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V L Woods, Jr; ... et. al

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IgD ALLOTYPIC DETERMINANTS

I. Determinants Expressed on Murine IgD of the *a* Allotype[1]

VIRGIL L. WOODS, JR.,[2] STEVEN W. KESSLER, FRED D. FINKELMAN, ROSE LIEBERMAN, IRWIN SCHER, AND WILLIAM E. PAUL[3]

From the Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20205; the Department of Medicine, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20014; and the Naval Medical Research Institute, Bethesda, Maryland 20014

Allotypes of membrane IgD of 27 strains of mice were determined with a series of anti-δ reagents, each capable of binding to the IgD of BALB/c spleen cells. One of these reagents is a newly defined allospecific rabbit anti-mouse-δ^a. Typing with these reagents reveals the strain distribution of the previously described IgD.36 (Ig5.1) and IgD.43 (Ig5.4) specificities and demonstrates the existence of a new specificity, IgD.45 (Ig5.5). The results confirm the previous subdivision of the *Igh-C*^{e} haplotype into *Igh-C*^{e}, to which A mice are assigned, and *Igh-C*^{n}, to which NZB and NZW mice are assigned. In addition, it is shown that the *Igh-C*^{d} haplotype must also be subdivided. AKR mice, which express the *a* allele at the *Igh-5(δ)* locus, are designated as possessing the *Igh-C*^{d} haplotype while AL/N and C.AL20 mice, which have the *e* allele at the *Igh-5(δ)* locus, are assigned a new *Igh-C* haplotype designation, *o*.

IgD is one of the two principal membrane (m)[4] immunoglobulin (Ig) molecules expressed on lymphocytes (1–4). It is of particular interest because, in contrast to other Ig, its principal function appears to be related to its membrane expression (5, 6). Furthermore, mIgD appears to be expressed only after a certain degree of maturation has been attained by a B lymphocyte (3, 7, 8), and it is co-expressed with IgM over a substantial period of time (2). This suggests that the molecular genetic events involved in the expression of IgD will be of considerable complexity and that mIgD has a very specific receptor function.

The existence of alternative allotypic forms of the IgD H chain constant region provides a powerful tool for the study of the genetic regulation of IgD expression at the level of gene product analysis. Indeed, among the first specific anti-mouse IgD reagents which were prepared were allospecific mouse anti-mouse δ antibodies (9). Several monoclonal antibodies that recognize allotypic antigenic determinants of IgD are now available (10, 11). This has allowed the description of a series of allotypic IgD determinants (12).

In this and the companion paper (13), we make use of a large panel of anti-IgD allotype reagents to more fully characterize the IgD expressed by a large number of inbred mouse strains, representing the major *Igh-C* haplotype groups found in inbred mice. This paper describes the allotypic antigenic determinants expressed on IgD of the *Igh-C*^{a} haplotype and by mice that express similar IgD. The second paper of this series (13) describes allotypic determinants expressed by the IgD of mice of the *b* and *e* *Igh-C* haplotypes. In addition to characterizing the IgD expressed in each of these strains, our results indicate the existence of a new *Igh-C* haplotype, *o*, of which AL/N and C.AL20 are the prototype strains and of two new IgD allotypic determinants.

