a]Underlined groups are not significantly different from each other ($p > 0.05$).

[b]Mean, ± standard error of the mean.

[c]Number serum samples.

consisted of 2-4 millimeter spherical, white firm nodules.
Necropsy of poults that died during the course of the
experiment revealed extensive hemorrhage of both lungs and
severe congestion of visceral organs.

Histopathology Findings

Bursa of Fabricius

For histological evaluation of morphological changes
in BF, atrophy was classified into 5 grades on the basis of:
(1) size and shape of BF folds, (2) degree of damage in
affected follicles including their size and cellular content
of cortices and medullae, and (3) the amount of interfollicular
connective tissue and degree of fold fibrosis. Bursa with
grade I atrophy had the least changes while BF with grade V
atrophy had the most marked changes.

Normal BF from control poults (Figures 1,2) had elongated
folds filled with polyhedral-shaped follicles. Each follicle
consisted of a well-defined cortex and medulla. Folds were
separated by a thin connective tissue stroma.

Grade I atrophy (Figures 3,4): Mucosal surface was
irregular due to invaginations over atrophied follicles
which resulted in formation of villous-like protrusions on
the mucosal surface. The size and shape of most follicles

was normal. They possessed a well-defined cortex and medulla although the cortex in some was reduced being only 3-4 lymphocytes wide. Other follicles were depleted of lymphocytes and populated entirely with reticulo-epithelial cells and a few lymphocytes. Connective tissue around atrophied follicles was slightly increased.

Grade II atrophy (Figures 5,6): The shape of folds was slightly altered due to more pronounced invaginations of the mucosal surfaces and attenuation. Villous-like protrusions of mucosal surfaces were more prominent. Two to 4 normal lymphoid follicles existed in each fold while the remaining follicles were atrophied. In most atrophied follicles, a distinct cortex could not be identified. It had been replaced by fibrous tissue which was directly adjacent to the undifferentiated epithelium of the medulla. Medullae of affected follicles were entirely populated with reticulo-epithelial cells. Fibrous tissue was moderately increased between atrophied follicles.

Grade III atrophy (Figures 7,8,9): Invagination of the mucosal surface was more extensive and the shape of folds moderately altered. Folds were reduced in size. Follicles could still be identified in all folds but none had normal follicular structure. A few follicles possessed a cortex 1-3 lymphocytes wide. Medullae of all follicles were completely

depleted of lymphocytes and populated entirely with a reticuloepithelial cell network. Interfollicular connective tissue was increased.

Grade IV atrophy (Figures 10,11): Folds were obviously atrophied. Interfollicular epithelium was depressed into the fold because of reduction in size and depletion of follicles. Mucosal surfaces were segmented due to extensive invagination causing marked distortion. There was a decrease in the number of follicles in each fold. Follicles that could still be identified were markedly reduced in size and composed of reticulo-epithelial cells in which no or few scattered lymphocytes could be identified. Interfollicular connective tissue was increased.

Grade V atrophy (Figures 12,13): Normal architecture of folds was severely disrupted. Folds were markedly reduced in size. In some folds, follicular damage had been so severe that the epithelial surface covered only a narrow band of fibrous tissue. A few folds contained small, rudimentary follicles populated entirely with reticulo-epithelial cells. In many folds, no follicles could be identified.

Bursal atrophy was not observed in control poults. In the 2 mg x 3 group there were 2 BF with grade I atrophy, 3 BF with grade II atrophy, and 4 BF with grade III atrophy.

In the 2 mg x 6 group, there were 8 BF with grade II atrophy
and 1 BF with grade IV atrophy. In the 4 mg x 3 group, there
were 5 BF with grade III atrophy and 3 BF with grade IV
atrophy. In the 6 mg x 3 group, there were 5 BF with grade IV
atrophy and 2 BF with grade V atrophy (Figure 14).

Lungs

Microscopically, the previously described gross nodules
were granulomas consisting of a dense focal aggregation of
lymphocytes, macrophages, and a few multinucleated giant
cells. Septate, branching hyphae could be identified in the
granulomatous lesion. Mosphology was compatible with
Aspergillus sp.

Figure 1.   Normal bursa, control poult. Folds are
            elongated and filled with lymphoid follicles
            separated by a thin stroma. Surface
            epithelium is smooth and extends evenly
            along the folds.

Figure 2.   Bursal follicles, control poult. Follicles
            are polyhedral in shape and consist of well
            defined cortex and medulla. Little inter-
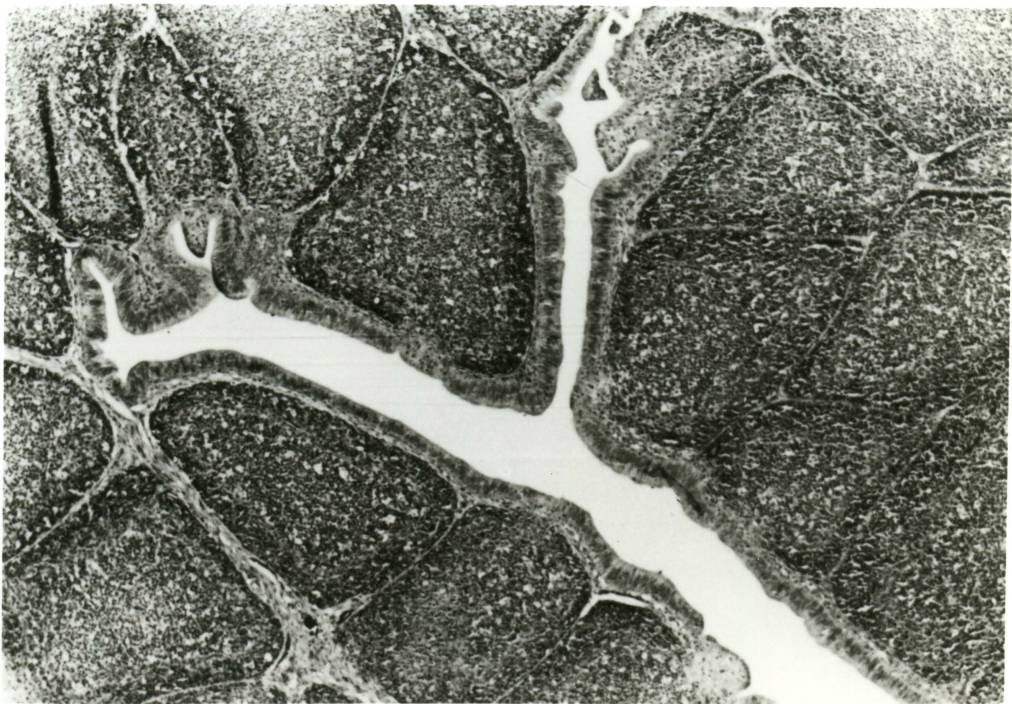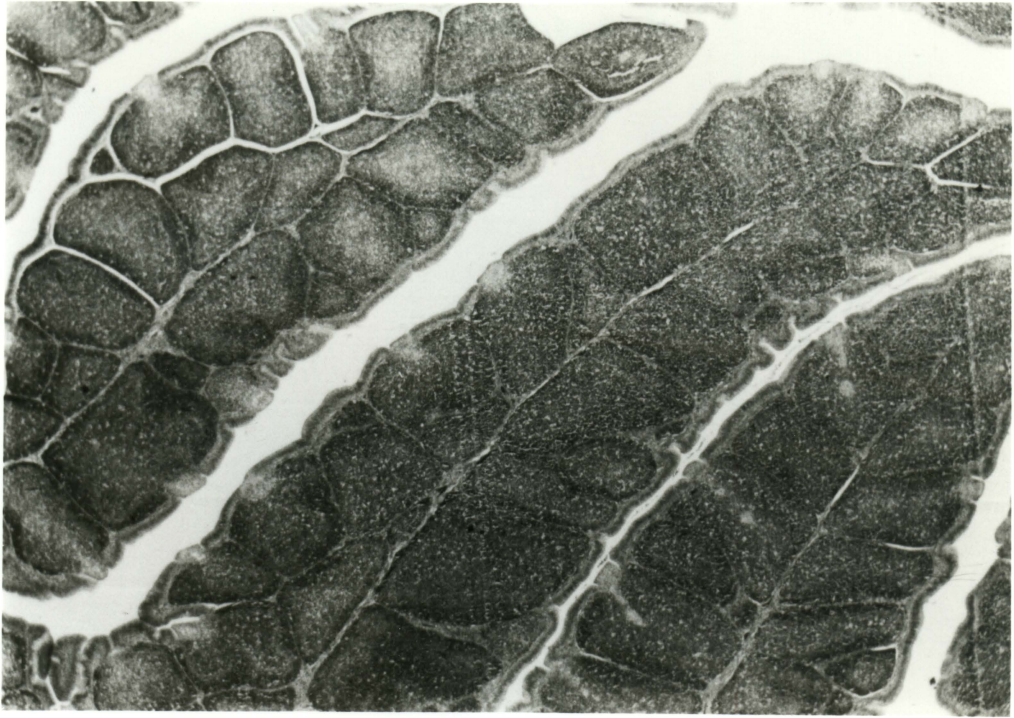   follicular connective tissue is present.

Figure 3. Bursa of Fabricius, CY-treated poult, grade I atrophy. Folds are small and contain a mixture of normal polyhedral follicles and atrophied follicles that lack density of normal follicles. Surface epithelium is irregular in some areas.

Figure 4. Lymphoid follicles from bursa of Fabricius with grade I atrophy. Medullae of some follicles are depleted of lymphocytes and populated with reticulo-epithelial cells. Cortical zone is narrow and contains few lymphocytes. To the left are relatively normal follicles.
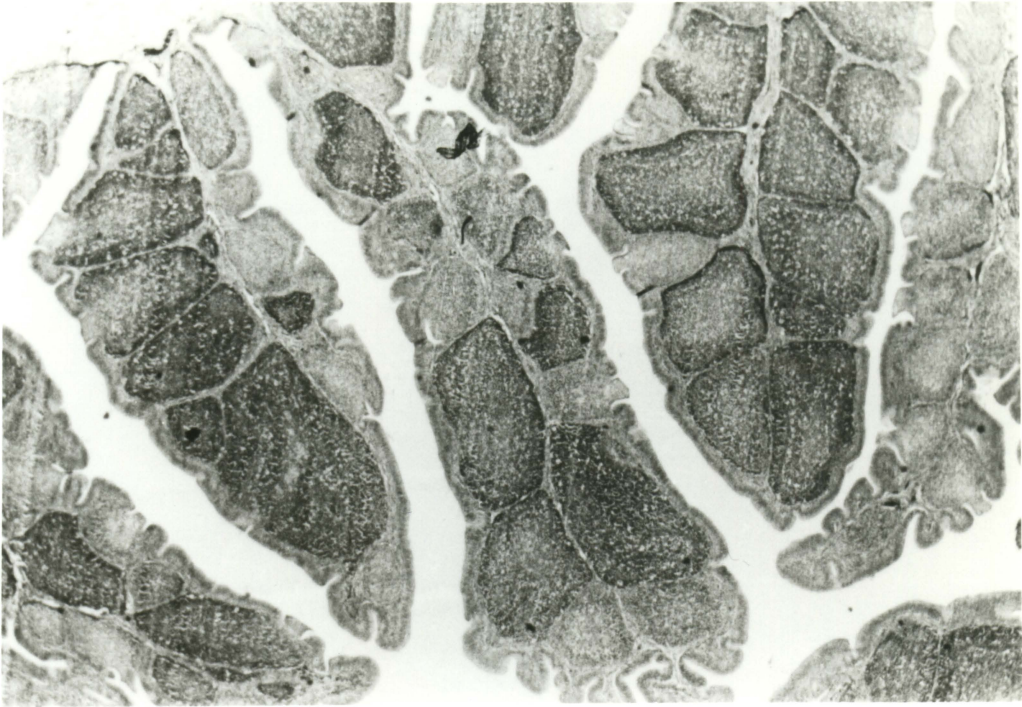
Figure 5. Bursa of Fabricius, CY-treated poult, grade II atrophy. Folds are moderately attenuated and contain few normal follicles. Many atrophied follicles are present. Surface epithelium is invaginated forming fissures and villous-like protrusions.

Figure 6. Lymphoid follicles from bursa of Fabricius with grade II atrophy. Two normal follicles with well defined cortex and medulla are present. Atrophied follicles are small, round in shape, and lack a lymphocyte population. Other areas of the fold are replaced by fibrous tissue. Invagination of the mucosal surface is marked.
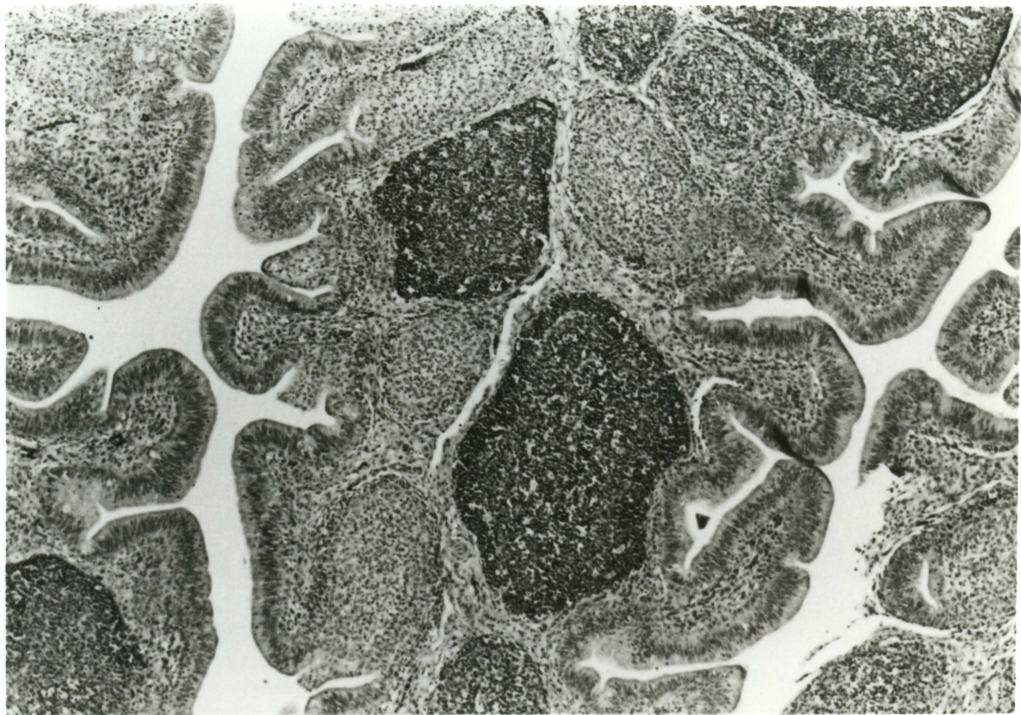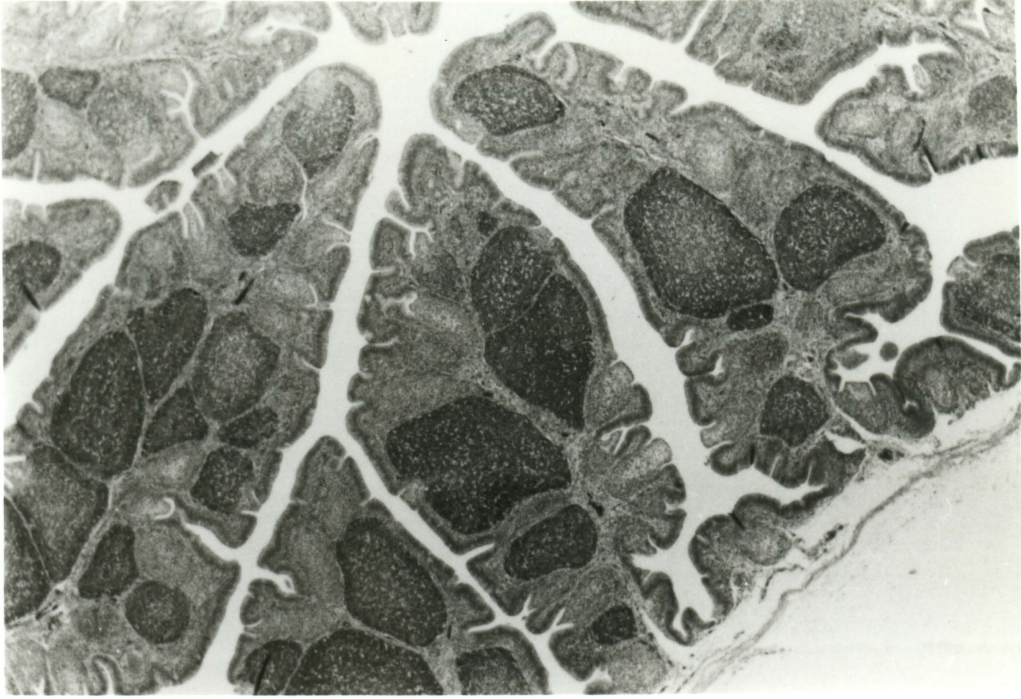
Figure 7. Bursa of Fabricius, CY-treated poult, grade III atrophy. Folds are reduced in size and lack normal follicles. Only small atrophied follicles are present. Invagination of the surface epithelium is marked.

