IMMUNOLOGICAL CHARACTERIZATION OF BLOOD GROUP A EPITOPES EXPRESSED ON CELLS AND TISSUES WITH A MONOCLONAL ANTI-CEA ANTIBODY

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ABSTRACT

Background and Methods. **Monoclonal antibodies (mAb) specific for the oligosaccharidic epitopes of glycoproteins or glycolipids, such as blood group antigens, are powerful tools for studying the antigenic structure of normal and pathological cells and tissues. Anti-A human red blood cell monoclonal antibodies were produced by immunizing mice with normal cells, but only a few fulfilled the conditions necessary for revealing qualitative differences among A-antigens. Only those produced by hybridomas obtained from mice immunized with human tumor antigens specifically recognize A_1 and A_2 blood group antigens. We report here several immunological properties of the A-antigen defined by a mAb raised against the tumor-associated carcinoembryonic antigen.**

Results and Conclusions. **The hybridoma B2C114, obtained as a result of the fusion of spleen cells from mice immunized with the carcinoembryonic antigen and a murine myeloma cell line, produces a mAb which reacts specifically against erythrocytes bearing the A blood-group antigen. The monoclonal antibody showed a high stability and a low dissociation rate from the antigen/antibody complex formed with adult A_1, A_2, A_2B and cord blood samples. The antibody was able not only to discriminate between A_1- and A_2-RBC but also to detect kinetic differences among A-sites. On the one hand B2C114, reactive with the glycosidic moiety of the A-antigen, can discern at least two qualitatively different epitopes expressed on the A_1-RBC surface, with a total number of A sites that is in close agreement with the figures already described for A_1-RBC. On the other hand, A2-RBC shows a single phenotype that is kinetically similar to A_1-low affinity binding sites. This antibody also labelled spontaneous and chemically-induced murine tumors as well as human tumors. Its reactivity with colon carcinoma frozen specimens obtained from O- and B-blood group patients indicated expression of an incompatible A-antigen. The immunochemical properties of B2C114 described here give support to our purpose of employing this mAb as a blood group reagent as well as a histopathological probe for *in vitro* and *in vivo* cancer diagnosis.**

Key words: monoclonal antibody, blood group reagent, A-site properties, histopathology

The production of monoclonal antibodies (mAb) directed against blood cells is of both scientific and practical interest. On the practical side it is very important to have homogeneous reagents with high specificity and avidity, capable of recognising weak variants, that are useful for blood typing and for histopathological studies in cancer diagnosis. A great deal of mAb directed against A-human erythrocytes have been produced, but none ful-

fils the conditions necessary for revealing qualitative differences among A_1 and A_2 antigens. Moreover, all successful anti-A reagents for routine tests are of the IgM and IgA classes.[1] Several mAb produced by hybridomas obtained from mice immunized with human tumor cells react specifically against blood group antigens.[1,2] This is due to the fact that certain tumor antigens are glycolipids or glycoproteins that are very similar to the blood group system present on the human RBC surfaces.[3] The hybridoma B2C114, obtained as a result of the fusion of spleen cells from mice immunized with the carcinoembryonic antigen (CEA) and a murine myeloma cell line, produces a mAb which reacts specifically against erythrocytes bearing the A blood-group antigen,[4,5] and it was therefore used to analyze the immunological and histochemical properties of the A-antigen expressed on RBC and normal and pathological tissues.

Materials and methods

Monoclonal antibodies

Hybridomas were generated by the fusion of lymphocytes from the spleen of a Balb/c mouse, immunized with purified CEA, with the X63-Ag8.653 mouse myeloma cell line, as previously described.[6] One hybrid subclone, designated B2C114, was expanded as subcutaneous solid or ascitic tumors in Balb/c mice and stored in liquid nitrogen. The culture supernatants and the ascitic fluids were stored at −20°C with sodium azide as preservative. The IgG1 isotype of B2C114 monoclonal immunoglobulin was determined by gel immunodiffusion assay and monoclonality was confirmed by internal labelling with ^{14}C-leucine followed by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate and autoradiography at −70°C using Kodak X-Omat film and Dupont intensifying screen.