MATERIALS AND METHODS

Animals. All mice were 9- to 12-wk-old females. BALB/cJ, ST/6J, C57BL/6J, LP/J, SJL/J, DBA/2J, DBA/1J, RF/J, AKR/J, CE/J, P/J, CBA/J, C3H/HeJ, PL/J, A/J, and A/HeJ mice were purchased from The Jackson Laboratory, Bar Harbor, ME. C.B20 , B.C8, C.AL20, and C57BL/Ka breeding stock were obtained from Dr. Michael Potter, National Cancer Institute, Bethesda, MD, and were bred in the Laboratory of Immunology, National Institute of Allergy and Infectious Diseases (NIAID). NZB/N, NZW/N, C3H/HeN, CBA/N, AL/N, A/HeN, and (CBA/N × BALB/c)F_1 mice were obtained from the Small Animal Section, Division of Research Services, NIH, Bethesda, MD. SJA and RIII mice were bred in the Laboratory of Immunology, NIAID. C.B20 mice are congenic to BALB/c mice but are of C57BL (b) Igh-C type; B.C8 mice are congenic to C57BL/Ka mice but are of the BALB/c (a) Igh-C type; C.AL20 mice are congenic to BALB/c mice but are of the AL/N Igh-C type; SJA mice are congenic to SJL but are of the BALB/c Igh-C type. New Zealand White (NZW) rabbits, 3 to 4 mo old, were obtained from Dr. Rose Mage, NIAID.

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[2] Present address: University Hospital, San Diego, California.
[3] Address reprint requests to: Dr. William E. Paul, National Institutes of Health, Building 10, Room 11N-311, Bethesda, Maryland 20205.

[4] Abbreviations used in this paper: CSA, channel scale area; FACS, fluorescence-activated cell sorter; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; Fl, fluoresceinated; KLH, keyhole limpet hemocyanin; m, membrane; MHC, major histocompatibility complex; RαM, rabbit anti-mouse; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Antisera. Rabbit anti-mouse-δ (RαMδ) antiserum was produced by immunizing NZW rabbits with detergent-solubilized mouse lymphocyte mIgD (14). Batches of 400 spleens from (CBA/N × BALB/c)F_1 female mice were each disrupted and solubilized in 1 liter of 0.5% NP-40 in phosphate-buffered saline (PBS), pH 7.4. The resulting solution was clarified by centrifugation at 50,000 × G for 2 hr, and then passed sequentially over three columns: the first consisting of Sepharose 4B coupled to a normal rabbit serum euglobulin fraction (15 mg/ml Sepharose, bed volume 50 ml); the second consisting of Sepharose 4B coupled to a 50% saturated $(NH_4)_2SO_4$ fraction of rabbit anti-mouse-μ antiserum (Litton Bionetics, Rockville, MD) (15 mg/ml Sepharose, bed volume 20 ml); and the third consisting of Sepharose 4B coupled to affinity purified rabbit anti-mouse κ antibody (5 mg/ml Sepharose, bed volume 4.0 ml). The final Sepharose conjugate was then extensively washed with 0.5% NP-40 in PBS, mixed with an equal volume of complete Freund's adjuvant (Difco Labs., Detroit, MI), and used to immunize four NZW rabbits in multiple subcutaneous and intramuscular sites. Each rabbit received the affinity-purified IgD prepared from 100 mouse spleens per immunization. Subsequent immunizations were done every 2 wk in the same manner, except that incomplete Freund's adjuvant was used. Rabbits were bled 1 wk after each immunization. Serum from these bleeds was passed over columns of Sepharose 4B-bound BALB/c serum euglobulin to remove IgM reactive specificities as assayed by immunoprecipitation of NP-40 extracts of ^{125}I surface-labeled BALB/c spleen cells and subsequent sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

A C57BL/Ka anti-BALB/c spleen cell antiserum was prepared by a procedure similar to that of Goding *et al.* (9) and Zitron *et al.* (15). Mice received i.p. injections of 10×10^6 spleen cells every 2 wk for a total of five injections. Ascitic fluid was prepared by the procedure of Tung *et al.* (16) and harvested 2 wk after the fifth immunization. Antibody was partially purified from the ascites by precipitation with 40% $(NH_4)_2SO_4$. This antiserum when used against lymphocytes of B.C8 mice reacted only with antigenic determinants coded for by genes in the *Igh-C* complex. Immunoprecipitation and SDS-PAGE demonstrated that most lots of ascites precipitated only material with mobility of δ and L chains. Occasional lots were, in addition, able to precipitate material of μ chain mobility. These lots were absorbed with limited amounts of BALB/c euglobulin-Sepharose 4B until rendered specific for IgD as tested by immunoprecipitation and SDS-PAGE. This serum was designated mouse anti-mouse δ^a (MαMδ^a).

