Blood Group A Active Glycoproteins of Respiratory Mucus and Their Synthesis by an N-Acetylgalactosaminyltransferase*

(Received for publication, September 21, 1972)

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SUMMARY

An N-acetylgalactosaminyltransferase has been found in canine respiratory tissue which catalyzes the transfer of GalNAc from UDP-GalNAc to a blood group H substance with the formation of a substance with human blood group A activity. The transfer was dependent upon Mn²⁺ and was stimulated several fold by Triton X-100. The optimal pH was between 5 and 6. The approximate Kₐ values for UDP-GalNAc and the acceptor (porcine submaxillary mucin deficient in GalNAc residues at the nonreducing end of the sugar chains) were 10⁻³ M and 10⁻² M, respectively. The enzyme was found only in the respiratory tissue of dogs whose tracheal mucus inhibited the agglutination of human red cells (type A) by human anti-A serum. In addition, mucins isolated from mucus which inhibited the agglutination had higher ratios of GalNAc to GlcNAc than those for mucins isolated from mucus which did not inhibit the hemagglutination. These studies indicate that glycoproteins with human blood group A activity are produced by canine respiratory tissue.

The rheological properties of respiratory mucus are of great importance for its normal function. Changes in these properties are accompanied by changes in ciliary movement of mucus as is frequently encountered in chronic obstructive lung diseases. By and large it is the glycoproteins present in these secretions which impart the necessary viscoelasticity, so it is possible that the altered physical state of respiratory mucins in these pathological conditions may arise from the presence of either abnormal glycoproteins or abnormal proportions of the usual glycoproteins. A prerequisite for an examination of differences in disease states is a characterization of the glycoproteins found in normal secretions. The present studies indicate that glycoproteins with human blood group A activity are present in human and canine respiratory mucus, and in addition, an enzyme is present in the mucosal layer of the respiratory tract of dogs which catalyzes the transfer of N-acetylgalactosamine to a blood group H substance with the formation of a blood group A-active substance.

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EXPERIMENTAL PROCEDURE

MATERIALS—UDP-N-acetyl-β-[1-¹⁴C]galactosamine was purchased from New England Nuclear, and the corresponding non-radioactive substance prepared as previously described (2) was used to adjust the specific activity. The submaxillary mucins, fetuin, and human α-1-acid glycoprotein were prepared and the enzymatic removal of the various sugars from these glycoproteins was performed by previously described methods (2, 3). Unless stated differently, the concentrations of all glycoproteins are expressed as the number of positions available for sugar attachment on each of the modified glycoproteins.

Canine tracheal mucus was collected from a tracheal pouch which was surgically prepared in purebred, male beagles (White Eagle Farms) as described by Wardell et al. (4). Human respiratory secretions were obtained from volunteers, either by aspiration through a tracheotomy or endotracheal tube, or by collection of sputum produced by chronic cough. Specimens were frozen immediately after collection.

Fresh frozen canine tracheas were obtained from Rockland, and human anti-A blood grouping serum was purchased from Ortho Diagnostics.

METHODS—Aqueous extracts (5) of canine tracheas were assayed for their ability to inhibit the human A-anti-A hemagglutination (6). The tracheas were combined according to their ability to inhibit this assay, and an enzymatic preparation was prepared from the pooled tracheas as described previously (2).

Saliva and tracheobronchial secretions were also typed for their ability to inhibit hemagglutination using standard antisera. Tracheobronchial secretions (1 mg) were dissolved by adding 0.01 ml of 0.01 M phosphate buffer, pH 7.0, in 0.15 M NaCl containing 0.02% NaN₃ and 0.1% dithioerythritol. After heating the samples for 10 min at 100°, they were centrifuged for 20 min at 32,000 × g, and the resulting supernatant fluids were assayed for blood group activity (6).

The standard assay for the estimation of the activity of N-acetylgalactosaminyltransferase was performed at 37° in a final volume of 0.05 ml, containing 0.09 µmole of PSM(A⁻)¹, 0.025 µmole of UDP-GalNAc (10⁶ dpm per µmole), 2.5 µmole of N-

¹ The abbreviations used are: PSM(A⁻), porcine submaxillary mucin which does not inhibit human A-anti-A hemagglutination; PSM(A⁺), porcine submaxillary mucin which does inhibit human A-anti-A hemagglutination; OSM-(NAN, GalNAc), sialidase-N-acetylgalactosaminidase-treated ovine submaxillary mucin; NAN, N-acetylnueuraminic acid.
2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 5.5, 0.25 μmole MnCl₂, 0.01 ml of 5% Triton X-100, and 50 μg of enzyme protein. The reaction was terminated by adding 0.01 ml of 0.3 m EDTA to the reaction mixture. Product formation was measured by subjecting an aliquot of the incubation mixture to high voltage electrophoresis for 45 min on Whatman No. 3MM paper with a Gilson high voltage electrophorator. A 1% solution of sodium tetraborate, pH 9.0, was the buffer. Following electrophoresis, descending chromatography was performed in 80% ethanol for 16 hours. The paper was dried, and the area around the origin was counted by liquid scintillation methods (2). The amount of radioactivity remaining at the origin after electrophoresis and chromatography was a measure of the amount of product formed (7).