Agglutination tests

Hemagglutination tests were carried out in U-shaped microtitration 96-well plates (Corning Incorp, USA). Fifty μL of serially diluted culture supernatants were incubated with 50 μL of a 2 % A, B or O RBC suspension in phosphate-buffered saline (PBS), pH 7.2, for 1h at room temperature. The settling patterns of red cells were judged visually against a well-lit background for the determination of agglutination titre defined as the reciprocal of the last dilution giving macroscopic agglutinates. Commercial reagents were used as controls for blood-group typing (Seraclone, Biotest, USA). MAb activity was also evaluated in plasma-suspended cells and immediate spin tests by mixing 0.1 mL culture supernatant or ascitic fluid with 0.1 mL cellular suspension at 3%. The test tubes were centrifuged for 20 sec at 500×g and the strength of agglutination determined visually under the microscope after gentle resuspension of the RBC pellets. Antibody avidity was determined on glass slides by mixing equal volumes of culture supernatants and 10% suspensions of different blood group RBC in saline. The time required for visible agglutination to appear was determined.

Inhibition tests using A, B and H soluble blood group substances from ABH saliva secretors or non-secretors, and competitive assays with galactose (Gal) and N-acetylgalactosamine (GalNAc) (Sigma Chemical Co, St Louis, Mo, USA) were used and performed according to classical methods.[7] Enhancement agglutination tests were carried out using bromelain, papain, low ionic strenght saline (LISS), Polybrene and the bovine serum albumin (BSA).[7]

Table 1. Anti-A titrations under different conditions. Reciprocal hemagglutination titres of monoclonal anti-A antibody performed on microtitre plates as described under Materials and Methods. n: number of samples tested. ND: not determined.

Cells	n	2% in saline	37°C 15min	pH 5	pH 9
A1	56	512	32,000	32	64
A2	31	256	8,000	32	64
A2B	15	64	128	2	8
Cord blood	10	256	ND	ND	ND

Radiobinding assays

The B2C114 mAb was purified from ascitic fluid by ammonium sulphate precipitation followed by affinity chromatography on Protein A-Sepharose CL-4B (Pharmacia, Uppsala, Sweden). The purified mAb was labelled with ^{125}I in Iodogen-coated tubes.[8] The reaction mixture was chromatographed on a Sephadex G-25 (Pharmacia, Uppsala, Sweden) column to remove the unincorporated ^{125}I. ^{125}I incorporation onto the protein was evaluated by trichloroacetic acid precipitation, and was always >95%. The specific activity of the labelled mAb ranged from 6-9 mCi/mg. The specificity of ^{125}I-B2C114 was determined by measuring the binding of the mAb to A, B, O-types of RBC and peripheral blood lymphocytes, polymorphs and monocytes.

The immunoreactivity of the labelled mAb was evaluated by linear extrapolation to the binding at infinite antigen excess.[9] Briefly, 10% packed RBC, washed with PBS and containing 0.1% BSA, were mixed with increasing concentrations of the labelled mAb, incubated for 30 min at room temperature and then washed with PBS by centrifugation. The RBC pellets were counted for radioactivity in a Gamma Counter (Packard, PRIAS). Data were expressed as the ratio of total applied to specifically bound mAb radioactivity (T/B) and plotted against the antibody-antigen ratio (mAb/Ag). The immunoreactivity of the radioiodinated product was defined as the y-intercept of the linear regression of the plot.

The dissociation rates of the antibody-antigen complex were measured by preincubating a 5% A-RBC suspension in 0.15 M NaCl with 50,000 cpm of ^{125}I-B2C114 and measuring the fall in binding after resuspending the cells at different times in the presence of an excess (~1 mg) of unlabelled mAb. Pellet-associated radioactivity was counted in a Gamma Counter.