The hybridoma-produced monoclonal IgG_{2a} mouse anti-mouse-δ antibody 10-4.22 was prepared from cells obtained from the Salk Institute Cell Distribution Center, LaJolla, CA, that were originally derived by Oi and colleagues (11). The hybridoma was grown *in vitro*, and antibody was purified from culture supernatants by adsorption to and elution from protein-A Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) as previously described (17).

Normal rabbit serum and rabbit anti-mouse-κ antiserum were gifts from Dr. Rose Mage. Affinity-purified rabbit anti-mouse κ antibody was a gift from Dr. Richard Asofsky, NIAID.

Preparation of fluorescent antibodies. A 40% saturated $(NH_4)_2SO_4$ fraction of each antiserum was dialyzed against 0.15 M NaCl, brought to a concentration of 10 mg/ml, and reacted with 6 mg/ml of fluorescein isothiocyanate (FITC) bound to celite (Calbiochem, LaJolla, CA) in the presence of 0.05 M Na_2CO_3, pH 9.5. Sephadex G-25 gel filtration was used to separate fluoresceinated (Fl) antibody from unconjugated FITC. Fluorescein:protein ratios varied from 3 to 5 as measured by optical density. Affinity purified 10-4.22 was fluoresceinated as above. Fl-rabbit anti-keyhole limpet hemocyanin (Fl-RαKLH) and Fl-human IgM were prepared as previously described (18, 19).

Binding of Fl-antibody to cells. Cells were teased from mouse spleens, treated with NH_4Cl erythrocyte-lysing buffer, and washed in Hanks' balanced salt solution with 10% fetal bovine serum (FBS) and 0.2% NaN_3 (HFA). 2×10^6 cells were pelleted, resuspended in a total volume of 100 μl of HFA containing Fl-antiserum, and incubated on ice for 30 min. The cells were then washed 3 times in HFA and resuspended in 1.0 ml of HFA before analysis.

Fluorescence analysis. A fluorescence-activated cell sorter (FACS) (Becton-Dickinson, Mountain View, CA) was used, as previously described (20), to analyze the fluorescence intensity of 40,000 spleen cells. Cells were assigned to fluorescence channels from 1 to 1000 in some experiments, and 1 to 256 in others, on the basis of linearly increasing fluorescence intensity, and the number of cells in each fluorescence channel was determined. The term channel scale area (CSA) was used to provide a measure of the total amount of Fl antibody bound by a cell population. CSA is the sum of the products of the number of cells in each fluorescence channel multiplied by that fluorescence channel number.

Titration of Fl-antisera. 2×10^6 spleen cells were incubated with varying concentrations of each Fl-αMδ antibody in order to determine the concentration of antibody necessary for optimum staining. This concentration was used in all studies other than blocking experiments. For blocking experiments, the lowest amount of Fl-αMδ that produced a typical IgD fluorescence profile when analyzed on the highest gain setting on the FACS was used.

Blocking studies. B.C8 splenic lymphocytes (8×10^6) were preincubated with one of a series of nonfluoresceinated anti-δ or control reagents in 400 μl of HFA for 2 hr on ice. The amount of anti-δ used in these preincubations was determined by prior experiments in which the volume necessary to completely block the capacity of the fluorescein conjugate of the same anti-δ to bind to lymphocytes was measured. Control reagents were used in amounts identical to the corresponding anti-δ reagent. 100 μl aliquots of cells preincubated in anti-δ or control reagent were incubated with one of a series of Fl-αMδ antibodies for 30 min on ice, in the presence of an excess of the reagent used in the preincubation. Cells were then washed 3 times with HFA and analyzed for fluorescence intensity. Percent inhibition of staining was determined as follows:

$$\%\ \text{Inhibition of binding of Fl-}\alpha M\delta(A) \text{ after preincubation with nonfluorescent } \alpha M\delta(B) = 100 \times \frac{\text{CSA of cells incubated with Fl-}\alpha M\delta(A) \text{ after preincubation with control reagent for } \alpha M\delta(B) - \text{CSA of cells incubated with Fl-}\alpha M\delta(A) \text{ after preincubation with } \alpha M\delta(B)}{\text{CSA of cells incubated with Fl-}\alpha M\delta(A) \text{ after preincubation with control reagent for } \alpha M\delta(A) - \text{CSA of cells incubated with Fl-}\alpha M\delta(A) \text{ after preincubation with } \alpha M\delta(A)}$$

Typing by absorption. Cells were teased from spleens of the appropriate mouse strains, washed 3 times with HFA, and numbers of lymphocytes were determined on a Coulter counter. Measured numbers of cells were aliquoted into tubes and pelleted by centrifugation. Supernatants were aspirated and 100 μl HFA plus a fixed amount of Fl-αMδ added to each tube. Cell pellets were resuspended and incubated on ice for 2 hr with agitation every 10 min. Cells were then pelleted and 90 μl of supernatant withdrawn from each tube and placed in a fresh tube. 2×10^6 erythrocyte-free splenic lymphocytes from the appropriate strain were then added to each tube of absorbed antisera. This mixture was incubated for 30 min on ice, then washed 3 times with HFA. Fluorescence intensity was then analyzed on the FACS.

Lactoperoxidase-catalyzed iodination and cell lysis. Cells were radiolabeled according to methods described in detail elsewhere (16). Cell lysis was performed at a maximum density of 20×10^6 cells/ml with 0.5% NP-40 in a saline-EDTA-Tris buffer that contained 0.001 molar phenylmethyl sulfonyl fluoride (Sigma, St. Louis, MO). Cell lysates were centrifuged at 40,000 × G for 30 min.

Immunoprecipitation and analysis by SDS-PAGE. Immunoprecipitation was performed by incubation on ice for 1 hr with 25 to 35 μg of antibody or 10 to 29 μl of antiserum. Then, 200 μl of 10% (V/V) protein A bearing *Staphylococcus aureus* (21) were added. The complexes of radiolabeled antigens plus antibody bound to staphylococci were washed and eluted by boiling for 2 min in 2% SDS, 5% mercaptoethanol.

Electrophoresis of eluates was performed in a discontinuous SDS-buffer system on vertical 10% polyacrylamide tube gels (22, 23). Reduced Fl-human IgM was added to each eluate as an internal marker of the mobility of secreted μ-chains. The location of μ-chain at the end of each electrophoresis was determined by visual inspection under a UV lamp (18). Gels were frozen and then sliced on a 50-slice gel slicing apparatus. Radioactivity in each slice was determined with the use of a Packard gamma counter.

RESULTS

Allospecific RαMδ. Membrane Ig from which mIgM had been removed was prepared from (CBA/N × BALB/c)F_1 female spleen cells by a procedure similar to that of Abney *et al.* (14). Rabbits immunized with this mIg produced antisera that had considerable anti-δ activity and were quite specific. This was shown by analyzing immunoprecipitates of NP-40 extracts of ^{125}I-labeled BALB/c spleen cells formed with RαMδ. On SDS-PAGE, molecules with the mobilities of δ and L chains were observed, but no evidence of μ chains was noted (Fig. 1). To our surprise, this RαMδ precipitated only a barely detectable amount of IgD from an NP-40 lysate of radioiodinated C57BL/Ka cells (Fig. 1). A rabbit anti-mouse κ chain antiserum precipitated essentially equivalent amounts of μ, δ, and L chains from BALB/c and C57BL/Ka lysates.