The carbohydrate analyses were determined by gas-liquid chromatography of the alditol acetates as described by Griggs et al. (8). Previously described methods were used for the assay of the UDP-N-acetylgalactosamine, polypeptide N-acetylgalactosaminyltransferase, protein determination, and estimation of $K_m$ (2).

**RESULTS**

**Blood Group A Activity and N-Acetylgalactosaminyltransferases**

When aqueous extracts of 20 canine tracheas were tested for their ability to inhibit the human A-anti-A hemagglutination system, it was found that A activity was present in 40% of them. Particulate enzymatic preparations prepared from mucosal scrapings of the active tracheas catalyzed the transfer of N-acetylgalactosamine from UDP-GalNAc to PSM(A⁻), a blood group H substance, while the particulate enzymatic preparation from the other tracheas did not. In contrast, scrapings from all tracheas catalyzed the transfer of GalNAc from its respective sugar nucleotide to ovine submaxillary mucin which had been sequentially treated with sialidase and N-acetylgalactosaminidase.

The above results suggested the presence of glycoproteins with blood group A activity in respiratory secretions and led to an examination of canine mucus collected from tracheal pouches for its ability to inhibit the human A-anti-A hemagglutination system. Thirteen of the 21 pouch dogs tested produced a secretion which inhibited the hemagglutination. Several of the samples of canine mucus were dialyzed (9), lyophilized, and the residues were hydrolyzed and analyzed for carbohydrates. The lyophilized residues from mucus with human blood group A activity showed higher ratios of GalNAc to GlcNAc than those found for mucus which did not inhibit the hemagglutination. In addition, several of these pouch dogs were killed, and the tracheas were removed. A cross section of each trachea was homogenized, and particulate preparations were prepared, as described for mucosal scrapings of the frozen tracheas, and analyzed for their ability to catalyze the transfer of GalNAc to PSM(A⁻). As expected, only the respiratory tissue from those dogs secreting A-active substances contained the enzyme which converted PSM(A⁻) to PSM(A+). A summary of these results is presented in Table I.

One sample of mucus from a secretor dog was chromatographed on a 1% agarose column (9), and the immunological activity was found to reside in only the fraction that contained the major mucins.

Human saliva and tracheobronchial secretions were also examined for their ability to inhibit the human ABO hemagglutination system, and the results are summarized in Table II. In all cases in which the subject was determined to be a secretor the antigenicity of the respiratory mucus was identical with that of saliva and corresponded with the blood type. However, it was not uncommon to find subjects with mixed A or B and O activities, a situation which has not been noted to date with the dog.

**Properties of Enzyme**—The transfer of N-acetylgalactosamine to PSM(A⁻) by a particulate preparation prepared from the mucosal fraction prepared from canine mucus as described in the text. 4 ND = Not determined.

| Blood group A activity | GalNAc transferred | Molar ratio of GalNAc to GlcNAc |
|------------------------|-------------------|-------------------------------|
| Blood type              | A                 | A                             |
| Saliva                  | A                 | A                             |
| Tracheobronchial secretions | A               | A                             |

* The ability to inhibit and not to inhibit the agglutination of red cells by human anti-A serum is indicated by + and −, respectively.

* The particulate enzymatic preparation was prepared from a cross-section of the trachea.

* Carbohydrate analyses were performed on the macromolecular fraction prepared from canine mucus as described in the text.

