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Pigeon breeders' lung: pigeon intestinal mucin, an antigen distinct from pigeon IgA

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SUMMARY

Antigens identified by indirect immunofluorescence staining and specific for sera from patients with pigeon breeders' lung or healthy pigeon breeders, have been isolated from pigeon intestinal mucus. Two antigenic peaks, one pigeon intestinal mucin and the other IgA, were separated by equilibrium centrifugation of water-soluble mucus in a caesium chloride density gradient. Antigenic positive material was identified by a modified double-sandwich ELISA, by inhibition of haemagglutination of turkey erythrocytes and by gel diffusion. Antigenic-positive material co-fractionated on gel-filtration with purified intact and papain digested pigeon mucin, both free of IgA. These studies demonstrate antibodies to two quite different antigens are associated specifically with sera from pigeon breeders, a novel antigen pigeon intestinal mucin and the previously documented pigeon IgA.

Keywords pigeon breeders' lung pigeon intestinal mucin pigeon IgA

INTRODUCTION

Extrinsic allergic alveolitis, a progressive chronic inflammatory disease of the gas exchange structures of the lung is known to be induced by the inhalation of airborne organic particles (Turner-Warwick, 1978). One form of this disease may be found among susceptible individuals who are regularly exposed to pigeons (Reed, Sosman & Barbee, 1965; Fink *et al.*, 1968). The causative agents arise from avian secretory materials found among the dusts encountered in the pursuit of this avocation, but these are not well defined in terms of the specific antigens.

The disease is characterized by non-caseating granulomata, a feature of type IV immunological reactions (Hensley *et al.*, 1969). Circulating antibodies to provoking agents, typical of type III immunological reactions, can also be demonstrated in the sera of exposed individuals (Barboriak, Sosman & Reed, 1965; Tebo, Moore & Fink, 1977; Banham *et al.*, 1982). Antigens defined by these antibodies have been identified in pigeon sera (Fink, Tebo & Barboriak, 1969; Faux, Wells & Pepys, 1971), intestinal mucosa (Sennekamp, Vogel & Stiens, 1976), droppings (Fredricks & Tebo, 1977, 1980) and more recently in pigeon bloom from feathers (Longbottom, 1989). Furthermore, in affected individuals, symptoms of the disease can be produced by the inhalation of nebulized pigeon sera or the saline extracts of pigeon faeces (Hargreave & Pepys, 1972).

Avian albumin and gamma globulins have been demonstrated as antigens in avian materials (Fink *et al.*, 1969; Faux *et al.*, 1971). However, in recent years attention has focused predominantly on secretory IgA and its subfractions as the major antigenic components in pigeon secretory materials. IgA has been demonstrated in feather bloom (Longbottom, 1989) and pigeon droppings (Fredricks & Tebo, 1977) with fractionation of the latter revealing at least four related antigenic components (Fredricks & Tebo, 1980). Little attention has been given so far to other antigenic materials in avian secretions. Here we describe a second antigen, quite distinct from pigeon IgA, which fractionates as pigeon intestinal mucin.

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MATERIALS AND METHODS

Sera

Sera were obtained from three groups of subjects.

The first group (group A) comprised 15 symptomatic pigeon breeders with clinically diagnosed pigeon breeders' lung who showed positive precipitins to pigeon sera, faecal extract and feather extract by counterimmunoelectrophoresis (CIE).

The second group (group B) was of 20 healthy pigeon breeders with no respiratory problems, who had precipitins to pigeon sera, faecal extract and feather extract. These individuals had no medical histories of pulmonary disease or reported breathlessness or influenza-like episodes in the year previous to the study.

The third group (group C) was of 40 volunteer individuals with no avian contact, 20 of whom demonstrated precipitins to teichoic acid extract and false-positive precipitins to pigeon faecal extract by CIE (Faux *et al.*, 1970).

Precipitin-positive sera were identified by CIE (Mackenzie, 1980) using antigens and positive control sera from Mercia Diagnostics, Guildford, Surrey, except for feather extract which was prepared directly (Banham *et al.*, 1982).

