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Original article

Selective in vitro degradation of the sialylated fraction of germ-free rat mucins by the caecal flora of the rat

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Abstract – Mucins, which are synthesized throughout the gastrointestinal tract, may be degraded by the microflora of the large intestine. The present study was undertaken to determine the differential fate of the various types of mucins. Mucins from germ-free rats were incubated in vitro in the presence of whole caecal flora from the conventional rat. Neutral, acidic and acidic sulphated mucins were spectrophotometrically assayed over time upon anaerobic incubation. Sialylated mucins were more rapidly degraded (90 %) than the other two types after 1 h and almost completely within 4 h. Neutral and acidic sulphated mucins, with a 10-fold and 30-fold lower content than the sialylated fraction in the original substrate, were more slowly degraded and to a lesser extent within 4 h, (55 and 40 %, respectively). The method used in the present study made it possible to investigate the activity of gut bacteria towards the various types of mucins. The degradation of the three mucin types was not uniform, the highest rate and extent of degradation being observed for sialylated mucins. © Inra/Elsevier, Paris

germ-free rat mucins / in vitro degradation / caecal microbiota

Résumé – Dégradation sélective de la fraction sialylée des mucines du rat *germ-free* par la flore cæcale du rat conventionnel. Les mucines qui sont synthétisées le long du tractus gastro-intestinal peuvent être dégradées par la microflore du côlon. La présente étude a été entreprise pour déterminer le devenir des divers types de mucines. Des mucines de rat axénique ont été incubées in vitro en présence de la flore cæcale totale de rat conventionnel. Les mucines neutres, sialylées et acides sulfatées ont été dosées par spectrophotométrie en fonction du temps après incubation en condition anaérobie. Les mucines sialylées ont été plus rapidement dégradées que les deux autres mucines après 1 h et presque complètement (98 %) après 4 h. Les mucines neutres et les mucines sulfatées respectivement 10 et 30 fois moins importantes quantitativement que les mucines sialylées dans le substrat de départ, ont été dégradées plus lentement et en moindre quantité (55 %

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et 40 %, respectivement) en 4 h. Cette méthode permet de suivre l'activité de dégradation des divers types de mucines par les bactéries intestinales. Cette activité n'a pas été uniforme pour les trois types de mucines, elle a été la plus importante pour les mucines sialylées. © Inra/Elsevier, Paris

mucines de rat axénique / dégradation in vitro / bactéries cæcales

1. INTRODUCTION

Mucins are synthesized and secreted by goblet cells in the gastrointestinal tract. There is an equilibrium between the biosynthesis of mucus by the goblet cells and its degradation by the gut microflora [1, 2]. Dietary variations and modification of the bacterial flora alter the distribution of mucin types [8, 17, 25–27]. Changes in the proportions of mucin types are also reported under certain pathological conditions [6, 15, 19–22].

The role of bacteria in the degradation of mucin and its alteration under pathological conditions has been recently reviewed [18]. A limited number of bacterial genera are able to degrade pig gastric mucin [23, 24] and rabbit intestinal mucin [9]. Mucin oligosaccharide degradation is thought to be associated with extracellular glycosidases of bacterial origin [11]. The faecal extracts from ulcerative colitis-affected patients had a higher sialate O-acetyl esterase and glycosulfatase activity, while mucin sialidase activity was unchanged. Moreover, mucin was degraded more efficiently and rapidly in patients than in healthy controls [4].

In the intestine of the germ-free (GF) rat, mucin is not degraded owing to the absence of bacteria; it is however slightly modified by endogenous enzymes. In contrast, in the conventional rat, intestinal mucin exists in the freshly secreted form and, most likely at various stages of degradation by bacterial enzymes. The complexity of this degradation has been recently reviewed [13]. We observed previously that mucolytic activities were found in the caecal contents of the heteroxenic (HE) rats harbouring a human faecal flora, showing that bacteria from humans can degrade carbohydrate chains of rat mucins. In these HE rats, the presence of a human flora alters the number of mucus-containing cells in the caecum, proximal and distal colon [17] and also mucin-type distribution both in the mucosa and intestinal contents, as compared to GF rats [8]. An in vitro kinetic study of the degradation of rat mucins by rat flora must be carried out and validated before the bacterial types involved in this degradation can be assessed or certain pure cultures used. The aim of the present study was to follow the kinetics of intestinal mucin degradation in vitro by the caecal bacterial flora of the rat and to determine whether there was a preferential degradation of neutral, acidic or sulphated mucins.

