Biochem. J. (1988) 254, 185–193 (Printed in Great Britain)

Immunochemical characterization of mucins

Polypeptide (M1) and polysaccharide (A and Le^b) antigens

Jacques BARA,*‡ Rodolphe GAUTIER,* Jacques LE PENDU† and Rafael ORIOL†

*Laboratoire d'Immunochimie des Mucines, Institut de Recherches sur le Cancer, ER-277 C.N.R.S., 94802 Villejuif Cedex, France, and †Institut d'Immunobiologie, ER-281 C.N.R.S., Hôpital Broussais, 75674 Paris Cedex 14, France

Seven monoclonal antibodies (MAbs) reacting with high-molecular-mass components (> 20000 kDa) isolated from an ovarian mucinous cyst of an A Le(a–b+) patient are described. By the use of immunoradiometric methods, these MAbs characterized seven different epitopes associated with components having a density of 1.45 g/ml by CsCl-density-gradient ultracentrifugation, like mucins. Two MAbs reacted with A and Lewis blood-group antigens respectively (polysaccharide epitopes). The five other MAbs characterized five M1 epitopes (called *a*, *b*, *c*, *d* and *e*), mainly associated with components of more than 20000 kDa and 2000 kDa. They were completely destroyed by papain and 2-mercaptoethanol treatment (polypeptide epitopes). Moreover, timed trypsin digestion of native mucin resulted in a progressive loss of M1 activity and degraded these mucins into smaller M1-positive fragments. The *a* and *c* epitopes were partially degraded from relatively high-molecular-mass fragments (2000 kDa to 500 kDa) into a 100 kDa fragment. The *b* and *d* epitopes were completely degraded into smaller fragments ranging from 100 kDa to 40 kDa. The *e* epitope was completely destroyed by trypsin. These different pathways of M1 antigen degradation suggest the occurrence of different epitopes located in separate regions of the mucin molecules.

INTRODUCTION

Although the amino acid and carbohydrate compositions of mucins have been determined (Pearson *et al.*, 1981), their structure is not yet well understood (Carlstedt *et al.*, 1985; Neutra & Forstner, 1986). Most authors agree (i) that the peptidic core contains heavily glycosylated regions (protected from proteolytic attack) and non-glycosylated regions (or 'naked regions') that are susceptible to proteinase digestion, and (ii) that these mucins may be built by assembly of subunits. But the arrangement and size of these subunits are controversial. After reduction with 0.2 M-2-mercaptoethanol, a protein (Pearson *et al.*, 1981) was released from mucins with an apparent molecular mass of 70 kDa for pig gastric mucins (Pearson *et al.*, 1981), 90 kDa for pig small-intestinal mucin (Mantle *et al.*, 1981) and 118 kDa for human small-intestinal mucin (Mantle *et al.*, 1984). These proteins could contribute to the internal assembly of mucin monomers. A 'windmill model' has been proposed (Allen, 1983), in which four highly glycosylated subunits are linked together by a central 70 kDa protein (link peptide) like the vanes of a windmill. A different model presented by Carlstedt *et al.* (1983) and Slayter *et al.* (1984) proposes a flexible thread model in which subunits containing both naked and glycosylated regions were attached end to end by disulphide bridges.

The best-known mucin antigens are the blood-group-related antigens associated with their carbohydrate moieties (Feizi *et al.*, 1984). However, some studies suggested eventual participation of the peptidic core in the antigenicity of mucins (Bhushana Rao *et al.*, 1973; Shochat *et al.*, 1981, 1982; Mantle *et al.*, 1984; Bara *et al.*, 1986; Fahim *et al.*, 1987). Fairly recently we obtained seven monoclonal antibodies by immunization of mice with mucins of ovarian mucinous-cyst fluid of an A Le(a−b+) patient (Bara *et al.*, 1986). By the use of immunoperoxidase, two of them (3-3A and 2-25LE) recognized the surface gastric epithelium of A- and Lewis-positive individuals respectively. The five other MAbs (1-13M1, 2-11M1, 2-12M1, 9-13M1 and 58M1) stained all mucous cells of the gastric surface epithelium independently of their ABO or Lewis phenotypes, and characterized epitopes that we called M1. In the present paper we demonstrate that these five anti-M1 MAbs characterize five different mucin M1 epitopes, associated with the peptidic core of mucins, as opposed to A and Le^b epitopes, which are associated with the polysaccharide moieties of mucins.

MATERIALS AND METHODS

Preparation of mucin antigens

M1 antigens were isolated from the fluid of an ovarian mucinous cyst of a pure endocerical type (Fenoglio *et al.*, 1975) of an A Le(a−b+) patient, as already described (Bara *et al.*, 1980). Fluid extract was chromatographed on a column (180 cm × 1.6 cm) of Sepharose CL-6B (Pharmacia, Uppsala, Sweden) eluted by upward flow with PBS. The material excluded from this gel was called 'M1 antigen preparation'. Before and after papain, trypsin and 2-mercaptoethanol treatment, 5 mg of this M1 antigen preparation in 1 ml was further filtered on a

Abbreviations: MAb, monoclonal antibody; i.r.m.a., immunoradiometric assay; M1, mucin antigens common to gastric epithelium and ovarian mucinous cysts; PBS, phosphate-buffered saline (0.15 M-NaCl/0.01 M-Na_2HPO_4/NaH_2PO_4 buffer, pH 7.4).

‡ To whom correspondence should be addressed.

Table 1. Immunoradiometric methods of M1 and A antigens

1. Antibody coating of stars (first layer)
   Mix 1000 stars with 500 ml of anti-A or anti-M1 MAb solution (5 mg of IgG in PBS)
   Incubate for 2 h with shaking
   Rinse stars three times with PBS
   Add 500 ml of 1% BSA
   Incubate overnight
   Wash three times with PBS
   Dry at 37 °C for 3 h (can be stored dried for several months)
2. Incubation of antigens (second layer)
   Add 300 μl of antigen solution in each tube containing a coated star
   Incubate for 24 h at 37 °C
   Aspirate and wash three times with PBS
3. ^{125}I-labelled antibodies (third layer)
   Add ^{125}I-labelled antibody (100000 c.p.m. per tube for anti-M1 MAb, 25000 c.p.m. per tube for anti-A Mab)
   Incubate for 24 h with Anti-M1 MAb or for 3 h with anti-A MAb at 37 °C
   Aspirate and wash three times with PBS
   Count radioactivity in each tube

180 cm × 1.6 cm Sepharose CL-2B column eluted as above with PBS.