Figure 8. Lymphoid follicles from bursa of Fabricius with grade III atrophy. All follicles are small and round to oval in shape. Medulla is populated entirely with reticulo-epithelial cells. Cortical zone is narrow and contains few lymphocytes. There is increased interfollicular connective tissue.
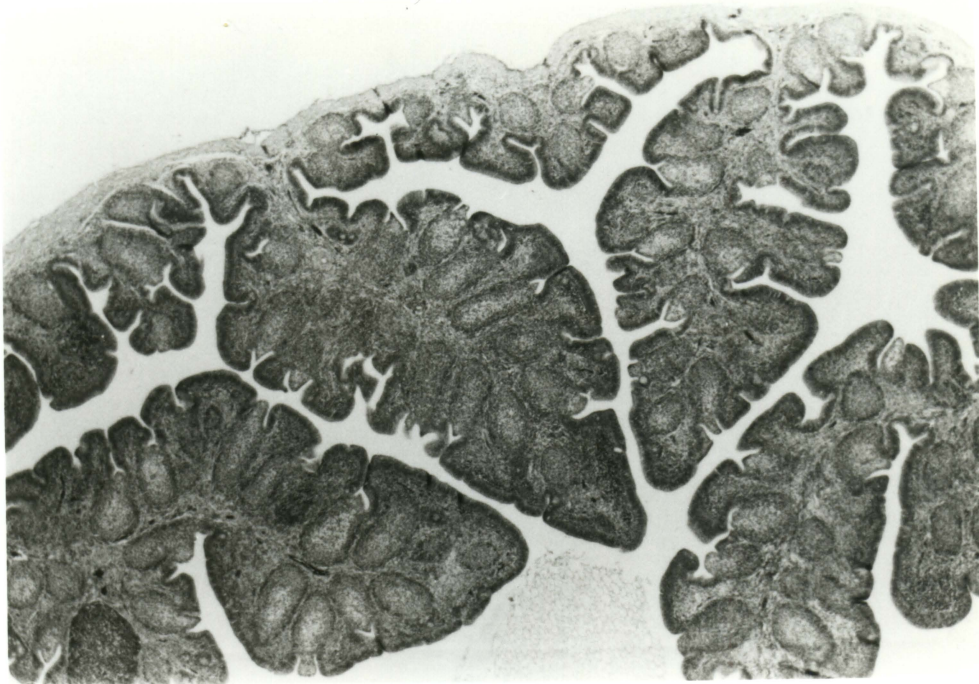
Figure 9.   Lymphoid follicles from bursa of Fabricius
            with Grade III atrophy. Medulla of follicles
            is entirely depleted of lymphocytes and
            populated with reticulo-epithelial cells.
            Cortices are composed of a narrow zone of
            1-3 lymphocytes.
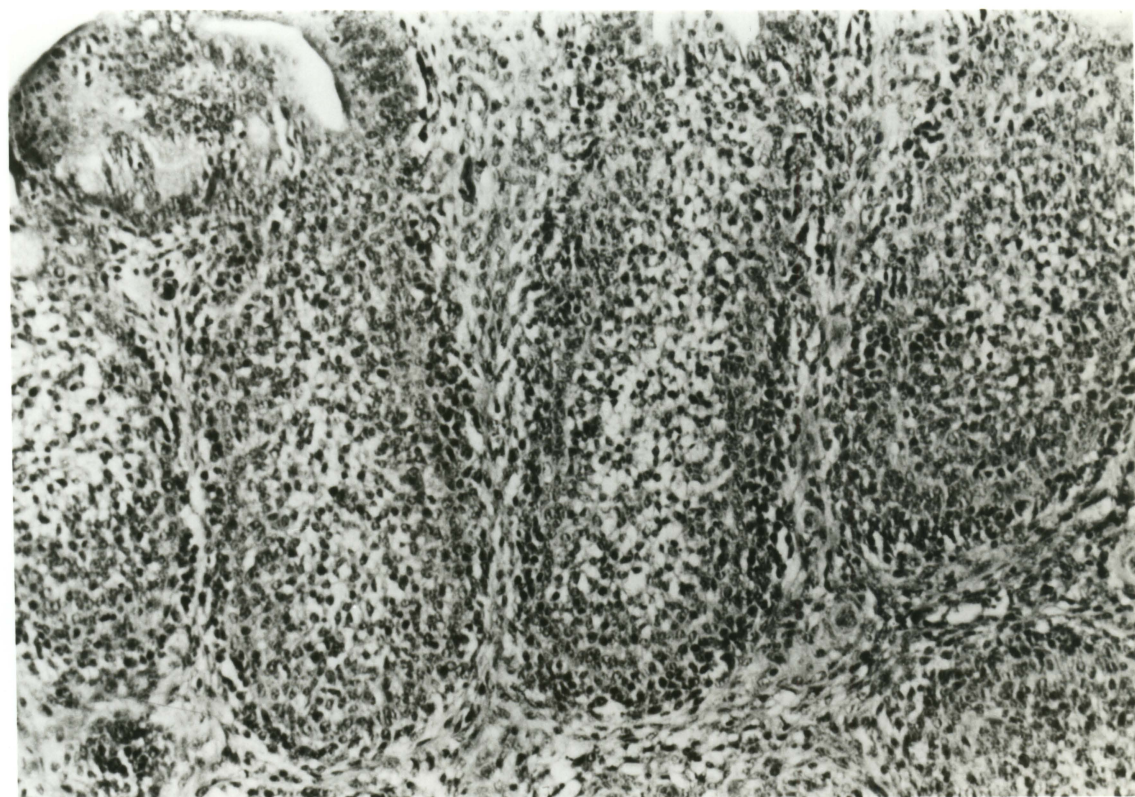
Figure 10.   Bursal of Fabricius, CY-treated poult,
             grade IV atrophy. Folds are small, narrow,
             and contain few, small atrophied follicles.

Figure 11.   A bursal fold, CY-treated poult, grade IV
             atrophy. Well-defined follicles can not be
             identified and are represented by areas of
             reticulo-endothelial cells. Invagination of
             surface epithelium is severe converting the
             normal smooth fold surface into one with
             extentive protrusions and fissures. Inter-
             follicular connective tissue is increased.
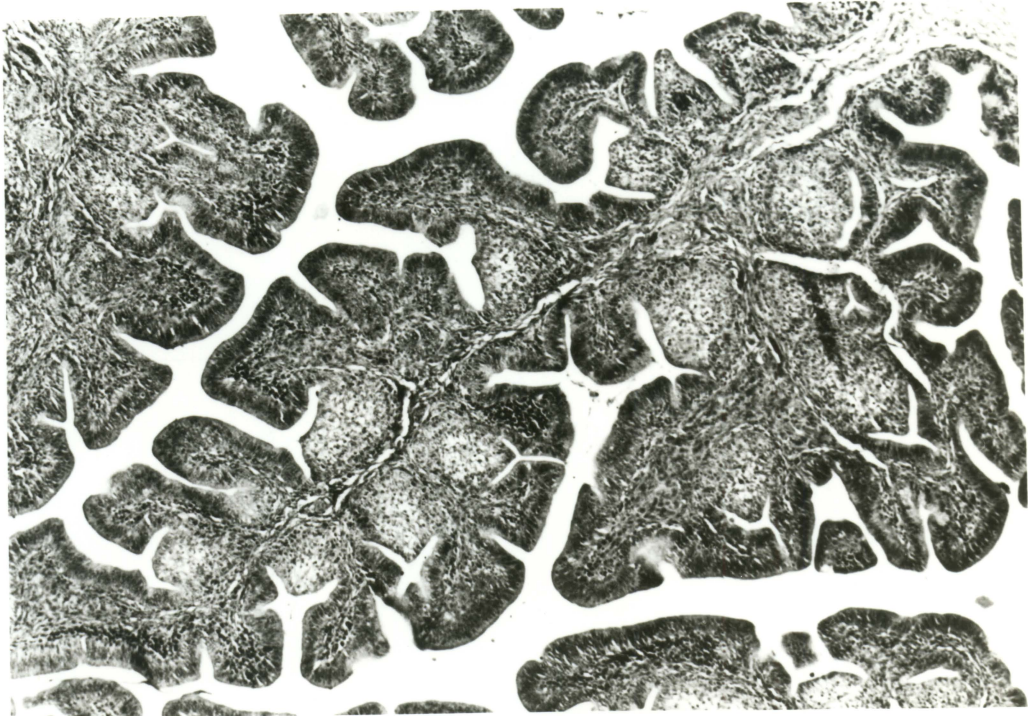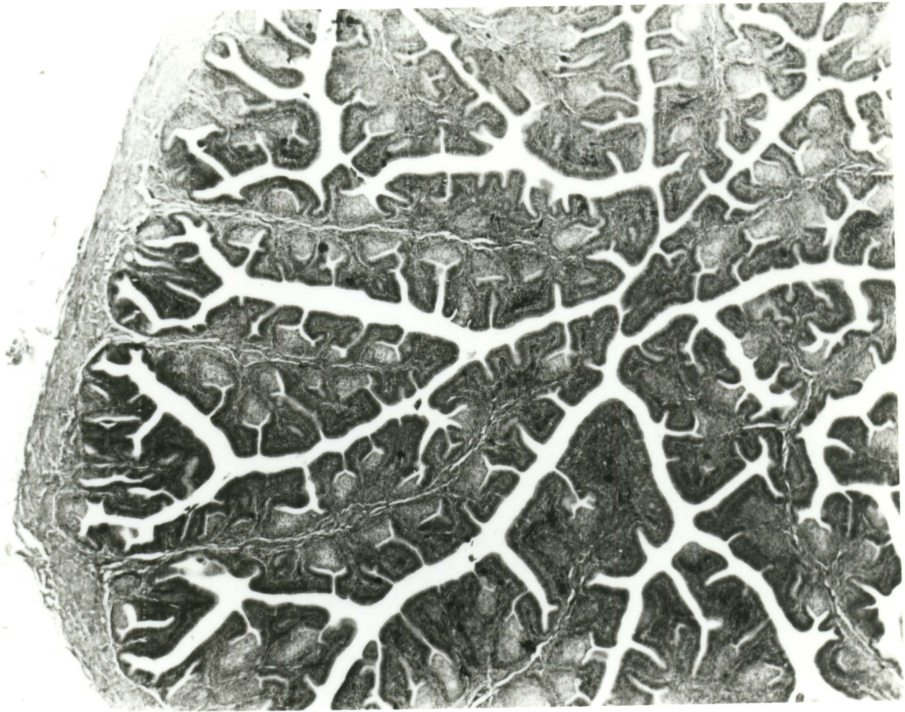
Figure 12.   Bursa of Fabricius, CY-treated poult,
              grade V atrophy. Architectural outline of
              folds is severely distorted.

Figure 13.   A bursal fold, CY-treated poult, grade V
              atrophy. Few rudimentary follicles are present.
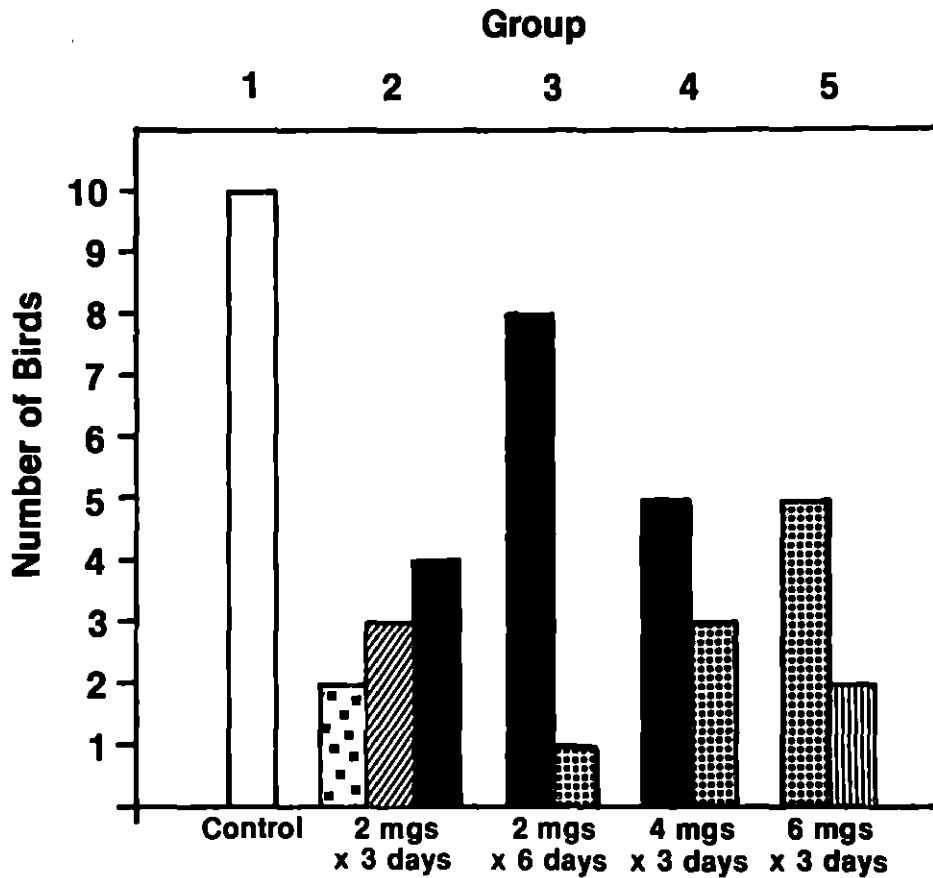   Interfollicular connective tissue is markedly
              increased.

Figure 14.   Degree of bursal atrophy in poults treated
            with cyclophosphamide.

Bursal Atrophy in CY Treated Turkeys

DISCUSSION

This experiment clearly demonstrated that CY profoundly suppresses growth, γ - globulin synthesis, and BF development in turkeys. The effect on body weight was dose-related while the effect on BF weight was not dose-related. Suppression of body weight following CY administration has been reported in chickens (Glick 1971, Hiraga et al. 1976, Prasad 1978) and ducks (Hashimoto and Sugimura 1976). Reduction in size and weight of BF was a consistent finding in CY-treated chickens (Glick 1971, Hiraga et al. 1976, Toma et al. 1977, Sharma and Lee 1977), ducks (Hashimoto and Sugimura 1976), and pigeons (Coignoul and Vindevogel 1980).

Bursae of Fabricius from control poults had normal histologic morphology as has been described previously (Hoskins 1977). Microscopic studies of BF from CY-treated poults revealed that CY induced marked changes. Reduction or absence of lymphocytes from follicles in CY-treated poults indicated that CY was cytotoxic for B lymphocytes since they make up the majority of lymphocytes in the BF (Sites et al. 1980). Presence of grade I atrophy in 2 mg x 3 group and grade V atrophy in 6 mg x 3 group indicates that a correlation between dose and degree of bursal damage exists although this was not reflected on bursal weight.

Presence of different grades of BF atrophy in the same group might be explained in 2 ways. First, it may have resulted from individual variation among poults in their ability to metabolize CY by microsomal enzymatic activity of the liver and generate cytotoxic metabolites. Second, individual poults may have differed in their ability to regenerate damaged lymphoid follicles. Distinguishing between these 2 possibilities would require answering whether the normal or relatively normal follicles observed at 28 days were original or regenerating follicles. A definite answer for this question cannot be obtained from this experiment. However, the irregular distribution of normal or relatively normal BF follicles in CY-treated poults favors the second explanation i.e., these follicles represent regenerating follicles.

Why some BF have more regenerating follicles and why some follicles appear to be regenerating faster and more efficiently are additional questions that need to be answered. It is possible that damaged follicles must regenerate themselves i.e., lymphocytes needed for repopulation of damaged follicles must be derived from remaining intact lymphocytes. This is likely since B lymphocyte precursors from bone marrow of 4-day-old chicks were unable to reconstitute BF follicles in CY-treated 4-day-old chicks

(Toivanen et al. 1972a). Thus, the degree of follicular damage and ability to regenerate are probably directly correlated and severely damaged follicles may not be able to regenerate at all or only regenerate slowly depending on the number of intact lymphocytes remaining in these follicles. Support for this concept is provided by the results of this experiment in that more normal follicles, as reflected by lower grades of atrophy, were found in poults receiving lower doses of CY. It would appear that rate of CY metabolism, size of lymphoid follicle, and number of lymphocytes in the follicle are the 3 most important factors which will determine the degree of lymphocyte depletion, severity of follicular damage and potential for follicle regeneration. Restoration of damaged lymphoid follicles by blood-born progenitor cells has been proposed by Linna et al. (1972). It is possible that some circulating B lymphocytes can survive CY-treatment and enter the BF. If circulating B lymphocytes are responsible for restoring damaged lymphoid follicles, why do some follicles regenerate while others do not in a particular bursa?

Among serum protein fractions, γ- globulin was the only fraction which was significantly lowered by CY-treatment. Hypo-γ-globulinemia has been reported in chickens and ducks treated with CY (Lerman and Weidanz 1970, Linna et al.

1972, Rouse and Szenberg 1974, Hashimoto and Sugimura 1976).
This selectivity of CY to suppress γ-globulin synthesis
results from the high toxicity of CY for B lymphocytes
(Spreafico and Anaclerio 1977) which markedly reduces
the number of their progeny (plasma cells) that synthesize
γ-globulin (Benacerraf and Unanue 1980). Albumin, α- and
β-globulins are synthesized in the liver (Cheville 1976).
Total serum protein was not significantly lowered in CY-
treated poults because γ-globulins comprise only 36.3% of
the total serum proteins in fowls (Rice 1968) and there were
compensatory changes in other protein fractions.

Surgical bursectomy of chickens at hatching had little
or no effect on γ-globulin synthesis (Cooper et al. 1966a,
Arnason and Jankovic 1967, Meter et al. 1969, Cooper et al.
1969). Failure of surgical bursectomy to induce hypo-γ-
globulinemia probably results from peripheralization of
B lymphocytes to secondary lymphoid organs in the early
phases of ontogenic development (Cooper et al. 1966a,
Cooper et al. 1966b, Linna et al. 1970, Linna et al. 1971).
The more severe CY-induced hypo-γ-globulinemia suggests that
CY destroys B lymphocyte populations in the bursa of Fabricius
as well as those in the secondary lymphoid organs.

The low antibody titers in both CY-treated and control
groups in this experiment may be due to either improper

route of immunization or the short period between the second booster dose and the collection of the sample.

Spontaneous aspergillosis and colibacillosis in CY-treated poults indicates resistance to these diseases may be directly related to efficiency of the immune system. High mortality has been reported in surgically bursectomized chicks infected with $\underline{E}$. $\underline{coli}$ (Heller and Perek 1974).

Results obtained from this experiment indicate that CY can be used in the turkey for inducing bursal atrophy and that the degree of atrophy can be modulated by controlling the dose.

EXPERIMENT II: SEQUENTIAL CHANGES IN THE BURSA OF
FABRICIUS OF TURKEYS FOLLOWING
CYCLOPHOSPHAMIDE TREATMENT

MATERIALS AND METHODS

Turkeys

Fertile eggs preincubated for 25 days were purchased from a commercial hatchery and incubated at the Veterinary Medical Research Institute. After hatching, 50 poults were identified with wing-tags, weighed, divided randomly into 2 groups, and transferred to 2 wire-floor battery units where they were kept under the same conditions and fed the same rations as experiment I.

Experimental Design

Poults were divided into 2 groups; 27 in group 1 and 23 in group 2. Poults in group 1 were treated with CY while group 2 birds served as untreated controls. Poults from each group were killed at 3, 7, 10, 14, 21, and 28 days of age. Parameters used for evaluation were: (1) daily clinical signs, (2) mortality, (3) body weight at necropsy, (4) BF weight expressed as a percentage of total body weight, (5) total serum protein, (6) serum protein electrophoresis, (7) gross and microscopic pathology, and (8) ultrastructure of BF.

Preparation and Use of Cyclophosphamide

100 mg CY (Cytoxan[R]) was dissolved in 12.5 ml sterile
distilled water. Poults in group 1 were injected intra-
peritoneally with 4 mg/poult/day (0.5 ml) for 3 successive
days starting from the hatching day. Poults in group 2 were
injected intraperitoneally with 0.5 ml sterile distilled
water on the same schedule as group 1 birds.

Necropsy

Table 1 indicates the number of poults necropsied at
each age. Before necropsy, body weight was recorded, blood
was collected from the jugular vein (except from the 3 day-
old group), and poults were killed by cervical dislocation.
The BF was removed from the body using the same technique as
in experiment I, weighed, injected with cold 3.5% gluteralde-
hyde in 0.1 M sodium cacodylate buffer (pH 7.2), and divided
by a transverse cut with a sharp razor blade into 2 halves.
One half was placed in gluteraldehyde solution; the other
half was opened and individual folds were carefully removed
with cuts by a sharp razor blade. Folds were placed in
gluteraldehyde solution for electron microscopic examination.

Poults were opened and examined for gross lesions. Any
abnormal tissue was removed and placed in 10 percent neutral
buffered formalin for light microscopic examination.

Table 1. Number of poults necropsied at each age

Age (day)	CY-group	Control group
3	4	4
7	6	4
10	4	4
14	5	4
21	3	3
28	3	4

Total Serum Protein Determination and
Serum Protein Electrophoresis

Total serum protein values and serum protein electrophoresis were determined by the same instruments and techniques used in experiment I.

Preparation of Tissues for Histologic Examination

The procedures were used as in experiment I except BF were fixed in gluteraldehyde solution for about 15 hours before being transferred to formalin.

