A new monoclonal antibody (A46-B/B10) highly specific for the blood group H type 2 epitope: Generation, epitope analysis, serological and histological evaluation

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Summary A monoclonal antibody recognizing the blood group H type 2 antigen has been obtained from a BALB/c mouse immunized with MCF-7 (human mammary carcinoma) cells. The specificity of this antibody (A46-B/B10, IgM, κ) has been identified by haemagglutination tests, immunohistochemistry, binding inhibition studies, and absorption experiments performed with synthetic oligosaccharides. The antibody is virtually nonreactive with H type 1 antigen or with closely related type 2 structures (e.g. Y antigen). A46-B/B10 strongly agglutinates human erythrocytes according to the amount of H substance expressed and can, therefore, easily discriminate between blood groups A₁ and A₂ as well as A₁, B and A₁, B (A₁ and B are not or only weakly agglutinated). In immunohistochemistry, this antibody seems to provide a highly specific reagent for a restricted number of carcinomas and epithelial lineages in tissue sections and in vitro.

Blood group-related carbohydrate antigens are important parts of membrane glycoproteins and glycolipids. They are thought to be involved in recognition processes. There is at present increasing interest in these substances because of accumulating evidence that they are characteristically modified during malignant transformation (Hakomori, 1984; Ginsburg et al., 1984; Feizi, 1985; Uhlenbruck, 1986). Monoclonal antibodies (mabs) are essential tools for the analysis of these phenomena. We here describe a new mab specific for the H type 2 epitope and show its applicability in blood group serology as well as in immunohistochemistry.

Materials and methods

Generation of hybridoma clone A46-B/B10

Methodical details were as described in Karsten et al. (1983). Briefly, BALB/c mice were immunized with live cells of the human mammary carcinoma cell line MCF-7, derived from a patient of blood group O (Soule et al., 1973). Spleen cells from one of these mice were fused with X63-Ag8.653 mouse myeloma cells (Kearney et al., 1979), and supernatants from hybridomas growing in HAT medium were screened by means of indirect immunofluorescence tests performed with MCF-7 and A-204 (human rhabdomyosarcoma, Giard et al., 1973) cells. Hybridoma A46-B/B10, which produced a mab reacting with a membrane antigen present on MCF-7 but absent on A-204 cells, was cloned repeatedly by limiting dilution on feeder cells, expanded and stored in liquid nitrogen. Cells of this clone were cultured in RPMI 1640 medium containing 5 x 10⁻⁵ M 2-mercaptoethanol and 10% foetal calf or horse serum without antibiotics. (Horse serum, although well suited for hybridoma growth, disturbs some tests due to intrinsic anti carbohydrate antibodies). Hybridoma cells were checked for mycoplasma contamination by DNA staining (Chen, 1977) and electron microscopy with negative results. Ascites fluid was obtained in BALB/c mice conditioned by pristane and transplanted with 1 x 10⁸ hybridoma cells. Isotype determination was done by immunodiffusion (heavy chain) and dot test (light chain) using specific antiserum (Miles, Slough, UK).

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Haematological methods

Culture supernatants of A46-B/B10 (titres ranging from 1:16 to 1:64) were used in standard agglutination tests with washed erythrocytes resuspended in isotonic NaCl solution (for details see Pilgrim & Karsten, 1987).

Immunohistochemistry

This was performed with either cell lines grown on multilayer slides (Flow Labs., Bonn, FRG) and analyzed by indirect immunofluorescence or with cryostat sections cut from frozen tissue samples (4μm; Frigocut, Reichert, Vienna, Austria) analyzed by means of a modified peroxidase anti peroxidase technique (Kupper et al., 1984). Sections were counterstained with Mayer’s haemalum. In some cases alternative techniques were employed, e.g. paraffin sections after formalin or methacarn fixation, or indirect immunoperoxidase staining. Peroxidase and FITC labelled second antibodies were from a commercial source (Staatliches Institut für Immunprüparate und Nährmedien, Berlin, GDR). Mabs with irrelevant specificity or culture medium alone served as negative controls.