Monoclonal antibodies

Five monoclonal antibodies (MAbs) against M1 antigens (1-13M1, 2-11M1, 2-12M1, 9-13M1 and 58M1) and two MAbs against blood-group-related antigens (3-3A and 2-25LE) were obtained as already described (Bara *et al.*, 1986). These seven anti-mucin MAbs were IgG_1.

Immunoradiometric methods

With the use of a solid phase (polystyrene stars and tubes, a gift from Oris Industrie Company, St.-Quentin-Yvelines, France), a double-antibody sandwich system was used. Pure IgG_1 was isolated from ascitic fluids by the use of Protein A–Sepharose (Ey *et al.*, 1978). All solutions were made in PBS. Purified IgG_1 was labelled with ^{125}I by the method of Greenwood *et al.* (1963). The procedure is outlined in Table 1.

I.r.m.a. of A and M1 antigens. For M1 antigen, the first layer was a mixture of the five anti-M1 MAbs. The second layer was the M1 antigen preparation. The third layer was the ^{125}I-IgG_1 from a mixture of the five anti-M1 MAbs. For A antigen dosage, the first layer and the third layer were unlabelled IgG_1 and ^{125}I-IgG_1 of anti-A Mab (3-3A) respectively. The second layer was the M1 antigen preparation. As a standard, the M1 antigen preparation was adjusted to the concentration of 1 μg/ml (carbohydrates); for comparison purposes, we arbitrary considered that the preparation contained 1000 units of A and M1 antigens.

Immunoradiometric fixation. These experiments were performed in order to determine whether the M1, A and Lewis antigens were associated with the same molecules in the M1 antigen preparation. The first layer was made up of each of the seven anti-mucin MAbs: then a colon mucin preparation of an O Le(a−b−) patient in 1% (w/v) bovine serum albumin was added in order to prevent non-specific binding, and the preparation was incubated overnight. The second layer was the M1 antigen preparation at a concentration of 1 μg/ml (carbohydrates), and the third layer was ^{125}I-IgG_1 from one of the seven anti-mucin MAbs. Consequently 49 combination experiments were performed, as reported in Table 2.

Inhibition of immunoradiometric fixation. This method was used in order to determine whether the seven anti-mucin MAbs characterized seven different epitopes. The first layer was IgG from 1-13M1 MAb, and the second layer was the M1 antigen preparation adjusted to an adequate concentration for the reaction with the MAb of the third layer. The third layer was a solution of MAb containing 100 μl of each particular radiolabelled antibody (100000 c.p.m. in 100 μl) and 200 μl of each of the seven unlabelled MAbs at different dilutions (between 1/1 and 1/1000), starting at an IgG_1 protein concentration of 10 μg/ml.

Western blot

Polyacrylamide-gel electrophoresis was carried out in 2–16%-(w/v)-gradient polyacrylamide-gels (4.9 mm × 82 mm × 82 mm) (Pharmacia, Uppsala, Sweden) in 0.04 M-Tris base/5 mM-Na_2EDTA/0.2% SDS/0.02 M-sodium acetate buffer, pH 8.35; 10 μl of mucin sample (1 mg of protein/ml) was applied and electrophoresis was performed for 2 h. The molecular masses of components were evaluated by using pre-stained protein high-molecular-mass standards (range 12300–200000 Da) (BRL, Gaithersburg, MD, U,S.A.). Mucins separated by electrophoresis were transferred to nitrocellulose filters at 0.1 A for 24 h. These filters were then incubated for 2 h in PBS containing 5% (w/v) bovine serum albumin, followed by incubation with anti-A or anti-M1 MAbs conjugated with ^{125}I (5×10^5 c.p.m./ml). Each antibody was incubated for 16 h at room temperature with constant rocking. The filters were then extensively washed with PBS containing 0.1% Tween-20. Western blots were air-dried and exposed to Amersham MP Hyperfilm at −80 °C overnight with the use of intensifying screens.

Determination of blood-group antigens

Blood-group-related aritifical antigens made with 15–20 synthetic oligosaccharide groups per molecule of bovine serum albumin were obtained from R.U. Lemieux (1978) and Chembiomed (Edmonton, Alberta, Canada). These blood-group-related antigens are type I precursor (Le^c), type 2 precursor (LacNAc), A disaccharide, B disaccharide, Lewis disaccharide, H type 1 (Le^d), H type 2, Le^a, X (Le^x), A trisaccharide, A type 1, A type 2, B trisaccharide, Le^b, Y (Le^y) and Ale^b. The structures of oligosaccharides bearing these antigens were reported previously (Bara *et al.*, 1988).

Antibody 2-25LE was tested by a radioimmunoassay as previously described (Le Pendu *et al.*, 1985). Antibody 3-3A was tested by enzyme-linked immunosorbent assays. Artificial antigens were coated on the plastic of microtitration plates (Immunoplate 1; NUNC, Roskilde, Denmark). Antibody 3-3A was then added in 2-fold serial dilutions in 0.2% gelatin/PBS containing 0.05% Tween-20. After incubation and washes with PBS, anti-(mouse immunoglobulin) antibodies labelled with per-

oxidase were added. The reaction was revealed by the use of *o*-phenylenediamine.

Biochemical analysis

Concentration of neutral sugars was determined by the method of Smith (Koch *et al.*, 1951), with galactose as standard. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Density-gradient ultracentrifugation

Ultracentrifugation of the M1 antigen preparation was carried out in 5 ml of a CsCl isopycnic density gradient. The M1 antigen preparation was dissolved in a CsCl solution containing 0.2 M-guanidinium chloride, 5 mM-EDTA and 10 mM-sodium phosphate buffer, pH 6.5 (Carlstedt *et al.*, 1983), with a starting density of 1.456 g/ml. The gradients were formed by centrifugation in a Beckman SW 50.1 rotor at 45000 rev./min for 72 h at 5 °C. Fractions (0.5 ml) were collected and diluted 1/1000 or 1/10000 for i.r.m.a.