Staining of sections

Cryostat sections of pigeon intestine were stained for IgA by indirect immunofluorescence using goat anti-chicken IgA (Nordic Immunologicals) and donkey anti-goat fluorescein conjugate (Sigma). Sections were also stained with haematoxylin : eosin and periodic acid–Schiff (PAS) reaction. Indirect immunofluorescence staining was performed on cryostat sections of pigeon intestinal mucosa with all sera and using antihuman globulin fluorescein conjugate, obtained from Dako (Sennekamp *et al.*, 1976).

Determination and quantification of antigenic components

All antibody–antigen reactions were performed at 40°C to avoid possible reactions with any P1-like antigen which may have been present in the pigeon material (Munro *et al.*, 1980).

Modified double-antibody sandwich ELISA. Conjugate: Horseradish peroxidase rabbit anti-human immunoglobulin (Dako) was used as a 1/2000 dilution in phosphate-buffered saline (PBS) Tween (0·5%) containing 1% bovine albumin.

Second antibody: Excess antibodies from groups A and B were used in separate assays diluted 1/100 with PBS Tween (0·5%), pH 7·4. Sera from group C were employed as controls.

Substrate: 3,3,5,5,tetramethylbenzidine (TMB) (Dynatech Laboratories) was used. Reactions were stopped after 20 min with 2·5 M H_2SO_4 and read against air using a Titertek Multiscan microplate reader (Flow Laboratories) at 450 nm.

Sensitized plates: Of various plates investigated, M29 from Sterilin were found to give an adequate coating of antibody and good reproducibility in this assay. Pre-washed, flat-bottomed microtitre plates were coated overnight with 0·1 ml carbonate–bicarbonate coating buffer (Na_2CO_3 1·59 g/*l*, $NaHCO_3$ 2·93 g/*l*, NaN_3 0·2 g/*l*), pH 9·6, containing 1/5 dilutions of either sheep anti-avian antibody (prepared by pigeon serum and faecal extract immunization, Mercia Diagnostics) for total antigen or the antichicken IgA for avian IgA determination.

A modified, double-antibody sandwich ELISA (Voller, Bidwell & Bartlett, 1979) was performed for the quantification of antigen. Initial runs and checkerboard titrations to determine optimal dilutions of reagents and incubation times were performed using unfractionated pigeon faecal extract as a source of antigen. Subsequent quantification of antigen in the various fractionations of mucus (described below) were determined by end-point titration using this assay. End-points were considered to be readings > 3 s.d. above the mean reading of 30 samples of elution buffer (0·85% saline) included in each assay.

Gel diffusion. Precipitating antibodies to antigenic components were demonstrated by the double diffusion technique of Ouchterlony (1953).

Haemagglutination inhibition of turkey erythrocytes (Diment & Pepys, 1977). Pooled sera from nine pigeon breeders from group A was diluted from 1/2 to 1/256 in PBS, pH 7·2, in 10 rows across a U-well microtitre plate in 0·025 ml volumes. Aliquots (0·025 ml) of fractions of a caesium chloride density of pigeon intestinal mucus were added to each well of the respective rows 1–9. PBS (0·025 ml) was added to the wells and an eleventh row contained PBS (0·05 ml) alone. The plate was incubated at 40°C for 3 h. Glutaraldehyde (0·5%) pretreated pigeon or turkey erythrocytes (0·025 ml) were added and incubated at 40°C.

Preparation and fractionation of pigeon intestinal mucus

The first 25 cm of intestines from freshly killed pigeons were gently washed through with water, opened and mucus gel carefully scraped from the mucosal surface (Hutton *et al.*, 1990). The mucus was immediately frozen at −20°C; in one preparation, mucus was collected in two volumes of 4 M guanidinium chloride solution.

Pigeon mucus (100 ml) was thawed at 4°C and mixed thoroughly with 200 ml of proteinase inhibitor, 1 mM/*l* phenyl methyl sulphonyl fluoride, 5 mmol/*l* EDTA, 5 mmol/*l* *N* ethylmaleimide and 10 mmol/*l* phosphate buffer, pH 6·5 (Mantle & Allen, 1981). The resulting mixture was homogenized for 1 min, centrifuged (10 000 ***g*** for 1 h at 4°C) and the supernatant collected as soluble mucus. A concentration of 4 M guanidinium chloride was maintained throughout the preparation and fractionation of the mucus sample collected in this solvent. Soluble mucus was fractionated in a CsCl gradient (starting density 1·42 g/ml) and centrifuged towards equilibrium (48 h, 4°C, 40 000 ***g***). The resultant gradients were fractionated into eight or nine fractions and density was determined by weighing a known volume (Mantle & Allen, 1981; Hutton *et al.*, 1990). Following dialysis, small aliquots were analysed for glycoprotein using a PAS method (Mantle & Allen, 1978), protein and nucleic acid by a 260 nm ratio.