Inhibition assays

Quantitative analysis of antibody binding to seminal plasma mucins (4 μg ml⁻¹) and binding inhibition were performed as enzyme immunoassays on Immulon plates (Dynatech, Plochingen, FRG) according to Voller et al. (1976). Antibody A46-B/B10 produced as ascites was diluted 1:40 and mixed with serial two-fold dilutions of saccharides (starting from 50μg well) or glycoprotein inhibitors (starting from 100 μg well). Lacto-N-fucopentaose I was purchased from BioCarb Chemicals, Lund, Sweden, and N-acetyllactosamine from Sigma, Munich, FRG. Glycoproteins from human secretions of serologically defined donors were enriched by hot phenol-saline extraction (Hanisch et al., 1985a), and the mucins contained in these fractions were separated by exclusion chromatography on Sephacryl S 400 (Hanisch et al., 1985b). Carbohydrate fractions and neutral saccharide alditols N4 and N8 with the primary structures Fuc(1-2) Gal(1-4)GlcNAc(1-3)Gal(1-3)GalNAc(1-3)GalNAc(1-3)GalNAc, respectively, were isolated from mucins of human seminal fluid (Hanisch et al., 1985a, 1986). Anti mouse IgG-phosphatase conjugate and p-nitrophenyl phosphate were
from Sigma, Munich, FRG. Lectin inhibition was performed using a cell line expressing the A46-B/B10 antigen, H184A1 (mammary epithelial cells, Smith et al., 1981). Cells were grown on multistest slides and stained in indirect immuno-fluorescence after preincubation with Ulex europaeus I lectin (Serva, Heidelberg, FRG) in serial dilutions (starting from 200 μg ml⁻¹) at 4°C for 1 h.

Absorption with synthetic oligosaccharides

Synthetic H type I and type 2 oligosaccharides bound to an inorganic carrier (Synsorb, Chemibiochem, Edmonton, Canada) were washed with PBS, degassed, and incubated with A46-B/B10 supernatant at room temperature for 16 h with gentle shaking. Antibody titres before and after absorption were evaluated by haemagglutination with 0 erythrocytes. The absorption capacity of the Synsorb material had been estimated in preliminary experiments. Accordingly, 25 mg dry weight of immunosorbent was reacted with 100 μl supernatant, titre 1:64. For comparison, an anti H mab commercially available from Chemibiochem (Edmonton, Canada) was included. This mab had an original haemagglutination titre of 1:16.

Results

The monoclonal antibody A46-B/B10 (IgM, κ) first occurred as an antibody strongly binding to a membrane antigen present on MCF-7 but absent on A-204 cells (Karsten et al., 1983). Further examination against a panel of human cell lines (Table I) revealed the expression of this antigen on 4 out of 5 mammary carcinoma cell lines and on a mammary epithelial cell line derived from normal breast tissue. However, the results obtained with A46-B/B10 were not consistent. In certain cases, the antigen was detected on normal epithelial cells, whereas it was not detected on the respective neoplastic cell lines. These results suggest that the antigen is associated with normal epithelial cells and that its expression is upregulated in neoplastic cells. The reactivity of A46-B/B10 with normal mammary tissues was variable, with some specimens showing a percentage of positive cells ranging from 10% to 90%.

| Cell line* | Tissue of origin | Donor blood group | Reactivityb |
|------------|------------------|--------------------|-------------|
| MCF-7      | Mammary carcinoma| 0                  | +           |
| T47D       | Mammary carcinoma| +                  |             |
| BT-20      | Mammary carcinoma| +                  |             |
| CAMA-1     | Mammary carcinoma| +                  |             |
| MaTu       | Mammary carcinoma| -                  |             |
| H184A1     | Mammary epithelium| +                  |             |
| Org8       | Mammary epithelium| A, +               |             |
| Hs578T     | Carcinosarcoma of breast| - |             |
| T-24       | Bladder carcinoma| +                  |             |
| HT-29      | Colon carcinoma  | A (+)              |             |
| LS-174T    | Colon carcinoma  | (+)                |             |
| HeLa-S     | Epithroderm carcinoma of cervix| (+) |             |
| A-431      | Epidermoid carcinoma of vulva| (+) |             |
| Tu197      | Ovarian carcinoma| (+)                |             |
| A-204      | Rhabdomyosarcoma | -                  |             |
| Tu256      | Osteogenic sarcoma| -                  |             |
| Tu131      | Ewing sarcoma    | -                  |             |
| HMy2       | Plasmacytoma      | -                  |             |
| Reh        | Null cell ALL    | -                  |             |
| Molt-4     | T-cell ALL       | -                  |             |
| SKW3       | T-cell leukaemia | -                  |             |

aSources, references, and cultivation conditions see Karsten et al., 1983, 1985. Org8 is one of a series of normal mammary epithelial cell lines described in Karsten et al. (1988); bReactivity indicated as follows: + = negative (no staining); + = weak staining; ++ = strong staining; +++++ = almost complete staining. *AL = acute lymphoblastic leukaemia.