2-Mercaptoethanol and proteinase treatments

Thiol reduction of mucin. The M1 antigen preparation (1 ml containing 5 mg of protein/ml) was reduced with 0.2 M-2-mercaptoethanol in 300 mM-Tris/HCl buffer, pH 8.6, containing 6 M-guanidinium chloride, 1 mM-phenylmethanesulphonyl fluoride (Sigma Chemical Co., St. Louis, MO, U.S.A.), 5 mM-Na_2EDTA and 5 mM-*N*-ethylmaleimide (Sigma Chemical Co.) for 72 h at 4 °C; then 0.4 M-iodoacetamide was added for 2 h at 4 °C. Mucin samples were dialysed exhaustively against PBS. Appropriate controls (without 2-mercaptoethanol) were done to determine the effect of guanidine and iodoacetamide on mucin antigenicity.

Papain digestion. The M1 antigen preparation (1 ml containing 5 mg of protein/ml) was incubated with 50 μg of water-soluble papain (Merck, Darmstadt, West Germany) in 0.2 M-sodium acetate buffer, pH 5.6, containing 5 mM-cysteine and 5 mM-Na_2EDTA and dialysed for 36 h at 65 °C against the same acetate buffer. A control M1 antigen preparation was incubated similarly but without enzyme.

Trypsin digestion. Trypsin (EC 3.4.21.4; type XI from Sigma Chemical Co.) digestion of M1 antigen preparation (1 ml containing 1 mg of protein/ml) was performed in 0.10 M-Tris/HCl buffer, pH 8.0, at 37 °C with the use of 50 μg of trypsin/mg of mucin (Carlstedt *et al.*, 1983). Incubations were performed for different times (30 min, 1 h, 3 h and 5 h). After each incubation, the trypsin activity was inhibited by type I-S trypsin inhibitor (Sigma Chemical Co.) (2 μg/μg of trypsin).

The apparent molecular masses of mucin fragments obtained after these treatments were analysed by chromatography on Sepharose 2B followed by i.r.m.a. and Western blotting of each eluted fraction.

RESULTS

I.r.m.a. of A and M1 antigens

The optimal conditions for i.r.m.a. were determined for both antigens. The antigen concentrations were 10–100 units/ml for the M1 antigen and 1–10 units/ml for the A antigen. These tests were suitable and reproducible only in these ranges of concentrations.

Characterization of 3-3A and 2-25LE epitopes (Fig. 1)

Antibody 3-3A bound to all antigens containing an A blood-group epitope (Fig. 1*a*). The strongest binding was obtained with the A trisaccharide and the A type 1 and A type 2 tetrasaccharides. The pentasaccharide Ale^b showed intermediate binding and the disaccharide GalNAc-α(1-3)Gal, devoid of fucose, had very weak binding. In a previous report (Mollicone *et al.*, 1986) this antibody, quoted as 33-C13, was shown to react with all the cells synthesizing A epitopes in the normal mucosa of the gastroduodenal junction.

As shown in Fig. 1(*b*), antibody 2-25LE bound to both

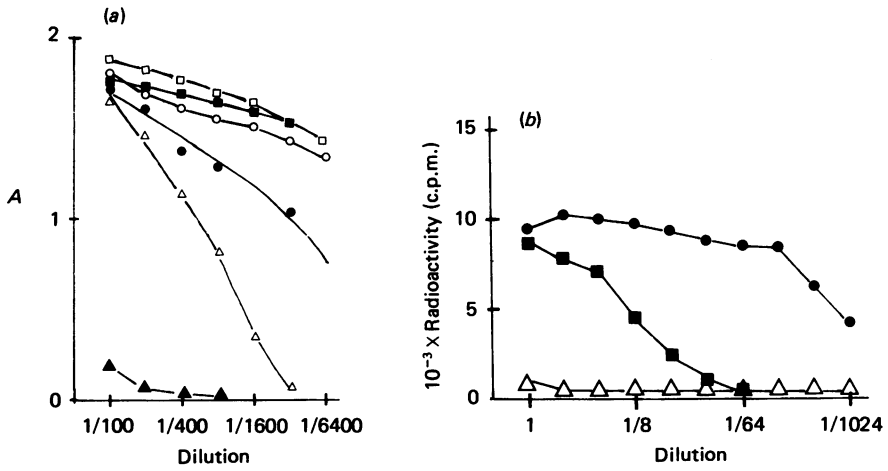

Fig. 1. Characterization of the 3-3A and 2-25LE MAbs by the use of different artificial oligosaccharides

(*a*) Characterization by enzyme-linked immunosorbent assay for 3-3A MAb; □, A trisaccharide; ■, A type 1; ○, A type 2; ●, Ale^b; △, A disaccharide; ▲, other polysaccharide antigens. (*b*) Characterization by i.r.m.a. for 2-25LE MAb: ■, Ale^a; ●, Ale^b; △, other polysaccharide antigens.

