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Monoclonal Anti-Human Aldolase C Antibodies That React to the Isozyme Group-Specific Sequences and Generally Conserved Sequences of Human Aldolase C[1]

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Nine monoclonal mouse anti-human aldolase C antibodies, mAbs A4, A8, B4, B7, B8, C1, D9, E10, and H1, were isolated and characterized. These mAbs fall substantially into four groups according to their reactivity with antigens. (i) Human aldolase C-specific mAbs (B8, D9, and H1). (ii) Type C aldolase-specific mAbs (B4 and E10). (iii) Ubiquitous mAbs, which react with vertebrate aldolases irrespective of type of isozyme and species (A4 and B7). (iv) Sub-ubiquitous mAbs, which are closely similar to the ubiquitous mAbs but differ slightly in terms of antigenic specificity (A8 and C1). Aldolase C-specific mAbs B8, H1, B4, and E10, but not D9, have their epitopes on a region within amino acid positions 79-193 of antigens, where the type-C isozyme group-specific sequence-3 (IGS-3) is situated. In contrast, ubiquitous mAbs A4 and B7 and sub-ubiquitous mAb A8 may have their epitopes on the commonly conserved regions of the three isozyme groups. The epitope of sub-ubiquitous mAb C1 appears to be on the IGS-2/3 but this is yet to be resolved. These nine mAbs can be classified into two groups based on the mode of epitope recognition, which was determined by ELISA, immunoblotting, and immunoprecipitation assays: (i) primary sequence-epitope mAbs such as B4, E10, and B7; and (ii) conformation-epitope mAbs (B8, D9, H1, A4, A8, and C1). Among these mAbs, aldolase C-specific mAbs H1 and E10 appear to be useful as probes for detection of conformational change around the type-C IGS-3 motif of human aldolase C because, when assessed by immunoprecipitation assay, mAb H1 reacts only with human aldolase C but not with CA250 and CA306, while mAb E10 reacts with CA250 and CA306 but not with aldolase C, even though these antigens have a common type-C IGS-3 motif. Similarly, the ubiquitous mAb B7 should serve as a probe for general use to detect vertebrate aldolases irrespective of isozyme groups and species.

Key words: aldolase, ELISA, epitope mapping, immunochemistry, isozyme.

Fructose bisphosphate aldolase [EC 4.1.2.13], a glycolytic enzyme, has three isozymic forms in vertebrates: A (muscle-type), B (liver-type), and C (brain-type) (*1, 2*). These three isozymes have nearly the same molecular size, but differ in substrate specificity, kinetic and immunological properties (*2*), and tissue-distribution (*1, 2*).

Vertebrate aldolase is a member of the α/β barrel enzyme family (*3*) and consists of three prominent types of sequences: (a) sequences conserved in common among three isozymic groups (CCS), (b) isozyme group-specific sequences (IGS), and (c) diverse sequences (DS) (Fig. 1). CCS are scattered in at least seven regions of the entire molecule and serve as the essential constituents of the basal α/β barrel structure. IGS are conserved in one or two isozyme groups. The motifs are located at a periphery of the molecule without disturbing the configuration of the regular barrel structure (*4, 5*) and are required for the exhibition of characteristics as type A, B, or C isozyme (*6-8*). So far, four IGS (IGS 1-4) have been identified. DS are located at several sites of the N-terminal, central, and C-terminal regions of the molecule (*6*). Aldolase C is known to be located in Purkinje cell perikarya, axons and dendrites in the central nervous system (*9, 10*) and thus is likely to interact tissue-specifically with other intracellular components in the tissues through the type-C IGS motifs.

In relation to the analysis of the role of these motifs in isozyme group-specific function of aldolase isozymes, we characterized three monoclonal anti-human aldolase A antibodies, mAbs 1A2, 3C5, and 4C2 (*11*). The mAb 4C2 recognizes an epitope of the enzyme present within amino acid residues 56-108 at the N-terminal region, where IGS-2 and 3 are located (*7*). In contrast, mAbs 1A2 and 3C5 react with sites located at the C-terminal region (amino acid residues 306-363) of the enzyme, where IGS-4 is situated. The epitopes for mAbs 3C5 and 4C2 are shown to

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Abbreviations: CNBr, cyanogen bromide; ELISA, enzyme-linked immunosorbent assay; FBP, fructose-1,6-bisphosphate; F1P, fructose-1-phosphate; mAb, monoclonal antibody; PVDF, polyvinylidene difluoride; TBS, Tris-buffered saline.