**TABLE I**

| Dog No. | Blood group A activity | GalNAc transferred | Molar ratio of GalNAc to GlcNAc |
|---------|------------------------|-------------------|-------------------------------|
| 556     | −                      | 80                | 0.27                          |
| 714     | −                      | 69                | 0.29                          |
| 854     | +                      | 87                | 0.65                          |
| 716     | +                      | 75                | 0.65                          |
| 497     | ND                     | 78                | 0.31                          |
| 458     | +                      | ND                | ND                            |
| 716     | −                      | ND                | ND                            |
| 176     | +                      | ND                | 1.0                           |
| 852     | ND                     | 65                | ND                            |
| 546     | ND                     | 72                | ND                            |
| 710     | ND                     | 81                | ND                            |
| 804     | ND                     | 77                | ND                            |
| 571     | ND                     | 67                | ND                            |

**TABLE II**

**Summary of blood type and antigenicity of respiratory mucus and saliva from humans**

| Subject code | Blood type | Secretor status |
|--------------|------------|-----------------|
| M. B         | A          | A               |
| J. B         | O          | O               |
| T. B         | O          | O               |
| H. C         | A          | A               |
| R. P         | O          | O               |
| R. G         | B          | B               |
| P. M         | A          | A               |
6. The rate of transfer of N-acetylgalactosamine to PSM(A⁻) as a function of the concentration of UDP-GalNAc and PSM(A⁻) is shown in Fig. 2. The approximate Kᵅ values for the sugar nucleotide and the submaxillary mucin were 10⁻⁴ M and 10⁻³ M, respectively.

The particulate enzymatic preparation was capable of catalyzing the transfer of GalNAc to OSM(-NAN, GalNAc) in addition to PSM(A⁻) (Table IV). The fact that the transfer of GalNAc to a mixture of OSM(-NAN, GalNAc) and PSM(A⁻) approached a summation suggested the presence of two enzymes or two active centers (Table V). No significant transfer was detected with the other glycoproteins tested.

The enzymatic reaction was further characterized by identifying the product of the reaction. A large scale incubation was performed, and the product of the reaction was isolated as previously described (2). The radioactive product formed was nondialyzable, was precipitated by 5% trichloroacetic acid containing 1% phosphotungstate, and did not migrate in electrophoresis in 1% borate, pH 9.0. After treating the ¹⁴C product (10⁵ cpm) with an N-acetylgalactosaminidase from Clostridium perfringens (2), all of the radioactivity was liberated and migrated with N-acetylgalactosamine when subjected to high voltage electrophoresis in 1% sodium tetraborate and paper chromatography in the following solvents: System A, 1-butanol-pyridine-water, 6:4:3; System B, 1-butanol-pyridine-water, 3:1:1; and System C, ethyl acetate-pyridine-water, 2:1:2 (upper phase). The product of the enzymatic reaction exhibited blood group A activity as it inhibited the human A-anti-A hemagglutination system. In addition, precipitin analysis (10) of the product demonstrated the net synthesis of blood group A activity (Fig. 3). Approximately 25% of the radioactivity incorporated into PSM(A⁻) was precipitated with human anti-A.

### TABLE III

**Requirements of canine tracheal UDP-N-acetylgalactosamine-mucin N-acetylgalactosaminyltransferase**

The standard assay as described in the text was used. The enzymatic preparation was prepared from the mucosal scrapings of A-active tracheas.

| Incubation mixture | GalNAc transferred (µmol/mg protein/hr) |
|--------------------|----------------------------------------|
| Complete           | 129                                    |
| Minus PSM(A⁻)      | 3                                      |
| Minus enzyme       | 2                                      |
| Minus enzyme plus boiled enzyme | 1                                    |
| Minus Triton       | 16                                     |
| Minus Mn²⁺         | 8                                      |

*Equivalent to 92,000 cpm incorporated per mg of protein per hour.

### TABLE IV

**Substrate specificity of canine tracheal N-acetylgalactosaminyltransferase**

The standard assay as described in the text was used, using the following macromolecules as acceptors. The enzymatic preparation was prepared from the mucosal scrapings of A-active tracheas.

| Acceptor | Concentrationa | GalNAc transferred (µmol/mg protein/hr) |
|----------|----------------|----------------------------------------|
| Porcine submaxillary mucin (A⁺) Native | 170 | 5.5 |
| Porcine submaxillary mucin (A⁻) Native | 170 | 120.4 |
| Fetuin Native | 250 | 3.1 |
| NAN free | 250 | 3.1 |
| NAN, Gal free | 250 | 3.2 |
| NAN, Gal, GlcNAc free | 250 | 3.8 |
| Human α-acid glycoprotein Native | 250 | 2.5 |
| NAN free | 250 | 1.6 |
| Ovine submaxillary mucin Native | 250 | 7.1 |
| NAN free | 250 | 18.5 |
| NAN, GalNAc free | 250 | 105 |

*Concentration is expressed as micrograms, dry wt, of the acceptors and not as acceptor site concentration.

**Fig. 1** (left). The effect of the concentration of Mn²⁺ (a) and the effect of pH (b) on the incorporation of GalNAc. The conditions of the assays were the same as described in the text. The enzymatic preparation was prepared from the mucosal scrapings of A-active tracheas.