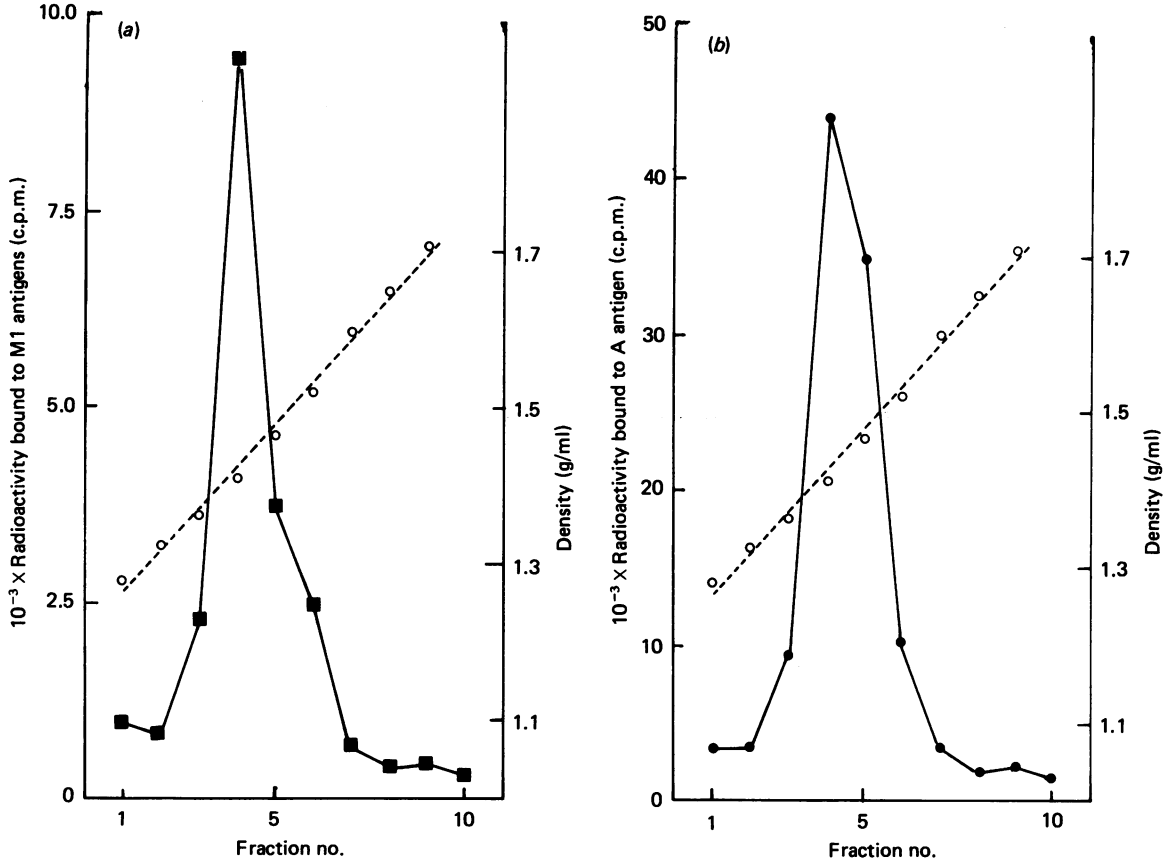

Fig. 2. Isopycnic density-gradient centrifugation in CsCl/0.2 M-guanidinium chloride of M1 antigen preparation

CsCl was added to an initial density of 1.456 g/ml. After centrifugation (at 150000 *g* for approx. 72 h at 5 °C in the SW 50.1 rotor of the Beckman L 65 ultracentrifuge), fractions were collected from the bottom of the tubes and were monitored for the presence of M1 antigen (■, in *a*) or A antigen (●, in *b*) by i.r.m.a., and density (○) was determined by refractive-index measurements.

Table 2. Immunoradiometric fixation of anti-A, anti-Lewis and anti-M1 MAbs over M1 antigen preparation

Values represent the radioactivity (c.p.m.) obtained after immunofixation of the different anti-A, anti-Lewis and anti-M1 MAb (third layer) on immunosorbents made with these seven anti-mucin MAbs (first layer) on the M1 antigen preparation (second layer).

MAb of the first layer	^{125}I-MAb of the third layer ...	Radioactivity obtained after immunofixation (c.p.m.)						
		I-13M1	2-11M1	2-12M1	2-25LE	3-3A	9-13M1	58M1
1-13M1		784	2233	2711	1798	9463	3666	488
2-11M1		2315	4	611	2393	10692	3881	222
2-12M1		604	117	103	1282	5667	331	55
2-25LE		246	723	106	6130	2989	680	20
3-3A		2017	706	1075	2007	17633	962	106
9-13M1		5156	2429	1285	2044	15894	152	579
58M1		1839	723	1514	595	12551	405	66

the Le^b tetrasaccharide and the Le^a trisaccharide, reacting more weakly with the latter. None of the other antigens tested reacted above the background value. Antibody 2-25LE was therefore a strong anti-Le^b antibody that cross-reacted with the Le^a structure.

Density-gradient centrifugation

The density of the A and M1 antigens present in the M1 antigen preparation was determined by equilibrium ultracentrifugation in CsCl as shown in Fig. 2. The gradient extended from 1.20 g/ml to 1.75 g/ml, and each

fraction was analysed for M1 and A content by i.r.m.a. The highest A and M1 activities were detected at the same density of 1.45 g/ml.

Immunoradiometric fixation

This method was used in order to determine whether the different M1 and blood-group-related epitopes were associated with the same mucin components. The results demonstrated that the five M1, the A and the Lewis related epitopes were indeed associated with the same high-molecular-mass mucin components (Table 2). When the MAb of the solid phase was identical with that of the third layer, immunofixation was negative with the 2-11M1, 2-12M1, 9-13M1 and 58M1 MAbs, slightly positive with the 1-13M1 MAb and strongly positive with the MAbs against blood-group-related antigens 3-3A and 2-25LE.

Competition of immunoradiometric fixation

The inhibition of radiolabelled MAb by unlabelled MAb occurred only when both MAbs were identical. Fig. 3(*a*) shows the dose effect of the inhibition curves on the concentration of unlabelled MAb when the unlabelled and radiolabelled MAbs were identical; 100% inhibition was obtained when the M1 antigen preparation was immunoabsorbed at a concentration of 1 μg/ml in these experimental conditions for MAbs 2-11M1, 2-12M1, 9-13M1 and 58M1. Fig. 3(*b*) shows that it was necessary to decrease the concentration of the M1 antigen preparation in order to obtain 100% inhibition of the radiolabelled 1-13M1, 2-25LE and 3-3A MAbs and the same unlabelled MAb at a concentration of 10 μg/ml of IgG. Then 50% inhibition was obtained with 1-13M1, 2-25LE and 3-3A for the M1 antigen preparation at concentrations of 3, 2.25 and 0.35 μg/ml respectively. These experiments demonstrate that the epitopes recognized by the seven MAbs are different.