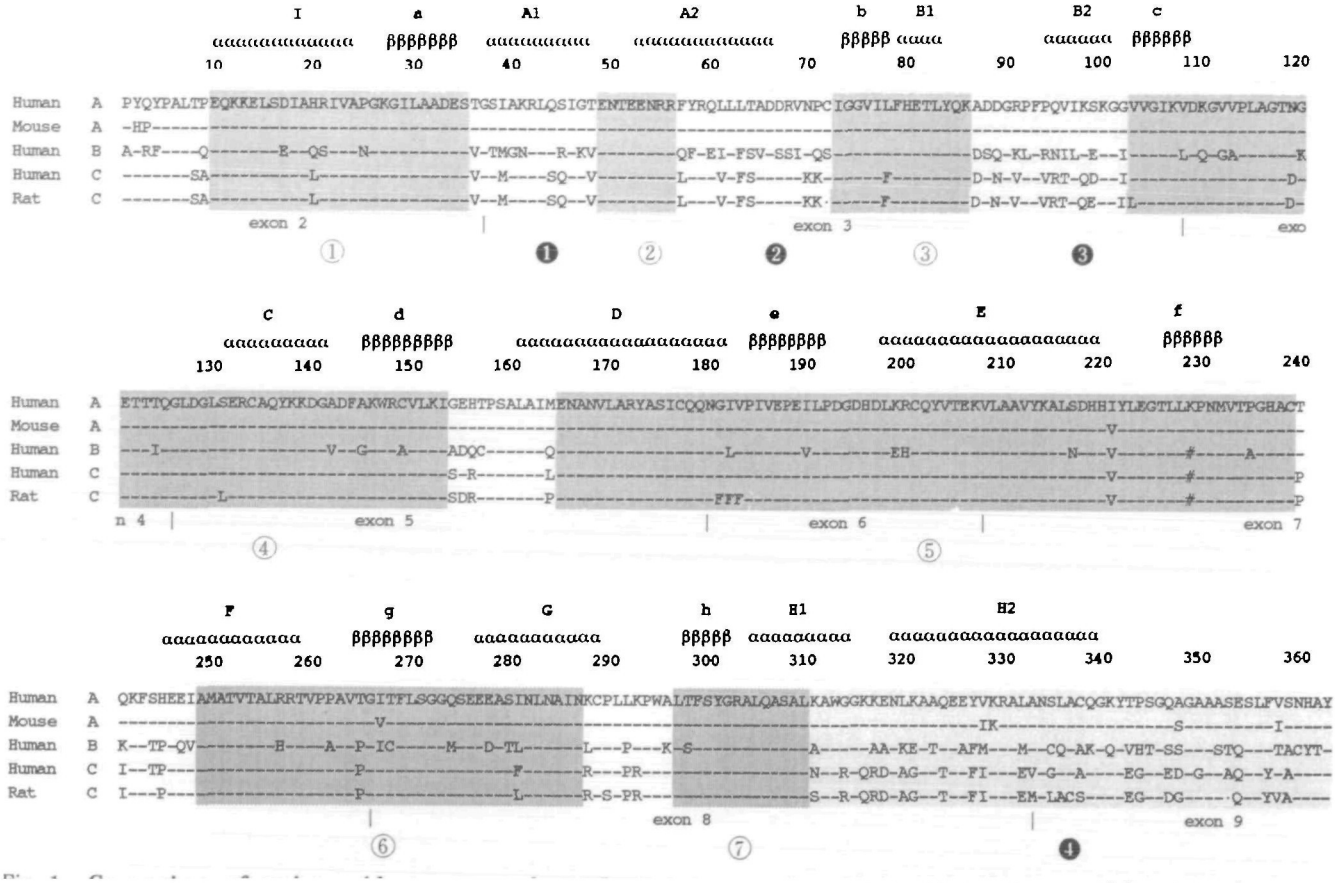

Fig. 1. **Comparison of amino acid sequences of vertebrate aldolase isozymes.** Amino acids identical to human aldolase A sequences are marked with dashes. Regions residing in commonly conserved amino acid sequences are boxed and heavily shaded (open circles 1-7) Similarly, isozyme group-specific residues are boxed and lightly shaded (filled circles 1-4) α-Helices (αI, and αA1-αH2) and β-strands (βa-βh) of rabbit and human aldolase A are diagrammed over the sequences. In this assignment, the N-terminal Met residue was omitted from the sequences. # represents the active site Lys-229. A dot at position 353 in rat aldolase C indicates the site where the amino acid is deleted. Vertical lines under the sequences represent boundaries between contiguous exons.

be highly sensitive to amino acid substitution, substrate-binding, or thermal treatment of the aldolase A molecule, whereas the epitope for mAb 1A2 remained unchanged under these conditions (*11*). Thus, these mAbs have been employed as probes of local conformational change in the IGS or CCS region or the entire molecular architecture of the human aldolase A (*7, 8, 11*). MAbs for human aldolase A were also shown to be useful tools for immunological experiments to examine the localization of human aldolase A in various normal and malignant tissues (*12*).

Meanwhile, the relationships between structure and function of human aldolase C are less understood than those of aldolases A and B. Monoclonal antibodies are particularly useful tools in studying these relationships. The object of the present study was to use monoclonal antibodies as probes directed against specific regions of the aldolase C molecule. Here we have established nine mouse hybridoma cell lines secreting monoclonal anti-human aldolase C antibodies and have characterized these mAbs. The results show that some of these mAbs react with an epitope present on the IGS motifs of aldolase C molecule. In particular, mAbs H1 and E10 are expected to help elucidate the role of the IGS-3 motif in the function of the isozyme. The usefulness of monospecific or monoclonal antibodies for human aldolase A, B, and C in diagnostic analyses have also been demonstrated by other groups (*9, 13-18*).

MATERIALS AND METHODS

Aldolases and Antibodies—Recombinant enzymes used in this work were as follows: human aldolase A (*19*), human aldolase B (*20*), human aldolase C (Motoki *et al.*, in preparation), rat aldolase C (*21*), and a mutant human aldolase C with a point mutation of Tyr-363 to Ser (HC-Y363S) (*22*). Mouse aldolase A was purified from the skeletal muscle by the method of Penhoet *et al.* (*23*). Chimeric enzymes between human aldolase A and C (Kusakabe *et al.*, in preparation), A and B (*6, 7*), and B and C (*8*) which were employed in this study were AC55, AC306, CA 55, CA250, CA306, ACA55-306, ACA250-306, CAC55-250, CAC55-306, AB34, ABA137-212, ABA137-306, ABA212-306, BC243, BC263, BC306, CB55, CB243, CB263, CB306, BCB55-193, BCB55-306, BCB79-193, BCB79-306, and CBC55-263 (see Fig. 6). Notations of chimeric enzymes are as follows: AC55 represents a chimera in which aldolase A and C are rejoined at position 55 with A on the N-terminal side and C on the C-terminal side. Similarly, ACA55-306 is a chimera in which a long

stretch of aldolase C from positions 55 to 306 is substituted for the corresponding type A stretch.

MAb 3C5, a monoclonal anti-human aldolase A antibody, and mAb C7C6-10-8, a mAb for a hydra membrane protein, were described previously (*11*).

Preparation of Monoclonal Mouse Anti-Human Aldolase C Antibodies—Monoclonal mouse anti-human aldolase C antibodies were prepared basically as described previously (*11, 24-26*). The purity of final preparations of mAbs was approximately 60%. The isotypes of individual mAbs were determined by using a mouse monoclonal sub-isotyping kit (American Qualex [International]).

Measurement of the Binding of Monoclonal Antibodies To Antigens—(1) ELISA : ELISA was carried out according to Kitajima *et al.* (*11*). A 100-μl portion of 5 μg/ml antigens (0.5 μg/well) in TBS was adsorbed onto wells of ELISA plates overnight at 4°C then remaining protein-binding sites were blocked with 1% gelatin in TBS. Immobilized antigens in the wells were incubated for 2 h at 25°C with 100 μl of TBS containing the antibodies indicated. After washing the wells with 0.05% Tween-20/TBS, binding of antibodies was determined as described previously (*11*). All data were determined by subtracting the reading of control mAb C7C6-10-8. The reactivity of each mAb with different antigens was expressed as the percent of the reactivity against human aldolase C.

