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IMMUNOHISTOCHEMICAL DETECTION OF FUNGAL ELEMENTS IN THE TISSUES OF GOSLINGS WITH PULMONARY AND SYSTEMIC ASPERGILLOSIS

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Nineteen goslings with pulmonary and systemic aspergillosis were the subject of the study. The lungs and air sacs were the main sites affected by the disease, and were generally characterised by diffuse yellowish-white granulomas. In 7 cases with pulmonary and air-sac involvement the granulomas were scattered to the serosal linings of the gastrointestinal and upper respiratory tracts, to the liver, spleen and kidneys, and in two cases also to the bursa of Fabricius, *musculus (m.) longus colli* and adventitia of aorta. The granulomas were often characterised by a necrotic centre surrounded by heterophils, macrophages, lymphocyte and plasma cells, and in late granulomas by multinucleated foreign-body giant cells, and again by an outer thin fibrous capsule. Numerous fungal hyphae were found within the necrotic debris of the granulomas by Gridley and PAS staining techniques. Immunohistochemistry reliably confirmed aspergillosis in all of the cases. Fungal elements in the lungs of goslings severely affected by the disease stained heavily within the centre of the granulomas, whereas few antigens reacted in the chronic cases. Fungal fragments, which were not discernible using routine fungal stains, reacted clearly in the cytoplasm of macrophages and giant cells. Thus, although fungal elements within the granulomas were histologically indicative of aspergillosis, immunohistochemistry also had to be applied to obtain a definitive diagnosis of the disease and to differentiate it from many of the filamentous fungi.

Key words: Immunohistochemistry, goslings, fungal elements, aspergillosis

Aspergillosis, defined as any disease caused by a member of the fungal genus *Aspergillus*, has been documented in many avian species (Richard, 1991; Carrasco et al., 1998) including the goose (Ulloa et al., 1987; Okoye et al., 1989; Turkutanit, 1999). Of all the fungal diseases, aspergillosis encompasses the broadest range of pathogenic mechanisms and clinical presentations, as *Aspergillus* species are common in the environment (Hawkey et al., 1984; Piérard et al., 1991; Machin et al., 1993; Singh et al., 1993). In birds, two major agents

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causing aspergillosis are *Aspergillus fumigatus* and *A. flavus* (Chute, 1965; Pandita et al., 1991; Richard, 1991), and two main types of the disease have been described (Chute, 1965; Richard, 1991; Machin et al., 1993). In young avian species, aspergillosis commonly occurs in acute and severe form with high mortality and morbidity, whereas it is usually sporadic and chronic in adult birds (Chute, 1965; Flach et al., 1990; Richard, 1991; Machin et al., 1993).

Since fungal infections of birds are difficult to diagnose clinically, most cases remain undiagnosed until a postmortem examination is performed (Kaufman, 1992; Buckley et al., 1992; Carrasco et al., 1993; Jensen et al., 1993; Jensen, 1994; Jensen et al., 1996*b*; Carrasco et al., 1998). Furthermore, even though manifest infections of particularly the lungs and air sacs typically display gross lesions with yellowish-white nodules (Dyar et al., 1984), minor gross lesions may be difficult to determine in young birds, and the impact on early mortality may consequently be underestimated. Thus, the presence of a mycotic disease is first recognised when histopathological examination is applied (Phillips and Weiner, 1987; Carrasco et al., 1993; Jensen et al., 1993; Carrasco et al., 1998).

Although typical hyphae of some fungi including *Aspergillus* species are easily recognised, obtaining an accurate identification of fungi by conventional histopathological methods is not always possible because of morphological similarities among the tissue forms of several fungal genera and the failure to find typical diagnostic elements (Phillips and Weiner, 1987; Piérard et al., 1991; Kaufman, 1992; Fenelon et al., 1999). Furthermore, some inflammatory reactions may suggest the possibility of a mycotic infection, even though no fungus is identified in tissue sections (Jensen, 1994; Jensen et al., 1996*a*). For these reasons, immunohistochemical techniques are increasingly used for obtaining specific diagnosis in cases of mycosis in birds (Carrasco et al., 1993; Jensen et al., 1997) as well as in mammals. Moreover, immunohistochemical methods are also highly suitable for the screening of tissues for fungal elements, and this is particularly true when only a few fragments of fungi are present (Jensen et al., 1993; Jensen, 1994; Carrasco et al., 1998).