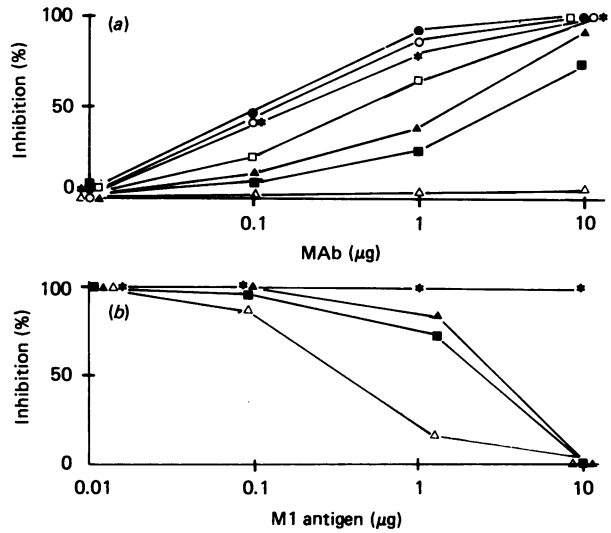

Fig. 3. Competition of immunoradiometric fixation

(*a*) shows the percentage inhibition obtained by incubation of 100 μl of ^{125}I-IgG containing 100000 c.p.m. with increasing concentrations of the same unlabelled anti-M1 MAb: ▲, 1-13M1; ●, 2-11M1; ✱, 2-12M1; □, 9-13M1; ○, 58M1; △, 3-3A; ■, 2-25LE. The solid phase was coated by using IgG of 1-13M1 MAb preincubated overnight with the M1 antigen preparation (1 μg/ml of carbohydrates). (*b*) shows the percentage inhibition obtained after incubation of 100 μl of ^{125}I-IgG (100000 c.p.m.) with 200 μl of the same unlabelled anti-M1 MAb (10 μg/ml of protein): ✱, 2-11M1 or 2-12M1 or 9-13M1 or 58M1; ▲, 1-13M1; △, 3-3A; ■, 2-25LE. Increasing amounts of the M1 antigen preparation (0.01 to 10 μg/ml of carbohydrates) were immunofixed on solid phase coated with 1-13M1 MAb.

Characterization of M1 native mucins

After chromatography on Sepharose CL-6B, M1 reactivity was found only in the fractions of the void volume (molecular masses < 10^6 Da). The components included in the gel did not show M1 immunoreactivities. The pool of these fractions, called the M1 antigen preparation, was rechromatographed on Sepharose CL-2B and had two main M1(+) peaks (Fig. 4): one that was excluded from the gel (molecular masses > 20000 kDa) and one that was included (molecular masses around 2000 kDa). A similar result was obtained by immunoblot. Fig. 5 (lane A) shows M1 immunoreactive material near the stack gel and between the stack gel and a component of molecular mass about 1000 kDa (^{125}I-IgM). The same pattern was observed with each anti-M1 MAb or with a mixture of the five anti-M1 MAbs.

Reduction and proteinase treatments

The M1 antigen preparation was divided into seven equivalent 2 ml fractions containing 5 mg of protein/ml and treated as follows: fraction A, untreated control; fraction B, incubated with the buffered solution containing 2-mercaptoethanol and then treated with iodoacetamide; fraction C, incubated similarly to fraction B but without 2-mercaptoethanol (2-mercaptoethanol control); fraction D, incubated for 36 h at 65 °C in buffered solution containing papain; fraction E, incubated under the same conditions as fraction D but without enzyme (papain control); fraction F, incubated for 5 h at 37 °C with trypsin; fraction G, incubated under the same conditions as fraction F but without enzyme (trypsin

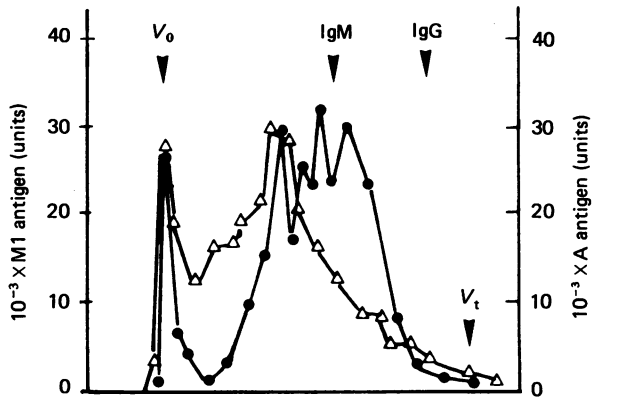

Fig. 4. Sepharose 2B chromatography of ovarian mucinous cyst crude extract

The material of the void volume of the Sepharose 6B chromatography (M1 antigen preparation) was chromatographed on Sepharose CL-2B column (180 cm × 1.6 cm), and i.r.m.a. of M1 (△) and A (●) antigens was performed in each eluted fraction. V_0 and V_t are the void volume and the excluded volume respectively. Protein molecular-mass standards: ^{125}I-IgG (180 kDa) and ^{125}I-IgM (980 kDa).

Table 3. Proteinases and 2-mercaptoethanol treatments of M1 antigen preparation

Protein, carbohydrates, A and M1 antigen concentrations in the M1 antigen preparation before and after reduction or proteinase treatment are given. Fraction A, original non-treated fractions; fraction B, treated for 72 h at 4 °C with 0.2 M-2-mercaptoethanol and then 2 h at 4 °C with 0.4 M-iodoacetamide; fraction C, treated similarly to the B fraction, but without 2-mercaptoethanol; fraction D, incubated with papain at 65 °C at pH 6.5 for 36 h; fraction E, incubated similarly to D fraction, but without papain; fraction E, incubated with trypsin at 37 °C for 5 h; fraction G, incubated similarly to the F fraction but without trypsin.

Fraction	Protein (μg/ml)	Carbohydrates (μg/ml)	Protein/ carbohydrate ratio	10^{-3} × A antigen (units/ml)	10^{-3} × M1 antigen (units/ml)
A	490	280	1.7	280	280
B	352	250	1.4	51	0.04
C	400	240	1.66	83	140
D	30	260	0.1	18	0.02
E	400	260	1.5	135	8.9
F	490	280	1.7	280	45
G	490	280	1.7	280	280

control). Table 3 shows that the M1 antigens were completely destroyed by papain and 2-mercaptoethanol treatments. Heating for 36 h at 65 °C destroyed 97% of M1 reactivity. In contrast, 6% and 18% of A reactivity remained after papain and 2-mercaptoethanol treatments respectively. Trypsin destroyed 84% of the M1 antigens but did not affect the A antigen. To determine the apparent molecular mass of immunoreactive components of the M1 antigen preparation before and after treatment, these fractions were analysed by immunoblot and by Sepharose CL-2B chromatography, followed by i.r.m.a. of each eluted fraction.

Effect of thiol reduction. In the immunoblot (Fig. 5, lane B) the M1 antigen preparation treated with 2-mercaptoethanol (fraction B) did not react with any anti-M1 MAb; in contrast, each anti-M1 MAb immunostained the control (fraction C) (Fig. 5, lane C). After chromatography on Sepharose 2B of fraction B, M1 activity could not be detected. On the contrary, the A antigen was found in the void volume as well as in a fraction included in the gel chromatography. The control (fraction C) showed the same M1 and A elution patterns as the fraction without treatment (fraction A).