Table II Staining characteristics of mab A46-B/B10 with cryostat sections of mammary carcinomas

| Blood group | No. | A | B | O | Total |
|-------------|-----|---|---|---|-------|
| A           | 8   | 8 | - | - | 16    |
| B           | 3   | 3 | - | - | 6     |
| O           | 6   | 2 | 2 | 2 | 12    |
| Total       | 17  | 13| 2 | 2 | 32    |

Numbers indicate specimens belonging to each group. Staining intensity is given as follows: 0 = negative (no staining); A = less than 10%; B = 10 to 90%; C = more than 90% of carcinoma cells stained.

unrelated mab, could be blocked by preincubation with the lectin in a dose-dependent manner. Pretreatment with 200 or 100 μg ml⁻¹ completely inhibited staining, whereas lower doses of the lectin were only partially effective. In a comparison of various human secretions, A46-B/B10 bound most strongly to glycoproteins from seminal fluid. These were accordingly used in binding inhibition studies. Binding was most effectively inhibited by gastric mucins from secretors or by neutral carbohydrates (SPL-N), which had been reductively cleaved from seminal plasma mucins (Figure 2). The corresponding sialic acid containing saccharide alditols from seminal mucins (SPL-A) were much less inhibitory. The inhibitory activity of neutral carbohydrates was abolished after chemical cleavage of L-fucose residues with mild acid. The involvement of L-fucose in the epitope structure recognized by mab A46-B/B10 was further examined with the following pure, chemically defined H type 2 related saccharides (Hanisch et al., 1986):

Fucα(1→2)Galβ(1→4)GlcNAcβ(1→3)Galβ(1→3)GalNAc-ol (N4; H type 2 epitope positive), and

Fucα(1→2)Galβ(1→4)(Fucα(1→3))GlcNAcβ(1→3) × Galβ(1→3)GalNAc-ol (N8; Y epitope positive).
Figure 1 Examples of cells and tissues expressing the A46-B/B10 antigen (H type 2). Bar = 20 μm. (a) Cryostat section of mammary carcinoma. Carcinoma cells focally positive, stromal cells negative; (b) Cryostat section of normal pancreas. Acinar epithelial cells (exocrine pancreas = ep) positive, islet cells (i) negative; (c) Methacarn fixed paraffin section from normal breast (reduction mammoplasty, blood group O). Endothelial cells positive, epithelial and stromal cells negative. (a)–(c) Peroxidase technique, counterstained with haemalum. In (c) a strong blue filter was applied in order to reduce the optical dominance of counterstained nuclei. (d) Cell line Org8 (mammary epithelial cell line derived from a patient blood group A1, undergoing reduction mammoplasty). Indirect immunofluorescence technique.

Table III Agglutination of a total of 1138 samples of known blood groups with mab A46-B/B10

| Agglutination strength | O   | A₂  | A₂B | A₁  | A₁B | B   |
|------------------------|-----|-----|-----|-----|-----|-----|
| + + + +                | 181 | 69  | 19  | 3   | 0   | 113 |
|                        | (87.4) |    | (41.3) | (1.2) | (0.0) | (26.1) |
| + + +                  | 26  | 23  | 23  | 18  | 0   | 151 |
|                        | (12.6) |    | (50.0) | (7.2) | (0.0) | (34.9) |
| + +                    | 0   | 4   | 4   | 39  | 18  | 102 |
|                        | (0.0) |    | (8.7) | (15.6) | (17.0) | (23.6) |
| +                      | 0   | 0   | 0   | 89  | 27  | 56  |
|                        | (0.0) |    | (0.0) | (35.6) | (25.5) | (12.9) |
| Ø                      | 0   | 0   | 0   | 101 | 61  | 11  |
|                        | (0.0) |    | (0.0) | (40.4) | (57.5) | (2.5) |
| Total                  | 207 | 96  | 46  | 250 | 106 | 433 |

*Agglutination strength scored as follows: 0 = negative; + = very small agglutinates; + + = small but definite agglutinates; + + + = several clots in clear fluid; + + + + = clot in clear fluid; *Numbers of samples examined; *Per cent of samples per blood group reacting according to indicated the agglutination strength; *These numbers do not reflect the relative proportions of blood groups in the population.
In addition, a defined H type 1 oligosaccharide and the simple disaccharide, N-acetyl-lactosamine, were also included:

Fuc(1–2)Galβ(1–3)GlcNAcβ(1–3)Galβ(1–4)Glc

(Lacto-N-fucopentaose I; H type 1 epitope; Le*)

Galβ(1–4)GlcNAc

(N-acetyl-lactosamine).