2. MATERIALS AND METHODS

2.1. Preparation of germ-free rat caecal mucins

Five male germ-free inbred Fischer rats were used. They were 3 months old, with a mean body weight of 350 ± 22 g. Animals were cared for in agreement with the guidelines set out by our institute and adapted to conform to those established by the Canadian Council for Animal Care (Ottawa, Ontario, 1984). Temperature and relative humidity of the animal room were controlled (21 ± 2 °C, 60 ± 5 %). The lighting schedule was also controlled (12 h light–12 h dark). Rats were placed in wire-mesh cages and kept in vinyl isolators. They received a commercial diet (UAR) ad libitum,

sterilized by gamma irradiation (40 kGy) in vacuum-sealed plastic bags. The rats were killed by an overdose of diethyl ether. Caecal contents were obtained by washing the caecal lumen with 3 to 4 mL of a NaCl (9 gL^{-1})-EDTA (2 mmol L^{-1}) buffer, at pH 7.0, containing bacterial protease inhibitors (Na azide 0.02 gL^{-1}), phenylmethylsulphonyl fluoride [PMSF] (0.1 mmol L^{-1}). Pooled samples of these caecal contents were homogenized for a few seconds before centrifugation at 27 000 *g* for 20 min at 4 °C. The supernatant was dialysed against 5 × 2 L water containing Na azide 0.001 gL^{-1}. The water soluble mucin preparation was then lyophilized and stored at –20 °C until further use. The resulting mucin preparation used as substrate for bacterial fermentation was obtained from the caecal contents of germ-free rats in order to have a greater quantity of crude mucin fraction. It was devoid of bacterial substrates and contained more sulphated mucins than that of the conventional or heteroxenic rats. Moreover, we previously showed that centrifugation and dialysis removes dietary components which might interfere with the results [8].

2.2. Anaerobic incubations

All procedures were performed using strictly anaerobic conditions with 100 % CO_2 gas [14]. AC21 medium [3], modified as described [5], was supplemented prior to inoculation with the water soluble mucin preparation made from the lyophilized germ-free rat caecal mucin powder by dilution (20 mg mL^{-1}, 1 mL in 5 mL culture medium, final concentration 3.4 mg mL^{-1}) in the incubation medium.

Microbiological inocula were prepared by diluting (10 fold w/v) in a substrate-free incubation medium, the entire content of a caecum ligated and removed from a conventional rat. Incubations were initiated less than 1 h after death of the animal by addition of 0.5 mL caecal suspension in Balch-type serum-stoppered culture tubes containing 1 mL mucin dilution in 5 mL culture medium.

Incubations were performed for 30 min, 1, 2 and 4 h. Zero time incubations with and without a microbial inoculum and 4 h incubations without the substrate were used as controls. Triplicate samples were inoculated for each condition. Incubations were stopped by adding 0.1 mL of a solution containing Na azide (0.02 gL^{-1}) and PMSF (0.1 mmol L^{-1}) with freezing at –20 °C.

For the mucin assays, samples were thawed and centrifuged at 27 000 *g* for 20 min at 4 °C to pellet the bacteria. Supernatants were dialysed against 5 × 5 L water containing Na azide (0.001 gL^{-1}). The initial volume and that after dialysis were measured.

2.3. Mucin assays

The absorbance for 1 mL of the initial culture (corresponding to an initial 3.4 mg mucin preparation) was determined for each mucin type from triplicate readings. Hence, the zero time value for mucin alone, i.e. before any contact with bacteria was measured for each mucin type.