Effect of papain digestion. Immunoblot of the papain control (fraction D) showed a very high-molecular-mass M1-positive component near the stack gel (Fig. 5, lane D). This result was confirmed by Sepharose CL-2B chromatography of fraction D, which showed M1 antigen exclusively in the void volume. M1 activity was not detected in fraction E (Fig. 5, lane E), irrespective of the immunochemical method used. On the other hand, A reactivity was found by immunoblot as well as by Sepharose 2B chromatography. In both fraction D and fraction E the A antigen pattern was similar to that of the untreated fraction pattern (Fig. 5, lane A), except that more A (+) reactivity was found in components between 2000 kDa and 100 kDa.

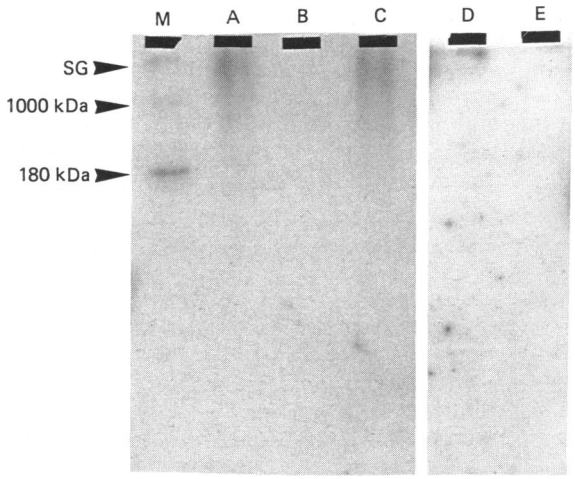

Fig. 5. SDS/polyacrylamide-gel electrophoresis and Western blot of M1 antigen preparation before and after thiol reduction or papain digestion

M1 antigen preparation (1.5 μg of protein in 1.0% SDS) was subjected to electrophoresis in a 2–16% polyacrylamide gel gradient, transferred to nitrocellulose, Western-blotted and incubated with a mixture of the five ^{125}I-labelled anti-M1 MAbs (500000 c.p.m./ml) and autoradiographed. Lanes A and B, before (lane A) and after (lane B) reduction for 72 h at 4 °C in 0.2 M-2-mercaptoethanol; lane C, M1 antigen preparation incubated without 2-mercaptoethanol; lane D, M1 antigen preparation incubated in the papain buffer at 65 °C for 36 h without enzyme; lane E, M1 antigen preparation digested with papain for 36 h at 65 °C. Samples contained 1.5 μg of protein. Note the loss of antigenicity after papain and 2-mercaptoethanol treatments, and M1 reactivity associated with components near the stack gel after heating (lane D). Protein molecular-mass marker positions are shown on the left: in the stack gel (SG), aggregate of IgM (> 2000 kDa), IgM (1000 kDa) and IgG (180 kDa).

Effect of trypsin reduction. Fig. 6 shows the percentage of M1 and A immunoreactivity left after trypsin treatment. After 30 min and 5 h of trypsin treatment 65% and 84% of the M1 reactivity were destroyed respectively. In contrast, A antigenic activity was not affected by this enzyme. Immunoblot shows that the *e* epitope characterized by the 58M1 MAb was completely destroyed after 30 min of trypsin digestion. As shown in

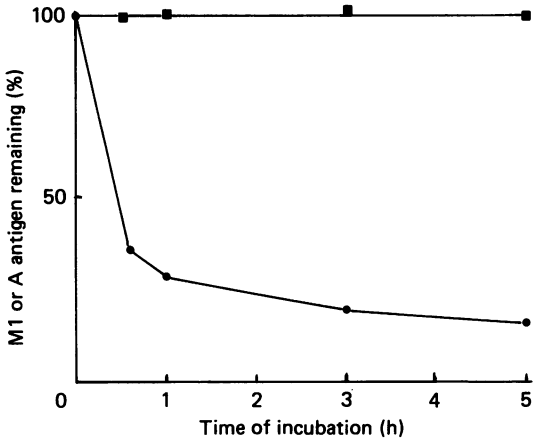

Fig. 6. Effect of trypsin digestion of A and M1 antigenicity

M1 antigen preparation containing 1000 μg of protein was incubated with trypsin at 37 °C at pH 7.2 for various periods, and i.r.m.a. of M1 (●) and A (■) antigen determinants was performed.

Western blots (Fig. 7), trypsin treatment produced increasing alterations in the apparent molecular masses of the components carrying the M1 epitopes as a function of digestion time (30 min, 1 h, 3 h and 5 h). The *a* and *c* epitopes (Fig. 7*a*) characterized by 1-13M1 and 2-12M1 MAbs respectively showed the same pattern. The M1(+) fragments showed three relatively-high-apparent-molecular-mass components (molecular masses between 2000 kDa and 200 kDa) and a component of molecular mass about 100 kDa. The amount of lower-molecular-mass components increased with the time of treatment. The *b* and *d* epitopes (Figs. 7*b* and 7*c*) were absent from the high-molecular-mass components after 5 h of trypsin digestion and were degraded into small fragments of about 60 kDa and 40 kDa for the *b* epitope and of about 100 kDa and 70 kDa for the *d* epitope. No A(+) components were associated with molecules of molecular mass less than 200 kDa. On Sepharose 2B chromatography of M1 antigen preparation after 5 h trypsin digestion, all M1(+) fragments were included in this gel. The *a* and *c* epitopes showed a large polydispersed peak near the ^{125}I-IgM (molecular masses around 1000 kDa). In contrast, the *b* and *d* epitopes were associated with a peak located near the ^{125}I-IgG.

DISCUSSION

M1 antigens have been identified by their immunohistopathological pattern on human gastrointestinal tract; they have been shown to be associated with the cytoplasm of mucous cells of surface gastric epithelium, and have an oncofetal expression during colon carcinogenesis (Bara *et al.*, 1980, 1983, 1984; Decaens *et al.*, 1983). More recently (Bara *et al.*, 1986) we described five anti-M1 MAbs showing different immunoperoxidase patterns on the normal gastrointestinal, tracheal and endocervical epithelium, and we suggested that these five M1 epitopes could be different. Immunoradiometric competition assays with our anti-M1 MAbs strongly support the existence of at least five different M1 epitopes distant and distinct from one another, since only homologous unlabelled anti-M1 MAb can inhibit immunofixation of the corresponding radiolabelled anti-M1 MAb.