The affinity constant (K_a) of the binding reaction and the number of antibody binding sites per cell were determined by Scatchard analysis.[10] Kinetic parameters were calculated at
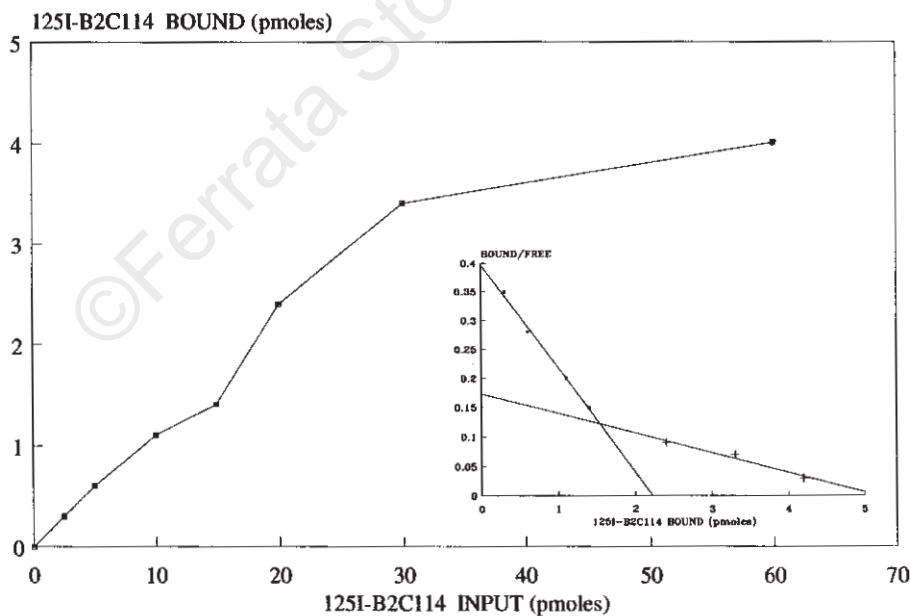

Figure 1. Specific binding of increasing concentrations of ^{125}I-B2C114 to A_1-RBC. Points, the mean of 2 observations within a single experiment, are representative of 3 separate experiments. The data, corrected for non-specific binding and for an immunoreactive fraction of 0.65, were used for Scatchard analysis (inset). The plot of the specific binding data shows the K_a and the maximum number of binding sites for the high- and the low-affinity sites.

Table 2. Enhancement of anti-A reactions by treatments of cells. All tests were performed on microtitre plates under conditions described in Materials and Methods. Agglutination strength was scored as follows: 0=negative; 1+: very small agglutinates; 2+: small but definite agglutinates; 3+: several clots in clear fluids; 4+: clot in clear fluid; s= strong.

Cells	A1		A2		O or B	
	Adult	Cord	Adult	Cord	Adult	Cord
Saline	3+	2+	2+	1+	0	0
Albumin	4+	3+	3+	2+	0	0
Papaine	4+	3+	4+	3+	0	0
Bromeline	4+s	3+	4+	2+	0	0
LISS	3+	2+	3+	1+	0	0
Polybrene	4+s	4+	3+	2+	0	0

saturation from: the radioactivity of mAb bound per cell minus the amount of mAb bound to the cell in the presence of unlabelled mAb (non-specific binding), the specific activity of the mAb, the molecular weight of the mouse immunoglobulin, Avogadro's number and the number of RBC.

Cell lines and tissues

The HT29 human colon adenocarcinoma cell line was grown as a monolayer in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (Bioser, Argentina). For the experiment, the cells were detached from the culture flask by a 10-min treatment with EDTA (0.2 mg/mL) and trypsin (2.5 mg/mL). Cell surface antigens were identified by mAb B2C114 binding as detected by indirect immunofluorescence (IIF) assays. Briefly, separated cells (5×10^5) were placed in test tubes and incubated with 1 μg of purified mAb at 4°C. After 30 min of incubation, cells were washed twice in cold PBS with 0.02% sodium azide, 1% BSA. Next, the washed cells were incubated (at 4°C for 30 min) with 100 μL of fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Cappel, Organon Teknika Corp, USA), then washed twice and analyzed for fluorescent staining by epifluorescence microscopy.

