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Human monoclonal antibodies specific for blood group antigens demonstrate multispecific properties characteristic of natural autoantibodies

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SUMMARY

A panel of 72 human monoclonal antibodies with specificities for blood group antigens, A, Rh D, Rh C, Rh c, Rh E, Rh e, Rh G, Jk^a and Jk^b, has been established from the peripheral blood of deliberately immunized donors. Previous work has established that the antibodies are highly specific for their respective blood group antigens, and a number of them are in routine clinical use as blood grouping reagents. This panel was screened for reactivity against six unrelated foreign and autoantigens by ELISA, for rheumatoid factor activity by ELISA and agglutination techniques, and for reactivity with a number of different tissues by immunofluorescence. Binding of the monoclonal antibodies to unrelated exo- and autoantigens was commonly seen amongst the antibodies of the IgM class, and to a lesser degree amongst the IgG class, with reaction patterns similar to those given by natural autoantibodies. Only five of the IgM antibodies failed to demonstrate any unexpected cross-reactivities and these included 1/13 anti-D, 2/7 anti-E, 1/13 anti-c and 1/2 anti-A. We propose that rather than natural autoantibodies representing a distinct population of immunoglobulins, multispecificity (polyspecificity, or polyreactivity) may be a feature of antibodies produced in response to exogenous antigens. The implications of this for the study of autoantibodies are discussed.

INTRODUCTION

The term 'natural antibody' was first used to describe 'those molecules present in the body fluids of normal animals having the capacity to combine specifically with potential antigens and being distinct to those produced in response to specific antigenic stimulus'.[1] Natural antibodies are usually multispecific and are able to recognize a number of different self and foreign antigens. They are often termed natural autoantibodies.[2] Natural autoantibodies reacting with tubulin, actin, myoglobin, thyroglobulin, fetuin, albumin and transferrin are present in normal human sera, and monoclonal immunoglobulins from multiple myeloma and Waldenström's macroglobulinaemia may express similar antibody specificities. Monoclonal antibodies particularly, but not exclusively of the multivalent IgM class, can often be shown to be multispecific,[3] and it is thought that these correspond to the natural autoantibodies present in the serum. It has been suggested that natural autoantibodies (multispecific, polyspecific or polyreactive antibodies) originate in humans from the $CD5^+$ subset of B cells which represent 10–25% of circulating and splenic B cells.[4] These multispecific antibodies are seen as a distinct population of antibodies from the monospecific antibodies produced in response to external antigens, having a possible role in the first line of defence against invading micro-organisms,[4] the elimination of dead tissues,[5] or being part of a primitive interconnecting set of B cells involved in setting up an idiotypic network early in life.[6] It has been suggested that these antibodies may give rise to high affinity, monospecific antibodies by the process of affinity maturation.[4,7,8]

Over the last few years many human monoclonal antibodies to blood group antigens have been produced for the development of reagents suitable for routine blood grouping.[9–11] These have been produced from the peripheral blood lymphocytes of donors deliberately immunized by mismatched red blood cells, and include examples of both IgG and IgM antibodies. In blood grouping practice antibodies of the IgM class are usually preferred as they are able to strongly agglutinate red blood cells directly in saline. To satisfy the requirements of a routine blood grouping reagent, absolute specificity and high avidity for the particular antigen is required. A number of human monoclonal antibodies are now in routine use, and one human monoclonal

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IgM anti-Rh D (MAD-2) has been in clinical use world-wide for a number of years as a routine grouping reagent for the Rh D antigen. Recently, out of six human monoclonal antibodies tested by immunofluorescence against a number of human and animal tissues, three were found to recognize antigens present in non-erythroid cells.[12] Included in this group of antibodies was MAD-2 which was found to recognize the strucurally unrelated intermediate filament protein, vimentin.[13] This apparent paradox of high specificity and multispecificity was further investigated using a large panel of human monoclonal antibodies in tests against a number of foreign and autoantigens.

MATERIALS AND METHODS

Human monoclonal antibodies

Heterohybridomas secreting human monoclonal antibodies against human erythrocyte blood group antigens were produced from immunized donors as described previously.[14] The blood group specificities of these monoclonal antibodies (mAb) are shown in Tables 1 and 2. The panel consisted of 72 mAb derived from 34 different donors, and included specificities against the A, Rh C, Rh D, Rh E, Rh c, Rh e, Rh G, Jk^a and Jk^b blood group antigens. Forty-four of these were of the IgM class and 28 were of the IgG class.

Immunofluorescence microscopy

Indirect immunofluorescence microscopy of acetone-fixed cryostat sections of animal tissues (rabbit stomach, heart and kidney, rat cerebellum and forebrain) using culture supernatants containing human monoclonal antibodies in conjunction with fluorescein isothiocyanate (FITC)-labelled anti-human IgM or IgG (Sigma Chemical Co., Poole, Dorset, U.K.) was carried out as described previously.[12] In addition, a rat glioma cell line (C6) was grown on glass coverslips until subconfluent and then fixed with 4% w/v formaldehyde and 0·2% v/v Triton X-100, for 5 min. Immunofluorescence was then carried out as for the tissue sections.

Absorption of anti-blood group mAb with erythrocytes

A number of human anti-blood group mAb (HAM-2, MAD-2, NELP-4, NELP-6, MS-12, MS-14, MS-7 and MS-8) were retested for tissue reactivity after absorption with erythrocytes positive for and negative for the blood group antigen they recognize. In each case, one volume of culture supernatant containing the mAb was mixed with 2 volumes of washed, packed red cells and incubated for 45 min at 37°. The cells were sedimented by centrifugation and the supernatants tested by immunofluorescence of tissue sections.