Using artificial antigens made with synthetic oligosaccharides, we demonstrate that 3-3A and 2-25LE MAbs characterize structures corresponding to the A and Le^b blood-group antigens, respectively.

Our results demonstrate that molecules carrying the

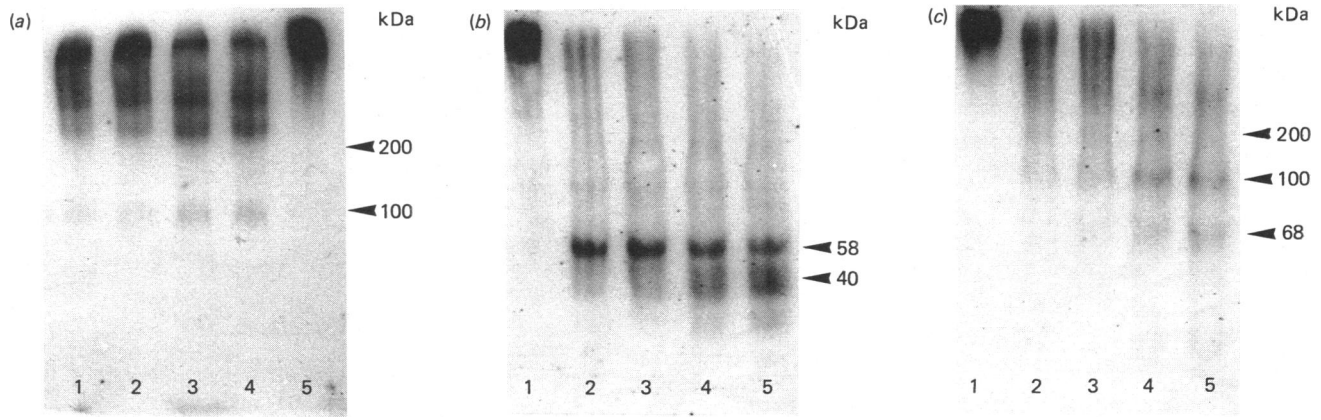

Fig. 7. Western blot of trypsin-digested M1 antigen preparation

(*a*) Western blot incubated with ^{125}I-1-13M1 MAb or ^{125}I-2-12M1 MAb, showing the association of the *a* and *c* epitopes on the fragments obtained after trypsin digestion for 0 min (lane 5), 30 min (lane 1), 1 h (lane 2), 3 h (lane 3) and 5 h (lane 4). Three main M1(+) high-molecular-mass components (between 200 kDa and 2000 kDa) and a component of about 100 kDa were observed. (*b*) Western blot of the same polyacrylamide-gel electrophoresis as in (*a*), but incubated with ^{125}I-2-11M1 MAb, showing the association of the *b* epitope with very-high-molecular-mass components (2000 kDa) that were degraded into small M1(+) fragments of 58 kDa and 40 kDa by trypsin digestion for 0 min (lane 1), 30 min (lane 2), 1 h (lane 3), 3 h (lane 4) and 5 h (lane 5). (*c*) Western blot of the same polyacrylamide-gel electrophoresis as in (*a*), but incubated with ^{125}I-9-13M1 MAb showing the association of *d* epitope with high-molecular-mass components between 2000 kDa and 500 kDa that were degraded into smaller M1(+) fragments of 100 kDa and 68 kDa by trypsin digestion for 0 min (lane 1), 30 min (lane 2), 1 h (lane 3), 3 h (lane 4) and 5 h (lane 5). Note, in each case, the increasing M1 immunoreactivities of the smaller fragments as a function of the time of trypsin treatment.

M1 epitopes are mucins. This conclusion is based on the following characteristics: (*a*) high molecular mass ($> 1 \times 10^6$ Da); (*b*) density of 1.45 g/ml, indicating a heavily glycosylated glycoprotein (Creeth & Denborough, 1970); (*c*) association with blood-group-related antigens commonly found on mucins (Creeth & Denborough, 1970).

The topographic density of the different epitopes could be classified as follows: epitopes *b*, *c*, *d* and *e* are scarce on the surface of mucins, because MAbs adsorbed on the solid phase (first layer) saturate these M1 epitopes, thereby inhibiting the fixation of the corresponding radiolabelled MAb. In contrast, the *a* epitope could be regarded as relatively abundant and the A and Lewis epitopes as largely distributed over the hyperglycosylated surface of mucins, since the fixation of the mucin by either of these two latter antibodies induced a clear increase in the fixation of the same antibody. This latter observation is in agreement with immunodiffusion results showing that only the 3-3A and 2-25LE MAbs precipitated the M1 antigen preparation (Bara *et al.*, 1986). Moreover, the competition of immunoradiometric fixation is also in favour of this hypothesis, since lower concentrations of M1 antigen preparation are necessary to obtain 50% inhibition with the 3-3A, 2-25LE and 1-13M1 MAbs compared with the four other anti-M1 MAbs (Fig. 3*b*).

In the model proposed by Carlstedt *et al.* (1983) mucins are made of flexible threads containing subunits. The subunits of about 2000 kDa containing four or five naked glycosylated regions (T domain of around 380 kDa with a length of 105 nm) are attached end to end by disulphide bridges. A glycosylated region may contain about 150–200 different saccharide chains if these chains are of four to 12 sugar units (Slomiany *et al.*, 1984). Most of these chains display blood-group ABH and Lewis antigens (Feizi *et al.*, 1984). Consequently, both the A- and Lewis-related epitopes must be located in these glycosylated T domains and are not destroyed by papain or 2-mercapetoethanol treatment. Conversely, M1 epitopes have a completely different immunochemical behaviour and could be associated with 'naked regions' of the mucin, which are of a peptidic nature (susceptible to papain or trypsin attack) and are rich in disulphide bridges (Carlstedt *et al.*, 1983) and consequently destroyed by 2-mercaptoethanol. Although 97% of M1 reactivities are destroyed by heating at 65 °C for 36 h, 3% of the remaining M1 reactivities are associated with high-molecular-mass components (excluded from Sepharose 2B) and are completely destroyed by papain. Trypsin treatment showed progressive loss of M1 immunoreactivity, and 84% of M1 antigens were destroyed after 5 h of proteolysis. However, at this time, three patterns of trypsin degradation were obtained by Western blot: (i) complete destruction of M1 epitope (*e* epitope), (ii) complete destruction of the M1 epitope associated with the relatively-high-molecular-mass components that were degraded into M1(+) fragments of 100 kDa to 40 kDa (*b* and *d* epitopes), and (iii) M1 epitopes essentially associated with the high-molecular-mass components and in addition with a 100 kDa (M1(+) fragment (*a* and *c* epitopes). These results suggest the occurrence of different M1 epitopes located in separate regions of the mucin molecule. Immunologically active fragments showing a small molecular mass (120 kDa) have been obtained after cleavage of colonic mucin (CSA_p) by trypsin (Shochat *et al.*, 1981). However, these observations do not permit any conclusion concerning the existence of a link peptide centrally joining the subunits.

