HUMAN MONOCLONAL MACROGLOBULINS WITH SPECIFICITY FOR *KLEBSIELLA* K POLYSACCHARIDES THAT CONTAIN 3,4-PYRUVYLATED-D-GALACTOSE AND 4,6-PYRUVYLATED-D-GALACTOSE*

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Monoclonal immunoglobulins with anti-carbohydrate specificity have been found more frequently in mice (1) than in humans (2–4). It must be recognized, however, that the detection of specificity depends upon the choice of possible antigens and the numbers and characteristics of the monoclonal proteins included in any screening study. Thus, in the survey carried out by Yoo and Franklin (2), 198 IgG, 3 IgA, and only 1 IgM were screened against pneumococcal C polysaccharide. Freedman et al. (4) screened 353 IgG, 87 IgA, and 54 IgM against 115 antigens, including pneumococcal polysaccharides, mucopolysaccharides, and plant gums. Two IgG and one IgM reacted with heparin, and another IgG reacted with several pneumococcal polysaccharides and two plant gums (4). In contrast, the anti-carbohydrate specificity of monoclonal IgM cold agglutinins with anti-I and anti-i specificity is well established (5, 6). Two other IgM cold agglutinins have been reported: (*a*) one bound *N*-acetylneuraminosyl residues (7) and (*b*) the other lacto-*N*-tetraose (8). Riesen et al. (9) screened 904 myeloma proteins, finding one IgM with specificity for phosphorylcholine that probably represents anti-carbohydrate. A monoclonal non-cold agglutinin IgM, reacting with Forssman antigen and with glycolipids with terminal nonreducing DGalNAc-linked α or β, has also been described (10, 11).

Employing a panel of nine acidic *Klebsiella* polysaccharides and one Enterobacter polysaccharide, Hannestad et al. (12) found 4 of 154 monoclonal IgM, each of which reacted specifically with a single *Klebsiella* polysaccharide. In contrast, 0 of 73 IgG were positive against the same antigens. All four IgM had kappa light chains. The serum of one case, Tö, contained two monoclonal IgM, one of which precipitated

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with *Klebsiella ozenae* type AE and with an acidic proteoglycan from cartilage, as well as with agar but not agarose; the other monoclonal IgM in the Tö serum was inactive. IgM in serum from patient B.E. also reacted with *K. ozenae*; $IgM^{Sö}$ reacted with *K. ozenae* type 6. A second serum, Nae (13), also contained two monoclonal IgMκ, both of which precipitated strongly with *Klebsiella* K11 and weakly with K35 and dextran sulfate. In a subsequent study (14), 116 sera that contained a monoclonal IgM were tested with 17 acidic *Klebsiella* polysaccharides. Three additional sera were identified: Th reacted with *Klebsiella* K35; We precipitated K13; Ro reacted with both K12 and K13. All three IgM, when added to suspensions of *Klebsiella*, caused capsular swelling. A finding of 7 proteins with specificity for *Klebsiella* polysaccharides of 270 would seem a relatively high frequency.

In the present study, we are concerned with two additional IgMκ that exhibit specificity for *Klebsiella* polysaccharides. One, IgM^{WEA}, was initially detected because of its interaction with Sepharose; the second, IgM^{MAY}, was discovered in the course of a screening study of monoclonal immunoglobulins of all classes against a diverse collection of polysaccharides and glycoproteins of bacterial, plant, and animal origin; it reacted with a galactan from the snail *Helix pomatia*. By quantitative precipitin and precipitin inhibition analyses, the specificity of both of these proteins has been shown to be most closely related to *Klebsiella* polysaccharides with pyruvylated galactoses. The combining sites of these two proteins, however, differ in specificity. Pyruvylated sugars are important antigenic determinants of some *Klebsiella* K antigens, several pneumococcal type-specific polysaccharides, and other microbial polysaccharides (15).

Materials and Methods

Case Descriptions. W.E.A. (information obtained courtesy of Dr. Richard J. Hirschman, Cabrini Health Care Center, N. Y.), a 33-yr-old white male, was in apparent good health until March 1976 when he developed persistent fevers to 103°F, nonproductive cough, anorexia, and weight loss. Physical examination disclosed generalized lymphoadenopathy and hepatosplenomegaly. Complete blood count: hemoglobin, 7.8 g/dl; white blood count, 6,300 with 47% lymphocytes and some atypical forms, platelets, 155,000. Total serum protein was 9.8 g/dl, and electrophoresis demonstrated a monoclonal spike, identified immunoelectrophoretically as an IgMκ. IgG and IgA levels were within normal limits. Lymph node biopsy showed a marked infiltration with plasmacytic forms, but the nodal architecture was well preserved. Bacterial cultures of the blood, urine, and stools were negative. One bone marrow culture revealed *Corynebacterium* sp:three other bone marrow cultures were negative.

Although an atypical occult infection could not be excluded, primary macroglobulinemia was considered to be the most likely diagnosis, and the patient was treated with chlorambucil and corticosteroids. This therapy produced a transient remission. He died three and one-half yr after onset of his illness; autopsy was not performed.

M.A.Y. is a 71-yr-old white male with a background of chronic peptic ulcer disease, chronic cholecystitis and cholelithiasis, and possible inactive pulmonary tuberculosis. He was hospitalized in August 1978 for an atypical pneumonia and sputum cultures showed *Haemophilus influenzae* and *Streptococcus pneumoniae*. Serum electrophoresis demonstrated a somewhat paucidispersed monoclonal peak (~1.5 g/dl) shown to be an IgMκ. The concentrations of the immunoglobulins were (in g/dl): IgM 1,512 (normal = 50–200), IgG 771 (normal = 700–1,700), IgA 150 (normal = 100–350). The peripheral blood count was normal except for a moderate anemia that cleared after resolution of the pneumonia. Bone marrow aspiration showed an increase in lymphocytes (~20%) consistent with lymphosarcoma. In the subsequent 18 mo, the patient has remained asymptomatic without specific therapy. There is no detectable lymphoadenopathy or hepatosplenomegaly and the serum concentration of IgM is unchanged. At this time (March 1980) this case is classified as an asymptomatic plasma cell dyscrasia or so-called benign monoclonal gammopathy.

IgM Myeloma Proteins. IgM from serum of patient W.E.A. (IgM^{WEA})[1] was purified as follows: serum was dialyzed against distilled water for 48 h. The precipitated euglobulin fraction that contained IgM was purified further on Sephadex G-200 in phosphate-buffered saline, pH 7.2. Tubes were pooled, dialyzed against distilled water, and lyophilized or centrifuged to recover the precipitated euglobulin. Purity was tested with antisera to γ-, α-, μ-, κ-, and λ-chains.