Mucin-containing fractions from the density gradient fractionation were analysed by gel filtration on Sepharose 2B (Pharmacia). Prior to gel filtration, some mucin samples were proteolytically digested with papain (EC 3.4.22.1) (Hutton *et al.*, 1990).

RESULTS

Localization of antigens in pigeon tissue

Strong staining of pigeon intestinal secretions within and emanating from the crypts was found to be a consistent and predominant feature of all 35 pigeon breeders with positive precipitins. Of these 35 sera, 28 also demonstrated staining of the mucosal stroma and epithelial cells of the intestine. Sera from 40 non-pigeon breeders (including 20 with positive precipitins to teichoic acid extract and to pigeon dropping extract) produced no positive staining of the pigeon intestinal mucosal sections.

Indirect immunofluorescence staining of the pigeon intestinal mucosal sections with anti-chicken IgA produced strong staining of the mucoid secretions but negligible tissue staining. As a result of this demonstrated reactivity of anti-chicken IgA with pigeon secretions and presumably pigeon IgA, this antibody was used in subsequent IgA ELISA capture assays. The PAS reaction demonstrated intense staining of the secretory material within the crypts as expected in an area of mucin production.

Isolation of antigens from pigeon intestinal mucus

The surface mucous secretions were gently scraped from washed pigeon intestinal mucosa. Following brief homogenization to solubilize the mucus gel, the soluble mucous secretions were

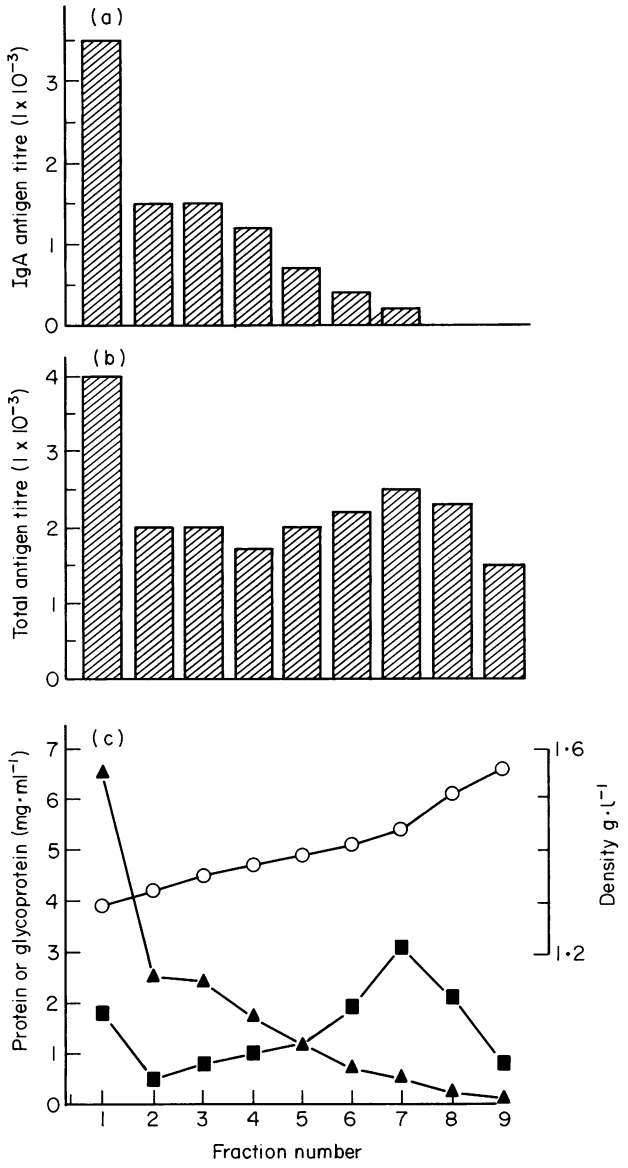

Fig. 1. IgA antigen titre (a); total antigen titre (b); and fractionation of pigeon intestinal mucus by equilibrium centrifugation in a CsCl density gradient (c). ▲, protein; ■, glycoprotein; ○, density. Quantification of antigen was by end-point titration in modified total antigen and IgA ELISA capture assay using sera from an individual with pigeon breeders' lung as second antibody.