Residual mucins (neutral, acidic and sulphated), after incubation, were measured on dialysates by spectrophotometric assays, i.e. by transposing the tests used in histochemistry to the mucins of the intestinal goblet cells, as previously described [7, 8]. They were diluted to 0.025–0.150 mg mL^{-1} for neutral mucin determination and to 0.1–0.5 mg mL^{-1} for acidic and sulphated mucins; histochemical reagents (periodic acid-Schiff (PAS) Mantle and Allen's method [16], or alcian blue (AB) pH 2.5 or AB pH 0.5, respectively) were added, and the pH adjusted as required. Acidic mucins were precipitated in the presence of modified Carnoy fixative (ethanol (950 mL L^{-1})-formaldehyde (350 mL L^{-1})-acetic acid (6:3:1, by vol.)). Centrifugation was carried out at 9 500 *g*, 10 min. The pellet was dissolved at the convenient pH in the presence of Triton X100 and sonicated for 1 min (Bransonic 52 tank, Biobloc Scientific, Paris, France). Absorbance was read with a Shimadzu spectrophotometer (Roucaire, Vélizy, France) at 600 nm for the AB and at 570 nm for the PAS reaction. Values are presented as absorbance per mL of culture medium. The mean values from triplicate readings gave estimates of the extent of degradation for each mucin type.

3. RESULTS

Kinetics of neutral, acidic (sialylated and sulphated) and sulphated mucin recovery from 1 mL of initial culture are

shown in *figures 1–4*. Values for sialylated mucins were calculated by the difference from staining with alcian blue at pH 2.5, which detected both sialylated and sulphated mucins, and staining with alcian blue at pH 0.5 for the sulphated mucins alone.

It appeared that contact with bacteria, followed by an immediate centrifugation, removed a significant portion of soluble mucin from the incubation medium. The level of removal was 37 ± 5 % irrespective of the mucin-type considered.

Rates of mucin degradation were different depending on the type of mucin considered. Neutral mucins (*figure 1*) remained undegraded during the first incubation hour and then decreased regularly until the fourth hour. The amount that had disappeared at 4 h was 55 %. Acidic mucins (both sialylated and sulphated; *figure 2*) were not modified during the first 30 min, although standard deviations were high. There was then a rapid decrease which reached 90 % disappearance after 1 h and 95 % after 4 h incubation. These modifications were due to sialylated mucins, 98 % of which had disappeared after 4 h incubation (*figure 3*). Sulphated mucins (*figure 4*) were not modified during the first hour of incubation, and were only slowly degraded thereafter to reach 40 % of the initial value after 4 h.

4. DISCUSSION

In the present work we report on the differential degradation of various mucin types by whole rat caecal bacteria incubated in vitro.

Preliminary studies with whole AC21 medium showed that certain components, most likely coming from rumen fluid, interfered with the staining procedures used for spectrophotometric assays of the three mucin types. Therefore we subsequently used a more defined modified

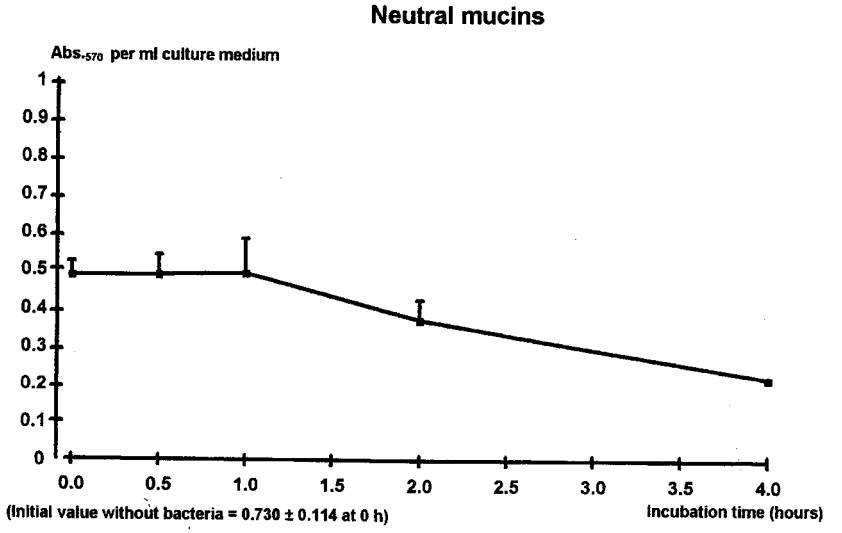

Figure 1. Bacterial degradation of neutral mucins in vitro. Neutral mucins are assayed spectrophotometrically and concentrations expressed in arbitrary units as absorbance per mL of the culture medium + SD from three assays at each incubation time. The value in parenthesis under the graph refers to mucin alone, i.e. before any contact with bacteria at zero time.