In the literature, aspergillosis in various avian species has frequently been reported in its pulmonary form, with involvement of the lungs and air sacs. However, there is a paucity of information on the systemic form of the disease in geese, as in other birds. Moreover, immunohistochemistry has not been applied for the diagnosis of aspergillosis in geese to date, even though in the recent past a few polyclonal and monoclonal antibodies have been raised against *Aspergillus* species for use in the detection of the fungal species in mammals and other avian species. The aim of the present study was therefore to detect immunohistochemically fungal elements in the tissues of goslings with pulmonary and systemic aspergillosis.

Materials and methods

Birds

The materials for this research were 19 one- to three-month-old, live (6) or dead (13) goslings with aspergillosis, submitted to the Department of Veterinary Pathology for diagnosis, or collected from hen houses where the disease caused high mortality, in the period of 2000–2002.

Histopathology

The goslings affected by the disease were macroscopically examined following necropsy and samples were taken from the lungs, air sacs, trachea, liver, spleen, kidneys, oesophagus, proventriculus, gizzard, small intestines, caecum, testis, bursa of Fabricius, *m. longus colli*, cerebrum and cerebellum. The samples were fixed in 10% buffered formalin, processed routinely, and stained with haematoxylin and eosin (HE), some of them with Gridley, periodic acid–Schiff (PAS) and Ziehl-Neelsen methods.

Mycology

From the lungs and air sacs of ten goslings, cultivation was attempted following surface decontamination by searing with a hot spatula. Tissue samples were inoculated onto two sets of Saboraud's dextrose agar (SDA) (Difco) plates, pH 5.7 and incubated for 7 days at 25 °C and 37 °C, respectively.

Immunohistochemistry

Specimens from 19 goslings were stained using the avidin-biotin-peroxidase complex (ABC) technique (Hsu et al., 1981), employing a monoclonal mouse anti-aspergillus (*A. fumigatus, A. flavus, A. niger*) IgM antibody (Dako, M 3564). All of the sections were dewaxed in xylene and hydrated through graded alcohols. Endogenous peroxidase was blocked with 3% hydrogen peroxide. The sections were incubated successively with phosphate-buffered saline (PBS) for 5 min, citrate buffer saline in a microwave oven for 50 min, monoclonal mouse anti-aspergillus IgM at a concentration of 1:50 in PBS for 1 h, biotinylated rabbit-anti mouse antibody at a concentration of 1:300 in PBS for 30 min, and peroxidase-conjugated streptavidin at a concentration of 1:300 in Tris-buffered saline for 30 min. The sections were washed in PBS after each step. Immunostaining was obtained using 3,3-diaminobenzidine as chromogen. Haematoxylin was used as counterstain.

Results

Clinical signs

The main clinical signs in the live goslings were dullness, depression, gasping, staggering and prostration. Goslings heavily affected by the disease showed marked difficulty in respiration and accelerated breathing.

Necropsy findings

Necropsy revealed that the lungs were the organ mainly affected by the disease, and were generally characterised by diffuse yellowish-white granulomas, the size of which varied from miliary to large nodules (about 1 cm in diameter), causing obliteration of a large portion of the lobes (Fig. 1). Inner surfaces of the air sacs were completely covered with a dense mat of white fungal material. The air sacs were visibly thickened, and in a few cases there were mycotic plaques. In addition to the involvement of the lungs and air sacs, in 7 goslings miliary granulomatous nodules were scattered to the serosal linings of the trachea, oesophagus, proventriculus, gizzard and small intestine. Also, small (pinpoint-sized) granulomas were detected in the liver, spleen, kidneys, and on the wall of the small intestines, causing complete obliteration of the wall. In addition, in 2 out of these 7 cases, nodules of pinpoint size were distributed to the adventitia of aorta, plicae of the bursa of Fabricius and the *m. longus colli*. Except for typical granulomas of the disease, in two cases the lesions appeared as mouldy-necrotic masses, greenish-yellow in colour, occluding the abdominal cavity air sacs (Fig. 2).