ELISA for reactivity with DNA, thyroglobulin, tetanus toxoid, DNP, hen egg lysozyme and haemoglobin

ELISA kits for detecting antibodies to DNA and human thyroglobulin were obtained from Bioscot Ltd (Edinburgh, U.K.). Calf thymus DNA, porcine thyroglobulin, bovine haemoglobin, hen egg lysozyme and DNP-human serum albumin were obtained from Sigma Chemical Co. Tetanus toxoid was the generous gift of Dr M. Corbell (NIBSC, Potters Bar, Herts, U.K.). Ninety-six-well flat-bottom microtitre trays (Greiner, Germany) were coated with 100 μl of phosphate-buffered saline (PBS) containing antigen at 10 μg/ml, with an overnight incubation at 4°. Before use the antigen-coated trays, together with uncoated trays, were blocked with 150 μl of PBS/0·5% Tween containing 5% foetal calf serum (FCS), with an incubation of 15 min at 37°. After three washes with PBS/Tween, 100 μl of culture supernatant containing the human monoclonal antibody was added to both the coated and uncoated trays, in duplicate, and the trays incubated for 1 hr at 37°. The trays were then washed three times with PBS/Tween, and either goat anti-human IgM (μ chain specific) or goat anti-human IgG (whole molecule) conjugated to alkaline phosphatase (Sigma) was added (1/1000 in PBS/Tween; 100 μl/well), and the trays incubated for 1 hr at 37°. The trays were then washed three times as before, and 100 μl of phosphatase substrate (Sigma 104) 1 mg/ml in 10% diethanolamine buffer (pH 9·6) was added. Following a 30-min incubation at 37°, the trays were read in a Dynatech ELISA reader at 405 nm. The mean difference in OD between the antigen-coated and uncoated wells was calculated for each monoclonal antibody to determine binding to antigen.

Rheumatoid factor assays

Rheumatoid factor (RF) activity of the human monoclonal antibodies was assessed by agglutination of human IgG-coated latex, rabbit IgG-coated sheep red blood cells (SRBC), and by ELISA. For the latex agglutination assay, 40 μl of culture supernatant was transferred to flat-bottomed microtitre trays and 5 μl of IgG-coated latex particles (Rapi Tex-RF, Boehring, Germany) added to each well and mixed. After 10 min of incubation at room temperature agglutination was assessed with the aid of a low power inverted microscope. A human monoclonal RF (RF-TS3) served as a positive control.

SRBC were coated with a subagglutinating dose of rabbit anti-SRBC antibodies (Gibco, Paisley, Renfrewshire, U.K.) by incubation at 37° for 30 min. The coated cells were then washed three times with PBS by centrifugation, and resuspended to a 1% v/v suspension in PBS. Twenty-five microlitre aliquots of culture supernatant were added to wells of V-bottomed microtitre trays, and equal volumes of coated SRBC added and mixed. The cells were allowed to settle at room temperature and agglutination assessed macroscopically. A human monoclonal RF (RF-SJ1) served as a positive control. Supernatants were tested in parallel with SRBC which had not been coated with rabbit IgG.

RF activity was also assessed using ELISA techniques. ELISA trays were coated with either human or rabbit IgG (Sigma), at a concentration of 20 μg/ml in PBS with an overnight incubation at 4°. For the IgM monoclonal anti-blood group antibodies, human IgG-coated trays were used together with goat anti-IgM alkaline phosphatase conjugate (Sigma). For the IgG monoclonal anti-blood group antibodies, rabbit IgG-coated trays were used together with rabbit anti-human IgG alkaline phosphatase conjugate (Dako, High Wycombe, Bucks, U.K.). The ELISA were carried out as described above.

Competitive inhibition ELISA

Tenfold dilution series of thyroglobulin and DNP/HSA were made in PBS/Tween, and 100 μl transferred to flat-bottom microtitre trays. To these were added 100 μl of culture supernatants, and the mixture incubated overnight at 4°. One

Table 1. The multispecificity of IgM blood group antibodies. IgM monoclonal anti-blood group antibodies were tested for reactivity against unrelated antigens by immunofluorescence, ELISA and agglutination assays. Reactivity against any of the tissue sections is indicated +, the number of antigens recognized by ELISA is shown, and positivity in any of the three assays for RF activity is indicated as +

Specificity	Clone	Class	Tissues	C6 cells	ELISA	RF
Anti-D	FOM-1	μλ	—	—	1	+
	FOM-A	μλ	+	—	—	—
	NELP-1	μλ	+	—	—	—
	NELP-2	μλ	+	—	—	—
	NELP-3	μλ	+	—	1	+
	NELP-4	μλ	+	—	—	—
	NELP-5	μλ	—	—	—	—
	NELP-6	μλ	+	+	—	—
	MAD-2	μλ	+	+	—	—
	HAM-B	μλ	—	—	2	+
	HAM-2	μλ	+	+	—	—
	HAM-1	μκ	+	+	3	+
	HAM-A	μκ	+	—	1	+
Anti-E	MS-12	μλ	+	+	2	+
	MS-5	μκ	—	—	—	—
	MS-6	μκ	—	—	1	—
	MS-10	μκ	+	+	2	—
	MS-11	μκ	—	+	—	—
	MS-14	μκ	+	—	—	—
	MS-27	μκ	—	—	—	—
Anti-e	MS-16	μλ	—	+	1	—
	MS-17	μκ	—	—	1	—
	MS-19	μκ	—	+	1	—
	MS-21	μκ	+	+	—	—
Anti-C	MS-24	μκ	+	—	—	—
Anti-c	MS-25	μκ	—	—	—	—
	MS-30	μλ	+	+	4	—
	MS-31	μλ	+	+	3	—
	MS-32	μλ	+	—	—	—
	MS-33	μλ	+	+	1	—
	MS-34	μλ	+	+	2	—
	MS-35	μλ	+	—	2	—
	MS-36	μλ	+	—	2	—
	MS-41	μλ	+	+	2	+
	MS-42	μλ	+	+	3	—
	MS-49	μλ	+	—	2	—
	MS-50	μλ	+	+	3	—
	MS-54	μλ	+	+	2	—
Anti-Jk[a]	MS-15	μκ	+	+	3	+
Anti-Jk[b]	MS-8	μλ	+	+	4	+
	MS-7	μκ	+	+	—	—
	MS-9	μκ	+	+	3	+
Anti-A	A-1	μλ	—	—	—	—
	A-2	μλ	—	—	—	+