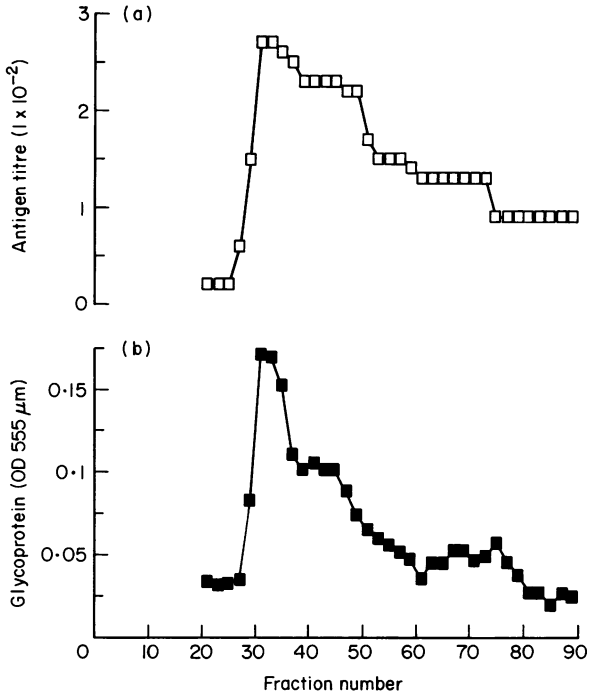

Fig. 2. Fractionation of purified pigeon small intestinal mucin by gel filtration on Sepharose 2B. Mucin-containing fractions from CsCl density gradient (Fig. 1) eluted with 0·2 M NaCl. Alternate fractions estimated for antigen (a) and glycoprotein (b) by the modified double-sandwich ELISA. Avian IgA could not be detected with anti-chicken IgA assay.

separated from remaining tissue debris by centrifugation. Proteinase inhibitors were present throughout all procedures and in one preparation 4 M guanidinium chloride was included.

The soluble mucous secretions were fractionated by equilibrium centrifugation in a caesium chloride density gradient (Mantle & Allen, 1981; Hutton *et al.*, 1990). Separation of the lower density protein containing fractions from the higher density fractions containing mucin glycoprotein was obtained (Fig. 1).

Using sera from 15 different symptomatic pigeon breeders, antigens were demonstrated by ELISA in all nine fractions from density gradient fractionation of the soluble pigeon intestinal mucous secretion (Fig. 1). The end-point titres of total antigen demonstrated a similar profile with all 15 sera (used as second antibody). This profile was consistent with separate antigenicity in both low-density protein and high-density mucin fractions. Similar profiles were obtained with sera from five asymptomatic pigeon breeders. No positive results were obtained using sera from unexposed individuals, irrespective of whether positive precipitins to teichoic acid extract and pigeon dropping extract had been previously demonstrated.

The capture ELISA showed IgA to be present only in the lower density protein-containing fractions and to be absent from the mucin glycoprotein-containing fractions.

Positive precipitins, assayed by double gel diffusion, were detected only against fractions 1–4 with 10 different sera from symptomatic pigeon breeders. These fractions also demonstrated reactions of partial identity with pigeon sera confirming the immunoglobulin nature of the antigen in fractions 1–4. No antigenic reaction was demonstrated by gel diffusion with the mucin-containing fractions 5–9, although these same fractions were antigen positive in the solution assay by ELISA. This failure to form precipitins can be explained by the mucin molecules being too large to diffuse through the gel (Allen, 1989).