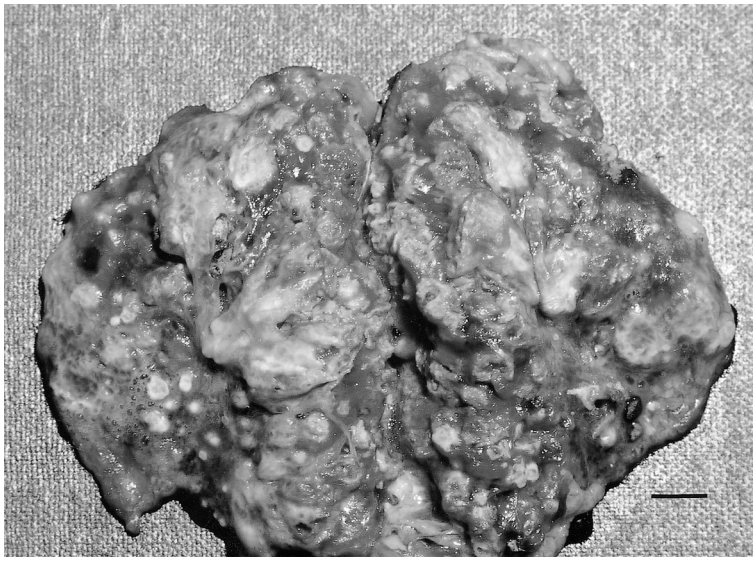

Fig. 1. Numerous granulomas of varying size distributed on the lung lobes

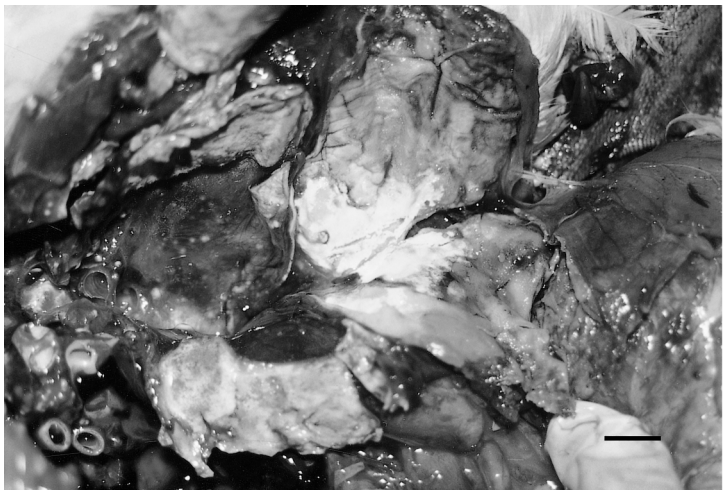

Fig. 2. Mouldy-necrotic masses in the abdominal cavity air sacs of a gosling with aspergillosis

Histopathology

Histopathological examination showed diffuse granulomas distributed throughout the pulmonary parenchyma. The lumina of bronchi and bronchioles, and alveoli were occluded by caseous masses, and the airways were narrowed occasionally because of compression by peripheral granulomas. In goslings severely affected by the disease it was found that large parts of the lungs were obliterated by large and coalescing granulomas. The granulomas were mainly characterised by a necrotic centre surrounded by macrophages, lymphocyte and plasma cells, and in late granulomas by multinucleated foreign-body giant cells, and again by an outer thin fibrous capsule. In particular, when the granulomas opened to the bronchial and bronchiolar lumina, a large number of hyphae, spores, and also conidiophores with radial and sunburst arrangement of mycelium were detected, with numerous spores releasing from the conidial heads. Several granulomas with histological characteristics were also found on the serosa of the trachea. The tracheal lumina contained a few hyphae and spores free within the exudate or attached to the cilia.

Numerous granulomas, varying in size, were detected in the liver parenchyma in all 7 cases. Late granulomas contained necrotic debris in the centre, surrounded by a line of multinucleated foreign-body giant cells, and by an outer thick fibrous capsule (Fig. 3). Also, thrombotic vasculitis containing fungal hyphae, severe congestion, haemorrhages and perivascular lympho-plasmocytic cell infiltration were found in the liver. In the kidneys, in addition to the granulomatous reaction on the capsule, severe necrosis was found to obliterate the capsule and a large portion of the subcapsular region, and to disseminate to the parenchyma along with thrombosed blood vessels containing fungal hyphae. In the ne-

crotic areas, there were also severe epithelial necrosis of proximal convoluted tubules, congestion, haemorrhages and mononuclear cell infiltration. It was noticed that a few fungal hyphae invaded the tubular lumina and intertubular regions (Fig. 4). In the spleen, small granulomas typical of the disease were seen to be attached to the capsule. Subcapsular and parenchymal necrosis, with no giant cell reaction or fungal elements, was also observed. Small granulomas with similar histological characteristics, severe necrosis and thrombosed vessels were detected within the wall of the gizzard and small intestine. Lesions in the bursa of Fabricius were characterised by severe caseous necrosis, heterophil and mononuclear cell infiltration and haemorrhages in plicae (Fig. 5). The *m. longus colli* showed widespread necrosis along with fungal elements and mononuclear cell infiltration.