Table 2. The multispecificity of IgG blood group antibodies. IgG monoclonal anti-blood group antibodies were tested for reactivity against unrelated antigens by immunofluorescence, ELISA and agglutination assays. Reactivity against any of the tissue sections is indicated +, the number of antigens recognized by ELISA is shown, and positivity in any of the three assays for RF activity is indicated as +

			Other reactivities			
Specificity	Clone	Class	Tissues	C6 cells	ELISA	RF
Anti-D	PAG-1	γ1λ	—	—	—	—
	FOG-B	γ1λ	—	—	—	—
	GAD-2	γ3λ	—	—	1	+
	MS-3	γ1κ	—	—	—	—
	BAG-1	γ1κ	—	—	—	—
	KEG-1	γ1κ	—	—	—	—
	GED-1	γ1κ	—	—	—	—
	FOG-1	γ1κ	+	—	—	+
	MS-26	γ1κ	+	—	1	—
	REG-A	γ1κ	—	—	—	—
	MS-2	γ3κ	—	—	—	—
	FOG-3	γ3κ	—	—	—	—
	FOG-A	γ3κ	—	—	—	—
Anti-G	MS-1	γ3λ	—	—	—	+
Anti-C	MS-4	γ1κ	—	—	—	+
	MS-23	γ1κ	—	—	—	+
Anti-e	MS-22	γ1κ	+	—	—	—
Anti-c	MS-45	γ1λ	—	—	—	—
	MS-43	γ3λ	+	—	1	+
	MS-44	γ3λ	—	—	—	—
	MS-46	γ3λ	—	—	1	—
	MS-47	γ3λ	—	—	—	+
	MS-51	γ3λ	—	—	—	+
	MS-40	γ1κ	—	—	—	—
	MS-52	γ1κ	+	+	3	—
	MS-28	γ3κ	+	—	3	—
	MS-37	γ3κ	—	—	—	—
	MS-48	γ3κ	—	—	—	—

hundred microlitres of the mixture was then transferred in duplicate to microtitre trays precoated with the same antigen used for the inhibition. The ELISA were then continued as above.

Inhibition of haemagglutination assays

Human monoclonal anti-blood group antibodies were diluted to give a 2 × minimum haemagglutinating dose (MHD) against human RBC of the appropriate phenotype. A dilution series of inhibiting antigen was then made in PBS/1%BSA, and 25 μl of each dilution transferred in duplicate to 96-well V-bottom microtitre trays (Greiner). To these were added 25 μl of human mAb, such that each well received 2 MHD of antibody. The trays were then mixed and incubated overnight at 4°. Twenty-five microlitres of a 2% suspension of RBC (in PBS) of appropriate phenotype was then added to each well, and after mixing the trays were centrifuged at 100 ***g*** for 20 seconds. Agglutination was assessed macroscopically.

RESULTS

The overall frequency of multispecificity

Of the 44 IgM anti-blood group antibodies, 37 (84%) reacted with other antigens in addition to a blood group antigen. Of the 28 IgG anti-blood group antibodies, 13 (46%) reacted with antigens in addition to a blood group antigen. The overall multispecificity of the monoclonal antibodies is summarized in Tables 1 and 2.

Immunofluorescence of tissue sections

Of the 44 IgM mAb with anti-blood group specificity 26 (59%) reacted with non-erythroid tissue components, and usually with more than one cell type. These are summarized in Table 3 and some examples of the staining are shown in Fig. 1. The components most commonly recognized were smooth muscle, the ependymal cells lining the ventricles of the brain and

Table 3. Reactivities of blood group mAb against fixed tissues. Anti-blood group antibodies were tested for reactivity with fixed tissues by immunofluorescence (see Materials and Methods). The tissue/cell types stained are shown by +

	Tissues/cells												
Clone	A	B	C	D	E	F	G	H	I	J	K	L	M
IgM													
Fom-A	+	—	—	—	—	—	—	—	—	—	—	—	—
NELP-1	—	—	—	—	+	—	—	—	—	—	—	—	—
NELP-2	+	+	—	—	—	—	—	—	—	—	—	—	—
NELP-3	+	+	—	—	—	—	—	+	—	—	—	—	—
NELP-4	+	+	+	—	+	—	+	—	—	+	—	—	—
NELP-6	+	+	+	+	+	—	+	+	—	+	—	+	—
MAD-2	+	+	+	—	+	+	—	—	—	+	—	—	—
HAM-2	+	+	+	—	—	—	—	—	+	—	—	—	—
HAM-1	+	+	+	—	+	+	—	—	+	+	—	—	—
HAM-A	+	—	+	—	—	—	—	—	—	—	—	—	—
MS-12	—	—	+	+	—	+	+	—	—	—	+	—	—
MS-10	+	+	+	—	—	—	+	—	+	—	—	—	—
MS-14	—	+	—	—	—	—	+	—	+	—	—	+	—
MS-16	—	—	—	+	—	—	—	—	—	—	—	—	—
MS-21	+	+	+	—	+	—	—	—	—	—	—	—	—
MS-24	+	+	+	—	+	—	—	—	—	—	—	—	—
MS-30	+	+	+	+	+	+	—	+	—	+	—	—	—
MS-31	+	+	+	+	+	+	—	—	—	+	—	—	—
MS-32	—	—	—	+	—	—	—	—	—	—	—	—	—
MS-33	—	—	—	+	—	—	—	—	—	—	—	—	—
MS-34	+	+	+	+	+	+	—	+	—	+	—	—	—
MS-35	—	—	—	+	+	+	—	—	—	—	—	—	—
MS-36	—	—	—	+	—	—	—	—	+	—	—	—	+
MS-41	+	+	+	+	+	+	—	+	—	+	—	—	—
MS-42	+	+	+	+	+	+	—	—	—	—	—	—	—
MS-49	—	—	—	+	—	—	—	—	—	—	—	—	—
MS-50	+	+	+	+	+	+	—	—	—	—	—	—	—
MS-54	—	—	—	+	—	—	—	—	—	—	—	—	—
MS-15	+	+	+	+	+	—	+	—	—	+	—	—	—
MS-8	+	+	+	+	+	+	+	+	+	—	—	—	—
MS-7	+	+	+	—	+	—	—	—	—	—	—	—	—
MS-9	+	+	+	+	+	+	+	+	+	+	—	—	—
IgG													
FOG-1	+	—	+	—	—	—	—	—	—	—	—	—	—
MS-26	+	+	+	—	—	—	—	—	—	—	—	—	—
MS-22	+	—	+	—	—	—	—	—	—	—	—	—	—
MS-43	+	—	—	—	—	—	—	—	—	—	—	—	—
MS-52	+	+	+	—	—	—	—	—	—	—	—	—	—
MS-28	—	—	+	—	—	—	—	—	—	—	—	—	—