IgM^{WEA} F(ab')$_2$. F(ab')$_2$ fragments were prepared as described by Miller and Metzger (16). The purified IgM^{WEA} was reacted with trypsin (EM Laboratories, Englewood Cliffs, N. J.) 1:100 wt/wt for 18 h, at 37°C, in 0.1 M Tris with 0.001 M $CaCl_2$ at pH 8. The reaction was stopped with soybean trypsin inhibitor and the digest put on a Sephadex G-200 column with 0.2 M Tris-buffer, pH 8.6. Four well-resolved peaks were obtained, of which the second was identified as the F(ab')$_2$ fragment by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and by immunological precipitation.

IgM^{WEA} (Fc)$_5\mu$. (Fc)$_5\mu$ was prepared as described by Plaut and Tomasi (17). IgM^{WEA} was digested with trypsin (1:25 wt/wt) for 30 min at 54°C in 0.05 M Tris-HCl buffer, pH 8.1, with 0.0115 M $CaCl_2$ and put on a Sephadex G-200 column in 0.14 M NaCl. Four peaks absorbing at 280 nm were found. The first peak, (Fc)$_5\mu$, identified with antiserum and by acrylamide electrophoresis, was pooled, dialyzed, and lyophilized; it contained a small amount of a second band on acrylamide gel electrophoresis.

IgM from serum of patient M.A.Y. (IgM^{MAY}) was studied in whole serum. IgM^{MAY} was originally detected by screening in agarose gel; it was recognized initially by its reaction with *Helix pomatia* galactan, a polysaccharide with which IgM^{WEA} also reacted. An IgMκ myeloma that did not react in screening tests, IgM^{SCO}, served as a control.

Polysaccharides. The following polysaccharides were studied: commercial Bacto and special agar (Difco Laboratories, Detroit, Mich.) and successive fractions prepared from it, as described by Duckworth and Yaphe (18), by extraction with water at 20 and 50°C, and the residue; commercial agarose (Fisher Scientific Co., Pittsburgh, Pa.); galactans from the snails *Helix pomatia* (19–22); *Arianta arbustorum* and their first stages of partial periodate oxidation and Smith degradation (23) *Cepaea nemoralis* and *Biomphalaria glabrata* (24); Larch arabinogalactan (25) and pig pneumogalactan (26, 27) from Dr. Niel Glaudemans (National Institutes of Health, Bethesda, Md.); polysaccharides from *Klebsiella* K7 (28), K21 (29), and K32 (30), *Rhizobium trifolii* TA1 (31), and types IV, XIV, and XXXII pneumococcal polysaccharides (32) through Dr. Michael Heidelberger, New York University College of Medicine, New York; *Rhizobium trifolii* strain U226 (33) and *Klebsiella* K30 (34) and K33 (35) from Professor Bengt Lindberg, University of Stockholm, Stockholm; pneumococcal group C polysaccharide from the late Professor Sam M. Beiser, Columbia University, New York; xanthan gums and their sulfomethylated products from Dr. I. W. Cottrell (36), Kelco Div., Merck & Co., Inc., Rahway, N. J.; dextran sulfate (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.); heparin, keratosulfate (37), a heparin analogue from alginic acid (38), and chondroitin sulfate (Sigma Chemical Co., St. Louis, Mo.); dextran (39) and various blood group B and I substances (40–44).

Inhibitors. Sodium pyruvate (ICN Nutritional Biochemicals Div., Cleveland, Ohio), sodium lactate (neutralized from Mallinckrodt lactic acid), sodium propionate (Fisher Scientific Co.), D- and L-fucose (Schwarz Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.), D-galactose (Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y.), L-galactose (Sigma Chemical Co.), D-mannose (Pfanstiehl Laboratories, Inc., Waukegan, Ill.), and methyl α- and β-D-galactosides from Dr. A. B. Pardee (Harvard University, Boston, Mass.) were used in various amounts as possible inhibitors of the various precipitin reactions. A sample of the R isomer of methyl 4,6-*O*-(1-carboxyethylidene) β-D-galactopyranoside (4,6pyDGalβ) (45) was kindly provided by Professor Bengt Lindberg and Dr. Ingemar Kvarnström (University of Stockholm), and a sample of the R and S isomers of methyl 4,6-*O*-(1-carboxyethylidine α-D-galactopyranoside (4,6pyDGalα) from these workers through Dr. Michael Heidelberger. D-

[1] *Abbreviations used in this paper:* DGal, D-galactose; DGlc, D-glucose; DMan, D-mannose; 3,4pyDGalα, 3,4-*O*-(1-carboxyethylidine) α-D-galactopyranoside, 3,4pyDGalβ, 3,4-*O*-(1-carboxyethylidine) β-D-galactopyranoside; 4,6pyDGalα, 4,6-*O*-(1-carboxyethylidine) α-D-galactopyranoside; 4,6pyDGalβ, 4,6-*O*-(1-carboxyethylidine) β-D-galactopyranoside, IgM^{MAY}, IgM from serum of patient M.A.Y.; IgM^{WEA}, IgM from serum of patient W.E.A.

Galactose-1-phosphate, D-galactose-6-phosphate, and D-glucose-1-phosphate (Sigma Chemical Co.) were used as the potassium salts.

Immunochemical Methods. Quantitative precipitin studies were carried out in the usual manner (46–48). Varying quantities of polysaccharide antigens were added to ~8 μg N of purified IgM^{WEA} or its $F(ab')_2$ and $(Fc)_5\mu$ fragments in a vol of 25 μl, and to 25 μl of a 1:5 dilution of IgM^{MAY} serum in 3.0-ml conical centrifuge tubes. The total volume was adjusted to 350 μl with 0.15 M NaCl. The contents of the tubes were mixed, placed at 37°C for 1 h, placed in the refrigerator for 1 wk and were mixed twice daily. The tubes were centrifuged, washed twice with saline, and analyzed for N by the ninhydrin method (44).

Quantitative precipitin inhibition tests were set up by adding various quantities of inhibitor to ~8 μg N purified IgM^{WEA} or to 25 μl of a 1:5 dilution of IgM^{MAY} serum. After 30 min at 37°C, a quantity of antigen giving maximum precipitation was added. The contents of the tubes were mixed, placed at 37°C for 1 h, and placed in the refrigerator for 1 wk. Centrifugation, washing, and analysis were as above. The capacity of the $F(ab')_2$ and $(Fc)_5\mu$ fragments of IgM^{WEA} to precipitate, to coprecipitate, and to inhibit precipitation was also assayed. For inhibition assays with purified IgM^{WEA} and the pyruvylated sugars and other charged compounds, special care must be taken to ensure that reactions are carried out close to neutrality. In some assays, at which the pH was 4.7, negative values for inhibition were obtained because a larger proportion of the added IgM was precipitated by antigen. Only determinations are reported that were at proper pH.