Tissue specimens removed at surgery were snap frozen in liquid-cooled isopentane and subsequently stored in liquid nitrogen until used. Malignant tumors were classified as colon carcinomas or transitional bladder carcinomas by immunohistochemistry performed on cryostat sections that were analyzed by means of the peroxidase-antiperoxidase technique. Sections were counterstained with hematoxylin. mAb with irrelevant specificity served as negative controls.
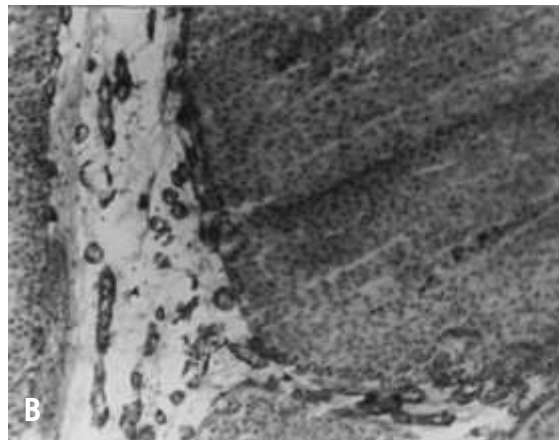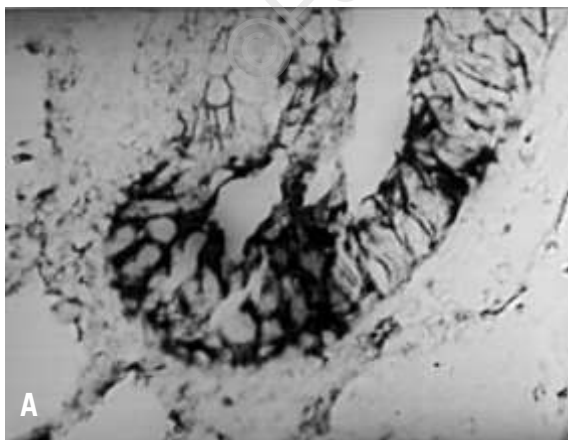
Figure 2. A) Well-differentiated colon adenocarcinoma of a group O patient; non-compatible blood group A present in tumor (x 400). B) Transitional bladder carcinoma grade I taken from a blood group A patient (x 120). The histological section shows blood group-A positivity on the transitional epithelial cells and on the underlying endothelium, while the connective tissue of the papilla is negative.

Table 3. Residual binding activity of B2C114 on HT29 cells after chemical (metaperiodate) or enzymatic treatment (trypsin) and after competitive assays with lectins (ConA and PHA). The assays were carried out as described under Materials and Methods.

Na IO4	50 mM	10 mM	2 mM
	25%	38%	57%
Trypsin	0.09%	0.03%	0.01%
	75%	95%	100%
ConA	5 mM	1 mM	0.5 mM
	90%	100%	100%
PHA	5 mM	1 mM	0.5 mM
	38%	43%	55%

Chemical and enzymatic assays

Chemical and enzymatic treatments were carried out on HT29 cells. Metaperiodate oxidation (NaIO4) at concentrations starting from 50 mM was carried out at 4°C for 2 h. Trypsin treatment was performed out for 1 h at 37°C. The residual binding activity of B2C114 was assessed by IIF as described above. Competitive binding experiments were done in the presence of increasing concentrations of concanavalin A (ConA) and phytohemagglutinin (PHA) (Sigma Chem Co, St. Louis, Mo, USA).

Results

The hemagglutination properties, analyzed in microplates, slides and test tubes, showed that B2C114 specifically recognizes type A RBC. Further studies confirmed that the mAb agglutinates A_1, A_2, A_2B and cord blood (Table 1). The reaction was only inhibited by A secretors, while no effect was observed with soluble substances from B- and H-saliva secretors. The avidity reaction observed for the mAb was quite potent for A_1 (3"), A_2 (5") and A_2B (12"). The hemagglutination tests done at different temperatures, pH and centrifuging conditions modified the titre of the mAb. When the temperature was raised to 37°C or spin tube tests were applied, higher agglutination titres were obtained. On the contrary, more acid or alkaline conditions reduced the titre determined at pH 7.2. The

agglutination activity of B2C114 was also shown to increase when adult or cord RBC were treated with proteolytic enzymes and other agglutination enhancers (Table 2).