A, smooth muscle; B, ependymal cells; C, astroglia; D, rabbit RBC; E, fibroblasts; F, endothelial cells; G, kidney tubule ducts; H, cardiac muscle; I, gastric epithelium; J, glomeruli; K, connective tissue; L, nuclei; M, Purkinje cells.

astroglia which were identified by 85, 81 and 77% of the tissue-reactive mAb respectively.

The proportions of the mAb of the different blood group specificities that reacted with tissue components were as follows; 10/13 (77%) of anti-Rh D; 3/7 (43%) of anti-Rh E; 2/4 (50%) of anti-Rh e; 12/13 (92%) of anti-Rh c; 3/3 (100%) of anti-Jk^b; 1/1 (100%) of anti-Jk^a, 1/1 (100%) of anti-Rh C; 0/2 (0%) of anti-A. None of the tissue-reactive mAb shared identical immunofluorescence profiles.

To confirm that the tissue reactivities were associated with the red cell binding fraction of the culture supernatants, some mAb were retested by immunofluorescence following absorption with erythrocytes. Tissue staining was unaffected by absorption with erythrocytes lacking the blood group antigen they recognize, but was abolished following absorption with erythrocytes expressing the appropriate blood group (Fig. 2).

Fifty per cent (22/44) of the IgM anti-blood group mAb reacted with fixed monolayers of C6 cells (Tables 1 and 2). The structures identified appeared to be intracellular and included intermediate (vimentin) filaments, stress fibres, mitochondria

Table 4. Reactivity of blood group antibodies in ELISA. Monoclonal anti-blood group antibodies were tested for reactivity with six unrelated antigens by ELISA. The absorbance was determined as OD of mAb versus Ag-coated well, minus OD of the same mAb versus uncoated well

	Reactivity by ELISA with:					
Clone	DNA	TG	TT	Hb	DNP	HEL
IgM						
FOM-1	—	—	—	—	++	—
NELP-3	++	—	—	—	—	—
HAM-B	+	—	—	—	++	—
HAM-1	+++	+	+	—	—	—
HAM-A	—	—	—	—	++	—
MS-6	—	—	—	—	++	—
MS-10	+	—	—	—	+	—
MS-12	—	—	—	+	—	+
MS-16	—	—	—	+	+	—
MS-17	—	—	—	++	—	—
MS-19	—	—	—	+	—	—
MS-30	+	++	+++	—	+++	—
MS-31	—	++	+	—	++	—
MS-33	—	++	—	—	—	—
MS-34	—	+	++	—	++	—
MS-35	—	++	+	—	—	—
MS-36	+	++	—	—	—	—
MS-41	+	++	—	—	—	—
MS-42	+	++	++	—	—	—
MS-49	+	+	—	—	—	—
MS-50	+	++	—	—	+	—
MS-54	+	+	—	—	—	—
MS-15	+++	+	—	—	+	—
MS-8	+++	+++	+++	—	+++	—
MS-9	+++	+++	++	—	—	—
IgG						
GAD-2	—	—	—	—	+++	—
MS-28	+	—	+	—	+	—
MS-43	—	—	—	—	+	—
MS-46	—	—	—	—	++	—
MS-26	—	—	—	—	++	—
MS-52	++	—	++	—	+++	—

0·1–0·2 OD (+); 0·2–0·5 OD (++); 0·5–1·5 OD (+++).
TG, thyroglobulin; TT, tetanus toxoid; Hb, haemoglobin; DNP, dinitrophenol; HEL, hen egg lysozyme.

and cytoplasmic vesicles/granules (Fig. 3). At least eight mAb appeared to recognize more than one cellular component.

Six out of 28 (21%) of the IgG mAb reacted with tissue components in the tissue panel studied (Table 3). These mAb showed a similar distribution of staining, identifying smooth muscle, ependymal cells and a proportion of astrocytes and Bergman glia.

ELISA for reactivity with DNA, TT, Tg, DNP, HEL, Hb

Fifty-seven per cent (25/44) of the IgM mAb and 6/28 (21%) of the IgG mAb reacted with one or more of the six unrelated antigens tested by ELISA (Table 4). The majority of the IgM mAb (19/25) reacted with more than one antigen, while only 2/28 of the IgG mAb did so. The most frequently reactive antigens were DNP, thyroglobulin and DNA. The reactivities of three mAb against thyroglobulin and DNP are presented as titrations of antibody concentration *vs* OD (Fig. 4). All mAb which were reactive with human thyroglobulin were also found to bind porcine thyroglobulin. The titrations and inhibition studies were carried out using porcine thyroglobulin.

Rheumatoid factor assays

Twenty-five per cent (11/44) of the IgM mAb, and 8/26 (31%) of the IgG mAb demonstrated RF-like activity by at least one of the three assays used. Only two mAb (MS-1 and MS-4) were judged positive by more than one assay (Table 5). The majority of the IgM mAb (9/11) were judged positive in agglutination assays either of human IgG-coated latex, or of rabbit IgG-sensitized SRBC. The majority of the IgG mAb (7/8) were judged positive by ELISA using rabbit IgG as antigen.