The allospecific character of RαMδ antisera, hereafter designated RαMδ^a, was a common feature of early post immunization bleedings of each of four rabbits. However, after the third boost with mIg, the RαMδ antisera were found to contain substantial reactivity to C57BL/Ka IgD, which rose in titer with subsequent boosts. Sera from these later bleedings could be rendered allospecific for BALB/c IgD by absorption with C57BL/Ka spleen cells. The anti-δ antibodies present in the later bleedings that precipitated C57BL/Ka IgD were directed against an antigenic determinant(s) common to both C57BL/Ka and BALB/c IgD, since absorption with BALB/c spleen cells completely removed the ability of the serum to precipitate C57BL/Ka IgD. Antisera with substantial non-allospecific ("common") anti-IgD reactivity are referred to as RαMδ$_c$.

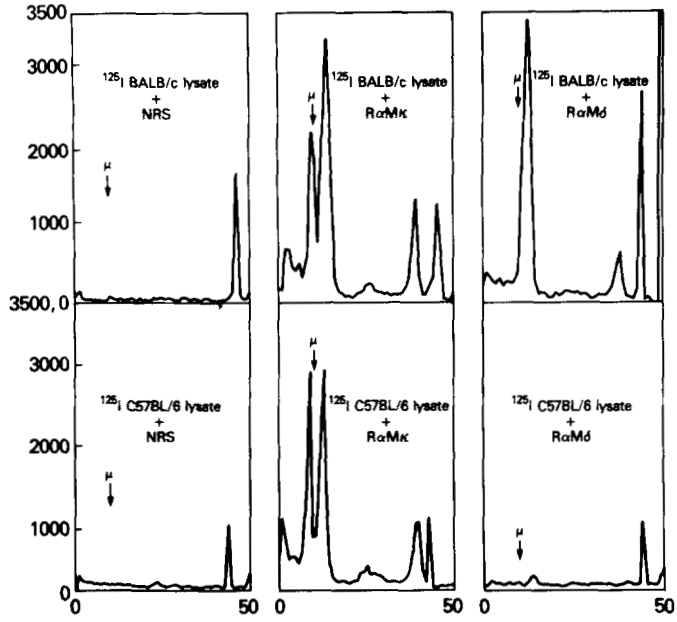

Figure 1. SDS-PAGE analysis of immunoprecipitates of ^{125}I-labeled BALB/c and C57BL/6 spleen cells. RαMδ^a and RαMκ were used for immunoprecipitation. μ refers to mobility of Fl-human μ-chain.

Binding of Fl-RαMδ^a to lymphocytes. A 40% ammonium sulfate fraction of RαMδ^a was fluoresceinated and its binding to splenic lymphocytes of BALB/c mice and of their *Igh-C^b* allotype congenic partners, C.B20 mice, was studied. After thorough washing, the number of fluorescent cells and their fluorescence intensity was analyzed on the FACS. The allospecific nature of the Fl-RαMδ^a was confirmed by the bright fluorescence exhibited by BALB/c (*Igh-C^a*) splenic lymphocytes, of which 49% were scored as positive, and the failure of splenic lymphocytes from C.B20 (*Igh-C^b*) mice to display fluorescence (Fig. 2). Indeed, the fluorescence profile of C.B20 spleen cells incubated with Fl-RαMδ^a was identical to that of spleen cells incubated with Fl-RαKLH.

The binding of Fl-RαMδ^a to BALB/c spleen cells could not be blocked by prior capping of mIgM with a rabbit anti-mouse IgM or by prior incubation of the Fl-RαMδ^a with the BALB/c IgM κ myeloma protein TEPC-183. Both these observations indicate that the reagent is not specific for μ or κ-chains and reinforce the idea that it is δ-specific.

The amount of Fl-RαMδ^a bound by a given cell population can be measured by the use of a parameter, CSA, which is the summation for all fluorescence channels of the product of the fluorescence intensity of a given channel and the number of cells in that channel. The CSA of the BALB/c splenic lymphocyte population incubated with Fl-RαMδ^a (Fig. 2) was 79.2×10^5 units whereas the C.B20 splenic lymphocytes had a CSA of 15.9×10^5 units. This quantitative measure of the binding of Fl-RαMδ^a to splenic lymphocytes reinforces the conclusion that the reagent is allospecific and provides a useful approach to the study of the binding of anti-δ to cells.