(2) Immunoblotting assays: The enzymes as antigens were separated by 10% SDS-PAGE (*27*), then electrophoretically transferred to a nitrocellulose membrane (Schleicher & Schuell) according to Kitajima *et al.* (*11*) by using a Sartoblot II Semi-Dry Electroblotting unit (Sartorius GmbH) under the specified conditions of 0.8 mA/cm^2 for 2 h. The membrane was incubated with 0.05% Tween-20/TBS containing 3% gelatin for 30 min at 37°C, washed three times with the Tween-20/TBS, then placed in a solution containing appropriate concentrations of mAb in TBS for 2 h at room temperature. The bands that had reacted with the mAbs were visualized by using sheep anti-mouse Ig coupled with biotin (Amersham), streptoavidin-horseradish peroxidase conjugate (Amersham), and 3,3′-diaminnobenzidine tetrahydrochloride as described previously (*11*).

(3) Immunoprecipitation assays: A 10-μg portion mAb was incubated with 5 μg of human aldolase C in 300 μl of TBS for 2 h at room temperature, then recombinant protein G-Sepharose 4B conjugates (ZYMED LABORATORIES) were added and the reaction mixtures were incubated with rotation for 1 h at 4°C. After extensive washing in the Tween-20/TBS, the antigen trapped on the beads was extracted with SDS-buffer (25 mM Tris-HCl, 200 mM Glycine, 3.5 mM SDS) and analyzed by immunoblotting using biotinyl-mAb E10. The mAb was biotinylated according to Bayer and Wilchek (*28*) using biotin succinimide ester (Biotin-OSu, DOJINDO). It was confirmed that all of the IgG class mAbs were bound to protein G before immunoprecipitation assays.

Cyanogen Bromide Cleavage of Human Aldolase C—Cyanogen bromide cleavage of human aldolase C was performed as described previously (*11, 29*) with slight modifications. Recombinant human aldolase C was extensively dialyzed against distilled water and lyophilized. Six hundred micrograms of the enzyme was dissolved in 60 μl of 70% formic acid, 12 μl of 20 mg/ml CNBr in formic acid was added to the solution and then the reaction mixture was incubated for 24 h, rotating the head over the bottom of the tube at room temperature. The peptides cleaved at methionine residues by CNBr were separated from each other on 12.5% SDS-PAGE. Each fragment was judged from its relative electrophoretic mobility, and was identified by analyzing the N-terminal amino acid sequence as described previously (*11*) (see Fig. 5).

Determinations—Aldolase activity was determined by a spectrophotometric method (*30*). Protein concentrations of enzyme and mAb preparations were determined from their absorbancies at 280 and 260 nm according to Layne (*31*) or by the method of Lowry *et al.* (*32*) with bovine serum albumin as a standard.

RESULTS

Monoclonal Mouse Anti-Human Aldolase C Antibodies—Nine hybridoma cell lines producing monoclonal anti-human aldolase C antibodies, mAbs A4, A8, B4, B7, B8, C1, D9, E10, and H1, were isolated by fusing mouse P3UI myeloma cells with spleen cells from BALB/c mice immunized with the recombinant human aldolase C (Table I). The mAbs produced by the hybridomas were prepared peritoneally in mice and partially purified as described previously (*11*). The isotype of the mAbs was determined by using a mouse monoclonal sub-isotyping kit. MAbs B8, D9, and A4 were of the IgM class while mAbs H1, B4, E10, B7, A8, and C1 were of the IgG class (Table I).

Characterization of Nine mAbs

Reactivity of the MAbs with Vertebrate Aldolases—The reactivity of the nine mAbs with aldolases was examined in three ways: ELISA, immunoblotting, and immunoprecipitation assays.

(a) ELISA: To determine the optimal concentration of individual mAbs to be used in ELISA, the binding of mAbs to antigens was determined using different concentrations of mAbs. The half-maximum binding concentrations (μg protein/ml) of the mAbs to human aldolase C are listed in Table I. The reactivity of the mAbs to different antigens such as human aldolase A, B, and C, mouse aldolase A, rat aldolase C and HC-Y363S was also determined at their optimal concentrations (Table I). Under the conditions used, mAbs B8, D9, and H1 were highly specific to human aldolase C and HC-Y363S. MAbs B4 and E10 reacted with both human and rat aldolases C but not with other aldolase isozymes. Of the other mAbs so far tested, mAbs A4 and B7 interacted efficiently with all the enzymes examined. MAb A8 showed a preference for human aldolase C and also reacted with human aldolases A and B and rat aldolase C with a binding efficiency of 63-77% that of human aldolase C. Interestingly, this mAb did not react with HC-Y363S and mouse aldolase A. MAb C1 reacted with both human aldolases B and C but preferred aldolase B to C.

(b) Immunoblotting: To test whether they recognize the primary structure of aldolases as antigens, the mAbs were subjected to immunoblotting assays using vertebrate aldolases as antigens under their optimum concentrations (Fig. 2). MAbs B4 and E10 reacted only with human and rat aldolases C and HC-Y363S, whereas mAb B7 reacted with all the vertebrate aldolases examined irrespective of isozyme groups and species. In contrast, the six other mAbs (B8, D9, H1, A4, A8, and C1) failed to give any significant

bands of the enzymes examined. Consistent results were obtained when the assays were repeated with 4-fold amounts of mAbs. Under the same conditions, mAb 3C5, a monoclonal anti-human aldolase A antibody (*11*) as a control, reacted specifically with human aldolase A but not with mouse aldolase A and other types of isozymes (Fig. 2). MAbs B4, E10, and B7 all reacted with chimeric enzyme BCB79-193, in which a short stretch of aldolase C at positions 79 to 193 is substituted for the corresponding type B stretch, and which thus bears type-B IGS-1, 2, and 4 and type-C IGS-3 (see Table II). These results suggest that the mAbs B4 and E10 recognize the primary structure and react specifically with a region conserved in human and rat type-C isozymes (type-C IGS), whereas mAb B7 recognizes those conserved in common in all vertebrate aldolases examined (CCS). The specificity of mAbs B4, E10, and B7 observed in this study are entirely consistent with those obtained by ELISA (Table I).