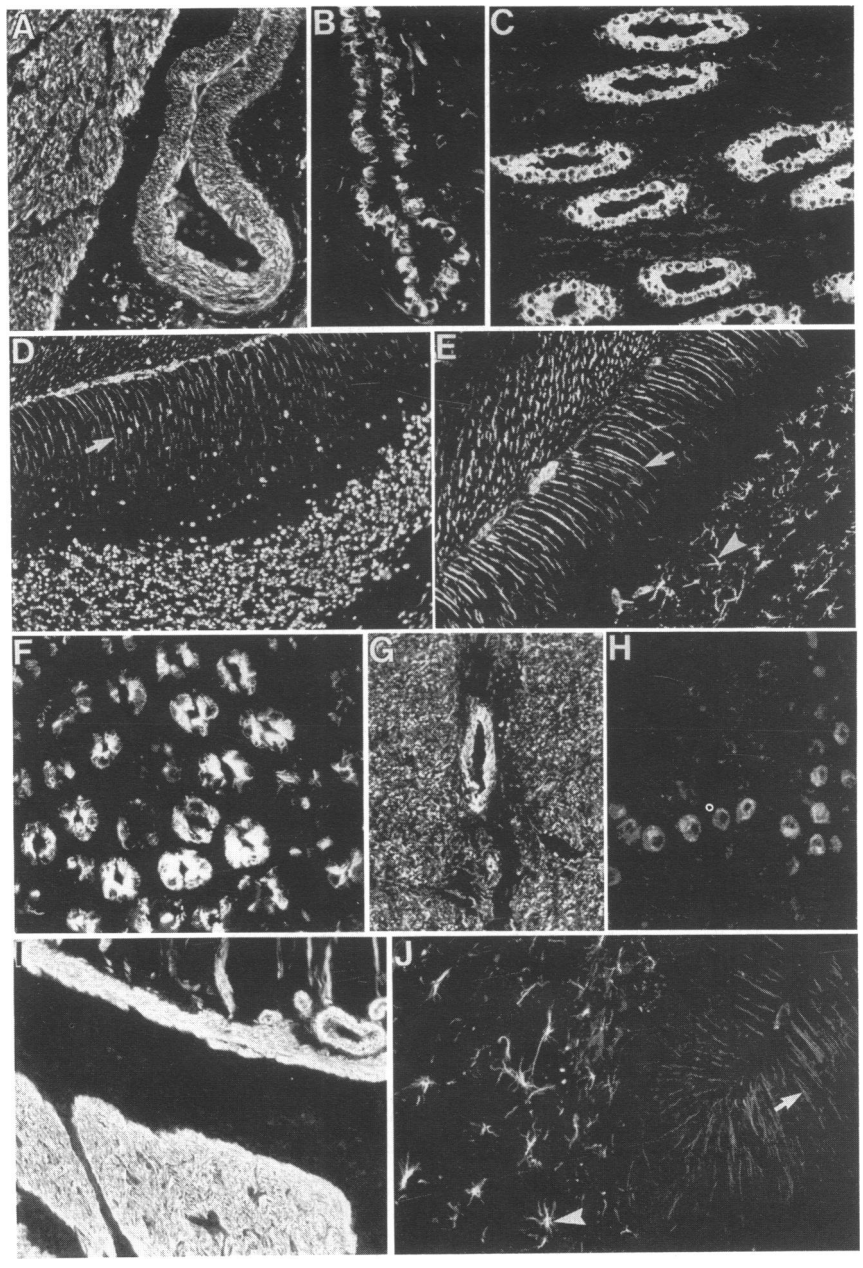

Figure 1. Examples of immunofluorescence of cryostat sections of rabbit (A, C, F, G, I) and rat (B, D, E, H, J) tissues using anti-blood group mAb of IgM (A–H) and IgG (I, J) class. Tissue components stained are as follows: (A) vascular and enteric smooth muscle using MS-34 (anti-Rh c); (B) ependymal cells of brain using HAM-1 (anti-Rh D); (C) collecting ducts of kidney using MS-12 (anti-Rh E); (D) cell nuclei and Bergman glia (arrow) in cerebellum using NELP-6 (anti-Rh D); (E) Bergman glia (arrow) and astrocyte processes (arrowhead) using MS-30 (anti-Rh c); (F) cells in the gastric epithelium using MS-10 (anti-Rh E); (G) cardiac muscle and vascular smooth muscle using MS-9 (anti-Jk^b); (H) Purkinje cells using MS-36 (anti-Rh c); (I) vascular and enteric smooth muscle using FOG-1 (anti-Rh D); (J) Bergman glia (arrow) and astocyte processes (arrowhead) using MS-26 (anti-Rh D).

Competitive inhibition ELISA

Competitive inhibition assays were carried out for a number of mAb to establish whether the reactivities with the unrelated antigens thyroglobulin and DNP in ELISA could be inhibited by free antigen. The binding of mAb to solid phase thyroglobulin was in each case inhibited by free thyroglobulin in a dose-dependent fashion. Similary, the binding of mAb to solid phase DNP/HSA could be inhibited by free DNP/HSA in a dose-dependent fashion. These inhibitions are presented in Fig. 5.

Inhibition of haemagglutination assays

All 17 mAb which reacted in ELISA with DNP/HSA were tested to see if DNP/HSA could inhibit specific haemagglutination of human RBC by the mAb. No inhibition was seen of any of the