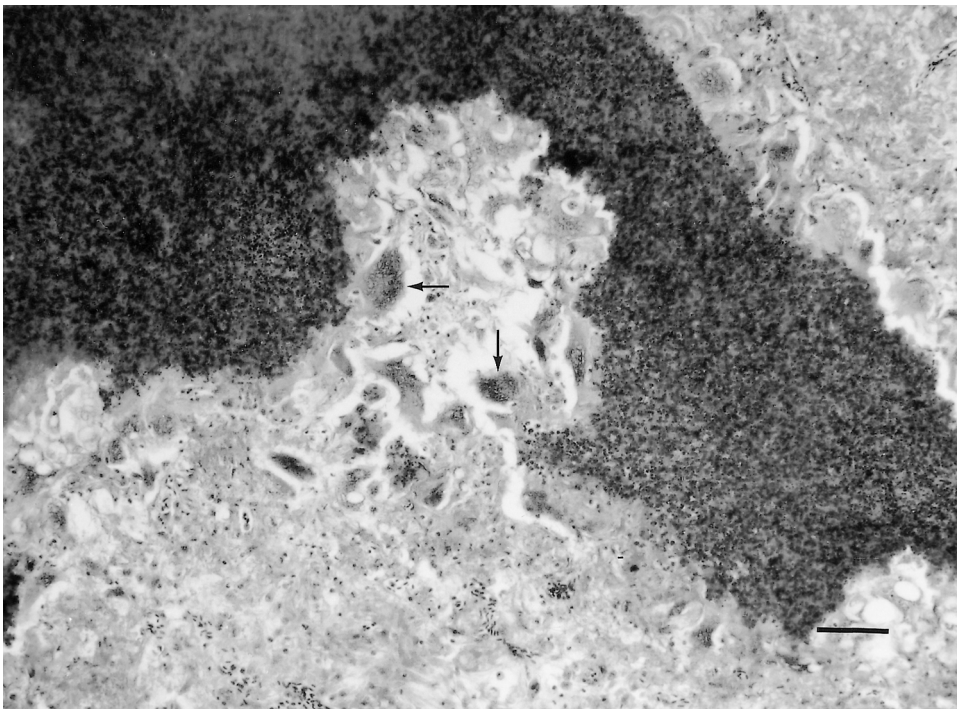

Fig. 3. Liver: a late granuloma showing central caseous necrosis is surrounded by a multinucleated foreign-body giant cell (arrows) reaction. Haematoxylin and eosin (HE). Bar = 303 µm

In the lungs and the abdominal viscera (except the liver in two cases), fungal elements were observed in the centre of the granulomas, necrotic areas and thrombosed vessels, using Gridley and PAS staining techniques. Ziehl-Neelsen staining found no acid-fast bacilli in any case.

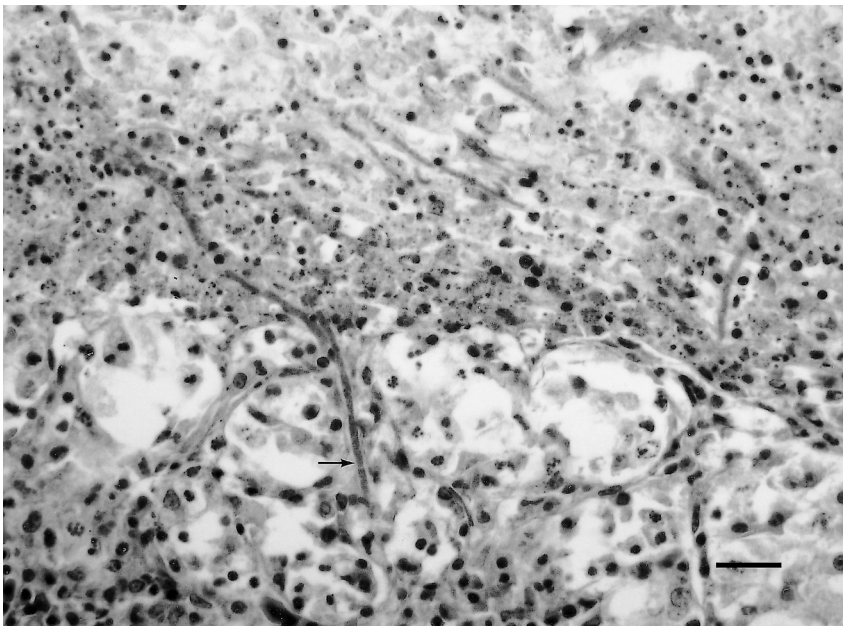

Fig. 4. Kidney: widespread necrosis along with fungal hyphae (arrow) in the interstitial areas. HE. Bar = 75 μm