Preparation of Tissues for
Transmission Electron Microscopic Examination

Tissues were fixed in gluteraldehyde solution for 6 hours, washed 3 times for 15 minutes each in 0.1 M sodium

cacodylate buffer (pH 7.2), post-fixed for 3 hours in 1 percent osmium tetroxide in 0.1 M cacodylate buffer, and washed twice with distilled water. They were then dehydrated in graded alcohols, placed overnight in a 1:1 mixture of propylene oxide and epoxy resin, placed in 100 percent epoxy resin for 2 hours, put into a vacuum chamber for 60 minutes, oriented in molds containing epoxy resin and held in the vacuum chamber for 4 hours. They were polymerized at 60°C for 48 hours and 4-6 folds were embedded for each BF. Thick sections were cut and examined by light microscopy. From selected blocks ultrathin sections were cut with an LKB 8802A ultratome[1] using a glass knife. Sections were placed on a 200 mesh copper grid, stained with uranyl acetate and post-stained with lead citrate. Sections were examined with Hitachi HS-9 electron microscope[2] at 50 KV. Negatives were printed on Kodachrome papers.[3]

Statistical Analysis

Testing for signifcance between CY-treated and control group means was performed by Student's t test.

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[1]LKB Producter AB, Stockholm, Sweden.
[2]Hitachi Scientific Instrument, Nissei Sangyo American, LTD, Lombard, Il.
[3]Eastman Kodak Company, Rochester, New York.

RESULTS

Clinical Signs and Mortality

Cyclophosphamide markedly suppressed growth. A significant difference in body weights was found between CY-treated and control groups at 7 and 14 days of age. Body weights of CY-treated poults killed at 3, 10, 21, and 28 days of age were consistently lower than those of the same age control poults although the difference was not significant (Table 2).

Two poults from the CY-treated group died during the first 14 days of the experiment and at day 14, one CY-treated poult had nervous signs of ataxia, falling over backwards, and paddling. Aspergillosis was diagnosed in all 3 poults on the basis of histologic findings.

Development of Bursa of Fabricius

The BF from CY-treated poults killed at different ages were obviously smaller than those from control poults. The BF weight of CY-treated poults killed at all ages was significantly different from BF weight of same age controls (Table 3).

Table 2. Mean body weight of CY-treated and control
          poults (gm)

Age (day)	CY-treated	Control
3	$73.72 \pm 2.83^{*}$	85.70 ± 2.33
7	$112.68 \pm 2.79^{\Delta}$	145.60 ± 6.02
10	153.05 ± 12.42	181.00 ± 21.59
14	$193.10 \pm 22.73^{\Delta}$	261.35 ± 16.82
21	433.47 ± 48.34	561.87 ± 8.35
28	469.43 ± 61.03	565.32 ± 36.44

*Mean ± standard error of the mean.

$^{\Delta}$Mean significantly differs from control ($p < 0.05$).

Table 3. Mean bursa percent in CY-treated and control
          groups (gm)

Age (day)	CY-treated	Control
3	$0.082 \pm 0.008^{\Delta *}$	0.108 ± 0.008
7	$0.077 \pm 0.002^{\Delta}$	0.153 ± 0.009
10	$0.054 \pm 0.010^{\Delta}$	0.171 ± 0.019
14	$0.067 \pm 0.007^{\Delta}$	0.193 ± 0.018
21	$0.043 \pm 0.003^{\Delta}$	0.191 ± 0.029
28	$0.043 \pm 0.008^{\Delta}$	0.154 ± 0.013

*Mean ± standard error of the mean.

$^{\Delta}$Mean significantly differs from control ($p < 0.05$).

Serum Proteins

The level of γ-globulin in all CY-treated groups
was consistently lower than its level in control groups.
At 28 days levels of α- and β-globulins and total proteins
were higher in the CY-treated group than control group.
At 7, 10, 14, and 21 days, levels of albumin, α- and β-
globulins and total proteins of CY-treated groups were
sometimes higher than their levels in control groups. No
difference in any serum protein value between CY-treated
and control groups was significant (Table 4).

Gross Pathology

Necropsy of the poult with nervous signs revealed
small grey nodules in both lungs and small white spots in
the cerebellum. Necropsy was performed on 1 poult that
died during the experiment; one lung was severely congested
and had a large focal area of pneumonia. The other bird
was putrified and not examined.

Histology of Bursa of Fabricius

Bursae from 3-day-old control poults had elongated
folds filled with oval to round lymphoid follicles separated
by a thin interfollicular connective tissue stroma. Cortices
and medullae were well demarcated and populated with

Table 4. Mean of serum proteins in CY-treated and control poults (gm/dl)

	7 days	10 days	14 days	21 days	28 days
Albumin					
Control	1.833±0.109[*]	1.618±0.051	1.767±0.051	1.543±0.043	1.560±0.101
CY-treated	1.605±0.106	1.686±0.108	1.342±0.180	1.597±0.086	1.034±0.169
α-globulin					
Control	0.486±0.023	0.522±0.104	0.529±0.057	0.450±0.047	0.501±0.040
CY-treated	0.533±0.025	0.413±0.031	0.567±0.096	0.469±0.095	1.001±0.287
β-globulin					
Control	0.616±0.041	0.604±0.067	0.710±0.015	0.532±0.014	0.595±0.037
CY-treated	0.536±0.043	0.359±0.055	0.592±0.059	0.635±0.066	0.930±0.257
γ-globulin					
Control	0.138±0.035	0.112±0.028	0.196±0.033	0.230±0.011	0.487±0.036
CY-treated	0.084±0.008	0.076±0.024	0.152±0.063	0.187±0.032	0.369±0.066
Total Proteins (gm)					
Control	3.058±0.170	2.829±0.168	3.199±0.131	2.727±0.087	3.118±0.085
CY-treated	2.734±0.150	2.524±0.138	2.633±0.153	2.858±0.145	3.304±0.467

[*]Mean ± standard deviation of the mean.

76

lymphocytes. Surface epithelium was smooth and composed of pseudostratified columnar interfollicular epithelium and polygonal-follicular epithelial cells (Figures 1,2,3).

Bursae from 3-day-old CY-treated poults were morphologically different from those of the same age control birds. Surface epithelium was normal in some folds, while in other folds it was invaginated forming fissures and protrusions on the surface. Invagination of the surface epithelium resulted in acinar-like formations within folds. This invagination was caused by a reduction in the amount of underlying supportive follicular tissue. Follicular pads were thin due to attenuation and decrease in the number of follicular epithelium cells (Figure 4).

Changes were most striking in the follicular portion of the folds. Development of lymphoid follicles was markedly suppressed and in some folds no follicle could be identified; instead fibrous tissue had replaced them. Within the fibrous tissue many acinar-like structures could be seen (Figure 5). They varied in size and shape and were lined with interfollicular epithelium. In other folds, follicles were small and had a round instead of polyhedral outline. In most follicles,the cortex and medulla were not demarcated, could not be identified, and the cortical zone had been replaced by a rim of connective tissue which gave follicles an encapsulated

appearance.

Cellular components of follicles, had been markedly
altered (Figure 6). Medullae were affected to various
degrees. In some follicles, the medulla was completely
depleted of lymphocytes and entirely populated with reticulo-
epithelial cells which gave the medulla a net-like appearance.
In other follicles the medulla was partially depleted and
contained only few scattered lymphocytes. In contrast to
the controls, mitotic figures were not seen in the few
remaining lymphocytes in the medulla. There was a marked
increase in interfollicular connective tissue.

Histology of BF from 7, 10, 14, 21, and 28-day-old
control poults was similar to that of BF from 3-day-old
control poults except lymphoid follicles were larger,
polyhedral, and had wider cortices (Figures 7,8).

At 7 days of age, invagination of BF surface epithelium
was more severe. Some plicae had gland-like structures
(Figure 9). Lymphoid follicles were small and round in
shape (Figure 10). Cellular components of these follicles
had been severely affected. The medulla of most follicles
was completely depleted of lymphocytes and populated with
reticulo-epithelial cells. Cortex was absent in most
follicles. Because of this, undifferentiated epithelium
between cortex and medulla became obvious forming a single

cell layer adjacent to the connective tissue rim which had replaced the cortex (Figure 11).

At 10 days of age, BF had the same histological architecture as BF from 7-day-old poults (Figure 12). In some follicles, many plasma cells were seen in both cortex and medulla. Some cortices were populated to a large extent by plasma cells (Figures 13,14). Interfollicular connective tissue was abundant and contained many plasma cells.

At 14 days of age, invagination of surface epithelium was obvious and lymphoid follicles had been variably depleted of lymphocytes. The cortex of most follicles had been replaced by fibrous tissue which directly abutted a clear zone of undifferentiated epithelium. In other follicles, the cortex was narrow and populated with numerous plasma cells. The medulla of most follicles was severely depleted of lymphocytes and populated predominantly with reticulo-epithelial cells and a few plasma cells. One BF had many relatively normal lymphoid follicles (Figure 15). Other follicles had a lymphocyte-depleted medulla populated entirely with reticulo-epithelial cells. An amorphous, eosinophilic material was present in affected follicles. Interfollicular connective tissue was abundant and contained many plasma cells (Figure 16).

At 21 days of age, no histologic changes distinct

from those observed previously were found.

At 28 days of age, the surface epithelium was less
invaginated and in most areas had a more normal appearance.
Interfollicular connective tissue was still increased in
some areas. Histologic architecture of follicles was quite
variable. Some follicles had a normal size, shape, and
lymphocyte population with well-defined cortex and medulla
while other follicles were small and depleted of lymphocytes
(Figure 17). In 1 BF, some follicles had intrafollicular
lakes of various sizes containing an homogenous, pale
eosinophilic material (Figure 18). The medulla of a few
follicles contained the same amorphous, eosinophilic
materials found in BF of 14-day-old poults. The cortex of
most depleted follicles was populated with numerous plasma
cells.

Ultrastructure of Bursa of Fabricius

Ultrastructure findings confirmed histological findings.
Additional subcellular changes were not identified. Ultra-
structural changes were seen exclusively in the follicular
epithelium, cortex, and medulla. No cytopathologic changes
were seen in the interfollicular epithelium.

Follicular epithelium from BF of 3, 7, 10, 14, 21,
and 28-day-old control poults was composed of several layers

Figure 1.   A bursal fold from 3-day-old control poult.
            The surface epithelium is smooth. The
            fold is filled with round to oval lymphoid
            follicles.

Figure 2.   Lymphoid follicles from bursa of Fabricius
            of 3-day-old control poult. Cortices and
            medullae are well demarcated. Cortices are
            narrow, not well-developed and 3-4 lymphocytes
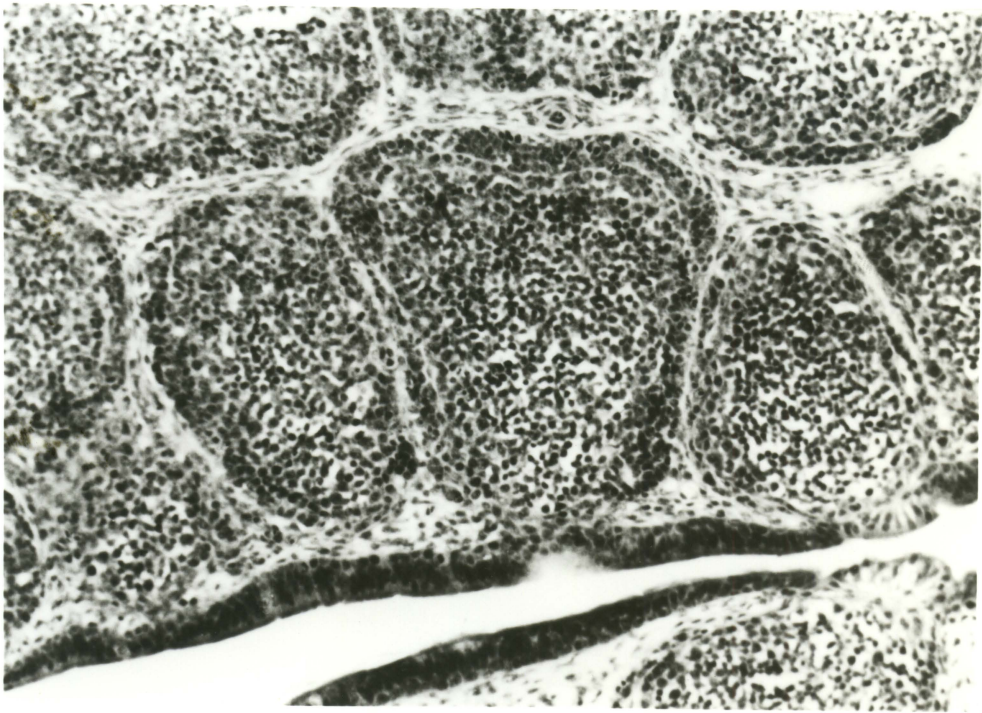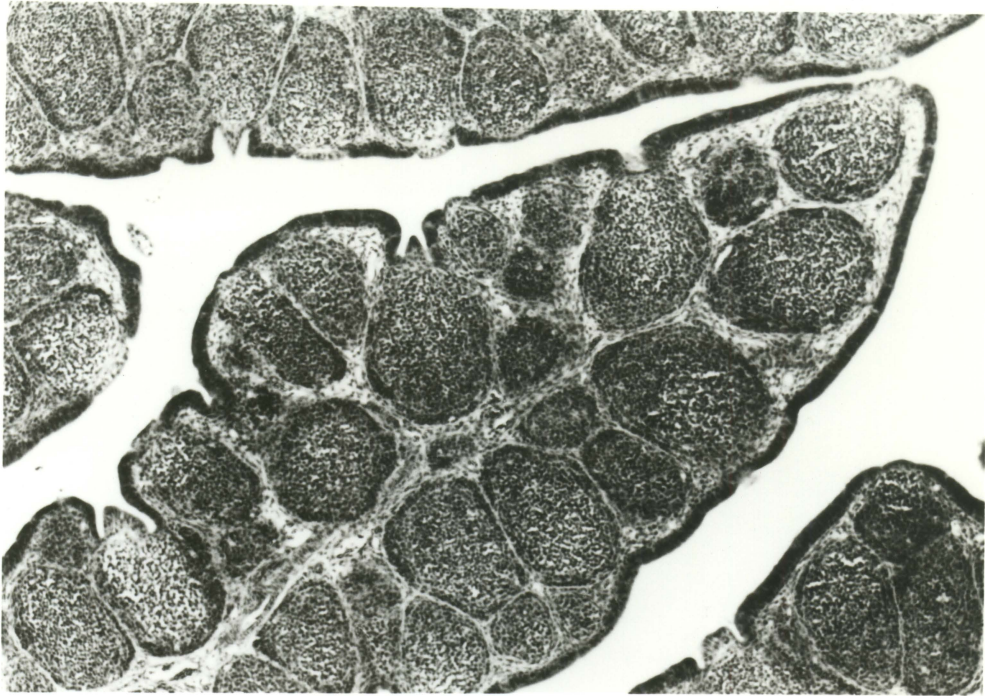   wide. Medullae are well-developed and
            populated with lymphocytes.

Figure 3.   Follicular epithelium from bursa of Fabricius
            of 3-day-old control poult. Epithelial cells
            are polygonal and form a continuous cellular
            layer over the follicle. Intercellular spaces
            are small.

Figure 4.   Follicular epithelium from bursa of Fabricius
            of 3-day-old CY-treated poult. Epithelial
            cells are attenuated and decreased in number.
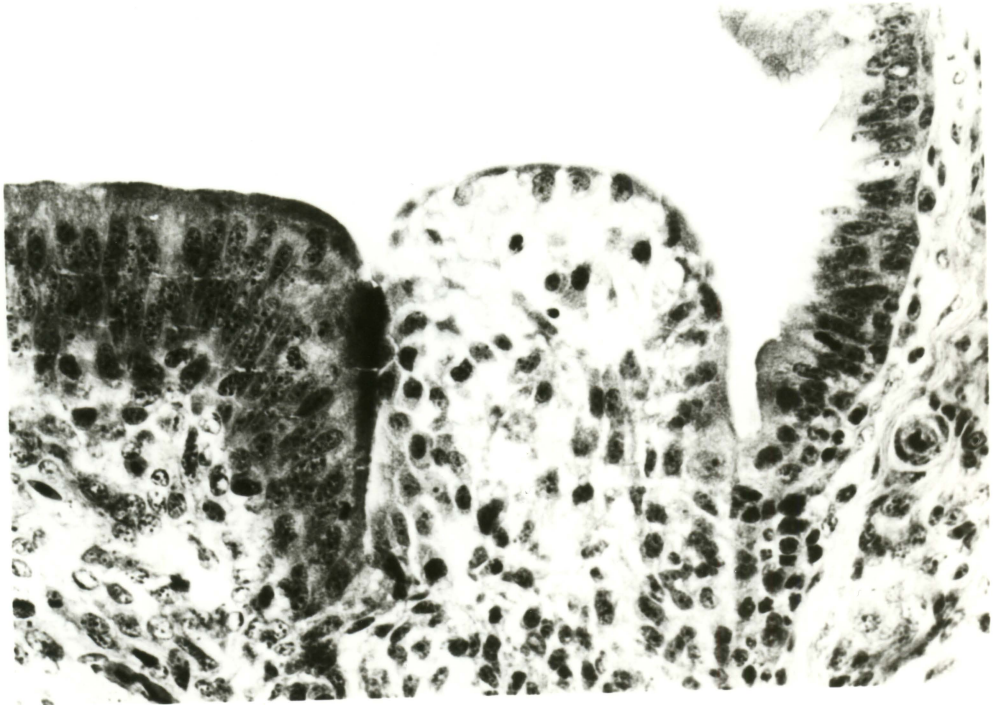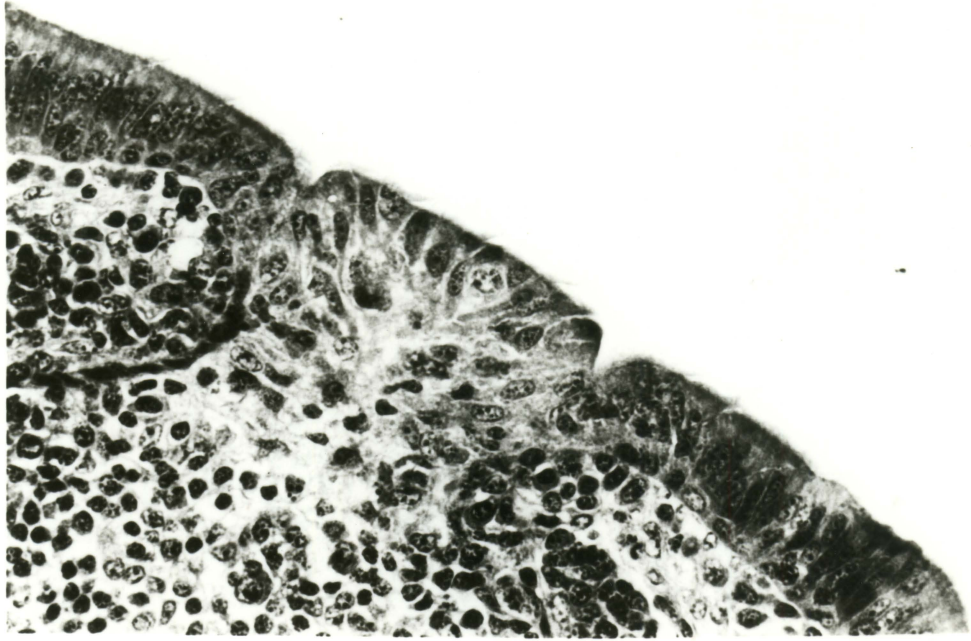   Intercellular spaces are large.