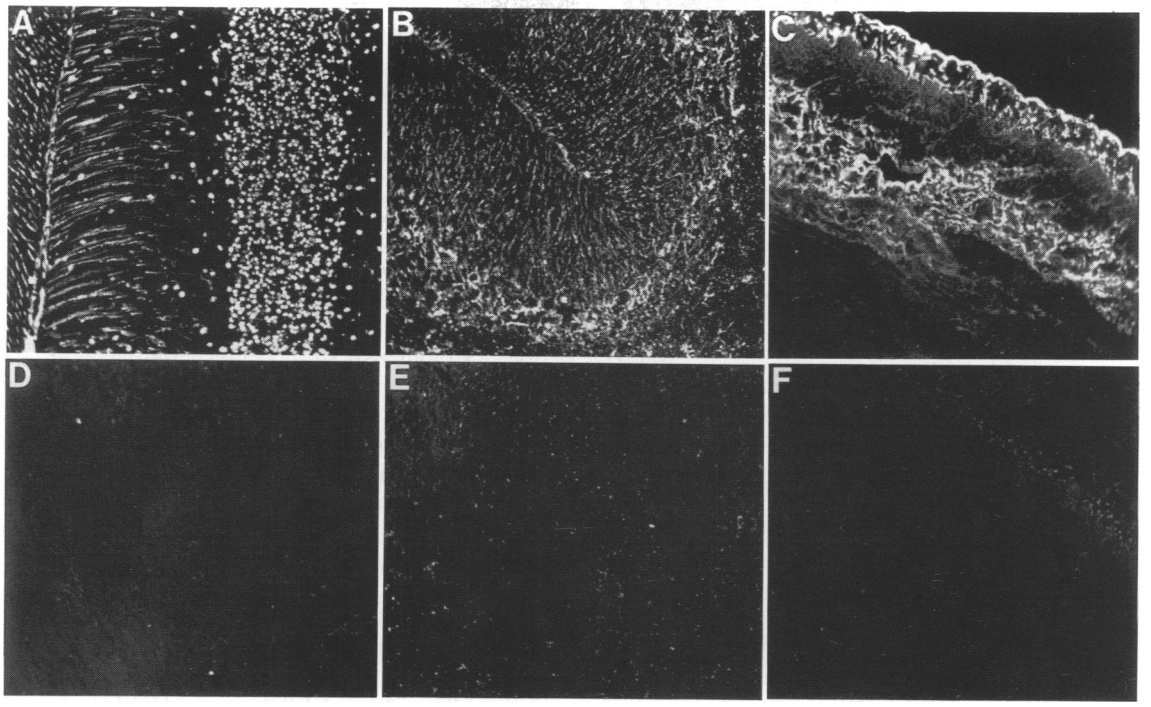

Figure 2. Immunofluorescence of cryostat sections of rat cerebellum (A, B, D, E) and rabbit stomach (C, F) using the IgM anti-blood group mAb NELP-6 (anti-Rh D; A, D), MS-8 (anti-Jk^b; B, E) and MS-12 (anti-Rh E; C, F) absorbed with erythrocytes lacking (A–C) or expressing (D–F) the blood group antigen they recognize.

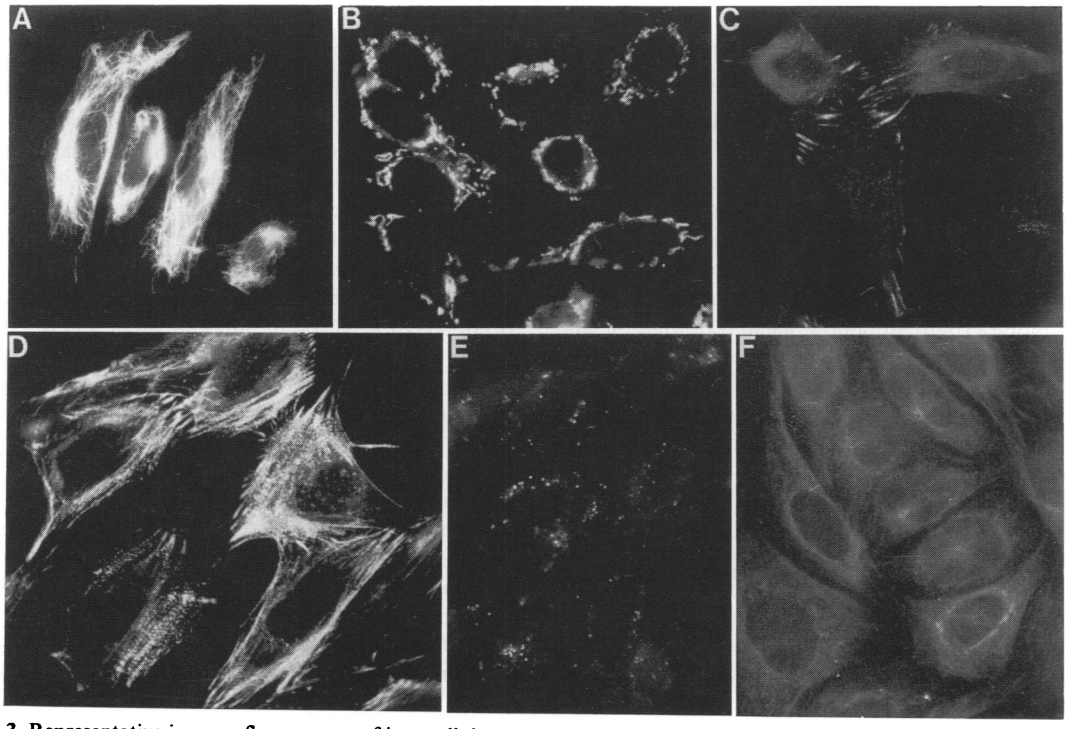

Figure 3. Representative immunofluorescence of intracellular components of C6 monolayers using IgM anti-blood group mAb. (A) Intermediate filament staining using MAD-2 (anti-Rh D); (B) staining of mitochondria using MS-10 (anti-Rh E); (C) staining of microspikes using HAM-1 (anti-Rh D); (D) stress fibre staining using MS-9 (anti-Jk^b); (E) staining of secretory vesicles using MS-19 (anti-Rh e); (F) cytoplasmic staining using MS-8 (anti-Jk^b).