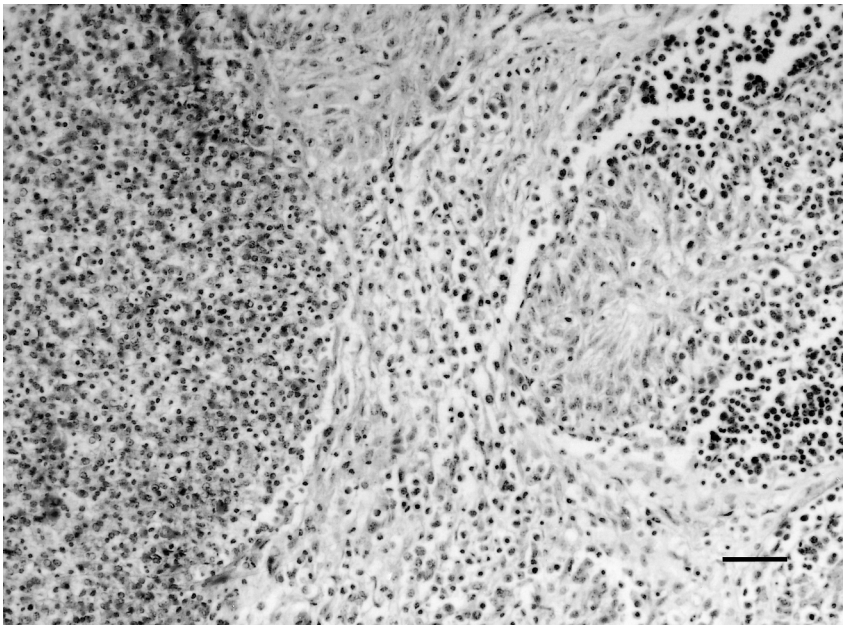

Fig. 5. Bursa of Fabricius: caseous necrosis and peripheral heterophil leukocyte infiltration. HE. Bar = 151 μm

Immunohistochemistry

Immunohistochemical staining, which was performed on the lung sections from all of the cases, revealed fungal elements within the granulomatous and necrotic areas (Fig. 6). Fungal elements were abundant within the granulomas with central necrosis, in comparison to young granulomas. The fungal elements stained heavily within the granulomas in the lungs of goslings severely affected by the disease, whereas few antigens were detected in the chronic cases. Fungal fragments, which were not discernible using HE and routine fungal staining techniques, reacted strongly in the cytoplasm of macrophages and foreign-body multinucleated giant cells. When the granulomas opened to the air spaces a large number of hyphae, conidial heads, often with radial and sunburst in shape, and spores releasing from the heads were easily detected (Fig. 7). A few hyphae and spores were seen on the cilia of the epithelium and free in the tracheal lumina. As in the lungs, the fungal elements were also found markedly within the granulomatous and widespread necrotic lesions in the liver (Fig. 8) (except in two cases), kidney, spleen, bursa of Fabricius, *m. longus colli*, and within the walls of the gizzard and small intestines. Furthermore, fungal elements were found in the granulomas on the serosal linings of the gastrointestinal and upper respiratory tracts, and in the adventitia of aorta.