Figure 5. Bursal folds of 3-day-old CY-treated poult.
          Histologic features of the folds are destroyed.
          Extensive plical fibrosis has replaced
          follicles. Acinar-like structures are seen
          within the fibrous tissue.

Figure 6. Lymphoid follicles from the bursa of Fabricius
          of a 3-day-old CY-treated poult. Size of
          follicles is reduced. Cortical zones have
          been replaced by a rim of fibrous tissue.
          Medullae are populated with reticulo-epithelial
          cells and largely depleted of lymphocytes
          although a few scattered ones can be seen.
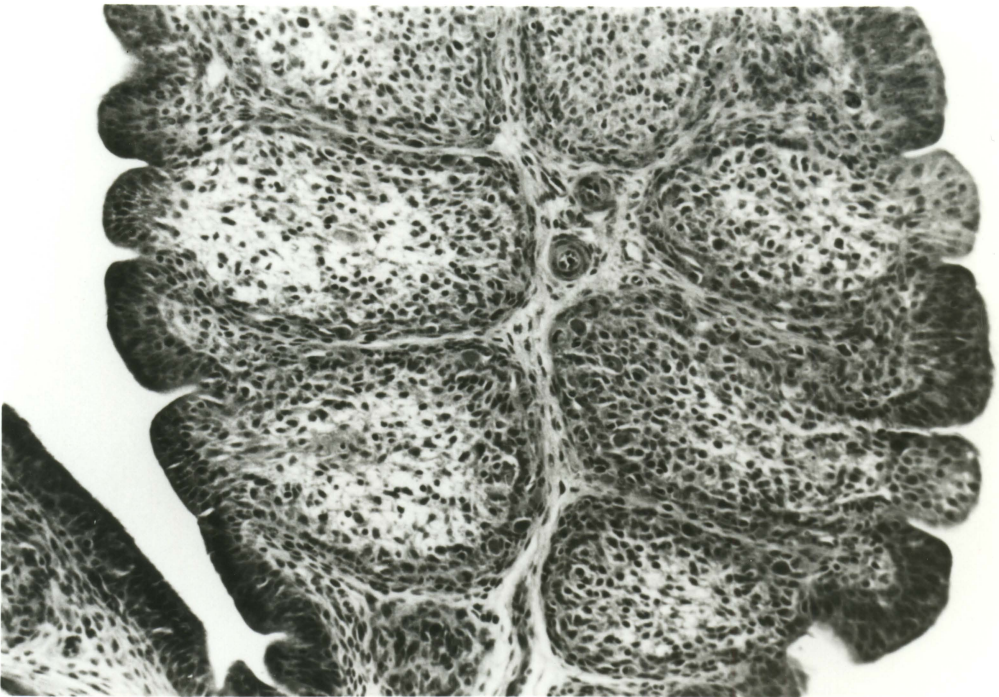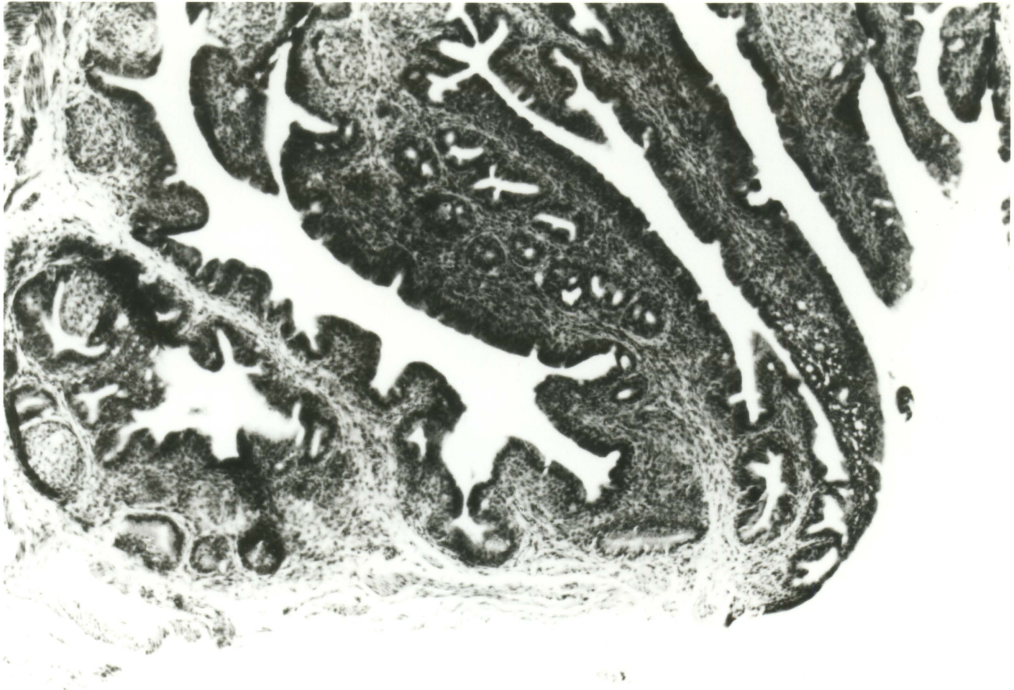
Figure 7.   Bursal folds of 14-day-old control poult.
             Folds are filled entirely with polyhedral
             lymphoid follicles. Cortices and medullae
             are well identified. A small amount of
             fibrous tissue separates follicles.

             Bursal folds of 7, 10, 14, 21 and 28-day-old
             control poults had the same histologic appearance.

Figure 8.   A lymphoid follicle from bursa of Fabricius
             of 14-day-old control poults. Cortex and medulla
             are well demarcated. The cortex is 8-9 lympho-
             cytes wide and populated with closely packed
             lymphocytes. Medulla is populated predominantly
             with a less dense lymphocyte population.

             Lymphoid follicles from the bursa of Fabricius
             of 7, 10, 14, 21 and 28-day-old control poult
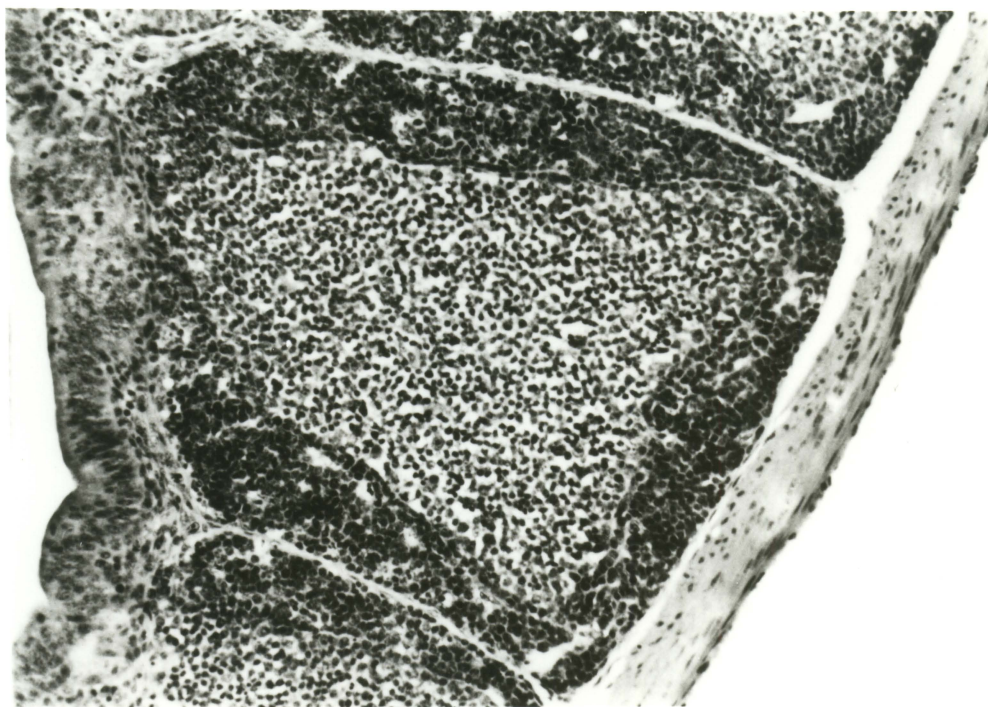   had the same histologic features.

Figure 9. A bursal fold of 7-day-old CY-treated poult.
Histological features of the fold are destroyed.
Extensive plical fibrosis replaces lymphoid
follicles. Gland-like structures are seen
within the fold.

Figure 10. Bursal folds of 7-day-old CY-treated poult.
Folds are markedly reduced in size and contain
many atrophied, round follicles which have
a decreased cellular density. There is increased
interfollicular connective tissue.

Figure 11. Lymphoid follicles from bursa of Fabricius of
7-day-old CY-treated poult. Cortices have
been replaced by a rim of fibrous tissue.
Undifferentiated epithelial cells are prominent,
have a dark nucleus, and form a zone between
the depleted medulla and cortical area.
Medullae are depleted of lymphocytes and
populated with reticulo-epithelial cells.
Interfollicular connective tissue is increased.
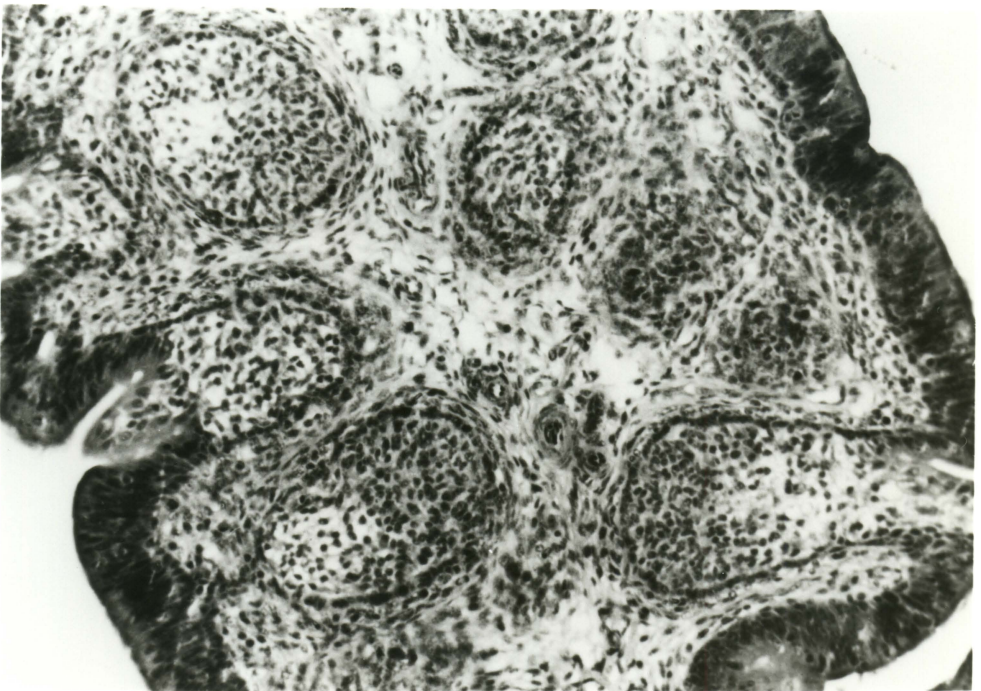
Figure 12.   Lymphoid follicles from bursa of Fabricius
             of 10-day-old CY-treated poult. Follicles
             are atrophied and round in shape. Cortices
             are replaced by a rim of fibrous tissue.
             Undifferentiated epithelial cells are prominent,
             have dark nuclei, and form a zone between
             the depleted medullae and the cortical areas.
             Medullae are depleted of lymphocytes and
             populated with reticulo-epithelial cells.
             There is an increase in interfollicular
             connective tissue.

             Some lymphoid follicles from bursa of Fabricius
             of 14 and 21-day-old CY-treated poult had the
             same histologic appearance.

Figure 13.   Lymphoid follicles from bursa of Fabricius of
             10-day-old CY-treated poult. Cortices and
             medullae can be identified. Cortices are narrow
             and populated to a large extent by plasma cells
             (dark nuclei). Medullae are depleted of lympho-
             cytes and populated with reticulo-epithelial
             cells and many plasma cells. Interfollicular
             connective tissue is abundant and contains
             scattered plasma cells.

             Some lymphoid follicles from bursa of Fabricius
             of 14-day-old CY-treated poult had the same
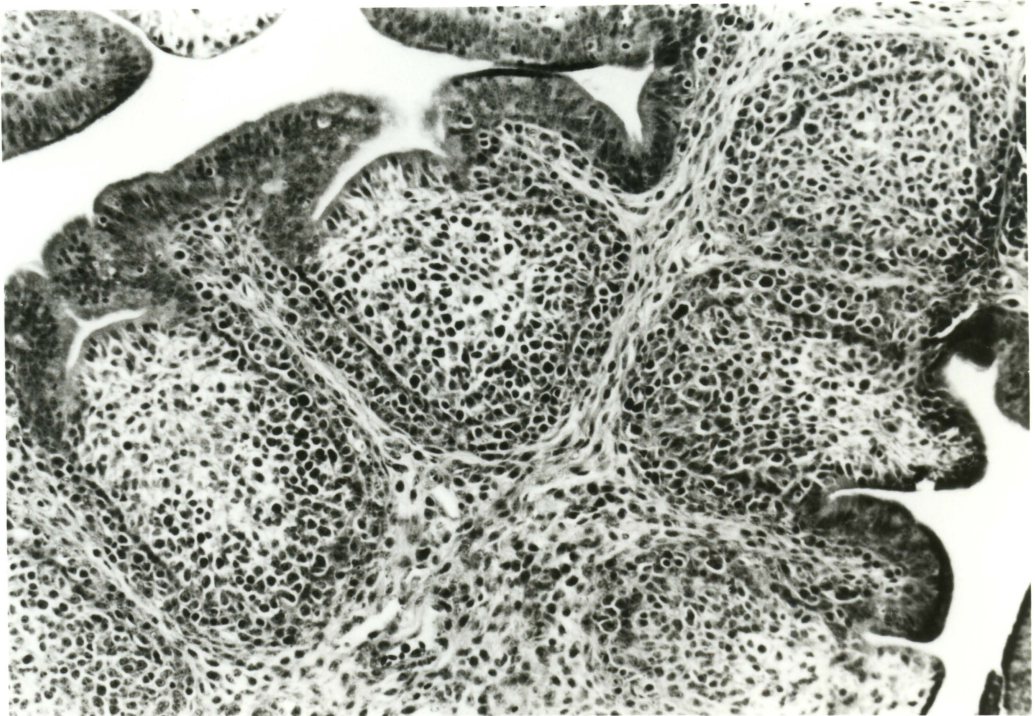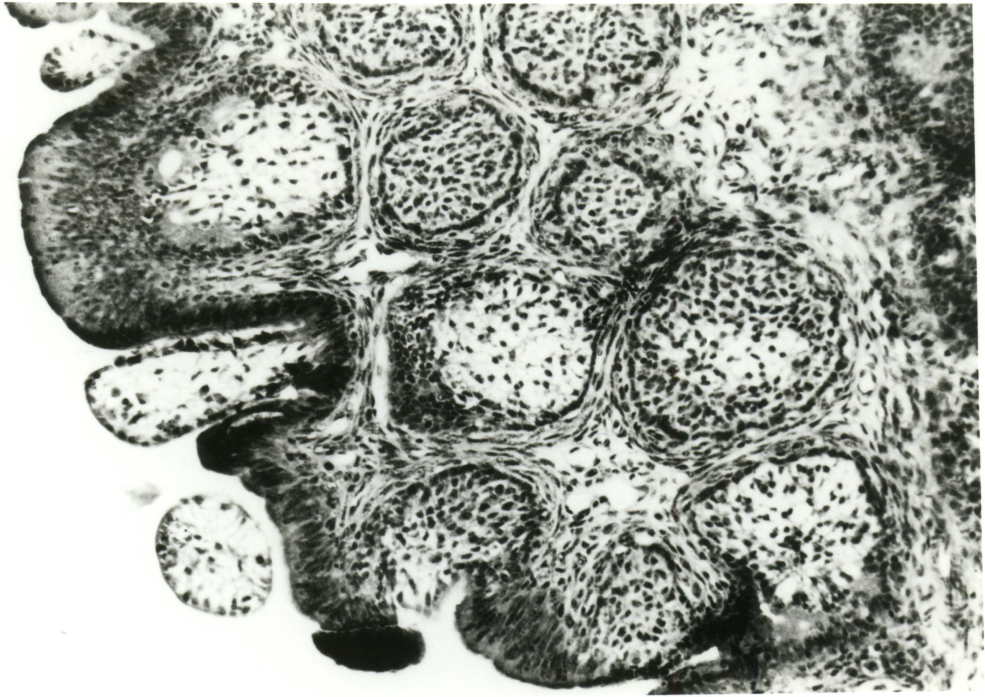   histologic appearance.

Figure 14. A lymphoid follicle from bursa of Fabricius
of 10-day-old CY-treated poult. Detail of
follicle in Figure 13. Cortex and medulla are
separated by a distinct zone of undifferentiated
epithelium. Cortex is narrow and populated with
plasma cells. Numerous plasma cells are also
seen in the depleted medulla and in the inter-
follicular connective tissue.

Plasma cell populated lymphoid follicles from
bursa of Fabricius of 14-day-old CY-treated
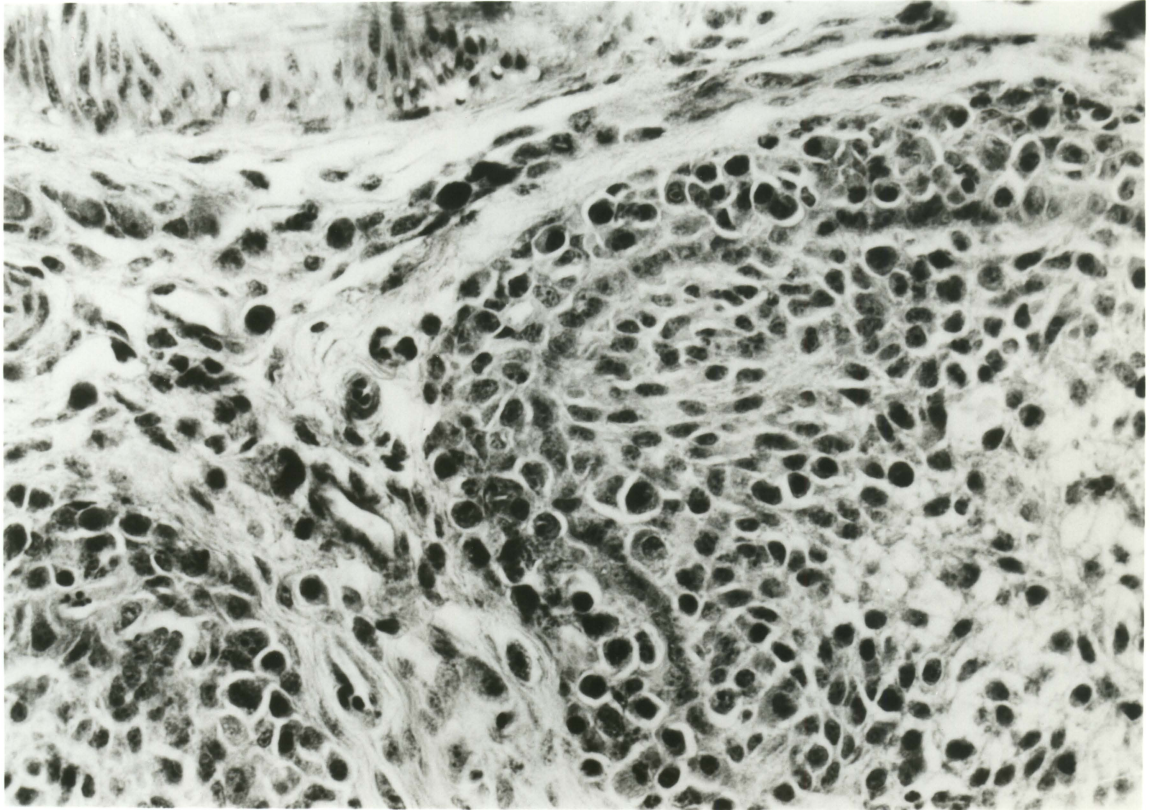poults had the same histologic detail.