Coprecipitation Studies (47). To 4-μg N portions of IgM^{WEA} in a series of 3-ml conical centrifuge tubes, 6.6 μg N of the $F(ab')_2$ of 6.4 μg N of the $(Fc)_5\mu$ fragments of IgM^{WEA} were added, followed by various quantities of *Klebsiella* K30. The tubes were then analyzed as described above.

Absorption Studies. The identification of the monoclonal peak ascribable to the anti-K21 in IgM^{MAY} serum was accomplished by the procedure used by Tiselius and Kabat (49): to 50 μl of undiluted IgM^{MAY} serum, 20 μg of *Klebsiella* K21 in 50 μl saline was added. To a second 50-μl sample of IgM^{MAY} serum, 50 μl of saline was added. After 1 wk in the refrigerator, the tubes were centrifuged, decanted, and the supernates examined by electrophoresis on cellulose acetate (50).

Immunoelectrophoretic Studies. Serum of IgM^{MAY} was placed in two wells and subjected to electrophoresis in agar. Arcs were developed in the bottom trough with horse anti-human serum, in the middle trough with goat anti-human IgM, and in the top trough with *Helix pomatia* galactan.

Helix pomatia, Cepaea nemoralis galactans, and dextran were placed in wells electrophoresed in agar, and IgM^{MAY} or QUPC52, an anti-α1→6 dextran myeloma protein, were placed in appropriate troughs to develop bands. Human serum was also subjected to electrophoresis to serve as a reference.

Results

Quantitative Precipitin Studies. Sepharose is manufactured from the agar component agarose, a linear polysaccharide with alternating D-galactose and 3,6 anhydro-L-galactose residues. Various agarose, agar, and galactan preparations were chosen, therefore, as the starting polysaccharides for analysis of the IgM^{WEA} binding specificity. The results of these precipitin analyses are shown in Fig. 1 A. It is evident that commercial agarose was inactive in the range tested, but the Bacto agar preparations reacted. The 20°C aqueous extract was more active than the subsequent 50°C extract (18), and both were more potent than the original Bacto agar on a weight basis. Identical results were obtained with fractions of Noble agar (Difco Laboratories) (data not shown).

IgM^{WEA} also reacted with the galactan from *Helix pomatia* (19–22) and its reactivity was increased by one stage of partial periodate oxidation and Smith degradation (23); periodate oxidation and Smith degradation removes all of the L-galactose, leaving a

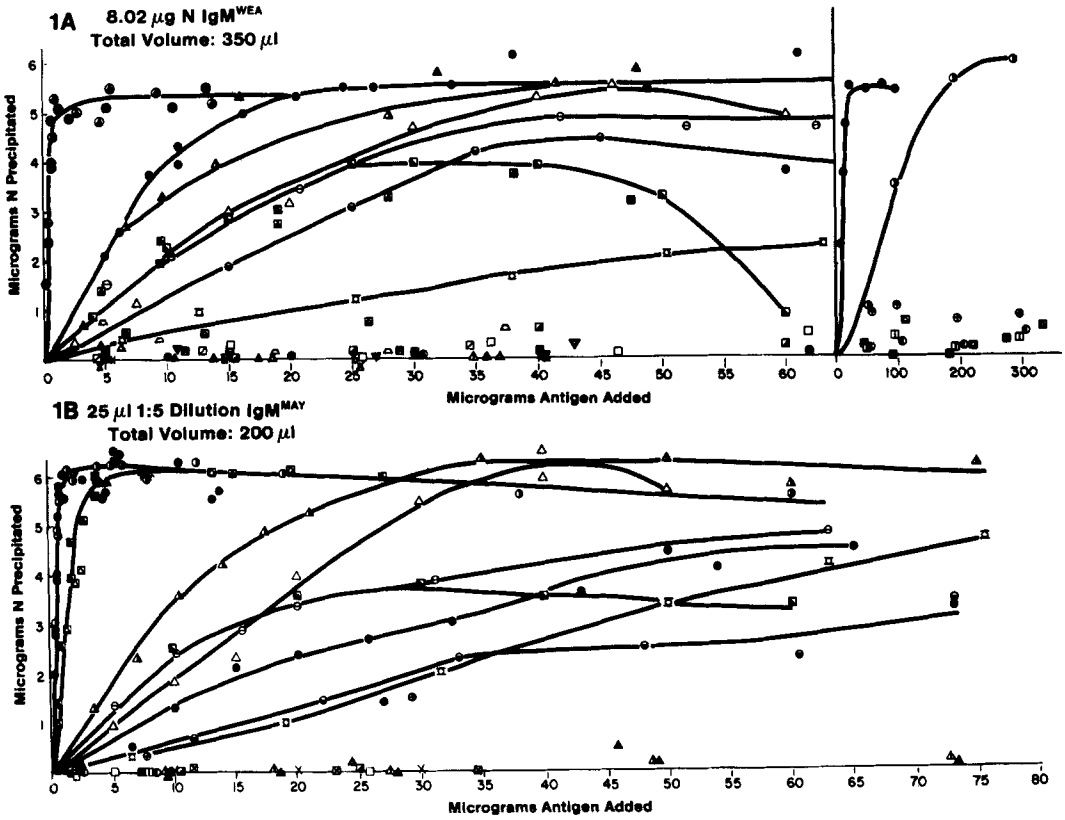

FIG. 1. Quantitative precipitin reactions of IgM^{WEA} (A) and IgM^{MAY} (B) with various polysaccharides. The graph at the right in (A) shows the reactions of the 20°C extract of agar and *Klebsiella* K21 on a scale using 20 times as much antigen. Symbols: ◑, *Klebsiella* K21; ●, *Klebsiella* K30 native; ◭, *Klebsiella* K33 native; ●, Bacto agar 20° extract; ▲, *Helix pomatia* galactan 1st Smith degradation; △, *Helix pomatia* galactan; ▲, *Capaea nemoralis* galactan; ⊖, Bacto agar 50°C extract; ▩, *Rhizobium trifolii* U226; ▣, dextran sulfate; ◒, Bacto agar; ◻, Bacto agar extracted residue; ◰, *Rhizobium trifolii* TA_1; ⊕, *Klebsiella* K32; ◐, *Klebsiella* K7; ◫, pneumococcus type S XXVII; ■, pneumococcus type S IV; ◪, pneumococcus type S XIV; ×, *Pneumococcus* C; □, agarose; ◉, heparin; ▼, heparin analogue from alginic acid; ▲, *Arianta arbustorum* galactan; ◭, *Arianta arbustorum* galactan 1st Smith degradation; ⊠, arabinogalactan (Larch); ◪, pneumogalactan; ⊠, keratosulfate; △, chondroitin sulfate; ◭, *Biomphalaria glabrata* galactan; ◫, gum ghatti α1→6.

polysaccharide composed entirely of D-galactose. The periodate-oxidized and Smith-degraded *Helix pomatia* galactan is about as active as the 20°C extract of agar. However, the galactan of *Cepaea nemoralis* without Smith degradation was as active as the 20°C agar extract. Galactans from the related species, *Arianta arbustorum* and its first stage of periodate oxidation, and Smith degradation (23) and a native galactan from *Biomphalaria glabrata* (24), were completely inactive. Larch arabinogalactan (25), known to react with various mouse myeloma proteins with anti-galactan activity (1), was inactive as were several blood group I (OG 20% from 10% [40, 41]); BI (Tij phenol insoluble [42]); and B (Beach phenol insoluble [41]) active substances with terminal nonreducing β- and α-linked D-galactose.