The results presented in Figure 3 show that the H type 2-blood group active saccharide N4 contained in the fraction of neutral carbohydrates (SPL-N) prepared from seminal plasma mucins was the only potent inhibitor of A46-B/B10 antibody binding. A second L-fucose residue linked to the subterminal N-acetyl-D-glucosamine (N8) rendered the molecule non-competitive for the A46-B/B10 epitope. The same was true for the H type 1 structure and the N-acetyl-lactosamine disaccharide lacking L-fucose. Absorption experiments performed with immobilized synthetic H type 1 and type 2 oligosaccharides (Table IV) gave results in accordance with the assumed H type 2 specificity, whereas the commercial anti H mab was less specific in this respect. In conclusion, the epitope structure recognized by antibody A46-B/B10 comprises at least a trisaccharide sequence based on the blood group H type 2 structure:

Fuc(1–2)Galβ(1–4)GlcNAc.

Discussion

One of the major advantages of the hybridoma technique developed by Köhler and Milstein (1975) consists in its ability to detect and define unknown tumour associated antigens. In pursuing this goal and using carcinoma cells or cell lines as immunogens, several groups arrived at mabs against blood group-related antigens (Ginsburg et al., 1984). At first sight, this was surprising but there is now growing awareness that these antigens are in fact among the most common and most potent carcinoma associated antigens (Hakomori, 1984; Ginsburg et al., 1984; Feizi, 1985; Uhlenbruck, 1986). From the biochemical point of view, different patterns of changes can be found depending on the tissue of origin. Mammary and prostate carcinomas are characterized by a loss of blood group isoantigens accompanied by the accumulation of precursor substances (Vowden et al., 1986a, b). On the contrary, distal colon and thyroid, which in normal adult life lack blood group isoantigens, may express these antigens in malignant state (Vowden et al., 1986c). Another type of deviation consists in the synthesis of modified (dimeric, sialylated, or more extensively fucosylated) carbohydrate chains (Hakomori, 1984). The H type 2...
antigen recognized by mab A46-B/B10 is a blood group precursor and can therefore be considered a carcinoma-associated antigen of tissues belonging to the first category. It has been reported to accumulate in mammary carcinomas (Vowden et al., 1986b), squamous cell carcinomas (Kimmel et al., 1986), prostate carcinomas (Vowden et al., 1986a), and precancerous oral epithelium (Dabelsteen et al., 1983). In such studies the use of strictly specific and thoroughly tested mabs is essential. A46-B/B10 seems to fulfill these criteria. Only few others of the same or similar specificity have been described (Young et al., 1981; Knowles et al., 1982; Fredman et al., 1983; Feizi, 1985; Kimmel et al., 1986). Among these, minor differences may exist. For instance, we cannot explain at present the reactivity of mammary carcinomas other than that from patients of blood group O with A46-B/B10 in contrast to the results of Vowden et al. (1986b). A direct comparison of the anti H type 2 mabs has not yet been performed. Furthermore, the different amount of H antigen expressed on subgroups (e.g. A₁ vs. A₂) has not yet been taken into account by either study. On the other hand, some preliminary data indicate that different methods of tissue processing may bring about not merely preservation or loss of an epitope but certain subtle changes in its pattern of distribution. As compared to the H type 2 binding Ulex europaeus I lectin, A46-B/B10 appears to be much more specific, because this lectin cannot discriminate between H2 and Le⁺ (Hindsdaul et al., 1985).

In principle, mab A46-B/B10 can be employed with cryostat or paraffin sections. Although methacarn fixation is to be preferred in the preparation of paraffin sections, formalin fixed material can be used.

In studies on cells cultured in vitro, A46-B/B10 seems to be a reagent able to identify certain epithelial lineages independent of the blood group of the donor. The rules governing carbohydrate antigen expression in vitro seem to differ from those acting in vivo and await further exploration. In recent experiments we were able, by using mab A46-B/B10, to define an H type 2 positive subpopulation of normal mammary epithelial cells cultured in vitro (see Figure 1d), which we consider to be stem cells (Karsen et al., 1988). Available data suggest an onco-fetal mode of expression of the H type 2 antigen in mammary epithelial cells in vitro.

A46-B/B10 is well suited in blood typing where it clearly distinguishes between A₁ and A₂ as well as between A, B and A₂B. Moreover, it seems to be able to reproducibly define subtypes of B differing in their amount of H substance (Pilgrim & Karsten, 1987).

Very recently it has been shown by Rodeck et al. (1987) that a mucin bearing X, Y and H type 2 determinants (different from the mucin carrying the 19-9 epitope) is shed by carcinoma cells. If this mucin can be found in patient sera, this could open up yet another field of application for anti H type 2 mabs such as A46-B/B10.

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