Figure 15.   Bursal fold from 14-day-old CY-treated
             poult. Fold contains many small round
             to oval follicles. Cortical and medullary
             outlines can be identified in those follicles.
             Medullae of some follicles have normal
             lymphocyte populations while others have a
             pale center indicative of lymphocyte depletion.

Figure 16.   Lymphoid follicles from bursa of Fabricius
             of 14-day-old CY-treated poult. Medullae
             contain amorphous, eosinophilic material and
             are populated with reticulo-epithelial cells
             and occasional plasma cells. Cortical areas
             are narrow and populated with numerous plasma
             cells. Interfollicular connective tissue
             is increased and contains many plasma cells.

             Some lymphoid follicles from bursa of Fabricius
             of 28-day-old CY-treated poults also had
             intrafollicular, amorphous, eosinophilic
             material.

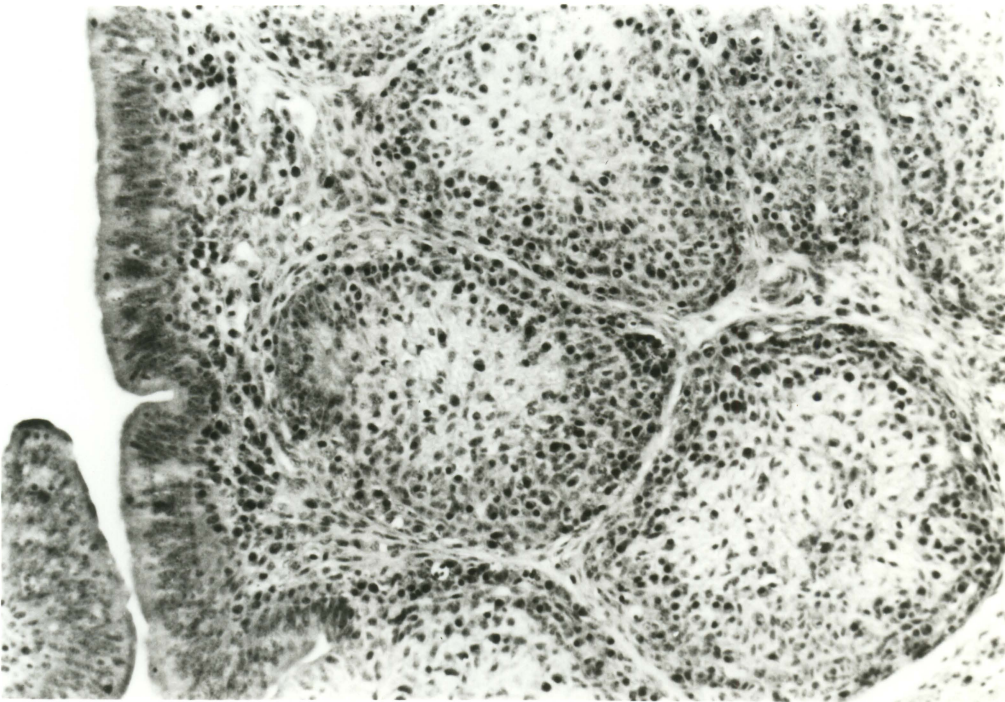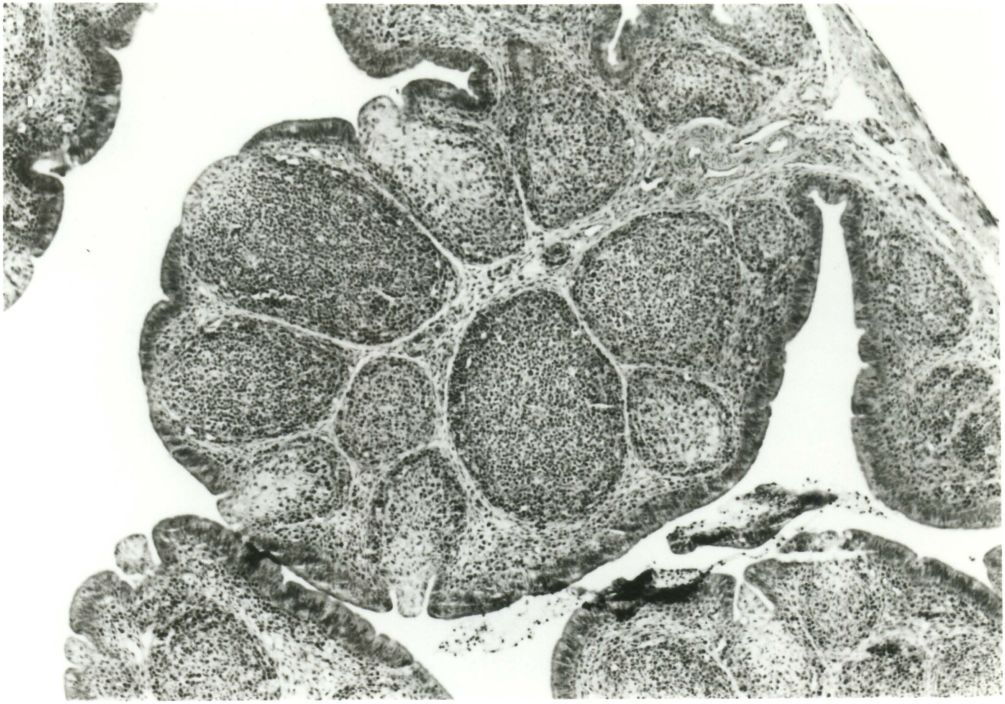Figure 17.   Bursal fold from 28-day-old CY-treated poult.
              Fold contains lymphoid follicles of variable
              size and shape.   Some follicles are large and
              have polyhedral outlines.   Small follicles
              are either round or polyhedral in shape.
              Medullae of some small follicles are devoid
              of cells.   Interfollicular connective tissue
              is moderately increased.

Figure 18.   Lymphoid follicles from bursa of Fabricius of
              28-day-old poult.   To the right is a normal
              follicle with well-defined cortex and medulla.
              In the middle is a depleted follicle; the
              medulla of which contains many intrafollicular
              lakes.   To the left is another depleted follicle.
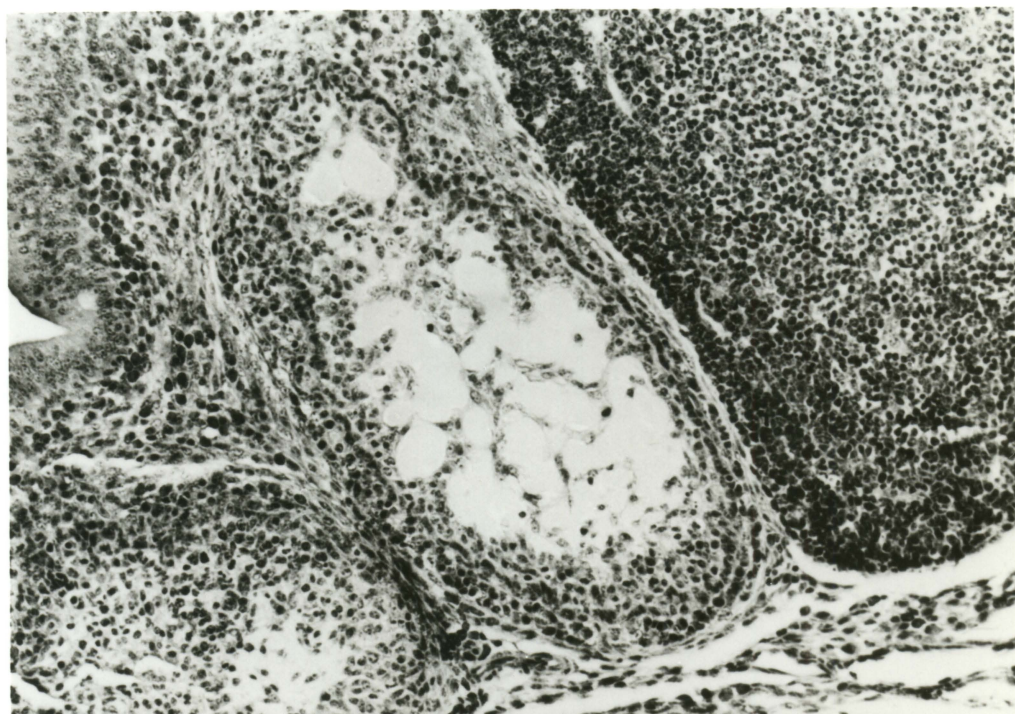
of polygonal-shaped epithelial cells which bore microvilli on their lumenal surface and joined each other by inter-cellular tight junctions (Figure 19).

In BF from 3-day-old CY-treated poults, the follicular epithelial cells were strikingly decreased in number leaving only a single cell layer over the follicle. This change was accompanied by a remarkable increase in intercellular space (Figure 20). The remaining cells had a misshapen appearance; some of them were stretched and elongated while others had irregular outlines instead of the normal polygonal appearance. Intercellular junctions between remaining cells appeared intact and tight junctions could still be identified at the apex of the cells.

Lymphoid follicular cortices of 3-day-old control poults were approximately 3-4 lymphocytes wide (Figure 21). Follicular cortex of the same age CY-treated poults was depleted of lymphocytes (Figure 22). Immediately beneath the basement membrane in the cortical region only fibro-blasts could be identified. In some areas of the depleted cortex, small groups of collagen fibrils were seen associated with fibroblasts.

Medullae of lymphoid follicles from 3-day-old control poults were populated with lymphocytes and lymphoblasts. Occasionally reticulo-epithelial cells were seen (Figure 23).

Medullae of different lymphoid follicles from the same age CY-treated poults had the entire spectrum of lymphocyte depletion and reticulo-epithelial cell population. Some follicles had a completely depleted medulla which was entirely populated with reticulo-epithelial cells (Figure 24). In other follicles, a few lymphocytes and lympho-blasts could be identified between reticulo-epithelial cells (Figure 25). The number and distribution pattern of reticulo-epithelial cells varied in different follicles. In some follicles only reticulo-epithelial cells were seen, and in some areas only their cytoplasmic processes could be identified. Intercellular spaces were large and empty (Figure 24). In other medullae, a large number of reticulo-epithelial cells were seen and intercellular spaces were small and contained sectional profiles of cytoplasmic processes (Figure 25). Reticulo-epithelial cells varied in morphology but all had tonofibrils in their cytoplasm, and long cytoplasmic processes which were often connected by desmosomes to processes of adjacent reticulo-epithelial cells.

Lymphoid follicles from the BF of 7, 10, 14, 21, and 28-day-old control poults had the same ultrastructural appearance as those of 30day-old control poults except cortex widened to approximately 8 lymphocytes (Figures 26,

27,28).

The same changes observed in follicular epithelium
of 3-day-old CY-treated poults were also seen in treated
poults killed at subsequent ages (Figure 29). The ultra-
structure of depleted lymphoid follicular cortices and
medullae from BF of older treated birds were also very
similar to the ultrastructure of depleted follicles from
BF of 3-day-old CY-treated poults (Figure 30).

Between 10 and 28 days, plasma cells in different
stages of differentiation could be found in cortices
(Figures 31,32) and medullae (Figures 33,34). Most plasma
cells were mature and contained rough endoplasmic reticulum
cisternae filled with an electron dense material. In the
cortex, plasma cells tended to lie along or in close
association with the basement membrane.

The amorphous eosinophilic material seen histologically
appeared in electron micrographs as uniform, electron dense
granular material between reticulo-epithelial cells (Figure
35). Intrafollicular lakes appeared ultrastructurally as
uniform, light granular material (Figure 36).

Figure 19. Follicular epithelium from bursa of Fabricius
of 10 day-old control poult. The epithelium
is composed of several layers of polygonal-
shaped epithelial cells. The luminal surface
of the epithelium bears irregularly-shaped
microvilli. Intercellular tight junctions
(arrows) are prominent. Mitochondria and
rough endoplasmic reticulum can be identified.
Intercellular spaces contain sectional profiles
of microvilli.

Follicular epithelium from bursa of Fabricius
of 3, 7, 14, 21, and 28 day-old control poults
had the same ultrastructural appearance.

Figure 20.   Follicular epithelium from bursa of Fabricius
             of 3-day-old CY-treated poult. There is a
             decrease in the number of cells. The epithelium
             is composed of a single cell layer. Cells are
             stretched and have irregular outline. Inter-
             cellular tight junctions (arrows) and cellular
             organelles appear intact. Intercellular spaces
             are large and contain a few sectional profiles
             of microvilli. The cell in the center is
             probably a heterophil.

             Follicular epithelium from bursa of Fabricius
             of 7, 10, 14, 21, and 28 day-old CY-treated
             poults had the same ultrastructural appearance
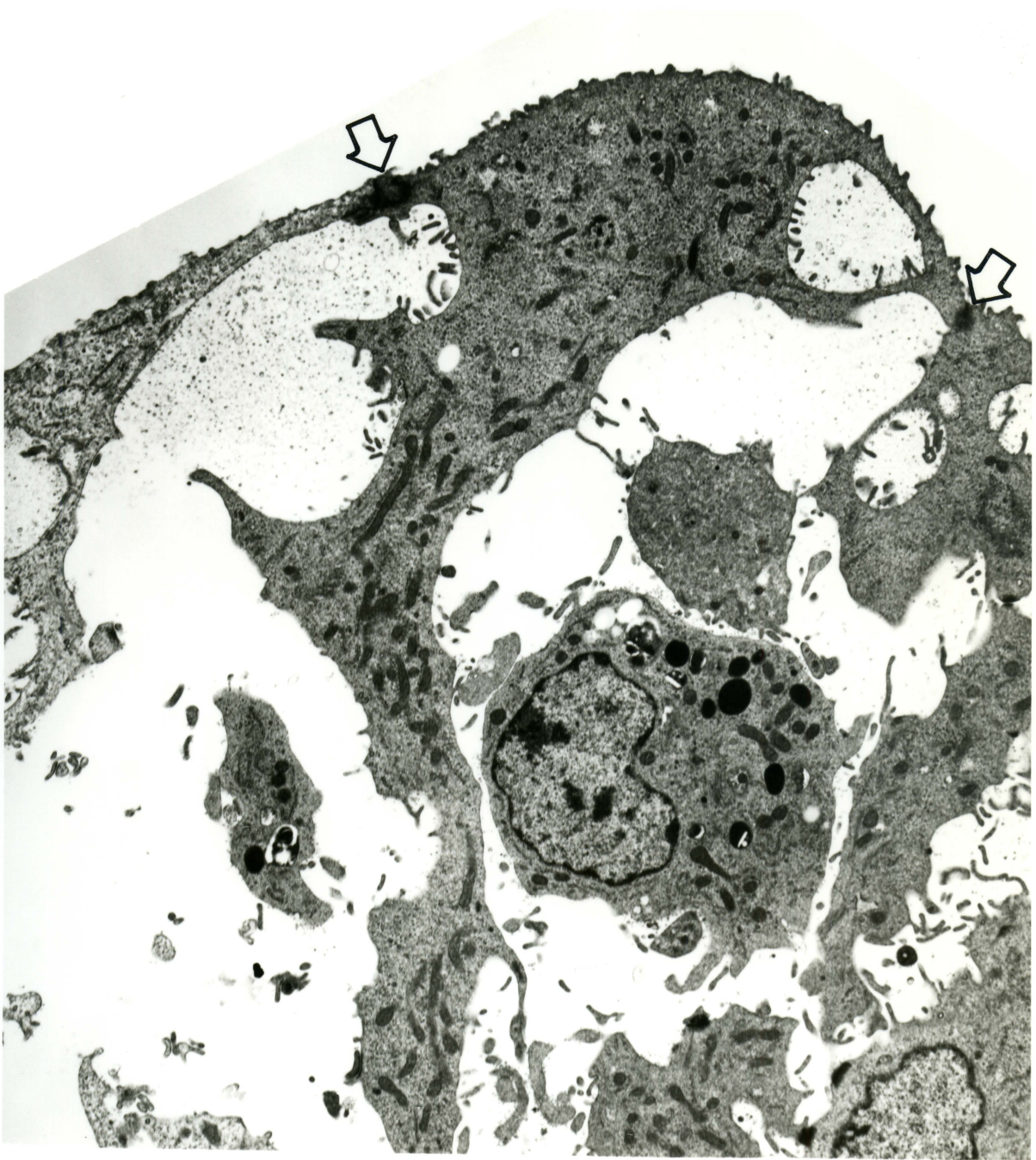   (see also Figure 26).