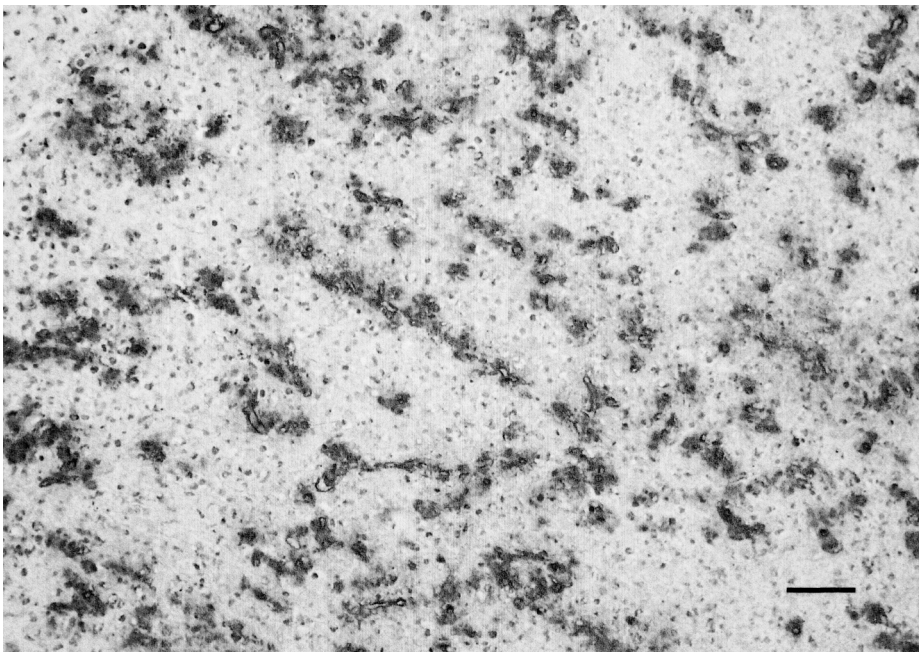

Fig. 6. A large number of hyphae and their remnants of *A. fumigatus* reacted with monoclonal antibody within the centre of a granuloma. Avidin-biotin-peroxidase complex (ABC) technique. Bar = 151 μm

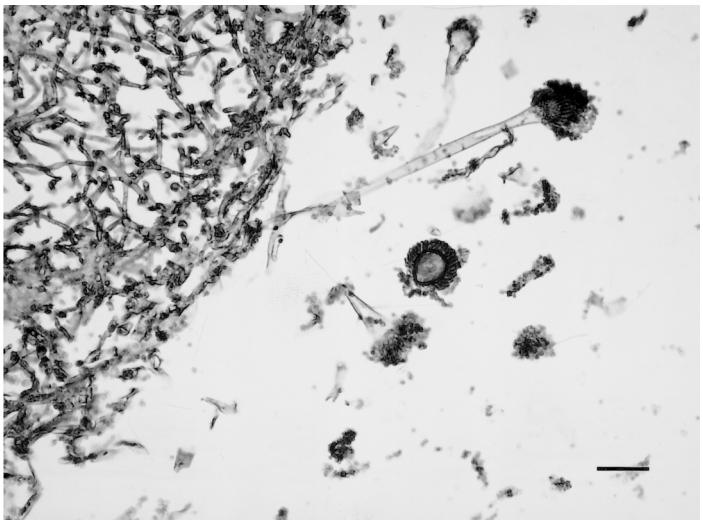

Fig. 7. Lungs: conidiophores bearing conidia of *A. fumigatus* in the airways. Vigorous reaction of fungal elements with monoclonal antibody is seen. ABC. Bar = 75 µm

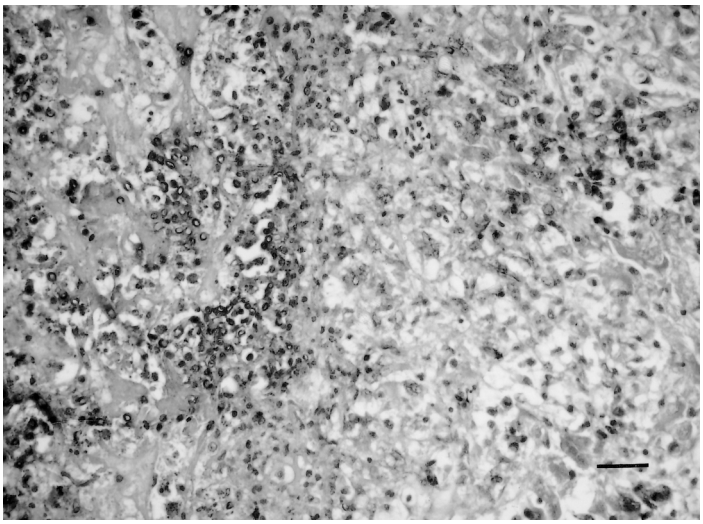

Fig. 8. Liver: numerous fungal hyphae and their fragments reacted with monoclonal antibody and their invasion are seen in the necrotic area. ABC. Bar = 75 µm

Mycology

Growth of bluish-green colonies, which darkened with age, were obtained from the specimens using Saboraud's dextrose agar as culture medium. The identification of *A. fumigatus* was made according to Quinn et al. (1998).

Discussion

Goose breeding is performed on small-scale family farms in the Kars region of Turkey and the industry is economically important for the farmers. However, as in other birds, the devastating disease aspergillosis has been responsible for high morbidity and mortality rates in goslings in the region.