IgD allotyping by direct binding of Fl-10-4.22 and Fl-RαMδ^a to spleen cells. The allotypes of mIgD of a series of 27 inbred mouse strains were determined by using Fl-10-4.22 and Fl-RαMδ^a. Spleen cells were obtained from ~3-mo-old female mice of each strain and were incubated with Fl-10-4.22 and Fl-

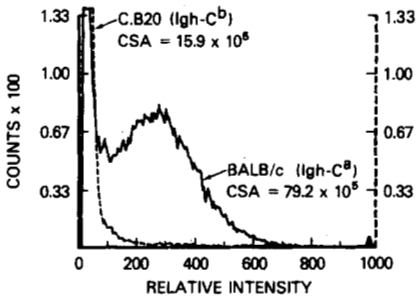

Figure 2. FACS analysis of BALB/c and C.B20 spleen cells incubated with Fl-RαMδ^a and extensively washed.

RαMδ^a. The cells were extensively washed and then analyzed on the FACS. Representative fluorescence profiles and CSA are presented in Figure 3*A*, for F-10-4.22, and Figure 3*B*, for Fl-RαMδ^a. CSA and percent positive cells are shown in Table I.

The Fl-10-4.22 reagent yielded a CSA of 6.9 × 10^5 units on BALB/c spleen cells and 2.1 × 10^5 on C.B20 spleen cells. The former is regarded as the index positive value and the latter, the index negative value. A parameter, percent specific fluorescence, was defined by using these index values. Of the mice studied, strains of *Igh-C a, c, f, g, h, j,* and *n* types were positive and exhibited percent specific fluorescence ≧72. The negative strains were those of the *b* and *e* types; they had percent specific fluorescences of 10 or less. Among strains previously typed as *Igh-C*^{d}, one, AKR, was positive with Fl-10-4.22 and two, AL/N and C.AL20, were negative. C.AL20 is a BALB/c congenic strain possessing *Igh-C* and *Igh-V* genes of AL/N.

The frequency of positive cells in these strains was also determined. This was done by subtracting the fluorescence profiles of the index negative population, C.B20, from the profiles of the experimental groups, as previously described (24). This method was chosen because of the lack of a clear inflection separating negative and positive cells. The frequency of positive cells in the various inbred strains paralleled the results obtained by CSA analysis.

The Fl-RαMδ^a reagent divided the inbred strains into three groups: those strongly positive, which displayed percent specific fluorescences ≧66; those clearly negative, which had percent specific fluorescences ≦6; and those weakly positive, which had percent specific fluorescences ranging from 19 to 46. The strains with strongly positive fluorescence included mice of the *Igh-C a, c, f, g, h, j,* and *n* types and the *Igh-C*^{d} strain AKR. The negative strains were all those of the *b* type. The intermediate strains were those of the *e* type and the two *d* strains, AL/N and C.AL20, which had been negative with Fl-10-4.22.

The percentage of cells that were calculated to be positive was generally lower in the intermediate strains than in the clearly positive strains. However, the positive cells in these intermediate strains have lower fluorescence intensities than do the positive cells in clearly positive strains (Fig. 3*B*). Thus, it is likely that we are underestimating the number of positive cells in the intermediate strains through the use of the subtraction analysis (24). Indeed, cells from strains that stained weakly or not at all with αMδ^a reagents stained well with αMδ^b or αMδ^e reagents, whereas such reagents failed to stain cells from mice of the *Igh-5*(δ)^a type (13). Furthermore, all strains tested had relatively similar proportions of mIgD$^+$ cells when the Fl-RαMδ_c reagent was used.