Figure 21. Cortex of lymphoid follicle from bursa of Fabricius of 3-day-old control poult. Cortex is not well developed (approximately 4 lymphoid cells wide), and populated entirely with lymphocytes (LY) and lymphoblasts (LB). Two cells in mitosis (MI) are present. Interfollicular area (INF) contains fibroblasts (FB) and collagen fibrils. Basement membrane (arrow) and part of the medulla can also be seen.
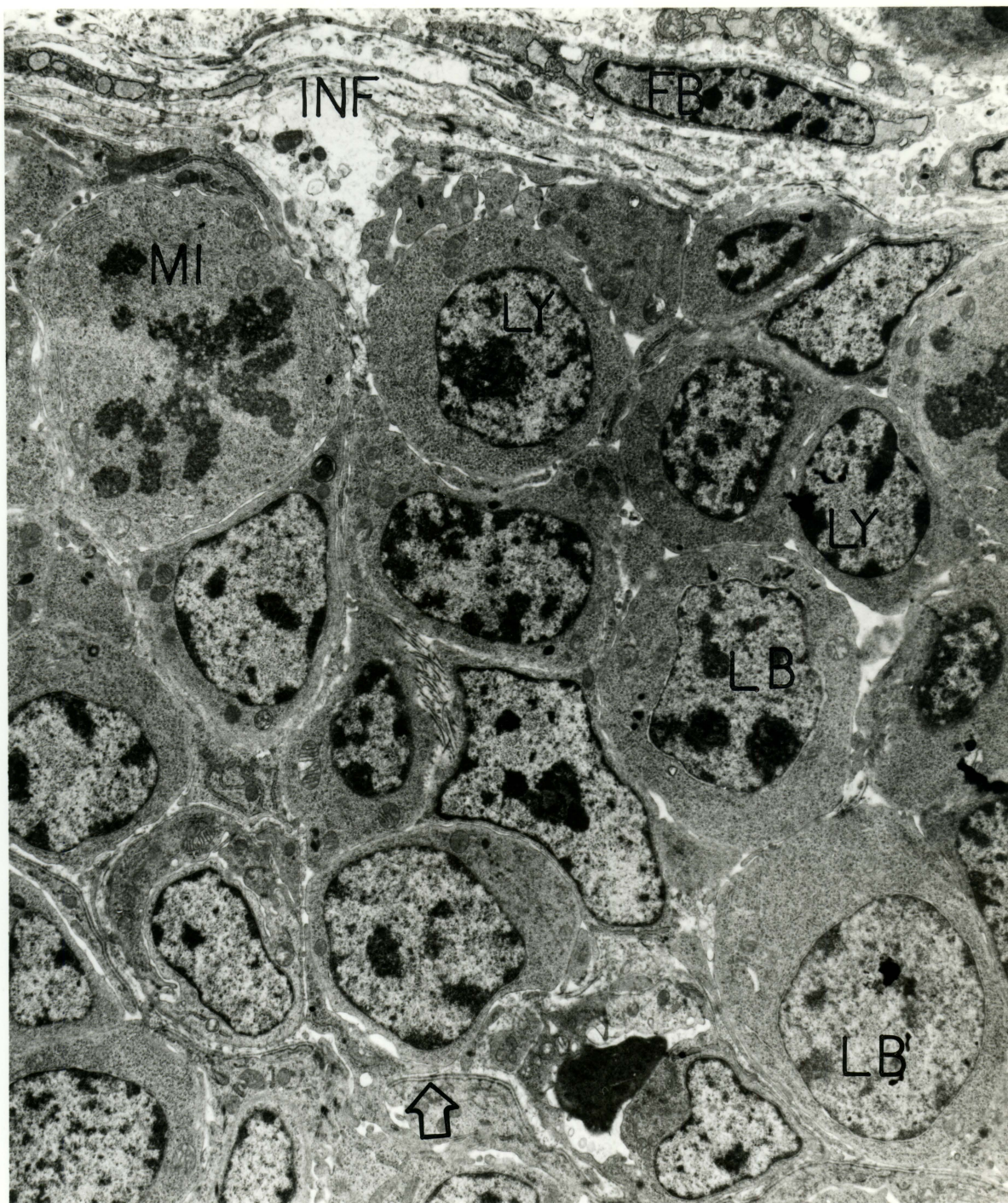
Figure 22. Cortex and cortico-medullary border of
lymphoid follicle from the bursa of Fabricius
of 3-day-old CY-treated poult. Cortex is
depleted of lymphocytes and populated with
fibroblasts (FB) which have long filopodia
and dilated cisternae of endoplasmic reticulum.
Collagen fibrils (C) are seen between fibro-
blasts. Undifferentiated epithelium (UE)
is located in the medullary region along
the basement membrane (arrow).

Cortex of some lymphoid follicles from bursa
of Fabricius of 7, 10, 14, 21, and 28 day-old
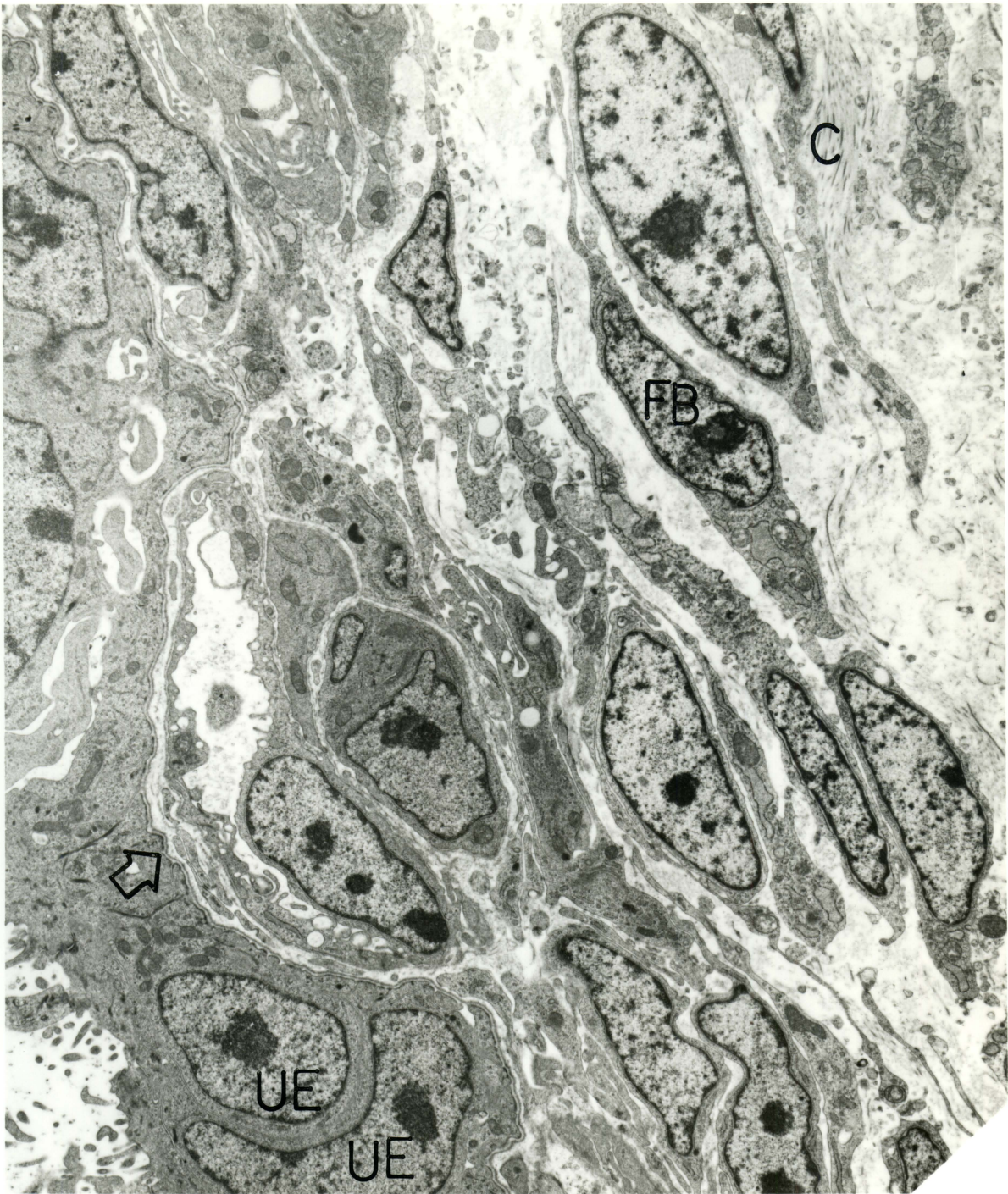CY-treated poults had a similar ultrastructural
appearance.

Figure 23.   Medulla of lymphoid follicle from bursa of
Fabricius of 3-day-old control poult. The
medulla is populated with lymphocytes (LY)
and lymphoblasts (LB). A reticulo-epithelial
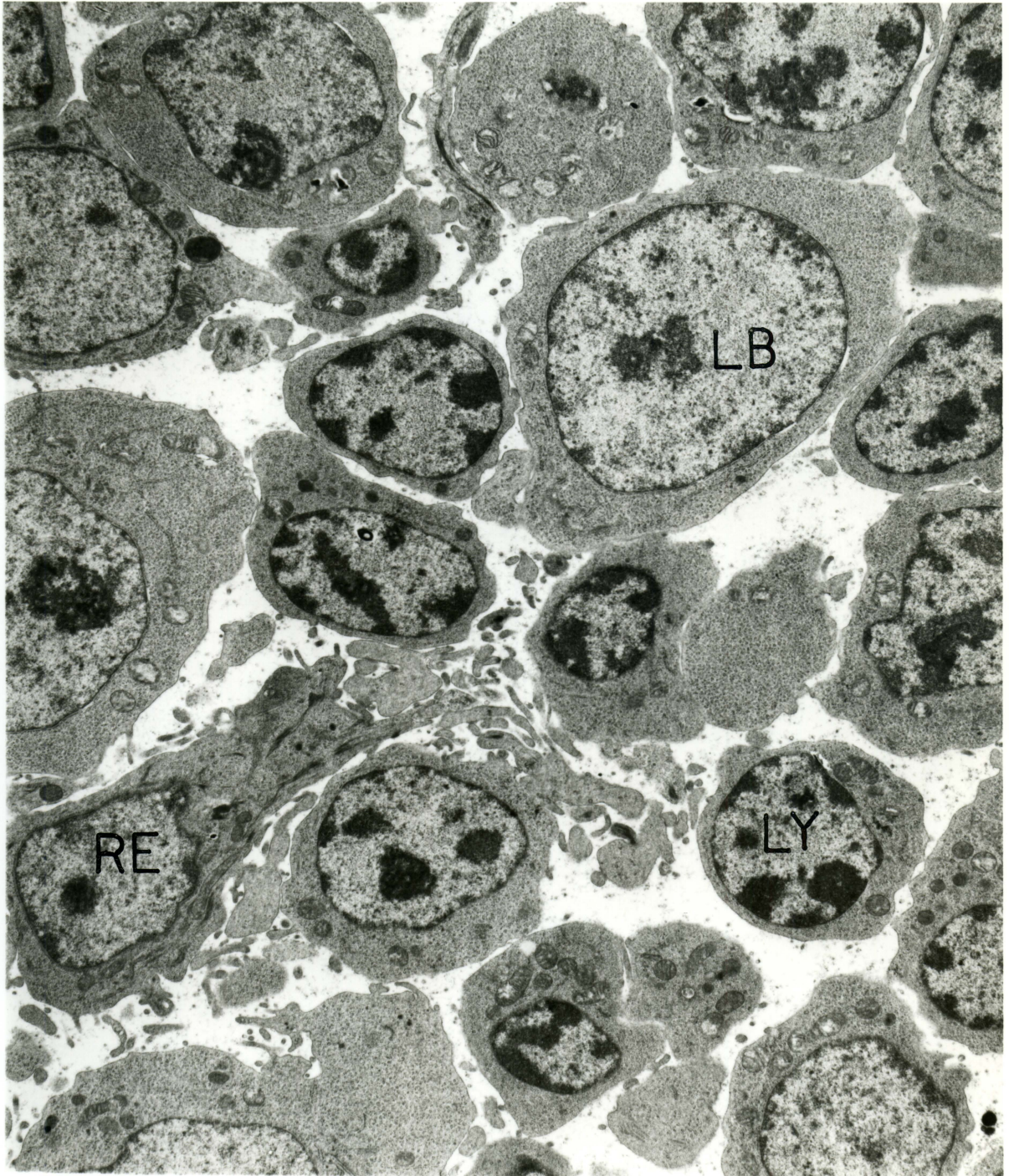cell (RE) is also present.

Figure 24.   Medulla of lymphoid follicle from bursa
of Fabricius of 3-day-old CY-treated poult.
Medulla is severely depleted of lymphocytes.
Only reticulo-epithelial cells (RE) or their
cytoplasmic processes (arrows) are seen.
Intercellular spaces are large and empty.

Medullae of some lymphoid follicles from
bursa of Fabricius of 7, 10, 14, 21 and
28-day-old CY-treated poults had a similar
ultrastructural appearance.   (See also
Figure 28.)

Figure 25. Medulla of lymphoid follicle from bursa
            of Fabricius of 3-day-old CY-treated poult.
            Medulla is depleted of lymphocytic series
            cells. Only few lymphocytes (LY) are seen
            which have intact organelles. Remainder of
            cells are large reticulo-epithelial cells
            which have long cytoplasmic processes and
            tonofibrils (arrows) in their cytoplasm.
            Intercellular spaces contain sectional profiles
            of microvilli.

            Medulla of some lymphoid follicles from
            bursa of Fabricius of 7, 10, 14, 21, and
            28-day-old CY-treated poults had a similar
            ultrastructure.
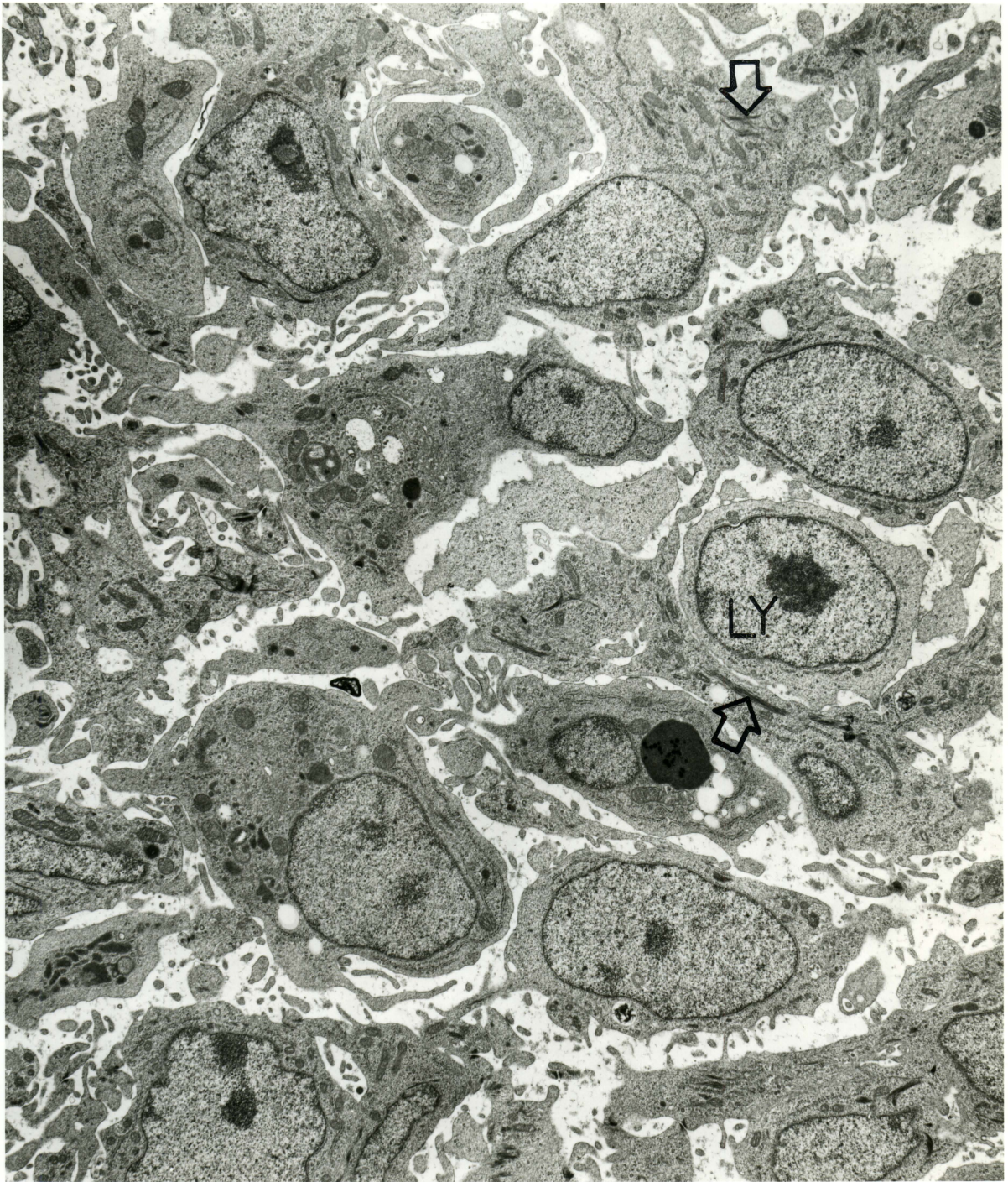
Figure 26.   Cortex of lymphoid follicle from bursa of
             Fabricius of 14-day-old control poult.
             Cortex is composed exclusively of several
             closely packed small to large lymphocytes
             (approximately 8 cells wide). Basement
             membrane (arrow) separates cortex from
             medulla (MED).

             Cortex of lymphoid follicles from bursa of
             Fabricius of 7, 10, 21, and 28-day-old
             CY-treated poults had the same ultrastructural
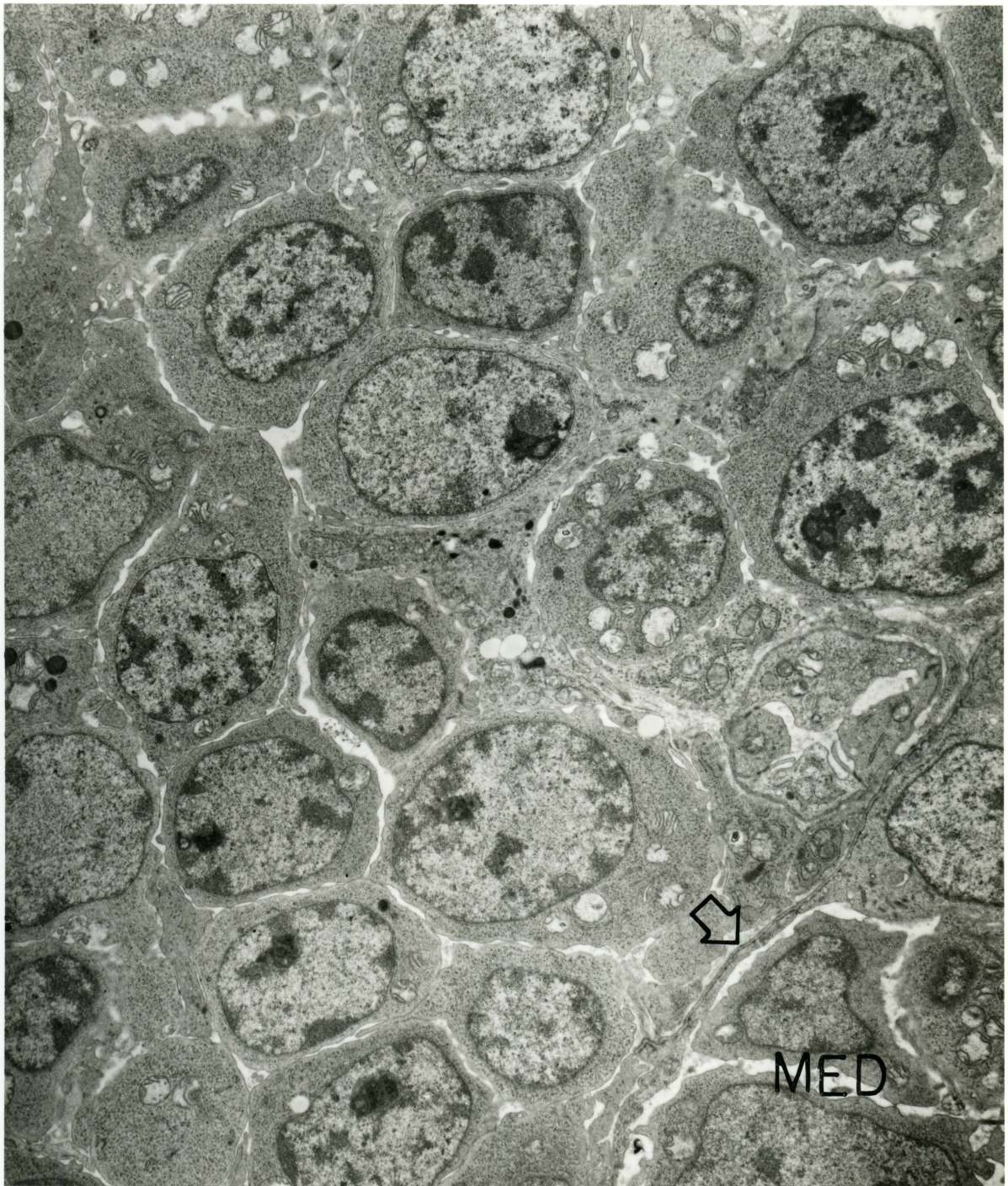   appearance.