The finding that IgM^{WEA} reacted strongly with the 20°C agar extract suggested that its specificity might involve sulfate or pyruvate groups known to be substituted

to varying extents on the linear agarose chain (18, 51). However, when a number of sulfated polysaccharides were examined, they were found to be completely inactive. For example, heparin, keratosulfate (37), chondroitin sulfate, xanthan gum (36), and their sulfomethylated compounds (36) all failed to precipitate IgM^{WEA}. However, IgM^{WEA} did react with dextran sulfate (Fig. 1 A). The reaction was much more readily inhibited by excess antigen and, at the maximum, precipitated only 73% as much N as did the 20°C and 50°C agar extracts and the *Helix* and *Cepaea* galactans.

Quite different results were obtained when eight polysaccharides known to contain pyruvylated sugars were studied. Only those with 3,4- and 4,6-pyruvylated-D-Gal residues were found to react with IgM^{WEA} (see Table I for structural comparisons). *Klebsiella* K30 and K33 were the most active, 1/10 as much being required for comparable precipitation as the 20°C aqueous agar extract, the first periodate stage of *Helix pomatia*, or *Cepaea nemoralis* polysaccharide (Fig. 1 A). The *Klebsiella* K30 and K33 ligands have 3,4-pyruvylated terminal D-Gal-linked β1→4 to D-Man in the main chain. The *Rhizobium trifolii* polysaccharides varied in activity; the U226 preparation reacted as well as whole agar, whereas the TA1 reacted very weakly despite the fact that both have pyruvate linked 4,6 to terminal nonreducing D-Gal. The *Klebsiella* K21 polysaccharide (29) also reacted, but ~10 times as much K21 as of 20°C aqueous agar extract was required for 50% precipitation of the myeloma antibody. In *Klebsiella* K21, the pyruvic acid is linked 4,6 to terminal nonreducing D-Gal-linked α1→4 to D-glucuronic acid in the main chain and, thus, shows similarities to the pyruvyl group in agar except for being terminal.

IgM^{MAY}: the second myeloma protein was detected in a screening program for identifying human myeloma proteins that react with various polysaccharides. One of the polysaccharides in the screening mixtures was the *Helix pomatia* galactan. IgM^{MAY} gave a good precipitate with the mixture and was found to precipitate only with the *Helix pomatia* polysaccharide. Fig. 1 B shows the quantitative precipitin findings with IgM^{MAY}. Reactions with the agar fractions differed from those of IgM^{WEA} in that the 50°C aqueous extract was more active than that made at 20°C. *Helix pomatia* and its

TABLE I

Structures of Pyruvylated Monosaccharides in Various Polysaccharides

Polysaccharide	Structure and linkage of pyruvylated sugar
1 *Klebsiella* K30 (34)	Pyruvate on 3,4 of D-Galβ1→6 linked to [→4DMan*p*→] of main chain
2 *Klebsiella* K33 (35)	Pyruvate on 3,4 of D-Galβ1→6 linked to [→4D-Man*p*→] of main chain
3 *Klebsiella* K21 (29)	Pyruvate on 4,6 of lateral nonreducing end group of D-Galα1→4 to [→3D-GlcA1→3]
4 *Rhizobium trifolii* (31,33)	Pyruvate on 4,6 of lateral nonreducing D-Gal linked β1→3 to a 4,6 pyruvylated βD-Glc*p* residue
5 Agar (51)	Pyruvate on 4,6 of [→3D-Gal1→] of main sugar chain
6 *Klebsiella* K7 (15)	Pyruvate on 4,6 of [→3D-Glc1→] of main chain
7 *Klebsiella* K32 (30)	Pyruvate on 3,4 of [→2L-Rhaα1→] of main chain
8 Pneumococcus type S IV (52–55)	Pyruvate presumably on 2,3 of D-Gal
9 Pneumococcus type S XXVII (56)	Pyruvate on 4,6 of [→3D-GlcNAcβ1→] of main chain

For a detailed analysis of structures of *Klebsiella* and other polysaccharides and their cross-reactions in anti-pneumococcal and anti-salmonella sera, see reference (15).

first stage of periodate oxidation and Smith degradation reacted as they did with IgM^{WEA}. IgM^{MAY} clearly differed from IgM^{WEA} in that *Klebsiella* K21 reacted 20 times better than the *Helix* first-stage periodate fraction and 80 times better than the 20°C aqueous agar extract. The most striking finding was that the *Klebsiella* K21, K30, and K33 polysaccharides were all equally active per unit weight, giving a single quantitative precipitin curve (Fig. 1 B). *Rhizobium trifolii* TA1 polysaccharide, which was essentially inactive with IgM^{WEA}, reacted strongly; it and U226 were equally potent and one-seventh as active as K21.

Purified (8.8 μg N)IgM^{SCO} did not give precipitin reactions with the various *Klebsiella* antigens over the range used in Fig. 1 A and B, nor with dextran sulfate, heparin, agar, etc.

Absorption Experiments. Because IgM^{MAY} was studied in whole serum, it was necessary to demonstrate specific removal of the monoclonal gamma peak from the serum electrophoretic patterns by addition of antigen. Addition of an amount of *Klebsiella* K21, which gave maximum precipitation, eliminated the monoclonal peak. Quantitative estimation of the concentrations of IgM, IgG, and IgA in the serum before and after addition of *Klebsiella* K21 confirmed the removal of >95% of the IgM. The IgG was decreased ~20%; IgA was unchanged. Addition of *Klebsiella* K21 to normal serum decreased the IgG concentration by 11% but did not change the IgM concentration. Addition of *Klebsiella* K7 to IgM^{MAY} serum or to normal serum did not reduce the concentrations of IgM, IgG, or IgA.

Immunoelectrophoretic Studies. The *Helix pomatia* galactan, with which the serum that contained IgM^{MAY} was found to react in the original screening, was shown by immunoelectrophoresis to be reacting specifically with the IgM.