IgD allotyping by absorption of RαMδ^a. Because Fl-RαMδ^a appeared to bind to splenic lymphocytes of A, AL/N, and C.AL20 to an intermediate degree, we wished to confirm the direct binding results by alternative approaches. To do so, we tested the capacity of spleen cells from a variety of strains to absorb the antibodies in Fl-RαMδ^a that could bind to BALB/c splenic lymphocytes. A fixed amount of Fl-RαMδ^a was incubated with 10, 30, or 100 × 10^6 spleen cells from each of the 27 inbred strains studied above. The resulting absorbed antisera were then incubated with BALB/c spleen cells, and fluorescence profiles and CSA were determined by FACS analysis (Fig. 4*A*). Unabsorbed Fl-RαMδ^a gave a strongly positive CSA with BALB/c spleen cells (93 × 10^5 units), whereas a control Fl-antibody (Fl-RαKLH) yielded a CSA of 40 × 10^5 units. Absorption of Fl-RαMδ^a with BALB/c spleen cells reduced the CSA yielded by the reagent to that of the control Fl-RαKLH. In contrast, absorption of Fl-RαMδ^a with C.B20 spleen cells caused only a minimal decrease in the CSA obtained on BALB/c spleen cells; this decrease most probably represents nonspecific absorption of RαMδ^a.

Spleen cells from all strains that had been typed as strongly positive by direct binding of Fl-RαMδ^a absorbed the binding activity of the reagent quite efficiently. These absorbed sera gave CSA on BALB/c spleen cells that fell within the shaded area in Figure 4*A*. Spleen cells from A, AL/N, and C.AL20 mice absorbed significantly more Fl-RαMδ^a than did C.B20 cells but

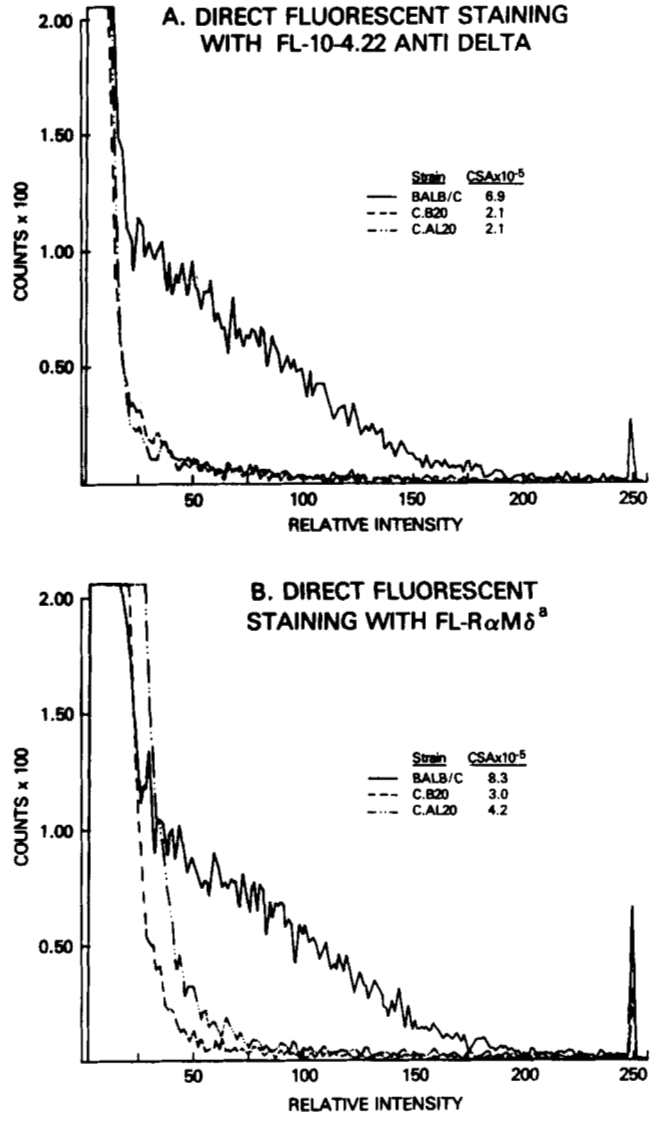

Figure 3. FACS analysis of BALB/c, C.B20, and C.AL20 spleen cells incubated with Fl-10-4.22 (*A*) or Fl-RαMδ^a (*B*) and extensively washed.