Further characterization of mucin glycoprotein antigen

Pigeon intestinal mucin, from the CsCl density gradient (fractions 5–7), was excluded by gel filtration on Sepharose 2B with a broad shoulder of material extending into the included volume (Fig. 2). A similar elution profile has been obtained using standard mammalian gastrointestinal mucin glycoproteins

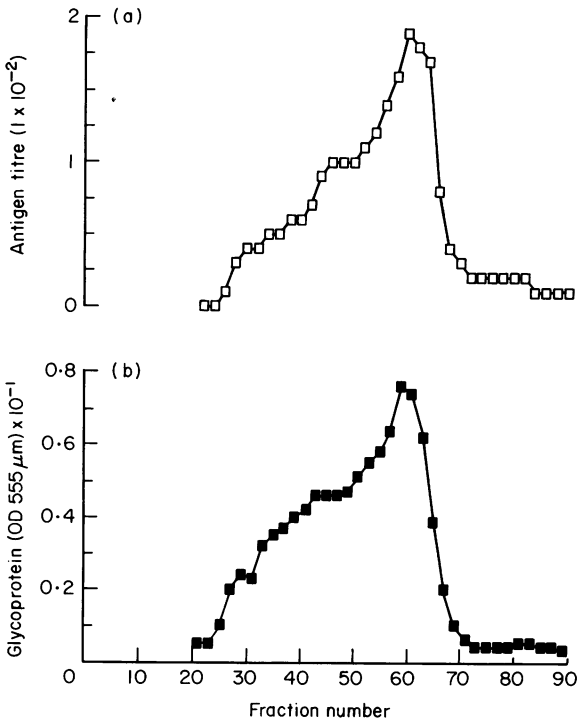

Fig. 3. Fractionation of papain-digested pigeon small intestinal mucin by gel filtration on Sepharose 2B. Mucin-containing fractions from CsCl density gradient (Fig. 1) were papain digested and eluted with 0·2 M NaCl. Alternate fractions were estimated for antigen (a) and glycoprotein (b) by the modified double-sandwich ELISA. Avian IgA could not be detected with anti-chicken IgA assay.

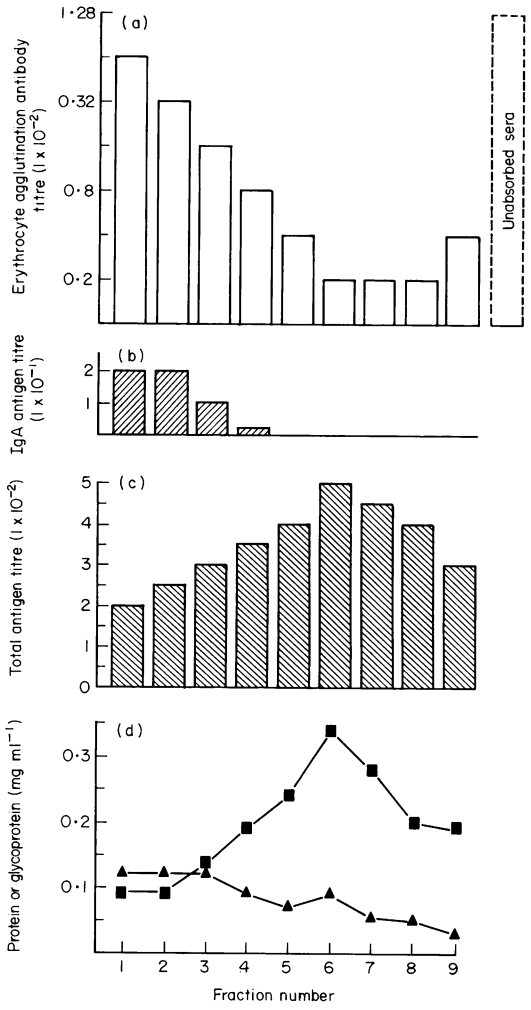

Fig. 4. Second successive fractionation of pigeon intestinal mucus by equilibrium centrifugation in a CsCl density gradient. (d). ▲, protein; ■, glycoprotein. Quantification of antigen was by end-point titration in the modified double-sandwich ELISAs for total antigen (c) and IgA (b). Inhibition of agglutination of turkey erythrocytes was with pooled sera from nine individuals with pigeon breeders' lung assay (a).