Our results demonstrate the association of the five M1 epitopes with the peptidic core of mucin, in good agreement with electron-microscopy observation showing a specific fixation of anti-M1 MAbs over the rough endoplasmic reticulum and the polyribosomes (Levrat *et al.*, 1987). Since the peptidic M1 epitopes are specific for gastric mucin and are not found in the intestine (Bara *et al.*, 1986), it is suggested that there may exist a gene coding for the gastric mucin distinct from a gene coding for the intestinal mucin, as pointed out by Wesley *et al.* (1985), who demonstrated differences in amino acid composition between small-intestinal and large-intestinal mucin, and by Fahim *et al.* (1987), who demonstrated organ-specific antigen associated with the 'link peptide' of rat intestinal mucin.

In conclusion, our results show the existence of five different peptidic M1 epitopes distant from one another in the peptidic core of gastric mucin.

This work was supported in part by a grant from the Caisse Nationale de l'Assurance Maladie des Travailleurs Salariés.

REFERENCES

Allen, A. (1983) Trends Biochem. Sci. **8**, 169–173
Bara, J., Loisillier, F. & Burtin, P. (1980) Br. J. Cancer **41**, 209–221
Bara, J., Languille, O., Gendron, M. C., Daher, N., Martin, E. & Burtin, P. (1983) Cancer Res. **43**, 3885–3891
Bara, J., André, J., Gautier, R. & Burtin, P. (1984) Cancer Res. **44**, 4040–4045
Bara, J., Gautier, R., Daher, N., Zaghouani, H. & Decaens, C. (1986) Cancer Res. **46**, 3983–3898
Bara, J., Mollicone, R., Herrero-Zabaleta, E., Gautier, R., Daher, N. & Oriol, R. (1988) Int. J. Cancer **41**, 683–689
Bhushana Rao, K. S. P., Van Roost, E., Masson, P. L., Heremans, J. F. & Andre, F. (1973) Biochim. Biophys. Acta **317**, 286–302
Carlstedt, I., Lindgren, H. & Sheehan, J. K. (1983) Biochem. J. **213**, 427–435
Carlstedt, I., Sheehan, J. K., Corfield, A. P. & Gallacher, J. T. (1985) Essays Biochem. **20**, 40–76
Creeth, J. M. & Denborough, M. A. (1970) Biochem. J. **117**, 879–891
Decaens, C., Bara, J., Rosa, B., Daher, N. & Burtin, P. (1983) Cancer Res. **43**, 355–362
Ey, P. L., Prowse, S. P. & Jenkin, C. R. (1978) Immunochemistry **15**, 429–436
Fahim, R. E. F., Specian, R. D., Forstner, G. G. & Forstner, J. F. (1987) Biochem. J. **243**, 631–640
Feizi, T., Gooi, H. C., Childs, R. A., Picard, J. K., Uemura, K., Loomes, L. M., Thorpe, S. J. & Hounsell, E. F. (1984) Biochem. Soc. Trans. **12**, 591–596
Fenoglio, C. M., Ferenczy, A. & Richart, R. M. (1975) Cancer **36**, 1709–1722
Greenwood, F. C., Hunter, W. M. & Glover, J. S. (1963) Biochem. J. **89**, 114–123
Koch, R. B., Geddes, W. F. & Smith, F. (1951) Cereal Chem. **28**, 424–434
Lemieux, R. U. (1978) Chem. Soc. Rev. **7**, 423–452
Le Pendu, J., Fredman, P., Richter, N. D., Magnani, J. L., Willingham, M. C., Pastan, I., Oriol, R. & Ginsburg, V. (1985) Carbohydr. Res. **141**, 347–349

Levrat, J. H., Faure, F., Palevody, C., Bara, J. & Hollande, E. (1987) Gastroenterol. Clin. Biol. **11**, 225

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275

Mantle, M., Mantle, D. & Allen, A. (1981) Biochem. J. **195**, 277–285

Mantle, M., Forstner, G. G. & Forstner, J. F. (1984) Biochem. J. **217**, 159–167

Mollicone, R., Le Pendu, J., Bara, J. & Oriol, R. (1986) Glycoconjugate J. **3**, 187–202

Neutra, M. R. & Forstner, J. F. (1986) in The Physiology of the Gastrointestinal Tract (Johnson, L. R., ed.), 2nd edn., vol. 2, pp. 975–1009, Raven Press, New York

Pearson, J. P., Allen, A. & Parry, S. (1981) Biochem. J. **197**, 155–162

Shochat, D., Archey, R. L., Pant, K. D., Dahlman, H. L., Gold, D. V. & Goldenberg, D. M. (1981) J. Immunol. **126**, 2284–2289

Shochat, D., Pant, D. & Goldenberg, D. M. (1982) Cancer **50**, 927–931

Slayter, H. S., Lamblin, G., Le Treut, A., Galabert, C., Houdret, N., Degand, P. & Roussel, P. (1984) Eur. J. Biochem. **142**, 209–218

Slomiany, B. L., Zdebska, E. & Slomiany, A. (1984) J. Biol. Chem. **259**, 2863–2869

Wesley, A., Mantle, M., Man, D., Qureshi, R., Forstner, G. & Forstner, J. (1985) J. Biol. Chem. **260**, 7955–7959

Received 5 October 1987/25 January 1988; accepted 6 May 1988