Pulmonary aspergillosis, which is characterised by fungal lesions in the lungs and air sacs, is the most commonly encountered form of the disease (Dyar et al., 1984; Erer et al., 1986; Okoye et al., 1989; Carrasco et al., 1993; Turkutanit, 1999). In this study, all of the goslings investigated were found to have the respiratory lesions of aspergillosis, with involvement of the lungs and air sacs. However, there is a paucity of information on systemic aspergillosis in geese (Ulloa et al., 1987), as in other birds (Pal et al., 1989; Bowes, 1990; Pandita et al., 1991), although the disease has been documented in sheep (Perez et al., 1998), cattle (Jensen et al., 1993) and humans (Buckley et al., 1992). Our study found systemic aspergillosis in 7 cases, with distribution of the nodules to the serosal linings and parenchymal organs of the abdominal cavity, bursa of Fabricius and *m. longus colli* along with lesions in the lungs and air sacs.

Gross postmortem examination of the goslings showed variously sized yellowish-white nodules scattered throughout the lungs and within the air sacs, in accordance with observations made on other avian species (Hawkey et al., 1984; Reece et al., 1986; Ulloa et al., 1987; Bowes, 1990; Flach et al., 1990; Pandita et al., 1991; Machin et al., 1993). In some cases, a large portion of the lung lobes was obliterated by large coalesced granulomas, as observed previously in Stellar's jays (Machin et al., 1993), turkeys (Dyar et al., 1984) and chickens (Chaudhary and Singh, 1983). It is most likely that the cause of death of the goslings was these pulmonary lesions. Likewise, it has been reported that the death of birds affected by aspergillosis was due to respiratory failure because of affection of a large portion of the lung parenchyma (Okoye and Okeke, 1986; Pandita et al., 1991).

In the study, in 7 out of the 19 goslings miliary white nodules were found on serous linings throughout the gastrointestinal tract, as well as in the liver, kidney and spleen, and in two cases also in the bursa of Fabricius, *m. longus colli* and adventitia of aorta, which are unusual locations for the disease. In the literature, however, aspergillus granulomas have rarely been reported in the abdominal viscera of geese (Ulloa et al., 1987), as in other avian species (Pal et al., 1989). It is well known that aspergillosis results mainly from inhalation of spores of the fungus, commonly found in the environment (Hawkey et al., 1984; Reece et al., 1986; Pal et al., 1989; Bowes, 1990; Richard, 1991; Machin et al., 1993; Singh et al., 1993). It has also been established that low humidity and excessive dust may damage the respiratory epithelium and increase susceptibility to the disease (Machin et al., 1993). Thus, the lungs are primarily affected by the disease and the agents spread by the haematogenous route to the brain and eye

which are the predominant organs for localisation of the agents, even though brain lesions were not detected in any cases in this study, and to the abdominal organs (Chute, 1965; Dyar et al., 1984; Richard, 1991; Buckley et al., 1992). In the literature (Chute, 1965; Richard, 1991), systemic aspergillosis has been reported in 5-week-old cockerels and it has been concluded that this resulted from a caponising infection. However, caponising had not been performed in our cases, and therefore it was most likely that systemic aspergillosis in goslings developed as a result of haematogenous spread of the fungus from the lungs, with reference to the thrombosed vessels containing fungal hyphae in the tissues in the abdominal viscera investigated. Consistent with our results, the systemic form of the disease has been documented in an oiled magallanic penguin (Carrasco et al., 2001), chickens (Erer et al., 1986; Pal et al., 1989) and ducks (Bowes, 1990), and has primarily been attributed to the haematogenous dissemination of the agents, as in systemic bovine (Jensen et al., 1993; Jensen, 1994) and ovine (Perez et al., 1998) aspergillosis. Nevertheless, as reported by Jensen (1994), finding of the granulomas on both serosal linings and abdominal air sacs may reveal a spread by implantation between facing serosal surfaces.

Histopathological examination of the lungs revealed multiple granulomas with the histopathological characteristics distributed throughout the pulmonary parenchyma. Fungal hyphae were often seen within the granulomatous areas using routine fungal stains, in particular when the granulomas opened to the air space, as reported by other authors (Chaudhary and Singh, 1983; Hawkey et al., 1984; Erer et al., 1986; Richard, 1991; Machin et al., 1993). In the cases with systemic aspergillosis, infection of the serosal linings was found to extend into the underlying liver, spleen and kidney parenchyma. As in other birds (Hawkey et al., 1984; Erer et al., 1986; Bowes, 1990; Jensen et al., 1997), the granulomas contained central necrosis surrounded by heterophils, macrophages, and particularly in late granulomas, multinucleated foreign-body giant cells and a scant amount of outer fibrous capsule. It is probable that the extension of serosal infection to the above-mentioned organs was due to the persistence of the infection, as stated by Okoye and Okeke (1986), who reported similar changes in the liver, kidney, spleen and bursa of Fabricius of chickens infected with *A. flavus*. However, it has been reported that mycotic lesions on the serosal linings of the gastrointestinal and upper respiratory tracts may result after a local transmural invasion of the agents (Carrasco et al., 1998).