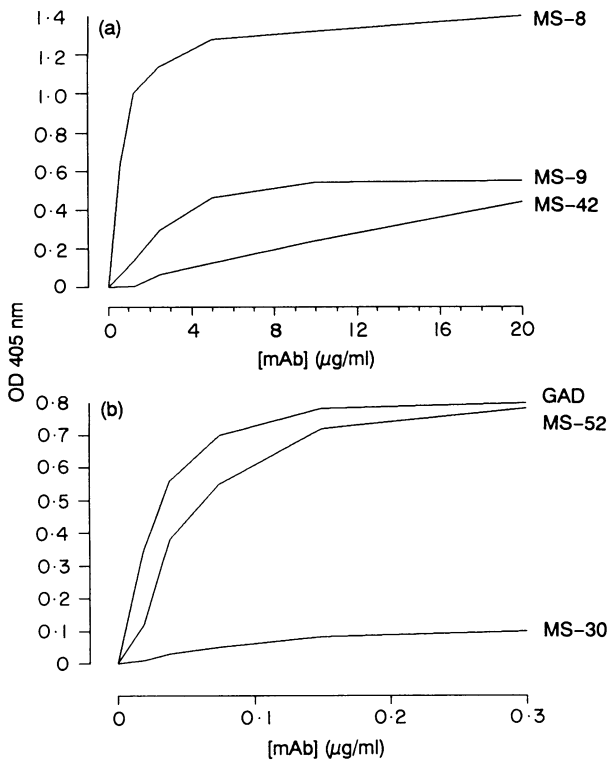

Figure 4. Human monoclonal antibodies were titrated against various antigens coated on ELISA trays. Examples of titration curves are shown against thyroglobulin [(a) MS-8 = IgM anti-Jk^b; MS-9 = IgM anti-Jk^b; MS-42 = IgM anti-c] and against DNP/HSA [(b) GAD = IgG anti-D; MS-52 = IgG anti-c; MS-30 = IgM anti-c].

mAb even with a concentration of DNP/HSA at 60 μg/ml. This represented a molar ratio of DNP to mAb of between 10^4:1 and 10^6:1 for the different antibodies. Similarly, thyroglobulin was unable to inhibit haemagglutination by those mAb capable of binding thyroglobulin, even at concentrations of 10 mg/ml.

The assay for inhibition of haemagglutination has been shown previously to be highly sensitive.[15] Using a rat monoclonal antibody directed to an epitope on the variable region of many of these blood group antibodies, haemagglutination can be completely inhibited with concentrations of rat antibody in the range of 0·01–8·75 μg/ml. This represents a molar ratio of inhibiting rat mAb to blood group mAb of approximately 1:1.

DISCUSSION

The concept that natural autoantibodies represent a distinct population of antibodies from those produced in response to a specific antigenic stimulus has been pervasive since the definition of the term.[1] Natural autoantibodies have been demonstrated in normal human serum,[16] and have been isolated in monoclonal form from non-immunized adult mice,[7,17] antigen-free mice,[18] newborn mice,[19] adult humans[4,20] and human foetal liver and cord blood.[21] Characteristically they are predominantly, but not exclusively of the IgM class, and have the capacity to bind a number of unrelated antigens, especially those with highly repeating structures such as DNA, LPS and cytoskeletal proteins. Although the intrinsic affinity may be low, where the antigen is densely packed or highly repeating, the functional affinity can be high.[22,23]

The results presented here clearly demonstrate that a considerable proportion of antibodies produced in response to exogenous antigens can show multispecific properties identical to natural autoantibodies. Eighty-four per cent of the panel of human IgM mAb against blood group red cell antigens studied show striking cross-reactions with a variety of antigens commonly recognized by natural autoantibodies. The IgG anti-blood group mAb showed more restricted and less frequent cross-reactions (46% of all IgG mAb).

In view of the diverse nature of the antigens recognized, and the lack of an association between anti-blood group specificity and distribution of antigens recognized, we conclude that these cross-reactions are due to the ability of certain human mAb to react with components structurally unrelated to the blood group antigen they recognize. Demonstration of multispecificity clearly depends on the concentration of mAb used and the number of antigens used to screen against. We have used fairly high concentrations of mAb (10–90 μg/ml), and the inclusion of fixed tissues provides a large number of antigens. Many of the mAb have low titres against the antigens tested, and would not have been identified as positive at concentrations below 10 μg/ml. Some however, have very high titres, e.g. GAD and MS-52 were still positive in ELISA against DNP/HSA at a concentration of 10 ng/ml.

Although it is unlikely that the cross-reactions seen here were due to structural homologies of the antigens, it is possible that the nature of the blood group antigens favours the production of antibodies with multispecific properties. There is also a possibility that the monoclonal antibodies we have isolated are not typical of the plasma anti-blood group antibodies. However, the anti-D antibodies studied in detail seem to correspond to polyclonal anti-D in terms of subclass distribution, affinity, specificity and functional activity.[24]

It might be argued that we have isolated a number of multispecific antibodies which fortuitously have blood group specificities. However, we have screened many tens of thousands of hybrids without ever finding an antibody recognizing a blood group antigen which the particular donor was not specifically immunized against. The blood group antibodies are therefore produced in response to immunization, and the occurrence of natural antibodies with Rh and Kidd blood group specificites in the donor population studied appears absent. Anti-blood group antibodies of the Rh and Kidd specificies are extremely rarely found in the plasma of individuals unless they have been exposed to the antigens by mismatched transfusions or through pregnancy.[25]

If it is accepted that the antibodies are produced in response to immunization, it is still possible that they may originate from the multispecific pool of B cells, and are undergoing a transition from multispecificity to monospecificity by the processes of somatic mutation and affinity maturation.[4,7,8] However, even the IgM mAb demonstrate exquisite specificities for their respective blood group antigens. They recognize a single blood group antigen out of over 600 described antigens on the human red blood cell[26] and are capable of making extremely fine allotypic distinctions. This is very difficult to reconcile with them being broadly reactive antibodies needing specificity refinement by somatic mutation. Furthermore, multispecificity is seen

Table 5. RF-like activity of monoclonal blood group antibodies. Human monoclonal anti-blood group antibodies were tested for RF-like activity by ELISA (using human IgG as antigen for the IgM antibodies and rabbit IgG as antigen for the IgG antibodies), agglutination of human IgG-coated latex particles and agglutination of rabbit IgG-sensitized SRBC. Reactivities in the ELISA are shown as the difference in OD between monoclonal antibody against antigen-coated trays minus the OD against uncoated (blocked) trays. A positive human monoclonal RF (RFAN) gave an OD in these conditions of 0·59