(c) Immunoprecipitation: To examine whether the IgG class mAbs react with a native antigen, each mAb was incubated with human aldolase C and the resultant antigen-antibody complexes were trapped on Protein G-bead conjugates. The proteins trapped on the beads were then analyzed by Western blotting using biotinyl-mAb E10 as a primary antibody as described under "MATERIALS AND METHODS" (Fig. 3). A positive band corresponding to aldolase C was observed when mAb H1 was incubated with the antigen. Other mAbs, irrespective of isotypes, failed to make a complex with human aldolase C under the same assay conditions (Fig. 3 and unpublished observation). These results indicate that all mAbs except for mAb H1 are unable to form a stable complex (or interact specifically) with the native form of human aldolase C under the conditions employed in this assay. Unexpectedly, mAb E10, but not other mAbs, reacted with chimeric enzymes CA250 and CA306, which are enzymatically active (data not shown) (Fig. 4).

Epitope Mapping Assessed by Using CNBr-Cleaved Fragments of Human Aldolase C—Because mAbs B4, E10, and B7 were found to react with human aldolase C when analyzed by Western blotting, we examined the epitope for these mAbs by using the CNBr-cleaved fragments of human aldolase C. Since human aldolase C has three methionine residues at positions 39, 232, and 250, it should be cleaved by CNBr into four peptides: One of 39 amino

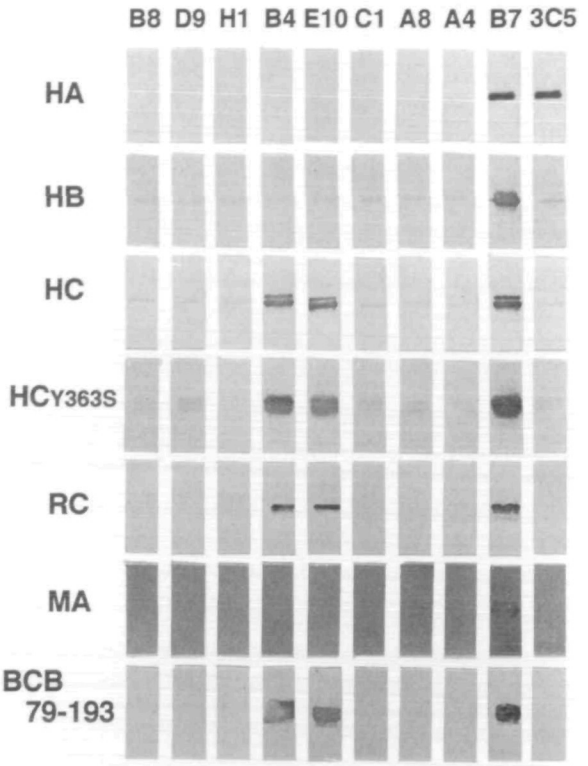

Fig. 2. **Reactivity of mAbs with vertebrate aldolases determined by immunoblotting assays.** Reactivities of mAbs with vertebrate aldolases were determined by immunoblotting (Western blotting) assay as described under "MATERIALS AND METHODS." In these assays 1 μg each of antigens and various amounts of mAbs, 2.5 μg/ml each of A4, A8, B4, D9, and E10, 5 μg/ml each of B7 and H1, and 20 μg/ml of C1, were used to determine the reactivity. Horizontal: anti-human aldolase C antibodies (mAbs B8, D9, H1, B4, E10, C1, A8, A4, and B7) and anti-human aldolase A mAb 3C5 as the control. Vertical: vertebrate aldolases: HA, human aldolase A; HB, human aldolase B; HC, human aldolase C; HC-Y363S, human aldolase C with the substitution of Tyr-363 to Ser; RC, rat aldolase C; MA, mouse aldolase A, and BCB79-193, a chimeric enzyme constructed between human aldolases B and C.

TABLE I. **Isotypes and antigen specificity of anti-human aldolase A antibodies.** Reactivity of mAbs with antigens was determined by ELISA as described under "MATERIALS AND METHODS" and expressed as the percent of that of human aldolase C. For half-maximum binding assay, human aldolase C adsorbed onto wells of ELISA plates was incubated with different amounts of the indicated antibodies in 100 μl of TBS, and binding of antibodies was determined as described under "MATERIALS AND METHODS." For binding assay of the indicated mAbs to six different antigens, the indicated antigens adsorbed onto wells of ELISA plates were incubated with 100 μl of the indicated antibodies in TBS (5 μg/ml of A4, A8, B4, D9, and E10; 10 μg/ml of B7 and H1; 40 μg/ml of C1) and their binding to antigens was also determined as described above. HA, human aldolase A; HB, human aldolase B; HC, human aldolase C; HC-Y363S, a mutant human aldolase C with the amino acid substitution of Ser for Tyr-363; RC, rat aldolase C; MA, mouse aldolase A.

mAb	Ig class	Aldolase						$B_{max}1/2^a$
		HA	HB	HC	HC-Y363S	RC	MA	
B8	IgM	4	7	100	79	11	8	<0.5
D9	IgM	13	20	100	79	12	3	<0.5
H1	IgG_{2b}	1	1	100	96	5	3	0.8
B4	IgG_1	1	1	100	104	99	1	<0.5
E10	IgG_{2a}	3	1	100	105	102	1	<0.5
A4	IgM	115	98	100	90	110	93	1.6
B7	IgG_{2a}	103	111	100	106	103	84	0.8
A8	IgG_1	63	67	100	7	77	2	<0.5
C1	IgG_1	24	144	100	107	122	15	18

[a]Immunoglobulin concentration showing half-maximum binding activity to plate-immobilized human aldolase C (μg/ml).

acid residues, 4 kDa (N); one of 193 amino acid residues, 21.6 kDa (A); one of 18 amino acid residues, 1.9 kDa (B); and one of 113 amino acid residues, 12 kDa (C). The CNBr cleavage may also generate intermediates of various sizes, such as N-A-B, A-B, B-C, and so on that are produced from the aldolase C protein (N-A-B-C) depending upon the cleavage conditions employed. The cleaved polypeptides were separated from each other on SDS-PAGE. The expected multiple bands were found and identified by both their relative electrophoretic mobility on a gel and their N-terminal amino acid sequences (Fig. 5A).

MAb B7 reacted with the N-A-B fragment but not with the A-B fragment of the CNBr-cleaved aldolase C, while

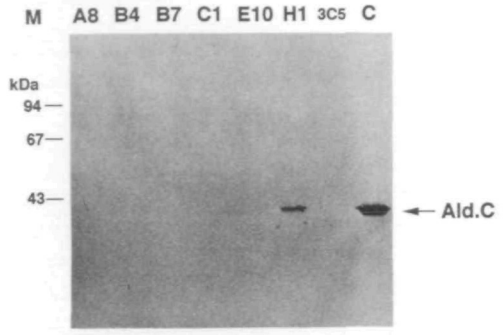

Fig. 3. **Immunoprecipitation of human aldolase C with mAbs.** Ten micrograms each of anti-human aldolase C mAbs was incubated with 5 μg of human aldolase C in 300 μl of TBS, then Protein G beads-anti-mouse IgG complex was added to the reaction mixture. The antigen trapped on the beads was then extracted with SDS-buffer and analyzed by immunoblotting using biotinyl-mAb E10 as described under "MATERIALS AND METHODS." M, molecular size marker; C, human aldolase C as the position marker; 3C5, an mAb for human aldolase A as the negative control.