Electrophoresis of the various galactans has shown them to be charged molecules (23). *Helix pomatia* galactans were highly negatively charged (23) and contained galactose-6-phosphate; Weinland (57) has isolated it and a trisaccharide that contains galactose-6-phosphate from the galactan. Fig. 2 shows that the galactans of *Helix pomatia* and *Cepaea nemoralis* are highly negatively charged, moving as rapidly as prealbumin in immunoelectrophoresis, whereas the dextran is seen to react with anti-dextran alongside of its well. The *Biomphalaria glabrata* (23) and *Arianta arbustorum* galactans may contain another constituent, the nature of which is unknown (Bretting, H., N. F. Whittaker, E. A. Kabat, K. Königsmann-Lange, and H.-J. Thiem. Manuscript in preparation).

Inhibition Assays. Inhibition studies of the precipitin reactions between IgM^{WEA} and the 20°C agar extract, the *Helix pomatia* galactan and its first stage of periodate oxidation and Smith degradation, were carried out with neutralized pyruvic, lactic, and propionic acids, with various monosaccharide glycosides and oligosaccharides, with the neutralized R isomers of methyl 4,6pyDGalα and 4,6pyDGalβ (45), with the S isomer of methyl 4,6pyDGalα, and with D-galactose-1-phosphate, D-galactose-6-phosphate, and D-galactose-1-phosphate.

Fig. 3 A shows that the precipitin reaction of IgM^{WEA} with the 20% agar extract was inhibited by sodium pyruvate and to a lesser degree by sodium lactate; sodium propionate was inactive. D-galactose-6-phosphate, D-galactose-1-phosphate, D-glucose-1-phosphate, and D-glucose-6-SO_4 inhibited less strongly than sodium pyruvate and about as well as sodium lactate. The precipitin reaction of IgM^{WEA}-*Helix pomatia* galactan was inhibited by sodium pyruvate but less effectively on a molar basis, and

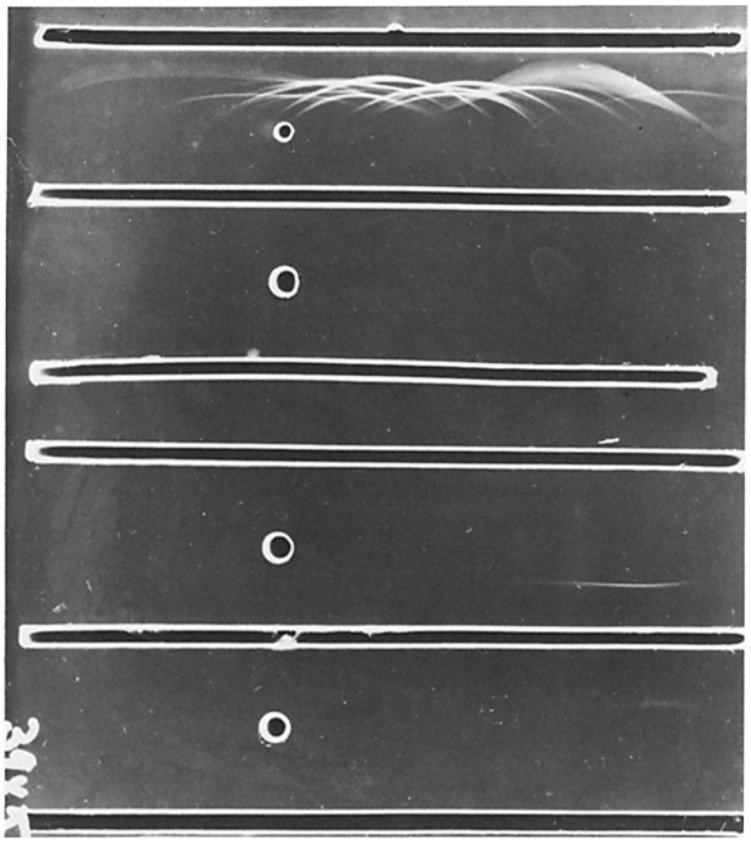

FIG. 2. Immunoelectrophoretic pattern showing charge on *Helix pomatia* and *Capaea nemoralis* galactans. The wells from top to bottom contain normal human serum, dextran N279, *Capaea nemoralis*, and *Helix pomatia* galactans. The top trough contains horse anti-human serum; the second trough is empty; the third contains myeloma anti-dextran QUPC-52; the fourth is empty; and the fifth contains IgM^{WEA}.

sodium lactate and sodium propionate were inactive (data not shown). The reaction of IgM^{WEA} with the first stage of periodate-oxidized and Smith-degraded *Helix* galactan was only minimally inhibited by sodium pyruvate, and the reaction appeared to be less specific (data not shown). No inhibition was obtained with the various monosaccharide glycosides and oligosaccharides (Fig. 3 A). The methyl 4,6pyDGalβ and 4,6pyDGalα R isomers were equally active and >20 times more active than sodium pyruvate on a molar basis in inhibiting precipitation of the IgM^{WEA} by agar, but the S isomer gave no inhibition. The small graph at the right of Fig. 3 A gives the inhibition by methyl 4,6pyDGalα and 4,6yDGalβ R isomer and methyl 4,6pyDGalα S isomer of precipitation of IgM^{WEA} by agar on a 25-fold expanded scale.

IgM^{MAY}. Fig. 3 B shows that pyruvate was better than lactate and propionate in inhibiting the precipitation of IgM^{WEA} by *Klebsiella* K21 polysaccharide. However, as with IgM^{WEA}, the R isomers of 4,6pyDGalα and 4,6pyDGalβ were equally potent and 150 times more active than pyruvate in inhibiting the *Klebsiella* K21 precipitin reaction; the S isomer of 4,6pyDGalα was inactive at an amount of R isomer, giving 50% inhibition. The graph at the right of Fig. 3 B gives the inhibition data with the