(Mantle & Allen, 1981; Mantle, Mantle & Allen, 1981; Hutton *et al.*, 1990). Alternate fractions assayed by ELISA with sera from five different symptomatic pigeon breeders gave a distribution of antigen coincident with that of the mucin. Pigeon IgA was absent in the gel filtration eluate (assayed every fifth fraction) of the mucin, consistent with its absence in these fractions from the CsCl density gradient.

The purified intestinal mucin from the density gradient was also exhaustively digested with papain, prior to fractionation by gel filtration. The papain-digested mucin eluted as a broad, polydisperse glycoprotein peak in the later fractions of the eluate following gel fractionation on Sepharose 2B and clearly distinct from the elution pattern seen for the undigested mucin (Fig. 3). This profile, which showed a substantial reduction in size of the mucin on papain digestion, was the same as that obtained following proteolytic digestion of mammalian gastrointestinal mucins (Mantle *et al.*, 1981; Hutton *et al.*, 1990). The antigenic profile determined by ELISA using sera from five symptomatic pigeon breeders mirrored the elution pattern for papain digested mucin. No detectable IgA was present in the fractions and the only detectable protein was a small peak eluting in the total volume, presumably papain.

Identification of total antigen by haemagglutination inhibition

Mucin prepared in the presence of 4 M guanidinium chloride and proteinase inhibitors was used for these studies, but it was first fractionated in a second successive CsCl density gradient because the initial fractionation gave incomplete separation of protein from the glycoprotein. Following the second fractionation in a CsCl density gradient, good separation was obtained with only small amounts of IgA and protein present in fractions 1 to 4. In this preparation, positive antigen reaction with sera gave a peak coincident with the mucin fractions alone (Fig. 4).

Antibody-positive sera from nine symptomatic pigeon breeders was shown to agglutinate pigeon or turkey erythrocytes (Diment & Pepys, 1977). This agglutination was inhibited by the mucin fractions from second density gradient fractionation with peak inhibition coinciding with those fractions containing the highest concentrations of mucin and the peak total antigen shown by the ELISA (Fig. 4).

DISCUSSION

Pigeon intestinal mucous secretions proved to be a good, novel source of antigenic material. Antibodies to these secretions were only shown by individuals with pigeon exposure. However, as previously shown (Sennekamp *et al.*, 1976), antibodies to these

mucous secretions failed to distinguish between symptomatic and asymptomatic pigeon breeders. Indirect immunofluorescent staining with anti-chicken IgA also stained the pigeon intestinal mucous secretions. This is consistent with previous work showing that IgA is a major antigen in pigeon faeces defined by antibodies in exposed pigeon breeders (Fredericks & Tebo, 1980). The demonstration of cross-reactivity of antibodies to IgA from different bird species is in contrast to the species specificity of avian gammaglobulins shown by other investigators with gel techniques (Faux *et al.*, 1971). The antigens in the pigeon intestinal mucous secretions were fractionated by equilibrium centrifugation in a CsCl density gradient, an effective method for mucin purification (Allen, 1989; Hutton *et al.*, 1990). Using this technique, pigeon IgA, identified in the lower density protein fractions by ELISA, was separated from the glycoprotein-positive, higher density intestinal mucin fractions.

Pigeon intestinal mucin has not (to our knowledge) been previously isolated but evidence showed it had a composition and macromolecular structure similar to the well-characterized mammalian small intestinal and other gastrointestinal mucins (Allen, 1989). Thus it fractionated in the characteristic density range of mucins (density 1·4–1·5) and this density is indicative of a high ratio of carbohydrate to protein (70–80% by weight carbohydrate) a hallmark of mucin glycoproteins. This high carbohydrate content of the pigeon mucin was also apparent from the ratio of PAS-positive material to protein (Fig. 1). Pigeon mucin was largely excluded following gel filtration on Sepharose 2B, giving a similar profile to that of the large molecular weight (about 10^4 kD) mammalian gastrointestinal polymeric mucins. Following exhaustive proteolytic digestion, pigeon mucin fractionated as a broad, polydisperse glycoprotein peak, included on Sepharose 2B. This demonstrated that while a substantial reduction in size of the polymeric pigeon mucin occurred on proteolytic digestion, the major component remaining was still of relatively large molecular size and resistant to further proteolysis. This pattern of mucin fragmentation is the same as that seen with mammalian gastrointestinal mucins, in particular small intestinal mucin, and reflects the presence of large (molecular weight about 500 kD), proteinase resistant, heavily glycosylated domains of the mucin (Mantle & Allen, 1981; Mantle *et al.*, 1981; Hutton *et al.*, 1990).