			Rheumatoid factor assay		
Specificity	Clone	Class	ELISA	Latex agglutination	IgG-SRBC
Anti-D	FOM 1	$\mu\lambda$	—	+	—
	HAM B	$\mu\lambda$	—	—	+
	HAM 1	$\mu\kappa$	—	+	—
	HAM A	$\mu\kappa$	—	+	—
	NELP 3	$\mu\lambda$	—	—	—
	GAD 2	$\gamma 3\lambda$	0·13	—	—
	FOG 1	$\gamma 1\kappa$	—	—	+
Anti-E	MS-12	$\mu\lambda$	—	+	—
Anti-C	MS-4	$\gamma 1\kappa$	0·12	—	+
	MS-23	$\gamma 1\kappa$	0·18	—	—
Anti-G	MS-1	$\gamma 3\lambda$	0·24	—	+
Anti-c	MS-41	$\mu\lambda$	—	—	+
	MS-43	$\gamma 3\lambda$	0·15	—	—
	MS-47	$\gamma 3\lambda$	0·12	—	—
	MS-51	$\gamma 3\lambda$	0·15	—	—
Anti-Jk^a	MS-15	$\mu\kappa$	—	+	—
Anti-Jk^b	MS-8	$\mu\lambda$	0·28	—	—
	MS-9	$\mu\kappa$	—	+	—
Anti-A	A-2	$\mu\lambda$	—	—	+

amongst the IgG antibodies, and the highest affinity IgG anti-Rh D[27] (Fog-1) reacts with smooth muscle and astroglia.

It has been suggested that multispecificity may be related to particular V_H gene usage.[28] Although the anti-blood group antibodies studied here demonstrate a strong bias in V_H gene usage (with the V_H 4-21 gene segment being particularly over represented),[15] multispecificity is expressed by blood group antibodies using a variety of different V-region gene families (data not shown). Similar findings have been reported for multispecific natural autoantibodies.[17,29]

The idea that natural autoantibodies represent a distinct population from those involved in the immune response to exogenous antigens relies on the studies which have demonstrated such antibodies in the repertoire of antigen-free mice, and neonatal and foetal animals and humans.[18,19,21] The present study indicates that multispecificity may also be a feature of antibodies produced in response to exogenous antigens. If multispecificity is found associated with antibodies from both the immune and pre-immune repertoires, it is simpler to explain this property as a feature of immunoglobulins, as previously suggested.[3,23,30] None of the 'irrelevant' antigens tested were capable of inhibiting agglutination of RBC by the mAb. This indicates that the interactions with the irrelevant antigens may occur outside the blood group antigen-binding sites, or with very low affinities.

There has been much interest over the last few years in using monoclonal antibody technology for studying the autoantibody response in autoimmune diseases. A number of autoantigens which are the targets of pathological autoimmune responses are also frequently recognized by multispecific antibodies (e.g. thyroglobulin, DNA, IgG). We have shown that monoclonal antibodies which are the product of immune responses to exogenous antigens can share these specificities. This poses some problems in determining whether monoclonal antibodies derived from autoimmune patients are typical of the disease, represent natural autoantibodies or are multispecific antibodies directed to exogenous antigens. We can see the possibility of three classes of 'autoantibodies'.

(1) Antibodies which are apparently monospecific for the autoantigen in question.

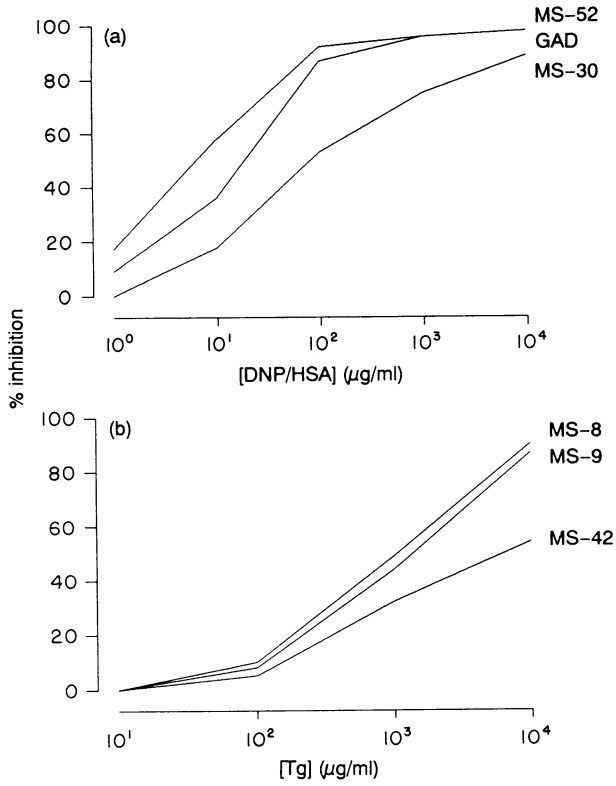

Figure 5. Competitive inhibition studies were performed to examine whether the binding of human monoclonal antibodies to thyroglobulin and DNP/HSA could be inhibited by free thyroglobulin and DNP/HSA respectively. Antibodies were incubated with varying concentrations of inhibiting antigen before being allowed to bind to solid phase antigen. The class and specificities of the examples of antibodies shown are indicated in Fig.4.

(2) Antibodies which show multispecific properties, but whose 'primary' antigen is the autoantigen.

(3) Antibodies which show multispecific properties, and whose autoreactivity is unrelated to the 'primary' antigen (e.g the blood group antibodies).

One would clearly have most confidence in studying autoantibodies of the first category, but as multispecificity may be a function of the number of antigens tested and the concentration of mAb, these may be rather few. Distinguishing whether a mAb selected on the basis of an autoreactivity belongs to the second (relevant) or third (irrelevant) categories is difficult. Studies of affinity and cross-inhibitions with other antigens the mAb binds may provide useful indications.

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