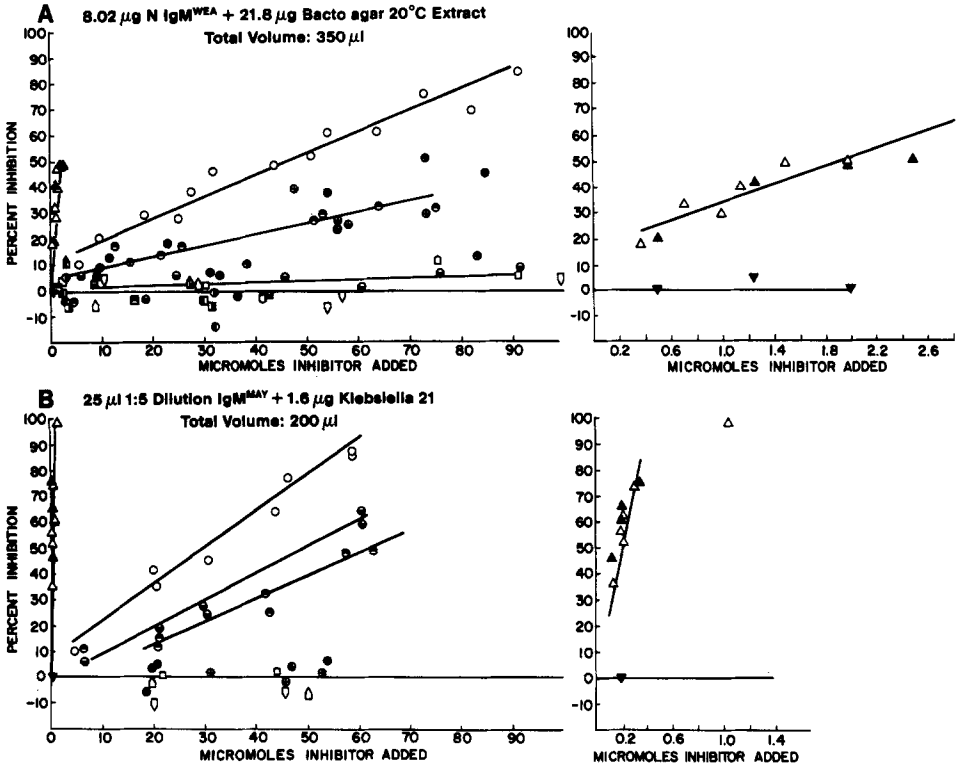

FIG. 3. Inhibition by methyl glycosides of neutralized pyruvylated DGal by sodium pyruvate, lactate, and propionate, and by various other compounds of precipitation of IgM^{WEA} by agar (A) and IgM^{MAY} (B) by *Klebsiella* K21. Symbols: ▲, methyl 4,6pyDGalα R isomer; △, methyl 4,6pyDGalβ R isomer; ▼, methyl 4,6pyDGalα S isomer; ○, sodium pyruvate; ⊕, glucuronic acid; ●, D-glucose-6-sulfate; ⊖, sodium lactate; ⊖, sodium propionate; ◐, L-fucose; ◑, D-fucose; □, DGal; ◧, L-galactose; ◨, DGlc; ▭, lactose; ▬, DMan; ⌂, methyl α-DGal; ▽, methyl β-DGal; ⏢, L-rhamnose; ⊕, D-galactose-6-phosphate; ⊕, D-glucose-1-phosphate; ⊕, D-galactose-1-phosphate.

methyl 4,6pyDGalα and 4,6DGalβ R isomer and methyl 4,6pyDGalα S isomer on a 25-fold expanded scale so that the precision of the data becomes evident. Unlike the findings with IgM^{WEA}, galactose-6-phosphate, galactose-1-phosphate, and glucose-1-phosphate gave no inhibition in the amounts used, nor did methyl α or βDGal.

Coprecipitation Studies. Fig. 4 shows the quantitative precipitin curves obtained by adding varying quantities of *Klebsiella* K30 polysaccharide to mixtures that contained 4.0 μg N of IgM^{WEA} plus 6.6 μg N of the $F(ab')_2$ or 6.4 μg N of the $(Fc)_5\mu$. It is evident that some of the $F(ab')_2$ coprecipitates in an IgM^{WEA}-*Klebsiella* K30-specific precipitate, whereas none of $(Fc)_5\mu$ coprecipitates. The $(Fab')_2$ fragment contained a second band on acrylamide. The proportions of antigen, precipitating antibody, and coprecipitating antibody for removing all coprecipitating antibody are difficult to establish. Fig. 4 indicates that the intact 2.75 μg N of intact IgM^{WEA} has a coprecipitating capacity for only 0.75 μg N $(Fab')_2$. If the amounts of IgM^{WEA} and *Klebsiella* K30 polysaccharide were doubled, more $(Fab')_2$ N but no Fc_5 was taken up (data not shown).

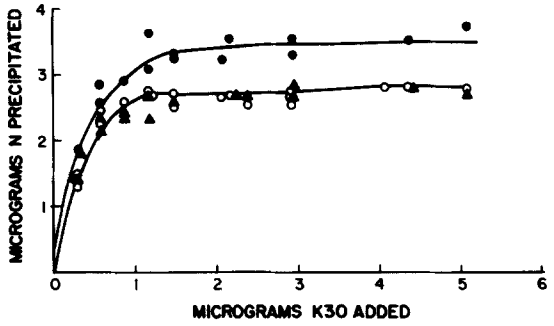

FIG. 4. Coprecipitation curves obtained by adding various amounts of *Klebsiella* K30 polysaccharide to 6.0 μg N of $(Fab')_2$ (●), and $(Fc)_5\mu$ (▲), of IgM^{WEA} plus 4.0 μg N of intact IgM^{WEA} as compared with that of 4.0 μg N of IgM^{WEA} alone (○).

Discussion

The specificity of IgM^{WEA} was first recognized by its absorption to Sepharose and its reaction with agar but not agarose. The various fractions of agar described by Duckworth and Yaphe (18) reacted differently, the most active being the 20°C aqueous extract. Agar has been shown to contain pyruvate-linked 4,6 to a 3-linked D-Gal in the main chain made up of alternating 1→3-linked β-D-Gal and 1→4-linked anhydro α-L-galactose. Agar also contains sulfate groups, and the various fractions of agar contain different proportions of sulfate and pyruvate; agarose is essentially free of both pyruvate and sulfate (51). The precipitin reactions of IgM^{WEA} with the various *Klebsiella* polysaccharides shown in Fig. 1 A provide strong evidence that the combining site of the IgM^{WEA} is most specific for a pyruvylated terminal nonreducing D-Gal-linked β, but do not permit a decision as to whether the pyruvate is 3,4- or 4,6-linked. Quantitative precipitin data with either polysaccharides from *Klebsiella* K30, K33, K21, K7, K32, and from pneumococci of types IV and S XXVII, all of which contain pyruvate linked in various ways (Table I), show that precipitation only occurred with K30 and K33 with 3,4-pyruvylated D-Gal linked β, one preparation of *Rhizobium trifolii* U226 with 4,6-pyruvylated D-Gal linked β, and K21 with 4,6-pyruvylated D-Gal linked α. K30 and K33 were ~10 times more potent, and *Rhizobium trifolii* U226 was 3 times less active/unit weight as compared with the 20°C extract of agar. *Klebsiella* K21[2] was ~1/100 as active/unit weight as *Klebsiella* K30 or K33. None of the sulfated polysaccharides except dextran sulfate precipitated with IgM^{WEA}, and precipitation with dextran sulfate did not resemble the usual precipitation curve with IgM and polysaccharides, in that it was readily inhibited by a relatively slight excess of dextran sulfate and all of the IgM^{WEA} was not precipitated. The precipitin data are best interpreted as indicating that the antibody-combining sites are most specific for a 3,4- or a 4,6-pyruvylated terminal nonreducing D-Gal linked β. The very poor reaction of K21 is probably attributable to the pyruvyl group of D-Gal being linked α. The poorer reaction of the *Rhizobium trifolii* polysaccharide U226, which also has terminal 4,6pyD-Galβ, is not clearly interpretable because it could be either attributable to