Upon staining with special fungal stains, numerous fungal elements were seen in the granulomas and necrotic areas, consistent with observations in turkeys infected with *A. flavus* (Okoye and Okeke, 1986). Unfortunately, many of the filamentous fungi cannot be reliably differentiated from one another with any of the routine fungal stains (Phillips and Weiner, 1987; Buckley et al., 1992; Reed et al., 1993; Jensen et al., 1993; Jensen et al., 1996*a*; Fenelon et al., 1999), because the appearance of fungi in sections is affected by steric orientation, age

of the fungi, type of infected tissue, the number of fungal elements present, and the host response (Jensen et al., 1993; Jensen, 1994). It has also been reported that sometimes, and most often in chronic lesions containing non-viable hyphae, staining may only be weak, or in overstained sections fungal fragments may be masked. Using the special fungal stains, calcified bodies and overstained empty small blood vessels may be misinterpreted as fungi (Reed et al., 1993; Jensen, 1994). Consequently, to improve identification of fungi in tissue sections a number of immunohistochemical staining systems using polyclonal and monoclonal antibodies have been developed (Reed et al., 1993; Jensen et al., 1993; Jensen et al., 1996*b*). Thus, the presumptive diagnosis of invasive fungal infections based on histologic examination ideally should be confirmed by immunological techniques using monoclonal antibodies (Piérard et al., 1991; Buckley et al., 1992; Jensen et al., 1993; Jensen et al., 1996*a*; Fenelon et al., 1999). Similarly, in this study, within the granulomas in both the lungs and abdominal viscera significantly more fungal elements were disclosed when immunostaining with monoclonal antibody was used compared to the conventional fungus staining methods, consistent with the observations of Jensen et al. (1997) in turkeys. In the lesions in the above-mentioned sites fungal hyphae, spores, and also conidiophores, which were especially observed when the granulomas were opened to the airways, reacted strongly with immunostaining using monoclonal antibody. In particular, in some cases late granulomas showed few fragments of the fungal hyphae using routine fungal stains, whereas numerous fungal fragments were clearly seen when immunostain was applied. In addition, in some cases showing a few fungal hyphae along with widespread necrosis or granulomatous reaction in the abdominal viscera numerous fungal elements reacted evidently with monoclonal antibody. Thus, upon staining with monoclonal antibody and given their typical morphological characteristics, all the cases were unequivocally identified as aspergillosis. In organs with access to air, especially within the upper parts of the respiratory tract, but also within the lungs, the morphology of the conidial heads may be very helpful in identifying the infecting agent (Cutsem and Rochette, 1991; Jensen, 1994; Dykstra et al., 1997; Carrasco et al., 2001). Similarly, in our research, based on the morphological characteristics of the branched and septate fungal hyphae and conidial heads reacted with monoclonal antibody in the airways, along with the growth of bluish-green colonies in SDA (Quinn et al., 1998), it was most likely that the disease was caused by *A. fumigatus* in all of the goslings, in accordance with data of the literature (Dyar et al., 1984; Reece et al., 1986; Pal et al., 1989; Machin et al., 1993; Singh et al., 1993; Jensen, 1994).

In conclusion, application of immunohistochemistry confirmed the diagnosis of aspergillosis in the goslings in a convincing manner despite the absence of the characteristic aspergillus granulomas in some cases, in accordance with the observations of Phillips and Weiner (1987). As stated by some authors

(Piérard et al., 1991; Kaufman, 1992; Buckley et al., 1992; Jensen et al., 1993; Jensen et al., 1997), when dealing with filamentous fungal infections, except for mycelia of *Mucor* spp., the differentiation of hyphal forms of *Aspergillus* spp. from *Fusarium* spp. and *Scedosporium* spp. in tissues normally cannot be done accurately. Furthermore, it is possible that in some cases hyphae of these fungi may be confused with those of the zygomycetes and *Candida* spp. (Jensen et al., 1993; Jensen, 1994). Likewise, systemic candidosis, concomitant aspergillosis and zygomycosis have been diagnosed immunohistochemically in the tissues of two Amazon parakeets (Carrasco et al., 1998) and three lovebirds (Carrasco et al., 1993). Thus, even though fungal elements within the granulomas in the lungs and other viscera of birds were indicative of aspergillosis histologically, as in the goslings, immunohistochemistry with monoclonal antibodies should be applied to obtain a definitive diagnosis of the disease and to differentiate it from many of the filamentous fungi.

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