The antigenic nature of pigeon intestinal mucin was shown by antibody-positive sera from patients with pigeon breeders' lung following clear separation of pigeon IgA from pigeon intestinal mucin by fractionation in a density gradient (Fig. 1). The clear distinction between this mucin antigen and pigeon IgA was confirmed by the sera reacting to both intact and papain-digested pigeon mucin following fractionation by gel filtration, and under conditions where IgA was shown to be absent. The reaction of the sera with the papain-digested mucin fragment strongly points to a carbohydrate antigen in the mucin since exhaustive proteolysis will have removed all the readily accessible protein core of the mucin (Allen, 1989). Further evidence that pigeon mucin was a distinct antigen was provided by its inhibition of antibodies demonstrating haemagglutination of turkey and pigeon erythrocytes. This was a different method of antigen detection from the double-sandwich ELISA used for the other assays. Thus in a density gradient fractionation of pigeon small intestinal mucin containing minimal amounts of pigeon IgA, the inhibition of turkey erythrocyte haemagglutination paralleled quantitatively the fractions containing mucin glycoprotein (Fig. 4).

Gel diffusion techniques have been used frequently to demonstrate the presence of IgA as the major antigen in sera from precipitin positive pigeon breeders (Fredricks & Tebo, 1977, 1980; Longbottom, 1989). In this study, using Ouchterlony plates, positive precipitin lines were confirmed with the purified IgA fractions but not with the purified mucin. The gel matrix in which double diffusion occurs would be expected to be impermeable to the large polymeric pigeon mucin (about 10^4 kD) (Mantle & Allen, 1981; Allen, 1989) which will therefore not diffuse and form precipitin lines within the gel. This would explain why other investigators have not observed a second major antigen, mucin, using the gel diffusion method.

This demonstration of an avian mucin antigen by precipitin positive sera from patients with pigeon breeders' lung is new. Other investigators have noticed antigenic material distinct from IgA, but ascribed this to IgA fragments (Fredricks & Tebo, 1977). All precipitin-positive sera from patients with diagnosed bird breeders' lung and healthy pigeon breeders reacted with both mucin and IgA antigens. Sera from non-exposed individuals reacted with neither. Antigenic material defined by precipitin-positive sera has been shown in the mucus secreting tissues of the pigeon intestinal (Sennekamp *et al.*, 1976) and respiratory tracts (Yang & Purtilo, 1975) as well as pigeon faeces and feather extract. IgA specifically has been shown to be present in pigeon faeces (Fredricks & Tebo, 1980) and feather bloom (Longbottom, 1989). Since IgA and mucin are part of the same secretion from gastrointestinal and respiratory epithelia, it might be expected that the mucin antigen is in faeces and by faecal contamination, dust or by direct application during preening, this antigen may also be present on the feathers.

The conclusion from this study is that there are two quite different antigenic components, pigeon intestinal mucin and secretory IgA, defined by precipitin-positive sera from pigeon breeders with bird breeders' lung or exposed healthy pigeon breeders. Mucins are over 70% by weight carbohydrate with the characteristic 'bottle brush' structure of many 'O'-linked oligosaccharide chains joined to a central protein core (Allen, 1989). Secretory IgA has a quite different structure with only small amounts by weight carbohydrate (12% by weight for pigeon IgA; Tebo, Fredricks & Roberts, 1977) linked as N-linked oligosaccharide chains and short O-linked monosaccharides and disaccharides (Mestecky & McGhee, 1987). This strongly suggests, although does not preclude different antigenic epitopes on IgA and mucin, respectively. Further support for different epitopes comes from the cross-reactivity of polyclonal chicken anti-IgA with pigeon IgA but not pigeon mucin. Studies are in progress to define further the individual epitopes in pigeon mucin and IgA to resolve this question.

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