[2] Dr. Arepalli S. Rao in this laboratory has subsequently noted that a solution of *Klebsiella* K21 prepared by Dr. M. Heidelberger ~8 yr ago reacts identically with IgM^{MAY} but does not react with IgM^{WEA}. It also may be that the reaction of IgM^{WEA} with *Klebsiella* K21 (Fig. 3, right hand graph) is more sensitive to pH; this is being studied.

cross-reactivity between 4,6pyDGalβ in a 3,4-*O*-(1-carboxyethylidine)β-D-galactopyranoside (3,4pyDGalβ) site or possibly to steric hindrance from the adjacent 4,6-pyruvylated-D-glucose (DGlc) residue. The difference in reactivity of the two strains of *Rhizobium trifolii* (31, 33) is not clear, although both have the same repeating unit structure, but Heidelberger et al. (15) have noted strain differences in the cross-reactions of *Rhizobia*.

That pyruvylated sugar is involved in the specificity of the reaction is supported by the finding that pyruvate is a better inhibitor than lactate, D-galactose-1-phosphate, D-galactose-6-phosphate, D-glucose-1-phosphate, and D-glucose-1-sulfate, and that propionate did not inhibit precipitation of IgM^{WEA} by agar over the range studied (Fig. 3A). The R isomers of methyl 4,6pyDGalβ and 4,6pyDGalα are >20 times as potent as pyruvic acid, whereas the S isomer of methyl 4,6pyDGalα did not inhibit; no inhibition was obtained by DGal and its methyl α and β galactosides, even at 100 μmol, although the R isomers of 4,6pyDGalα and 4,6pyDGalβ gave 50% inhibition at 0.2 μmol. Heidelberger et al. (15) showed that sodium pyruvate inhibited the cross-reaction of the UNZ strain of *Rhizobium trifolii* with type XXVII anti-pneumococcal horse sera more effectively than sodium lactate and sodium propionate. Despite the clear-cut involvement of the charged pyruvyl group, the IgM^{WEA} site may accommodate a DGal determinant substituted by other negatively charged groups as evidenced by the strong reaction with *Helix pomatia* and *Cepaea nemoralis* galactans, and by the increased reactivity of the *Helix pomatia* galactan that resulted from the removal of LGal by periodate oxidation and Smith degradation. The reaction with the *Helix* galactans was specific and charge-related because the uncharged Larch arabinogalactan and pneumogalactan did not react with IgM^{WEA}. The isolation of a trisaccharide from *Helix pomatia* (57), one residue of which was galactose-6-phosphate, together with the findings that galactose-6-phosphate, galactose-1-phosphate, and glucose-1-phosphate show some inhibition, although less than seen with sodium pyruvate, indicate that the phosphorylated galactose is probably reacting in the site. The positively charged moiety on the *Arianta arbustorum* and *Biomphalaria glabrata* polysaccharides may be a factor in their non-cross-reactivity.

The structure of the *Helix pomatia* galactan was inferred by O'Colla (21) to be a 3,6 branched dichotomous structure from four successive stages of periodate uptake and formic acid assays, each followed by phenylhydrazine treatment (Barry degradation) and from the finding of equal proportions of 2,4-dimethyl and 2,3,4,6-tetramethyl galactose. However, recent methylation studies have shown it to have a somewhat more complex structure and from immunochemical studies, (H. Bretting, N. F. Whittaker, E. A. Kabat, K. Königsmann-Lange, H.-J. Thiem. Manuscript in preparation.) the *Helix pomatia* and *Cepaea nemoralis* galactans must differ in structure from those of *Arianta arbustorum* and *Biomphalaria glabrata*.

The finding that the R isomers methyl 4,6pyDGalα and 4,6yDGalβ are of equal potency as inhibitors of the precipitin reaction of IgM^{WEA} by agar, whereas *Klebsiella* K21 polysaccharide with 4,6pyDGalα is 100 times less active[2] per unit weight than *Klebsiella* K30 and K33 with 3,4pyDGalβ structure, suggests either that the IgM^{WEA} site may be more specific for the 3,4pyDGal present in the K30 and K33 polysaccharides and that the poorer reaction with the K21 polysaccharide is due to the 4,6pyDGal not fitting as well into the site, and/or that a β-linkage creates less steric hindrance

than an α-linkage with the intact polysaccharides, which is not seen in inhibition assays with the methyl 4,6pyDGalα and 4,6pyDGalβ.

IgM^{MAY} has a combining site specific for pyruvylated DGal but differs markedly in its reaction pattern from IgM^{WEA}, although it reacts similarly to IgM^{WEA} with *Helix pomatia* galactan and its first stage of periodate oxidation and Smith degradation; IgM^{WEA} reacts better with the 50°C than with the 20°C agar extract. However, unlike IgM^{WEA}, glucose-1-phosphate, galactose-1-phosphate, and galactose-6-phosphate did not inhibit with IgM^{MAY} over the range in which they were as active as lactate. It also reacts very strongly with small amounts of *Klebsiella* K21, whereas IgM^{WEA} required very large amounts of K21 for precipitation[2] (Fig. 1 B). The combining site of IgM^{MAY} appears to be smaller and perhaps less specific than that of IgM^{WEA} in that *Klebsiella* K30 and K33 with 3,4-linked terminal nonreducing DGal-linked β react as well per unit weight as K21 with 4,6-linked terminal nonreducing DGal linked α. This finding would also be consistent with the strong reactivity of IgM^{MAY} with the polysaccharide of *Rhizobium trifolii* TA1, with which IgM^{WEA} did not react, and also with the poorer reactivity of IgM^{WEA} than IgM^{MAY} with U226; thus, if the failure to react and the poorer reactivity of IgM^{WEA} is caused by steric hindrance of the adjacent pyruvylated DGlc, this effect is not seen with IgM^{MAY}, suggesting a site more accessible or smaller than that of IgM^{WEA}. The inhibition data that show that the R isomers of methyl 4,6pyDGalα and 4,6pyDGalβ are equally effective as inhibitors on a molar basis (Fig. 3 B, right hand graph) also show that the IgM^{MAY} site is not sensitive as to whether the linkage of the 4,6-pyruvylated-DGal to the next sugar is α or β. Thus, with IgM^{MAY}, unlike IgM^{WEA}, the site is equally accessible to 4,6pyDGalα and 4,6pyDGalβ on a high molecular weight polysaccharide or as the methyl glycoside. The present study is limited because the 3,4pyDGalα and 3,4pyDGalβ were not available to compare their inhibiting power with the 4,6-pyruvylated-DGal. Because IgM^{WEA} and IgM^{MAY} are both myeloma proteins, their exact specificity is not known and the possibility always exists that some unexpected more highly specific reactant may be found. However, the amounts of the active *Klebsiella* antigens giving precipitation are so small, and the R isomers of 4,6pyDGalα and 4,6pyDGalβ as inhibitors, so active, whereas the S isomers are inactive, that this seems less likely for this system.