**Fig. 2** (center). The effect of the concentration of UDP-GalNAc (a) and the effect of the concentration of PSM(A⁻) (b) on the incorporation of GalNAc. The conditions of the assays were the same as described in the text. The enzymatic preparation was prepared from the mucosal scrapings of A-active tracheas.

**Fig. 3** (right). Precipitin analysis of the product formed when canine tracheal particles were incubated with UDP-GalNAc and PSM(A⁻). The standard reaction mixture was increased 5-fold and incubated at 37°C. At the indicated times 50 µl were removed and assayed for the formation of blood group A-active substance by the precipitin test described by Tuppy and Schenkel-Brunner (10). After washing the precipitates with cold 0.9% NaCl solution, they were then dissolved in 0.2 ml of Nuclear Chicago solubilizer and counted with a liquid scintillation spectrometer in toluene containing 4 g of 2,5-diphenyloxazole and 110 µg of p-bis-[2-(5-phenyloxazolyl)]benzene per liter.
Transfer of N-acetylgalactosamine to OSM(-NAN, GalNAc) and PSM(A-), singly and in a mixture

The standard assay as described in the text was used, except that the incubation was performed at pH 6.0. The enzymatic preparation was prepared from the murosa scrapings of A-active tracheae.

| Acceptor          | N-Acetylgalactosamine transferred |
|-------------------|----------------------------------|
| OSM(-NAN, GalNAc) |                                   |
| 0.25 μmoles        | 0.00 ng protein/hr               |
| 0.25 μmoles        | 0.09 ng protein/hr               |

**Distribution of Enzymatic Activity in Respiratory Tract**—Fourteen dogs were killed, the respiratory tracts were removed, and a particulate enzymatic preparation was prepared from the following areas: (a) cranial trachea, (b) caudal trachea, (c) bifurcation, (d) intrapulmonary bronchi, (e) peripheral lung. The particulate preparation from all fourteen dogs catalyzed the transfer of GalNAc to OSM(-X-NAN, GalNAc), but the particles from only 6 of the 14 catalyzed the transfer of GalNAc to PSM(A-). When present, the enzyme was found in all five sections with lower activities in the lung.

**Discussion**

Several of our findings suggest that glycoproteins containing the structural determinant of human blood group A specificity are elaborated by the mucus-producing structures of the airway passage of dogs. Preincubation of human anti-A serum with canine tracheal mucus prevented the hemagglutination upon the addition of human A erythrocytes. Carbohydrate analyses of respiratory mucins from A-active mucus showed higher ratios of GalNAc to GlcNAc than those for mucins from canine mucus which did not inhibit the hemagglutination. This is expected if glycoproteins with blood group A character are present, since it is the presence of terminal GalNAc residues linked to galactose residues of the oligosaccharide side chains which is responsible for the blood group A activity. There appears to be roughly three times the amount of GalNAc present in respiratory mucins from A-active secretor dogs, which suggests a branched chain structure similar to that suggested by Lloyd and Kabat (11).

In addition, an enzyme was found to be present in the mucosal layer of the respiratory tract of dogs which catalyzed the transfer of N acetylgalactosamine to a blood group H substance with the formation of a blood group A-active substance. This enzyme was found to be present in approximately 40% of the dogs examined and was only present in those dogs in which tracheal mucus inhibited the human A-anti-A hemagglutination system. Another N-acetylgalactosaminyltransferase which catalyzed the transfer of N-acetylgalactosamine from its uridine diphosphate derivative to the polypeptide core of ovine submaxillary mucin was present in all of the dogs examined. These findings, together with the results of the mixed substrate experiment (Table V), suggest that the former enzyme is a separate enzyme and is responsible for conferring human blood group A activity to canine respiratory mucus.

Human respiratory secretions were also found to inhibit the human A-anti-A hemagglutination system, as previously noted by Havez et al. (12). The finding that some humans secrete both A and O (II) or B and O (H) substances is probably due to a significant amount of incomplete biosynthesis. Our preliminary examination of human respiratory tissue for various glycosyltransferases have indicated that it, also, contains an N-acetyl-d-galactosaminyltransferase which transfers GalNAc to blood group H substances.

**Acknowledgments**—The guidance of Drs. Edward McGuire and Saul Rosenman of Johns Hopkins University and Drs. H. Green, V. Wielchaus, J. Keffin, and K. Holden of these laboratories is gratefully acknowledged. We wish to thank Dr. Lawrence Chakrin for providing us with canine mucus.

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J. Biol. Chem. 1973, 248:880-883.

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