The specificity of the mAb was also assessed by radiobinding assays. The direct radioactive binding assay with peripheral blood cells as targets showed that only RBC from group A donors bound B2C114 antibody. Among the A group RBC, the A_1 type donors (n=20) bound the mAb to an extent double that of the A_2 type (n=19) (13,200 cpm±460 vs 6,250 cpm±320). In the binding tests, reaction of A group RBC was observed in all cases, while no reaction was detected with B or O group cells, nor with lymphocytes, polymorphs or monocytes (1,260 cpm±625). The decrease in binding after the addition of an excess of unlabelled mAb was less than 25% of the total binding, indicating the high stability of the complex formed between the mAb and A-RBC.

Kinetic analysis of the mAb binding to A-RBC, based on measurement of the amount of bound and free mAb at various antibody concentrations, provides the number of anti-A sites per cell and the K_a of mAb binding for the A-epitope. Plotting these experimental data yielded a biphasic curve for A_1-RBC (Figure 1). The Figure 1 inset shows the presence of two different affinity components: one of high affinity ($K_a = 1 \times 10^9$ M^{-1}) involving 8×10^5 sites per cell and the other of lower affinity ($K_a = 1 \times 10^7$ M^{-1}) corresponding to 2×10^5 sites per cell. A_2-RBC presented a single phenotype that was kinetically similar to the A_1-low affinity binding sites (not shown).

B2C114 antigenic expression was also tested as a histochemical marker of malignancy. It is well known that normal tissues, such as the proximal (but not distal) colon in secretors, expresses the A-antigen. In tumors, there is a net loss of A-antigen expression in the proximal and a net gain in the distal colon.[3] We observed an inappropriate blood group expression in a colonic carcinoma detected with B2C114 (Figure 2A). Bladder tumors are known to originate in the transitional epithelium where the presence of ABO group antigens has been confirmed.[11] Accordingly, a histological section of a

bladder carcinoma showed blood group-A positivity on the transitional epithelial cells and on the underlying endothelium, while the connective tissue of the papilla was negative (Figure 2B). The reacting epitope is also expressed by the HT29 human tumor cell line (Figure 3). The reactivity of mAb B2C114 on HT29 cells was also tested in the presence of several reagents (Table 3). The inclusion of 2% (w/v) of GalNAc, almost completely abolished the binding of B2C114, while the addition of Gal had no effect. Reactivity was also unaffected by trypsin treatment but was inhibited in a dose-dependent way by sodium periodate. Assays with lectins showed that PHA behaved as a competitive inhibitor.

Discussion

The mAb B2C114 obtained from mice immunized with CEA presents immunological reactivity with human biopsies obtained from blood group A patients and, when further tested, with human A-RBC. This mAb does not show any false positive results. This was confirmed in a large number of blood group B and O cell donors by means of agglutination tests and an even far more sensitive test like radiobinding assays. Specific recognition of the A-antigen was further demonstrated through the inhibition of the B2C114/A-RBC agglutinating system by the saliva of A-secretors, while no inhibition was observed with the saliva of O-, B-secretors or with the saliva of non-secretors. The anti-A reagent has a high avidity as well as enough potency to give macroscopic reactions not only with A_1 and A_2 but also with weaker A_2B and A-cord blood at different agglutination conditions. B2C114 can be used for subtyping because it reacts more strongly with A_1 than A_2 cells. Since no activity was recorded with papainized group B cells, the mAb seems to be free from B(A) reactivity (i.e. the detection of A sites on blood group B cells). The optimum reactivity of the mAb occurred around neutral pH, suggesting that acid or alkaline conditions may alter the antigenic structure.