Neither DGal, its methyl α- and β-glycosides, nor any of the other neutral sugars tested gave any inhibition over the range used with either IgM^{WEA} or IgM^{MAY}, suggesting that the reaction with the *Helix pomatia* and *Cepaea nemoralis* galactans is attributable to the negative charges on these galactans. It will be important to determine whether removal of the charged groups eliminates their precipitin activity. Unlike the findings with IgM^{WEA}, galactose-6-phosphate, galactose-1-phosphate, and glucose-1-phosphate did not inhibit precipitation.

To map the combining sites more precisely, it will become of great importance to obtain oligosaccharides from various *Klebsiella* types containing 3,4pyDGalα, 3,4pyDGalβ, 4,6pyDGalα, and 4,6pyDGalβ and to compare these as inhibitors.

The 4,6 and 3,4 pyruvyl groups are chiral and each may be linked in two forms that are space isomers termed R and S (45). Garegg et al. (58) have recently determined the orientation of the 3,4- and 4,6-pyruvylated-DGal in various *Klebsiella* polysaccharides by nuclear magnetic resonance measurements. K30 and K33 with 3,4 pyruvate are both in the S configuration, whereas K21 and *Rhizobium trifolii* U226 with 4,6 pyruvate were in the R form; however, in the 4,6 polysaccharides, the

orientation of the methyl group was equatorial and in the 3,4 compounds was endo relative to the acetal ring so that they all have the same conformation despite the different linkage.

It is of significance that all of the pyruvylated polysaccharides that reacted with IgM^{WEA} and IgM^{MAY} are known to have the methyl group of the pyruvate moiety equatorial and the carboxyl group axial to the plane of the acetal ring (58) and that this is also the orientation in the R forms of methyl 4,6pyDGalα and 4,6pyDGalβ. Thus, the precipitation and inhibition involve the same spatial relationships of the determinant and inhibitors. Because the S isomer of methyl 4,6pyDGalα does not inhibit with IgM^{WEA} and IgM^{MAY} nor with *Klebsiella* K11-anti-*Klebsiella* K11 (59), these reactions are highly specific, providing unusually strong evidence for the specificity of the myeloma sites being actually directed toward 3,4- or 4,6-pyruvylated-DGal. *Klebsiella* K11 polysaccharide also contains the R form of 4,6pyDGalα (59) (Table I). Inhibition studies with the R and S isomers of methyl α- and β-glycosides of 3,4pyDGal will, therefore, be important for characterizing the sites of IgM^{WEA} and IgM^{MAY}.

The specificity of the previously reported IgM^{Nae} (12, 13) could also involve pyruvylated galactose because it reacted strongly with *Klebsiella* K11, which contains terminal 4,6-pyruvylated-DGal-linked α1→4 to a →3-linked D-glucuronic acid in the main chain (15), but a reaction was apparently not seen with K21 (12) in which the 4,6-pyruvylated-DGal is also joined α1→4 to a →3-linked D-glucuronic acid in the main chain (Table 1); K35, with which IgM^{Nae} and IgM^{Th} (60) reacted, and K12 and K13, with which IgM^{Ro} and IgM^{We} reacted, also contain pyruvic acid and galactose; in K12 (60) the 4,6-pyruvylated-DGal is linked β1→4 to D-glucuronic acid, which is linked β1→6 to a main chain. The structure of K13 has not been reported. The other three IgM proteins reacting with *Klebsiella* antigens appear not to involve a pyruvylated-DGal specificity.

The coprecipitation experiment (Fig. 4) shows that the unique reactivity of IgM^{WEA} with the polysaccharides is associated with the $F(ab')_2$ fragment and not with the $Fc_5\mu$ fragment and, thus, is consistent with an antibody activity. The finding of the two human IgM macroglobulins with combining sites that react specifically with 3,4- and 4,6-pyruvylated-DGal again raises the question (12, 13) of whether the frequent occurrence of *Klebsiella* in the nasopharynx and gastrointestinal tract expands *Klebsiella*-specific B cell clones and increases the probability of finding dyscrasias that involve a cell synthesizing anti-*Klebsiella* antibody. The frequent occurrence in mice of myelomas with antibody activity directed against antigens of the intestinal bacteria, hardwood bedding, and diet, to which mice are continually exposed, has been explained on this basis (61, 62). The failure to find a high incidence of myeloma antibodies in man may reflect the types of antigen used in previous screening studies.

Summary

Two human IgM myeloma proteins, IgM^{WEA} and IgM^{MAY}, were found to react with agar and *Klebsiella* polysaccharides that contain pyruvylated D-galactose (DGal). Quantitative precipitin data and precipitin inhibition studies with methyl α- and β-glycosides of 4,6-pyruvylated-D-galactose showed their combining sites to be different, although each was directed against the pyruvylated-D-Gal, one reacting most specifically with *Klebsiella* polysaccharides with terminal nonreducing β-linked 3,4 pyru-

vylated-D-Gal, whereas the other reacted equally well with *Klebsiella* polysaccharides that contain 3,4 β-linked and 4,6 α-linked terminal nonreducing pyruvylated-DGal. Inhibition studies showed that both sites are directed toward one of the two space isomers of 3,4- or 4,6-pyruvylated DGal, the form in which the methyl group of the pyruvate is equatorial, or endo, and its carboxyl group axial, or exo, to the plane of the acetal ring. Coprecipitation studies showed the combining site of IgM^{WEA} to be located on an $(Fab')_2$ fragment and not on the $(Fc)_5\mu$ fragment. The monoclonal peak in the serum of IgM^{MAY} was specifically precipitated by *Klebsiella* polysaccharide. Myeloma proteins with specificities of this type may occur with reasonable frequency in humans and may be a consequence of clonal expansion from inapparent infection, carrier states, or disease produced by various *Klebsiella* organisms.

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