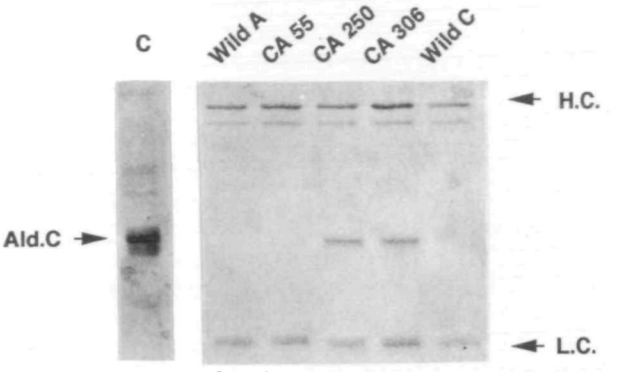

Fig 4. **Immunoprecipitation of human aldolases A and C and their chimeric enzymes with mAb E10.** Ten micrograms of mAb E10 was incubated with 5 μg of antigens to be assayed Protein G beads-anti-mouse IgG complex was then added to the reaction mixture containing mAb E10 and the antigen The antigen trapped on the beads was separated on 10% SDS-PAGE and analyzed as described in Fig. 3. Wild A and wild C represent human aldolases A and C, respectively. CA55, CA250, and CA306 are the chimeric enzymes used in this analysis C, human aldolase C as a marker: 1 μg of aldolase C was developed on 10% SDS-PAGE and stained by Coomassie Brilliant Blue. HC, LC a heavy chain and a light chain of IgG, respectively.

TABLE II. **Reactivity of nine mAbs with chimeric enzymes constructed between human aldolase A and B, B and C, and A and C.** Reactivity of mAbs with antigens was determined by ELISA as described under "MATERIALS AND METHODS" using the amounts of mAbs listed in Table I and expressed as the percent of that of human aldolase C as the control. Isozymic types of IGS-1 to 4 for each antigen are described in the second column from the left. For example, human aldolase C (C) has IGS-1, 2, 3, and 4 all of type C (CCCC).

Antigen	IGS 1234	mAb B8	D9	H1	B4	E10	A4	B7	A8	C1
C	CCCC	100	100	100	100	100	100	100	100	100
AC55	ACCC	80	100	101	105	116	83	81	113	87
AC306	AAAC	0	4	0	0	4	21	98	0	2
CA55	CAAA	5	20	5	3	1	81	88	6	30
CA250	CCCA	108	12	121	97	119	82	107	81	113
CA306	CCCA	112	19	118	100	114	120	113	50	110
ACA55-306	ACCA	98	15	97	95	109	84	102	84	97
ACA250-306	AAAA	12	23	0	0	0	64	108	52	10
CAC55-250	CAAC	10	13	0	1	0	124	104	140	17
CAC55-306	CAAC	0	18	1	0	0	133	87	132	27
AB34	BBBB	10	43	12	9	0	68	114	142	40
ABA137-212	AAAA	17	12	5	0	0	48	5	12	22
ABA137-306	AAAA	48	39	15	4	1	111	52	71	32
ABA212-306	AAAA	18	30	14	8	0	105	40	22	30
BC243	BBBC	25	26	8	1	0	148	106	154	28
BC263	BBBC	30	62	22	2	2	175	3	124	30
BC306	BBBC	11	12	0	0	0	34	107	0	15
CB55	CBBB	0	25	0	0	0	115	119	133	16
CB243	CCCB	78	51	130	102	115	104	116	117	168
CB263	CCCB	58	35	83	96	102	86	112	15	131
CB306	CCCB	138	54	168	101	106	133	122	18	151
BCB55-193	BCCB	216	3	181	102	111	108	114	156	153
BCB55-306	BCCB	0	2	3	37	64	1	11	0	5
BCB79-193	BBCB	129	33	138	116	102	90	123	58	133
BCB79-306	BBCB	121	37	126	107	104	103	120	65	110
CBC55-263	CBBC	11	7	0	1	1	20	4	0	10

both mAbs B4 and E10 reacted with both the N-A-B and A-B fragments (Fig. 5B). Therefore, we concluded that the epitope for B7 is located on the N-fragment of human aldolase C (amino acid residues 1-39), whereas the epitopes for mAbs B4 and E10 are on the A-B fragment (amino acid residues 40-250). Since B7 interacts with all of aldolases A, B, and C, whereas B4 and E10 are specific for aldolase C, the epitope for B7 may have a common location in aldolases A, B, and C (CCS-1), while those for B4 and E10 are in the aldolase C-specific region (IGS-1, 2, or 3).

Interaction of the Nine MAbs with Chimeric Human Aldolases—The reactivity of these mAbs with antigens was assessed by ELISA using various chimeric enzymes constructed with human aldolases A, B, and C as antigens (Fig. 6). These chimeras have different IGS motifs in the molecules as listed in Table II.

(a) MAbs B8, D9, and H1: MAbs B8 and H1, the human aldolase C-specific antibodies, were highly reactive with only the chimeras that share in common type-C IGS-3, *e.g.*, AC55, CA250, CA306, ACA55-306, CB243, CB263, CB306, BCB55-193, BCB79-193, and BCB79-306. These results indicate that the epitopes for mAbs B8 and H1 are located within the type-C IGS-3 motif. In contrast, mAb D9 showed somewhat different reactivity from mAbs B8 and H1. Although mAb D9 showed strong reactivity with human aldolase C and chimeric enzyme AC55, which both have type-C IGS-3, it also reacted significantly with BC263, CB243, and CB306, of which BC263 has type-B IGS-3 while CB243 and CB306 have type-C IGS-3 (Table II). Thus, the epitope for mAb D9 remains to be determined.