Figure 27. Medulla of lymphoid follicle from bursa of
Fabricius of 14-day-old control poult. The
medulla is populated predominantly with
different sized lymphocytes. They vary in
their nuclear diameter and amount of cytoplasm.
A lymphoblast (LB) is also present.

Medulla of lymphoid follicles from bursa of
Fabricius of 7, 10, 14, 21, and 28-day-old
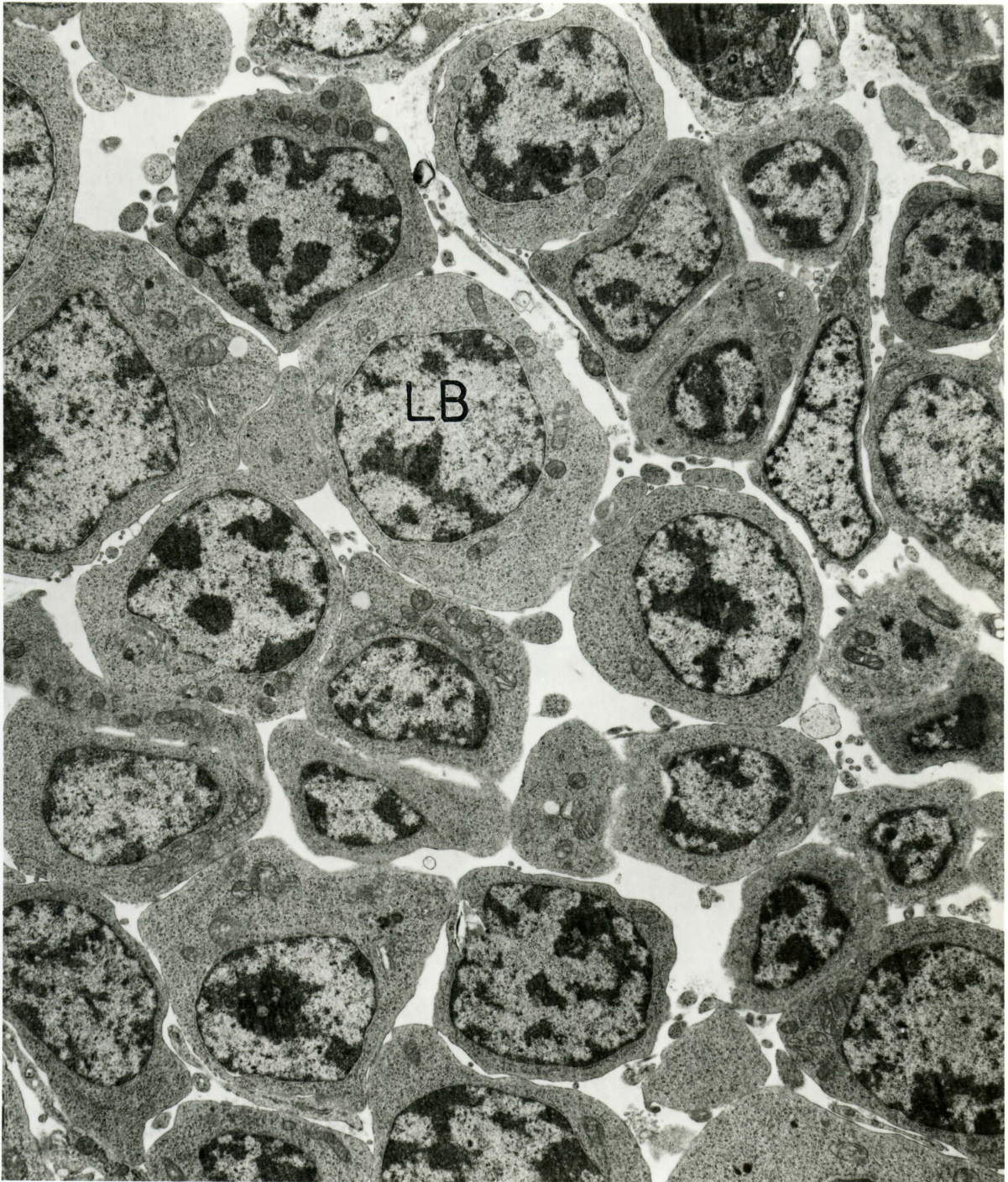control poults had the same ultrasturctural
appearance.

Figure 28. Medulla of lymphoid follicle from bursa of
Fabricius of 28 day-old control poult. Medulla
is populated with different sized lymphocytes.
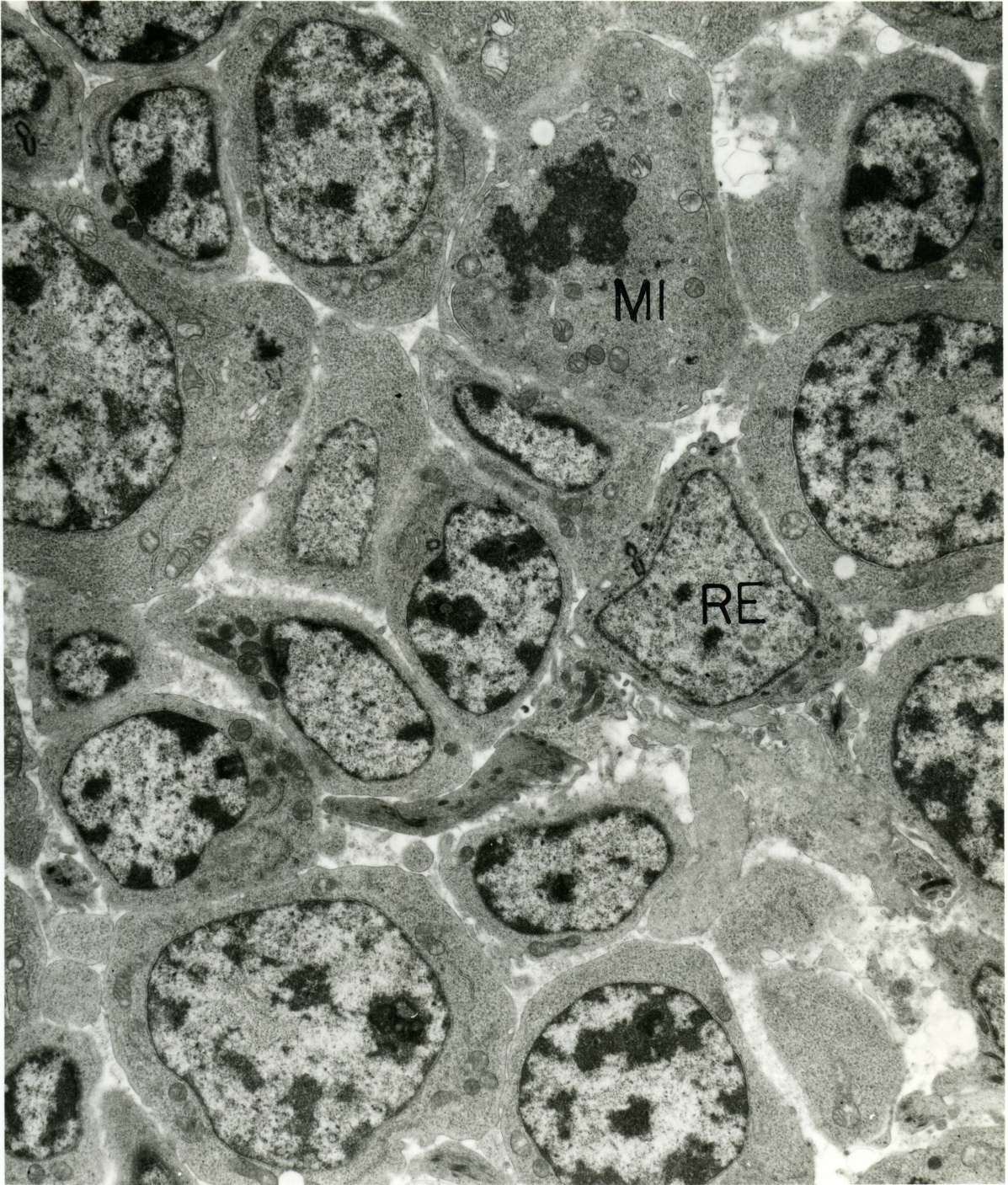A cell in mitosis (MI) and a reticulo-epithelial
cell (RE) can also be seen.

Figure 29. Follicular epithelium from bursa of Fabricius
of 21-day-old CY-treated poult. The epihtelium
is composed of a single cell layer. Cells are
stretched and have irregular outline. Inter-
cellular tight junctions are intact (arrows).
Intercellular spaces are large and empty.

Figure 30, Medulla of lymphoid follicle from bursa
of Fabricius of 28-day-old CY-treated poult.
Medulla is depleted of lymphocytes and
populated with reticulo-epithelial cells
characterized by long cytoplasmic processes,
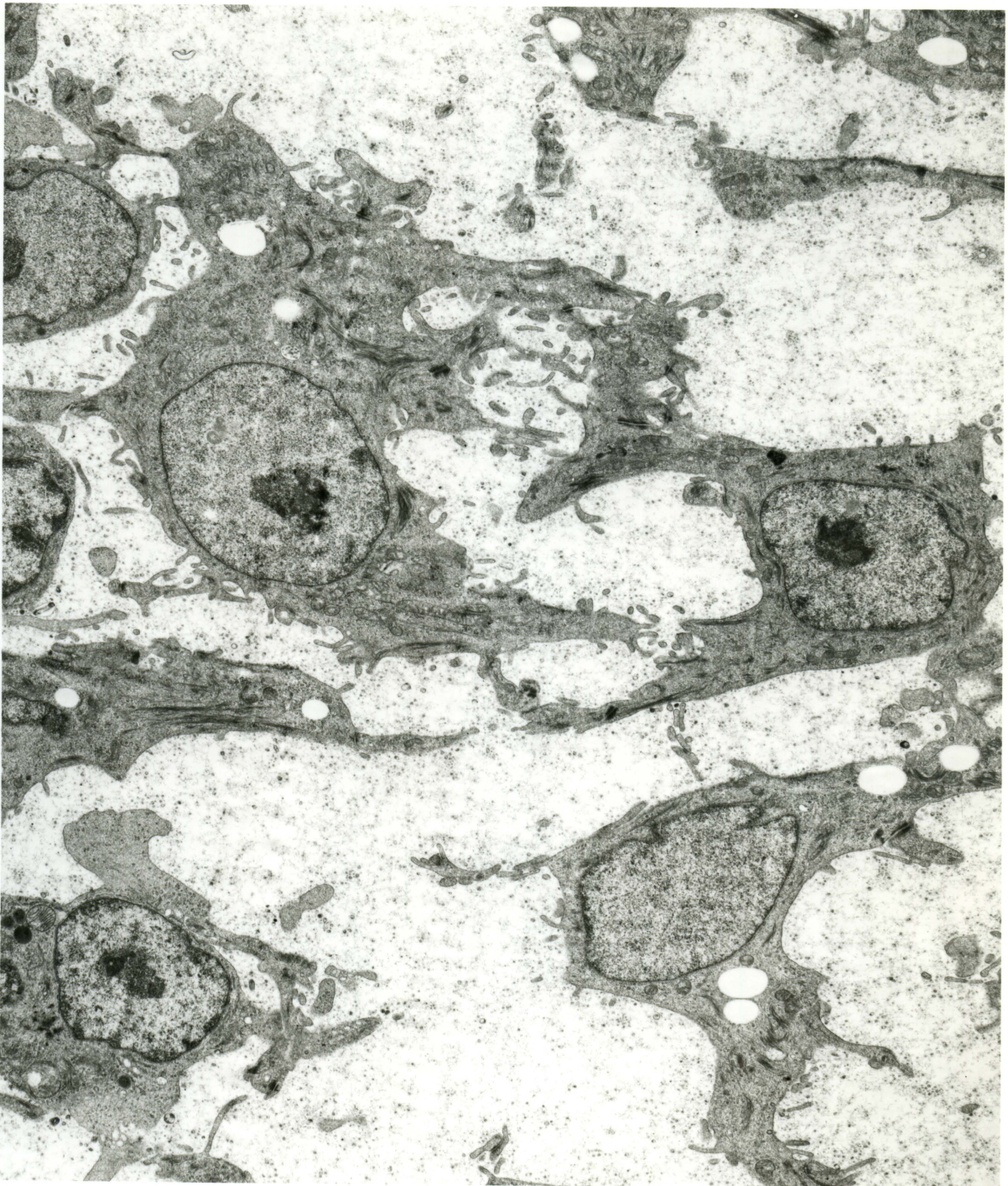intercellular desmosomal junctions, and
tonofibrils.

Figure 31. Cortex and cortico-medullary border of
lymphoid follicle from bursa of Fabricius
of 14-day-old CY-treated poult. Four plasma
cells (PC) are located along basement membrane
(arrows) in lymphocyte-depleted cortex. Two
lymphoblasts (LB) are present in cortical area.
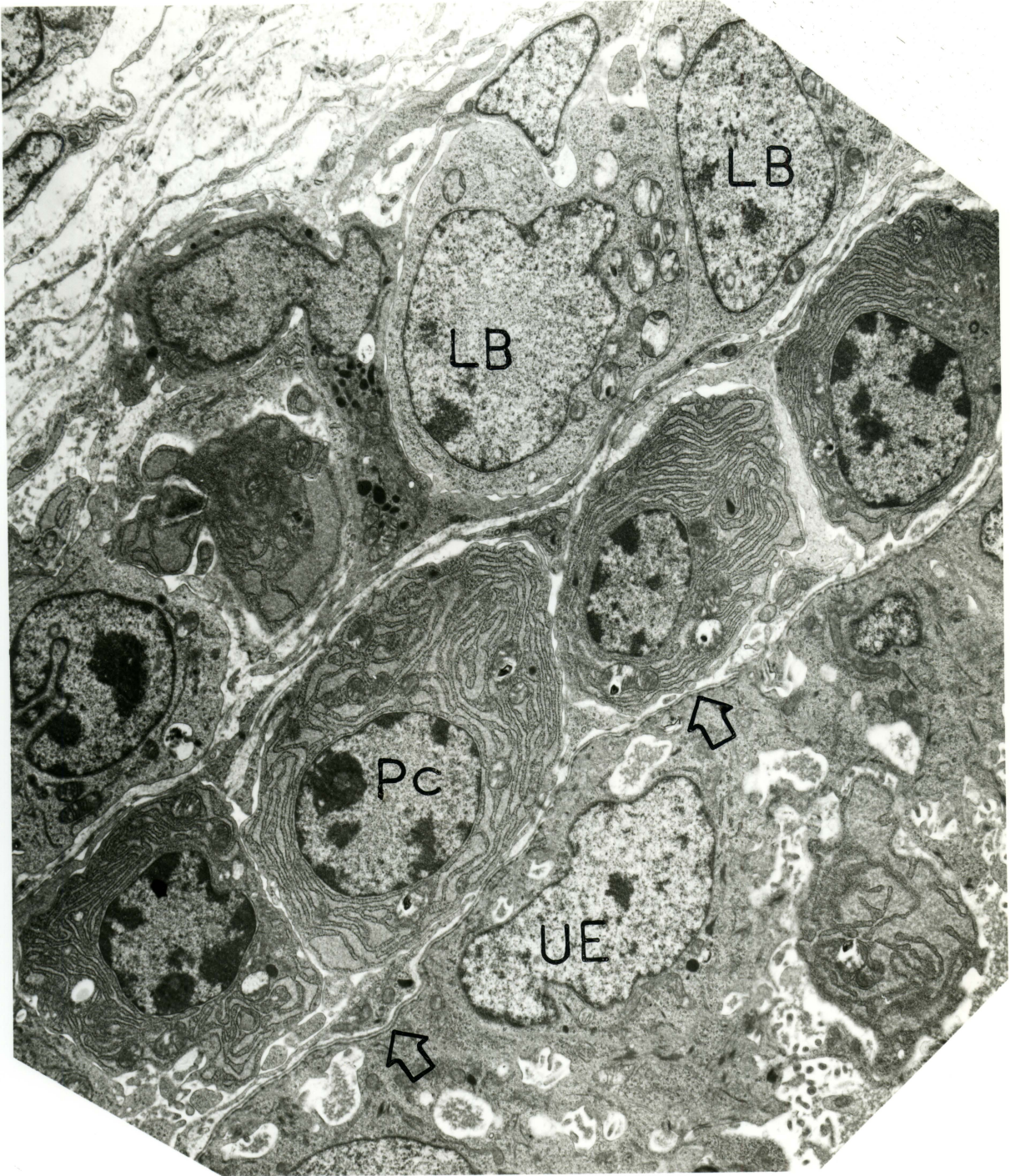Undifferentiated epithelium (UE) is seen in
the medulla.

Figure 32. Cortex and cortico-medullary border of
lymphoid follicle from bursa of Fabricius
of 28-day-old CY-treated poult. Cortex is
depleted of lymphocytes and populated with
mature plasma cells (PC) which lie along the
basement membrane (arrow). Fibroblasts (FB) and
undifferentiated epithelium (UE) are also present.

Figure 33.   Medulla of lymphoid follicle from bursa of
             Fabricius of 10-day-old CY-treated poult.
             Medulla is depleted of lymphocytes and
             populated predominantly with plasma cells
             (PC) in different stages of differentiation.
             Intercellular spaces are filled with a fine
             granular material.