It has been proved that high functional affinity mAb are required for strong agglutination of RBC. Low affinity leads to weak agglutination with rapid dissociation of the antibody-antigen
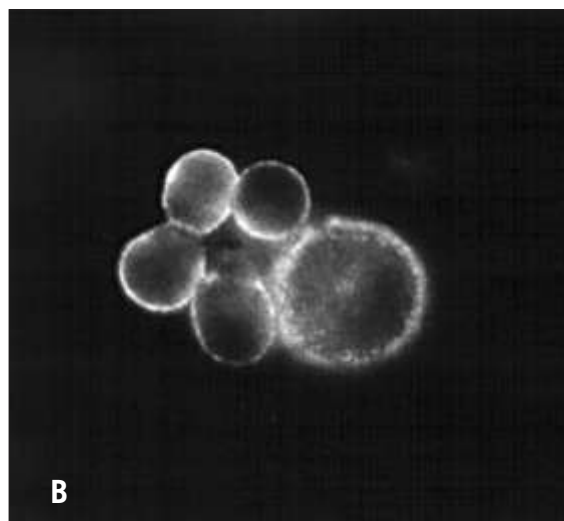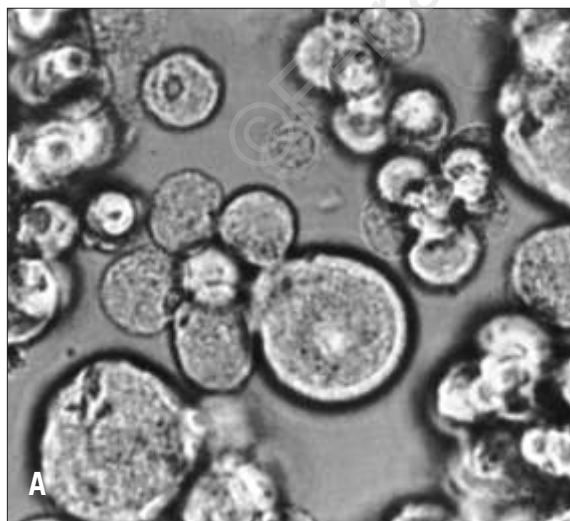

Figure 3. Fluorescence microscopy analizes of the B2C114 molecule expressed by HT29 cells in suspension. IIF tests were performed on 1×10^6 cells in suspension using culture supernatant of the B2C114 monoclonal antibody. (A= phase contrast; B= immunofluorescence).

complex.[12] Most successful anti-A reagents are of the IgM isotype.[1] Although they are mainly of low affinity ($K_a < 10^7$ M^{-1}), factors such as the *multiple binding bonus* due to the pentavalent binding of IgM mAb to the cellular target, account for their application in blood grouping. The IgG_1-B2C114 mAb binding data, analyzed by a Scatchard plot, demonstrated the presence of A-sites with a high K_a (1×10^9 M^{-1}) and with a lower K_a (1×10^7 M^{-1}). It was quite interesting to note that B2C114 was able to discern at least two different A-epitopes expressed on the A_1-RBC surface, with a total number of A sites that were in close agreement with the figures already described for A_1-RBC.[13] Since it has been shown that the K_a is independent of the number of sites, we assume that A_1-sites are qualitatively different. Elution studies showing qualitative differences between A-sites on A_x cells to those on A_1 and A_2 cells give support to this conclusion.[14]

With regard to changes in ABH determinants in colon cancer, we found incompatible A-antigen expression in two of five specimens from group B or O patients. The well-known localization of CEA in the lumen-oriented surface of tumor cells and detection of B2C114 over the whole cell suggested non-compatible expression of the antigen with the blood group of the patients, probably due to the activity of an aberrant transferase. Preliminary biochemical analysis suggests that the epitope recognized by mAb B2C114 is carbohydrate in nature. Since B2C114 activity was partially inhibited by GalNAc, an oligosaccharide present in the characteristic structure of the blood group A determinant, it was concluded that the mAb reacts with a cross-reacting epitope shared by the A antigen and CEA.[15,16]

Finally, after confirming its excellent properties as a blood group reagent, studies are in progress to demonstrate further the utility of the mAb B2C114 as a histopathological probe in cancer diagnosis. It is also noteworthy that using B2C114 anti-CEA mAb, which cross-reacts with blood group A, for *in vivo* imaging of colorectal cancer in non A-blood group antigen-bearing patients offers the chance of having a *tumor specific* radioimmunodetection approach.[17]

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