(b) MAbs B4 and E10: The mAbs B4 and E10, which recognize both human and rat aldolases C, were also highly reactive with chimeras that have in common type-C IGS-3, *e.g.*, AC55, CA250, CA306, ACA55-306, CB243, CB263, CB306, BCB55-193, BCB79-193, and BCB79-306, but definitely not with the chimeras that have the other types of IGS-3, *e.g.*, AC306, CA55, AB34, and BC306. These results, together with those obtained with Western blotting using CNBr fragments (Fig. 5B), led us to conclude that the epitopes for mAbs B4 and E10 could be within type-C IGS-3 of the antigens.

(c) MAbs A4 and B7: Although mAbs A4 and B7 have been found to react efficiently with human aldolases A, B, and C, rat aldolase C, mouse aldolase A and HC-Y363S in ELISA (Table I), they also reacted strongly with various chimeric enzymes, irrespective of the type of IGS motif, under the conditions described above. Thus, the epitopes for these mAbs appear to be a region conserved in common in the three isozymic groups (CCS). The data obtained with B7 particularly supports the result of Western blotting assay using CNBr fragments and led to the conclusion that the epitope for mAb B7 is within CCS-1 of antigens (Fig. 5B).

(d) MAbs A8 and C1: MAb A8 showed a broad antigen specificity (Table II). It reacted strongly with chimeric enzymes with different combinations of IGS, *e.g.*, AC55, CAC55-250, AB34, BC243, CB55, and CB243, and BCB55-193. It also interacted significantly with some chimeras, *e.g.*, CA306, ACA250-306, and BCB79-306. Thus, the epitope for mAb A8 may be located on CCS, although this remains unresolved. The mAb C1 was shown to have the highest reactivity with human aldolase B among antigens

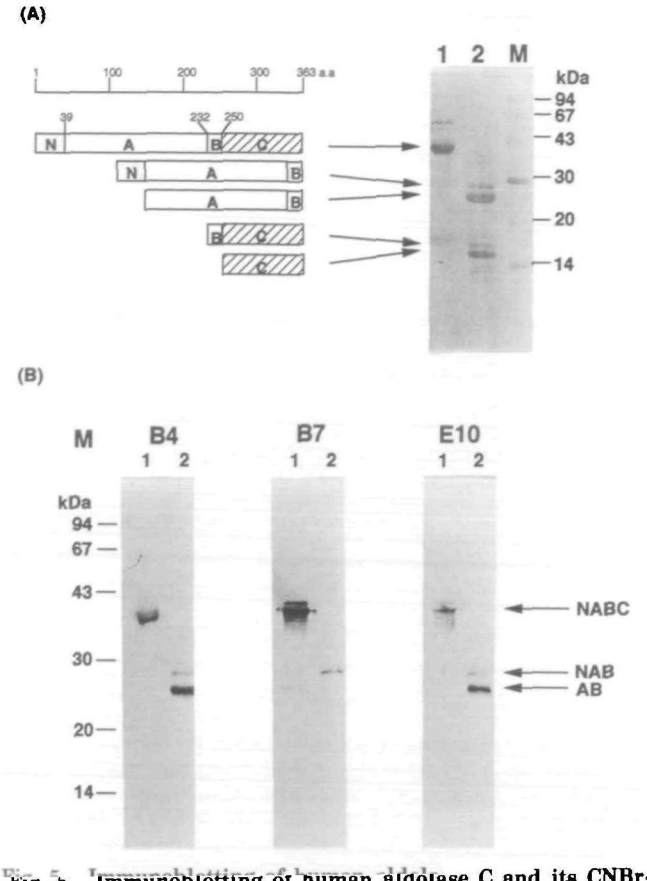

Fig. 5. Immunoblotting of human aldolase C and its CNBr-cleaved fragments with mAbs B4, B7, and E10. (A) Right panel SDS-PAGE patterns of purified human aldolase C and its CNBr-cleaved polypeptide fragments stained with Coomassie Brilliant Blue. One microgram of human aldolase C (lane 1) and 5 μg of the cleaved polypeptide fragments (lane 2) were separated by 10% SDS-PAGE. Left panel schematic presentation of the possible CNBr-cleaved fragments of human aldolase C. N, amino acid residues from 1 to 39, A, those from 40 to 232; B, those from 233 to 250, and C, those from 251 to 363. Each band was identified by the N-terminal amino acid sequence analysis. (B) Reactivity of mAbs B4, B7, and E10 with human aldolase C and its CNBr-cleaved fragments. One microgram of human aldolase C (lane 1) and 5 μg of the cleaved polypeptide fragments (lane 2) were developed on 12.5% SDS-PAGE and subjected to Western blotting using mAbs B4, B7, and E10 as probes. NABC, intact size aldolase C; NAB, the CNBr-cleaved fragments composed of N, A, and B peptides, AB, that composed of A and B fragments, M, molecular size markers.

tested and also considerably high reactivity with human and rat aldolases C and HC-Y363S in ELISA (Table I). This mAb also strongly reacted with chimeras which have in common type-C IGS-3, *e.g.*, AC55, CA250, CA306, ACA55-306, CB243, CB263, CB306, BCB55-193, BCB79-193, and BCB79-306, but not with chimeras that have type-B or type-A IGS-3 despite their binding to aldolase B. Thus, the reactivity of this mAb with chimeric enzymes appears to be slightly different from those of other IGS-3-specific mAbs (Table II).

DISCUSSION

Antigen-Specificity of Nine MAbs—In this paper, nine hybridomas producing monoclonal mouse anti-human