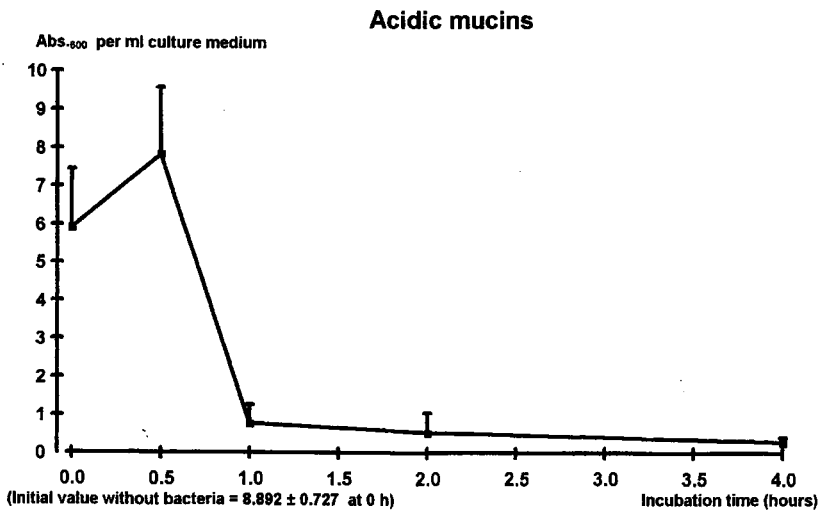

Figure 2. Bacterial degradation of acidic mucins in vitro. Acidic mucins are assayed spectrophotometrically and concentrations expressed in arbitrary units as absorbance per mL of the culture medium + SD from three assays at each incubation time. The value in parenthesis under the graph refers to mucin alone, i.e. before any contact with bacteria at zero time.

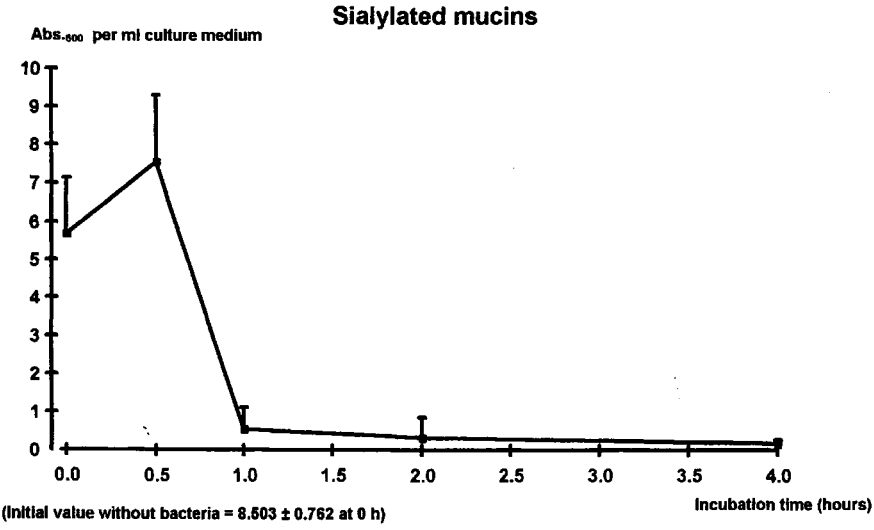

Figure 3. Bacterial degradation of sialylated mucins in vitro. Values for sialylated mucins are calculated by the difference from staining with alcian blue at pH 2.5 which detected both sialylated and sulphated mucins, and staining with alcian blue at pH 0.5 for the sulphated mucins. The concentrations are expressed in arbitrary units as absorbance per mL of the culture medium + SD from three assays at each incubation time. The value in parenthesis under the graph refers to mucin alone, i.e. before any contact with bacteria at zero time.

AC21 medium. A first trial with a rat caecal bacterial suspension, diluted to 1/10, with an incubation time of 24 h gave an almost complete bacterial degradation of the initial mucin sample. We therefore subsequently used shorter incubation times.

The comparison of free undegraded mucin contents, with or without contact with bacteria, (latter values in parenthesis at the bottom of the graphs *figures 1–4*), indicated that the centrifugation protocol used to sediment bacteria before the assays led to a significant decrease of free mucin content (37 ± 5 %). This suggested a very rapid adsorption of mucins of all types on the bacterial cell walls.