Figure 34.   Medulla of lymphoid follicle from bursa of
              Fabricius of 14-day-old CY-treated poult.
              Medulla is depleted of lymphocytes and
              populated with plasma cells (PC) in different
              stages of differentiation. Few reticulo-
              epithelial cells (RE) are also seen. Inter-
              cellular spaces contain granular material
              and sectional profiles of cytoplasmic processes
              of reticulo-epithelial cells. A small portion
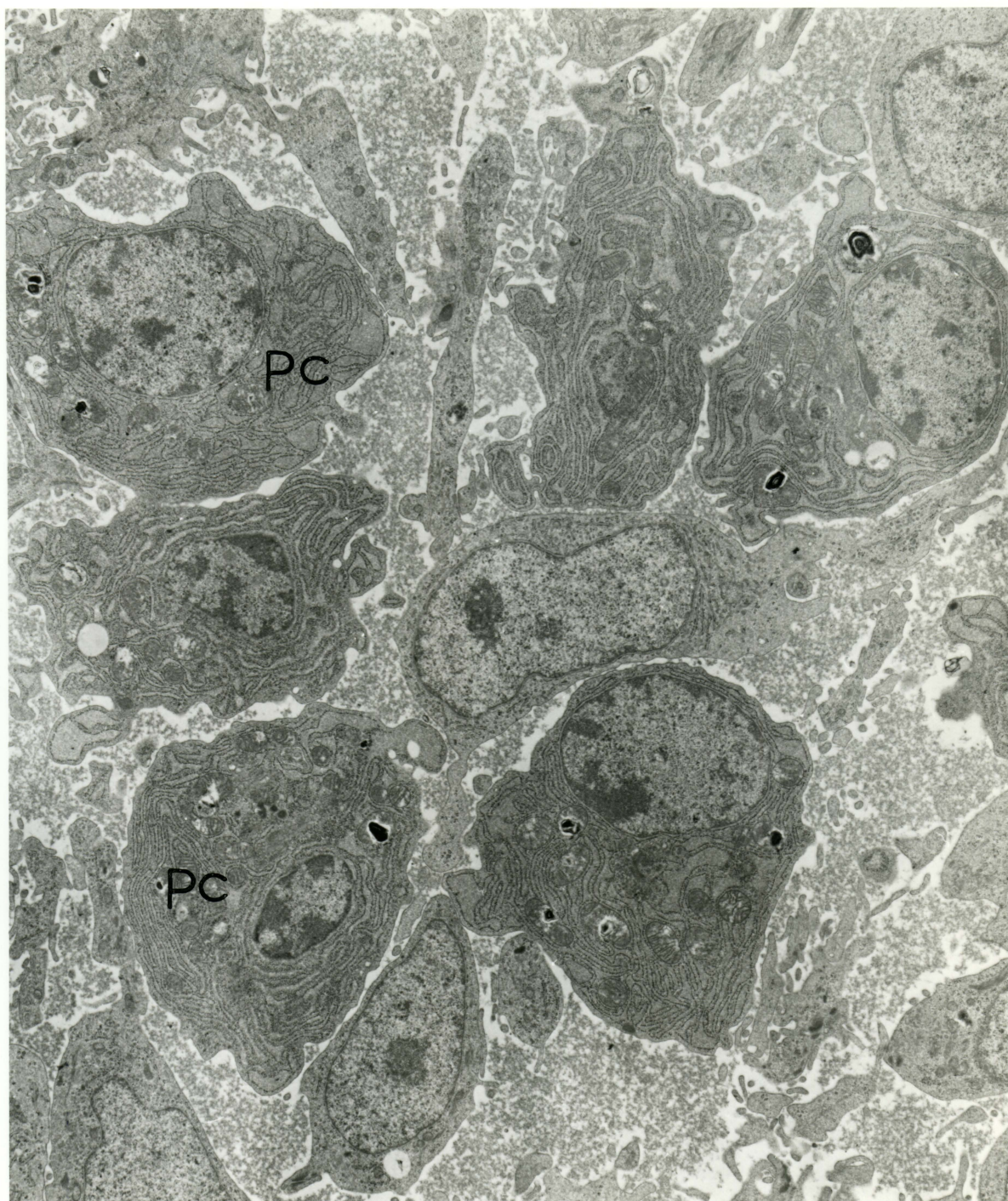   of the basement membrane (arrow) can be seen.
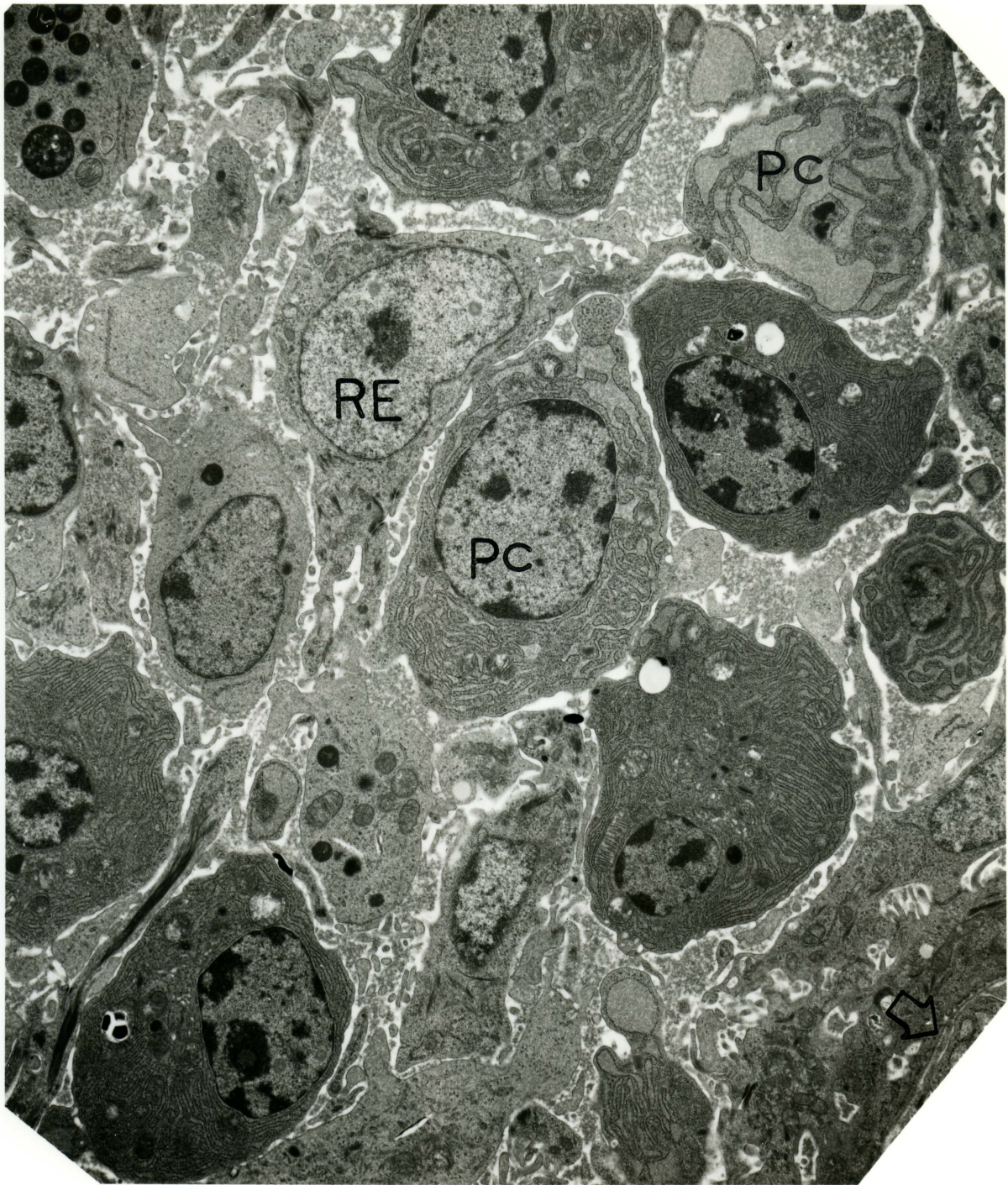
Figure 35. Medulla of lymphoid follicle from bursa of
Fabricius of 14-day-old CY-treated poult.
Medulla is depleted of lymphocytes and
populated with reticulo-epithelial cells
which have long cytoplasmic processes connected
by desmosomes (arrow) to processes of adjacent
cells. Intercellular spaces are large and
filled with granular material. A plasma cell
(PC) can be seen.
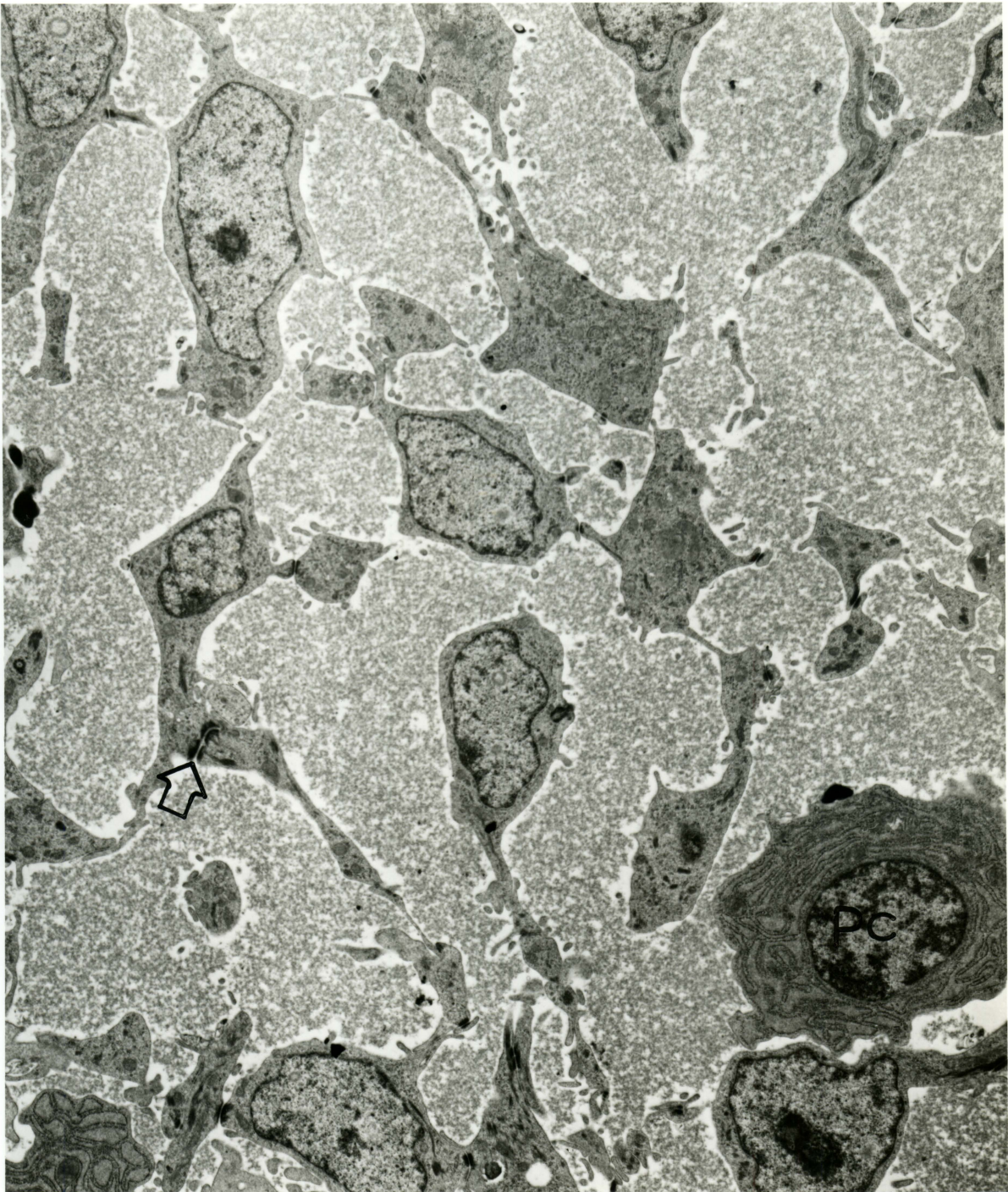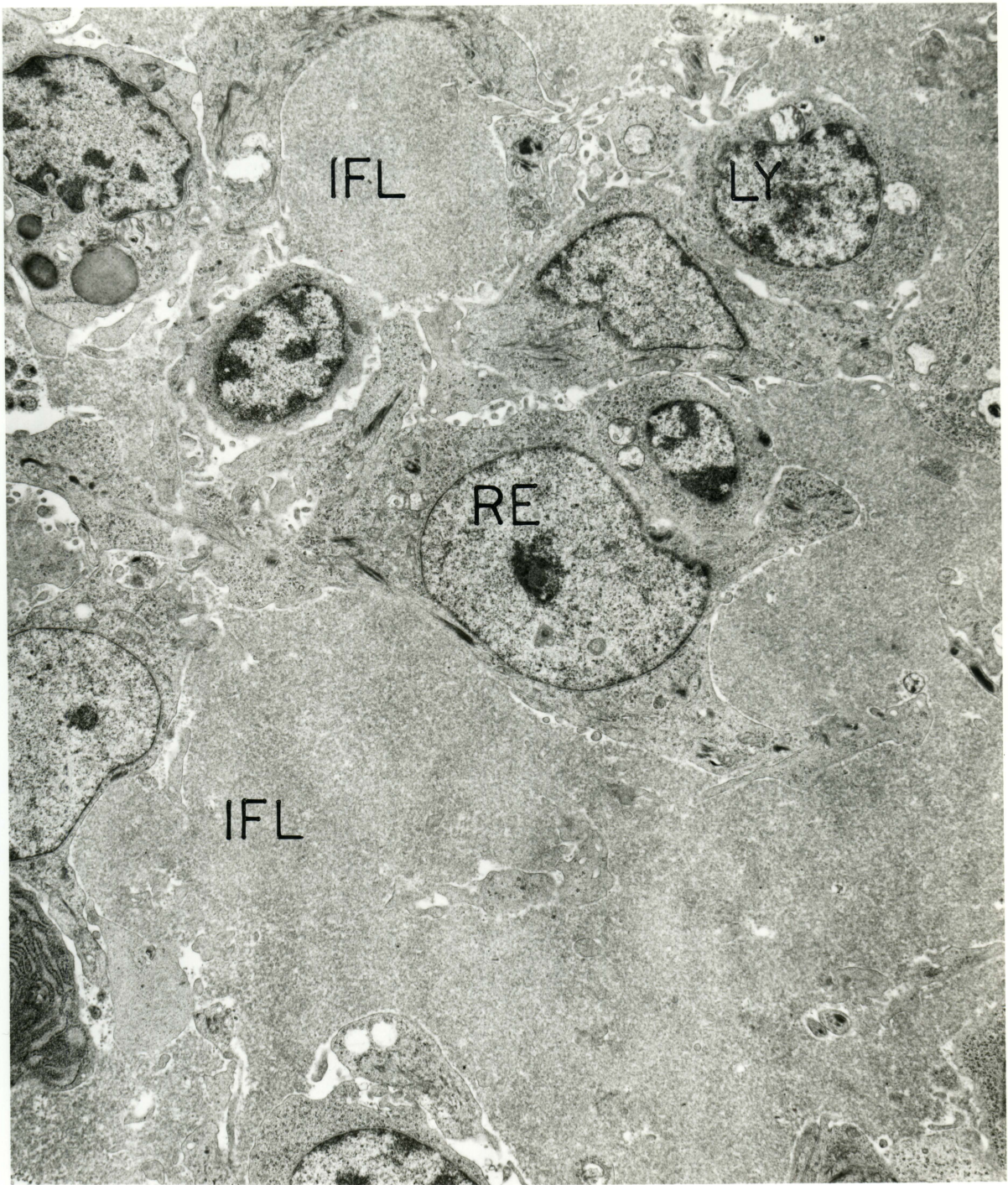
Figure 36. Medulla of lymphoid follicle from bursa of
            Fabricius of 28-day-old CY-treated poult.
            Medulla is depleted and contains intrafollicular
            lakes (IFL). These lakes contain uniform
            light granular material. Lymphocytes (LY)
            and reticulo-epithelial cells (RE) are located
            between lakes.

DISCUSSION

This experiment was designed to study sequential changes in the BF of turkeys following CY administration. Suppression of BF development in all CY-treated groups confirmed results of the first experiment. Lack of a statistically significant difference in body weight and γ -globulin level between control and CY-treated groups, in spite of a remarkable reduction of both in treated groups, was due to the small number of poults in each group.

In general, damaged unregenerating BF were seen in CY-treated poults killed at 3, 7, 10, and 21 days of age. At 14 days of age, only one BF had relatively normal regenerating lymphoid follicles. At 28 days there was substantial evidence of regeneration. This transitional phase from damaged BF at 21 days of age to regenerating BF at 28 days of age made it difficult to follow phases of follicular regeneration particularly with this small number of poults. Also, as discussed in the first experiment, BF from different poults of the same age and even individual follicles in a particular bursa differed in their response to the cytotoxic action of CY. This variability between follicles complicated interpretation of sequential changes. The

theoretical possibilities for regeneration of damaged
lymphoid follicles have been discussed in the first
experiment.

An increase in plasma cells in the BF was observed in
7-week-old ducks treated with CY during the first 2 or 4 days
after hatching (Sugimura and Hashimoto 1976). Hoffmann-Fezer
et al. (1977) reported an increase in plasma cells in the
late phase of regeneration of CY-damaged bursal follicles
in the chicken. In this experiment, plasma cells appeared
much sooner, even as early as 10 days of age.

Plasma cells develop from B lymphocytes following
antigenic stimulation (Cheville 1976). It is hard to determine
whether the plasma cells found in the lymphoid follicles
developed from bursal or blood B lymphocytes which survived
CY-treatment. It is possible that B lymphocytes in the
circulation are not in active state of proliferation and
therefore are more resistant to the cytotoxic action of
CY. If the plasma cells differentiated from blood B lympho-
cytes, they would have entered the BF via the blood stream.
The appearance of plasma cells predominantly in vascularized
areas of the BF (Cortex and interfollicular tissue) indicates
the likelihood of blood-origin precursor cells. Why the
B lymphocytes entered the BF to differentiate and develop
into plasma cells remains an unanswered question. Previous

reports have indicated that the BF can serve as a secondary
lymphoid organ (Sorvari et al. 1975, Sorvari and Sorvari
1977). If this is true, further investigation is needed
to explain the increase in the apparent immunologic
reactivity of the BF in CY-treated birds.

The amorphous, eosinophilic material observed in the
medullae of some follicles is histologically similar to
what has been described as amyloid-like substance in avian
species (Cowman and Johnson 1970, Wight and Shannon 1977).
This material has been reported to have a close resemblance
to fibrin and it is suggested that it is a plasma protein
derivative similar to fibrin (Wight and Shannon 1977). In
this experiment, the occurrence of this amyloid-like substance
predominantly in those BF which had numerous plasma cells
raises a question about a possible correlation between this
material and plasma cells since amyloidosis in mammalian
species is known to be associated with abnormal plasmacytosis
(Cheville 1976). Is it possible that this substance found
in the BF of some CY-treated poults represents another form
of "avian amyloid" which may be different chemically and
structurally from mammalian amyloid? Further biochemical
analysis is needed to reveal the exact chemical composition
of this substance.

The spontaneous occurrence of aspergillosis in CY-treated turkeys has been discussed in the first experiment.

SUMMARY

The effect of cyclophosphamide on bursa of Fabricius development and humoral immunity in turkeys was evaluated. Two experiments were done in which clinical signs, mortality, antibody response to sheep red blood cells, serum protein levels, and histology and ultrastructure of the bursa of Fabricius were evaluated.

In the first experiment, 4 groups of poults were injected intraperitoneally with 2, 4, or 6 mg cyclophosphamide per day for 3 days or 2 mg per day for 6 days beginning on the day of hatching. Histological analysis of bursal tissue was performed at 28 days of age. In the second experiment, poults were injected intraperitoneally with 4 mg cyclophosphamide per day during the first 3 days from the day of hatching. Bursae were collected sequentially at 3, 7, 10, 14, 21, and 28 days-of-age and examined by light and transmission electron microscopy.

In both experiments, cyclophosphamide had a marked suppressive effect on growth, bursa development and γ -globulin synthesis. Histological changes in the bursa included invagination of the surface epithelium, thinning of follicular epithelium, increase in interfollicular connective tissue with occasional plical fibrosis, and depletion of follicular lymphocytes in both cortices and

medullae with population of depleted follicles by reticulo-epithelial and plasma cells. Transmission electron microscopic examination confirmed histological findings with no additional cytopathologic changes identified. Early changes were seen at 3 days of age, and evidence of follicular regeneration was seen at 28 days of age.

In the sequential study, bursae from poults killed at various ages had different types of follicles. Moreover, in some bursae the different types of follicles were not regularly distributed in the bursal plicae.

The results of this study indicate that turkeys are affected by the immunosuppressive action of cyclophosphamide and that this chemical can be used for inducing bursal atrophy in turkeys.

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