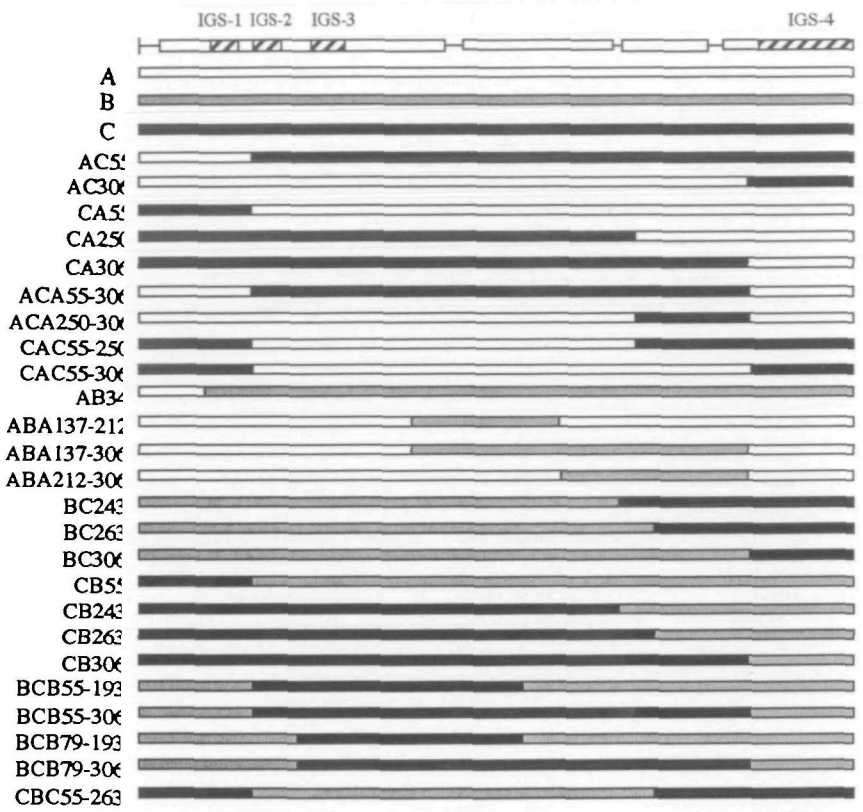

Fig. 6. **Molecular features of chimeric enzymes.** Top: the open boxes and shaded boxes represent the conserved common sequences (CCS) and isozyme group-specific sequences (IGS), respectively. Horizontal lines represent diverse sequences (DS). Others: the constructs of parent enzymes and their chimeric enzymes: A, aldolase A (open box); B, aldolase B (stippled box); C, aldolase C (closed box). AC55 to CBC55-263 are chimeric enzymes that are rejoined at the numbered positions and derived from two of the three aldolases A, B, and C. Notation of chimeric enzymes is described under "MATERIALS AND METHODS."

aldolase C antibodies were established and their reactivity with human aldolases A, B, and C, rat aldolase C, mouse aldolase A, and chimeric human aldolases was analyzed under their optimum assay conditions. They fall substantially into four groups according to their reactivity with the antigens. (i) Human aldolase C-specific mAbs; B8, D9, and H1 belong to this group. (ii) Type-C aldolase isozyme-specific mAbs; B4 and E10 belong to this group. (iii) Ubiquitous mAbs, which react with vertebrate aldolases irrespective of the isozyme group and species; A4 and B7 are involved in this group. (iv) The mAbs that are similar to group 3 mAbs in terms of antigen specificity but cannot strictly be classified into this group; A8 and C1 are in this group.

Epitopes of the MAbs—Nucleotide and amino acid sequence alignments of vertebrate aldolase isozymes revealed that aldolase molecules are composed of three prominent sequences (*6*): (a) sequences that are conserved in common in three isozyme groups (CCS), (b) isozyme group-specific sequences (IGS), and (c) diverse sequences (DS). From the structural point of view, one may speculate that the epitopes for ubiquitous mAbs are located on the CCS region while those for the type-C isozyme-specific antibodies are on type-C IGS motifs or the related conformation of antigens that covers IGS motifs. In fact, aldolase C-specific mAbs B8, H1, B4, and E10 all were found by three methods of assessment to have their epitopes in a region within positions 79 to 193 of antigens, in which the type-C IGS-3 is situated. By contrast, ubiquitous mAbs A4 and B7, and sub-ubiquitous mAb A8 were found to have epitopes in regions within the CCS of antigens. In particular, mAb B7 reacted with all the natural antigens examined, and the epitope for this mAb was mapped on the N-terminal region within amino acid residues 1-39, which is conserved in common in isozymes A, B, and C (CCS-1). The reactivity of mAb C1 with chimeric enzymes appears to be somewhat different from that of mAb A8, although they share some common antigens such as C, AC55, CA250, CB243, and BCB55-193 (Table II). MAb C1 reacted with aldolase B and antigens bearing type-C IGS-2 and 3 but not significantly with aldolase A. Although it is reasonable to believe that the epitope for mAb C1 may be located on IGS-motif 2 or 3, this has not been confirmed. The epitope recognition patterns of the nine mAbs are schematically summarized in Table III.

B8, H1, B4, and E10 have their own epitope within the type-C IGS-3 motif, whereas A4, B7, and A8 are on CCS regions. This is in contrast to three anti-human aldolase A antibodies established, of which two are of specific for IGS-4 at the C-terminal sequence (mAbs 1A2 and 3C5) and one for IGS-2 or 3 (mAb 4C2) (*11*). Since the IGS-3 motif is composed of α-helix B2 and a loop that connects α-helix B2 and the preceding α-helix B1 and is located on the periphery of the α/β barrel structure of the aldolase molecule (*4, 5*), IGS-3 might be the most available recognition site of the four IGS-motifs of aldolase C as epitope under the conditions used in this study. Recent studies strongly indicated that, for aldolases A, B, and C, their own IGS-3 together with IGS-1, 2, and 4 are required for expression of their characteristics as isozymes (*7, 8*), and a region within the IGS-3 motif serves as the molecular site to bind cytoskeleton (Kusakabe *et al.*, in preparation).

From the mode of epitope recognition, the nine established mAbs can be classified into two groups: (a) the primary sequence-epitope type, and (b) the conformation-epitope type. MAbs B4, E10, and B7 belong to the primary

sequence-epitope type because only these mAbs react with the epitopes present on the denatured forms of antigens and the CNBr-cleaved peptides of human aldolase C under the immunoblotting assay conditions. The remaining six mAbs probably belong to the conformation-epitope type, because none of them react with human aldolase C and its CNBr-cleaved peptides as antigen in the immunoblotting assay carried out under the same conditions. Instead they react with the natural antigens as well as those with the engineered sequences when assessed by ELISA. Related to this discussion, one may argue that antigens fixed to ELISA plates no longer keep their proper higher-order structure (*33-35*). As far as the antigens examined in this study are concerned, the antigens are able to maintain at least partially, a folded structure because most mAbs react equally and regularly with antigens in ELISA even if they are unable to react with the various enzymes and CNBr-cleaved peptides in the immunoblotting assay (Table I and Figs. 2, 3, and 5B).