The rapid degradation of mucin that we observed, particularly for sialylated mucin in 4 h, (nearly complete in 24 h in our preliminary study), must be related to the fact that mucins were incubated in the presence of a whole caecal microbial flora from the conventional rat. This is in agreement with the earlier study of Hoskins and Zamcheck [10] who observed practically no mucin compounds left in the faeces of the conventional rats, while they are always present in the faeces of germ-free rodents. Since the composition of mucin and the mucinolytic bacterial activities vary between species, our experiments with total bacterial flora were probably closer to physiological conditions than pure culture experiments. However, the validity of transposing such in vitro data to in vivo conditions is open to question.

Comparison of our results with pure culture studies reported in the literature is nevertheless interesting. Salyers et al. [23, 24 used very long incubation times (7 days). Oligosaccharide degradation from pig gastric mucin was associated with extracellular glycosidases produced by bacterial subpopulations [11]. Hill [9] used several mucinolytic bacterial isolates and incubation times of up to 5 days, and the hexose assays before and after incubation, showed variations, taking into account the origin of the mucin studied (pig gastric

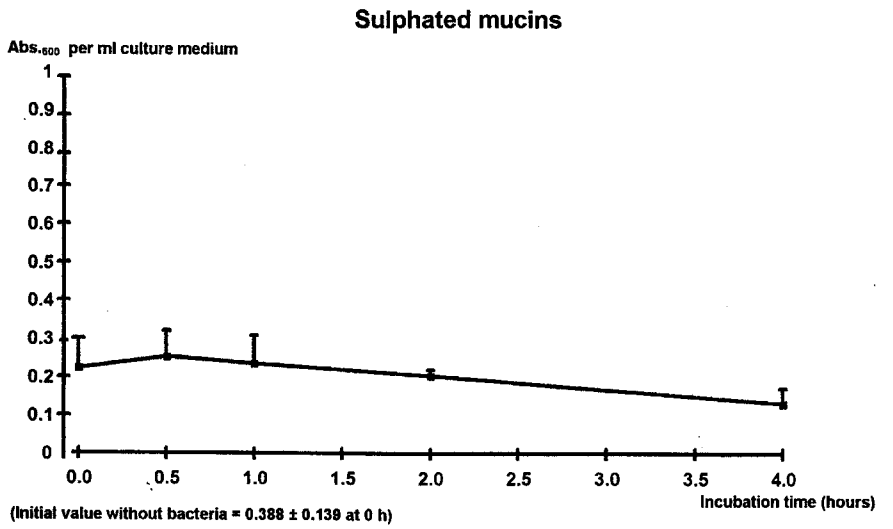

Figure 4. Bacterial degradation of sulphated mucins in vitro. Sulphated mucins are assayed spectrophotometrically and concentrations expressed in arbitrary units as absorbance per mL of the culture medium + SD from three assays at each incubation time. The value in parenthesis under the graph refers to mucin alone, i.e. before any contact with bacteria at zero time.

mucin or rabbit intestinal mucin). Variations in the number of mucin-containing cells occur between rats depending on their microbial status (conventional rats or human flora associated rats). This was related to the specific activities of these flora [17, 25, 26].

In a more recent study, the role of human faecal bacteria, particularly mucin oligosaccharide-degrading strains, was analysed. The cleavage products of polysaccharides were characterized and their potential role in nutritional support of larger faecal bacterial populations demonstrated [12]. Furthermore, Willis et al. [28] showed that batch culture incubation of pig gastric mucin with human faecal bacteria produced an increase in sulphide that was not seen with incubation of other fermentable carbohydrates. A variety of microorganisms are able to ferment mucin, in addition to its hydrolysis products, and our results strongly suggest the implication of a consortium of microorganisms in vivo.

The methodology used in the present study showed that the three mucin types were not degraded to a similar extent. Although the sialylated mucin was more abundant than the two other mucin classes, it was degraded more quickly. This can be related to the sialidase activity of the whole bacterial consortium. It would be of interest to determine whether the preferential degradation of sialylated mucin by the caecal flora differs with dietary constraints or under pathological situations.

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