Aldolase C-Specific MAbs: Those That Recognize the Type-C IGS-3 as Epitopes—All three human aldolase C-specific mAbs, B8, D9, and H1, are of the conformation-epitope type. MAbs B8 and D9 show a similar antigen specificity to each other toward natural antigens and chimeric enzymes, while H1 shows a more strict antigen specificity than they do. As can be seen in Fig. 3, mAb H1 appears to be truly a typical antibody of conformation-epitope type because it reacts with human aldolase C but not with chimeric enzymes CA250 and CA306 in the immunoprecipitation assay (data not shown). Moreover, mAb H1 interacts with mutant enzyme HC-Y363S and chimeric enzymes that carry strictly type-C IGS-3 when assessed by ELISA but not with these same antigens when examined by Western blotting. The mAb H1, therefore, might be used as a probe to detect the role of type-C IGS-3 motif or structural change around the motif of human aldolase C, although such modifications have not yet been reported.

In contrast to mAbs B8, D9, and H1, mAbs B4 and E10 appear to have their epitopes within the type-C IGS-3 of antigens, as judged by the analyses using chimeric enzymes in ELISA and Western blotting assays using the CNBr fragments. However, mAb E10 was unexpectedly found to form a stable antigen-antibody complex with CA250 and CA306 but not with aldolase C when examined by immunoprecipitation assays under the same conditions as those for mAb H1 (Fig. 4). Reactivity of the mAbs E10 and H1 with aldolase C and chimeric enzymes CA250 and CA306 varied inversely. This strongly suggests that the epitope for mAb E10 is hidden in the aldolase C molecule, whereas those for CA250 and CA306 remain exposed and enable this mAb to interact with these chimeric enzymes in the immunoprecipitation assay. Therefore, mAb E10 may also be useful as a probe for detection of conformational change around type-C IGS-3 motif of human aldolase C.

The mAbs B4 and E10 showed clearly different antigen specificities from mAbs B8 and H1 when tested by ELISA: the former two mAbs react with human and rat aldolase C, whereas the latter two react only with human aldolase C. The primary sequences around the IGS-3 motif of human and rat aldolases C are completely identical with each other except for position 99 in the α-helix B2 (Asp for human C and Glu for rat C) and position 103 in the β-strand c (Val for human C and Leu for rat C). One may speculate, therefore, that mAbs B4 and E10 recognize the same primary sequence for both antigens as epitopes, whereas mAbs B8 and H1 selectively interact with human C by recognizing the subtle difference in conformation between the two antigens

TABLE III. **Summary of antigen specificity of mAbs determined by using chimeric enzymes.** Combinations of mAb and antigen whose reactivity is more than 60% of that of human aldolase C as the control and are listed by showing isozymic types of IGS-1 to 4. Types of IGS deduced from ELISA using chimeric enzymes are shown in the upper row of the bottom box and a possible epitope deduced from ELISA and Western blotting assays using CNBr-cleaved fragments are in the lower row of the box. Isozymic types of sequence in IGS regions are denoted as follows: X, more than one isozymic type; B/C, type B or C; C, type C.

Antigen	mAb								
	B8	D9	H1	B4	E10	A4	B7	A8	C1
C	CCCC	CCCC	CCCC	CCCC	CCCC	CCCC	CCCC	CCCC	CCCC
AC55	ACCC	ACCC	ACCC	ACCC	ACCC	ACCC	ACCC	ACCC	ACCC
AC306							AAAC		
CA55						CAAA	CAAA		
CA250	CCCA		CCCA	CCCA	CCCA	CCCA	CCCA	CCCA	CCCA
CA306	CCCA		CCCA	CCCA	CCCA	CCCA	CCCA		CCCA
ACA55-306	ACCA		ACCA	ACCA	ACCA	ACCA	ACCA	ACCA	ACCA
CAC55-250						CAAC	CAAC	CAAC	
CAC55-306						CAAC	CAAC	CAAC	
ABA137-306						AAAA		AAAA	
ABA212-306						AAAA			
BC243						BBBC	BBBC	BBBC	
BC263		BBBC				BBBC		BBBC	
CB55						CBBB	CBBB	CBBB	
CB243	CCCB		CCCB	CCCB	CCCB	CCCB	CCCB	CCCB	CCCB
CB263			CCCB	CCCB	CCCB	CCCB	CCCB		CCCB
CB306	CCCB		CCCB	CCCB	CCCB	CCCB	CCCB		CCCB
BCB55-193	BCCB		BCCB	BCCB	BCCB	BCCB	BCCB	BCCB	BCCB
BCB55-306					BCCB				
BCB79-193	BBCB		BBCB	BBCB	BBCB	BBCB	BBCB		BBCB
BCB79-306	BBCB		BBCB	BBCB	BBCB	BBCB	BBCB	BBCB	BBCB
Epitope	XXCX (IGS-3)		XXCX (IGS-3)	XXCX (IGS-3)	XXCX (IGS-3)	XXXX (CCS)	XXXX (CCS-1)	XXXX (CCS)	

that might be caused by the amino acid substitutions in the vicinity of IGS-3, *e.g.*, at positions 99 and 103 described above and/or at position 131 in the α-helix C (Ser for human C and Leu for rat C).

Ubiquitous Type MAbs: Those That Interact with CCS as Epitopes—Despite being monoclonal antibodies secreted from hybridomas immunized with human aldolase C, mAbs A4 and B7 exhibit a broad specificity of antigen and their epitopes are on one of the seven CCS, and thus they belong to the ubiquitous mAbs. In addition, mAb B7 is available for assay both Western blotting and ELISA. Therefore, this mAb might be used as a ubiquitous mAb for the assessment of aldolases of various isozyme groups and species. By contrast, mAbs A4 is only available for assay by ELISA but not Western blotting and immunoprecipitation. The mAb C1 showed the highest reactivity toward human aldolase B among antigens examined and a significantly high reactivity toward human and rat aldolase C in addition to HC-Y363S in ELISA (Table I). This mAb also showed strong reactivity with chimeras that have in common type-C IGS-3 (Table II).

At present, however, we cannot exclude the possibility that the reactions of ubiquitous mAbs with the antigens are nonspecific, especially in the case of mAbs A4, A8, and C1, because competition experiments to ascertain whether their reactions reflect immunochemical significance are impossible with these mAbs.

Hoping to find useful antibodies for immunological study of human sera and tissues, we examined the immunoprecipitation of human aldolase C with the nine mAbs. However, mAb H1 was the only antibody which recognized apparently the native antigen when assessed by immunoprecipitation assays. This may be partly explained by assuming that the antigens undergo either partial denaturation when homogenized with Freund's incomplete adjuvant or proteolysis after injection in the mouse. An other reason may be that the antigens immobilized in the ELISA wells undergo partial or total unfolding during the coating procedure in the screening system of mAbs (*12, 33-35*).

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