TABLE I

IgD allotype determination by capacity of 10-4.22 and RαMδ[a] to bind to spleen cells

Strain	*Igh-C* Type	Fl-Anti-δ[a] Reagent					
		10-4.22			RαMδ[a]		
		CSA		% Positive cells	CSA		% Positive cells
		Units × 10^{-5}	% Specific fluorescence[a]		Units × 10^{-5}	% Specific fluorescence[a]	
BALB/cJ	*a*	6.9	100	38	8.3	100	36
ST/6J	*a*	7.2	106	36	7.9	92	40
SJA	*a*	7.2	106	38	7.8	90	40
C57BL/6J	*b*	2.0	−2	ND[b]	3.2	2	ND
LP/J	*b*	2.4	6	7	3.2	2	10
SJL/J	*b*	2.3	4	5	3.4	6	6
C.B20	*b*	2.1	0	0	3.1	0	0
DBA/2J	*c*	8.3	129	ND	7.7	89	ND
DBA/1J	*c*	13.2	231	48	12.6	182	ND
RF/J	*c*	7.1	104	29	7.1	77	34
AKR/J	*d*	8.1	125	42	7.7	89	47
CE/J	*f*	11.4	193	44	7.8	90	ND
RIII	*g*	9.2	148	43	7.1	77	ND
P/J	*h*	8.9	142	34	8.3	100	ND
CBA/J	*j*	8.8	140	34	10.5	142	29
CBA/N	*j*	7.1	104	22	6.7	69	28
C3H/HeJ	*j*	7.9	121	30	7.3	81	34
C3H/HeN	*j*	6.5	92	33	7.1	77	38
PL/J	*j*	7.6	114	31	8.2	98	36
NZB/N	*n*	5.5	72	22	6.5	66	24
NZW/N	*n*	8.2	127	34	7.0	75	35
A/J	*e*	2.6	10	2	4.1	19	15
A/HeN	*e*	2.0	−2	1	4.4	25	18
A/HeJ	*e*	2.4	6	2	5.1	38	22
AL/N	*d* (*o*)[c]	2.7	12	8	5.5	46	26
C.AL20	*d* (*o*)[c]	2.1	0	1	4.2	21	13

[a] % Specific fluorescence = $\frac{CSA_{test\ strain} - CSA_{C.B20}}{CSA_{BALB/c} - CSA_{C.B20}} \times 100$.

[b] Calculation of percent positive cells not done.

[c] AL/N and C.AL20 mice, both previously typed as *Igh-C*[d], are now designated as of the *Igh-C*[o] type based on the δ typing studies.

the reduction in CSA caused by cells of these strains was much less striking than that caused by the strongly positive strains. For example, 10×10^6 spleen cells from strongly absorbing strains removed approximately 70% of the anti-δ activity from Fl-RαMδ[a], whereas 100×10^6 spleen cells from AL/N, C.AL20, or A mice absorbed approximately 40% of the activity from the same reagent. These absorption studies indicate that spleen cells from the strongly positive strains bear all of the determinants that RαMδ[a] recognizes on BALB/c spleen cells, since spleen cells from the strongly positive strains can completely absorb the capacity of Fl-RαMδ[a] to bind to BALB/c spleen cells. In contrast, we failed to observe complete absorption of Fl-RαMδ[a] with spleen cells from the intermediate strains, but since we did not reach a plateau value we cannot be certain whether the intermediate strains possess all or only some of the determinants that RαMδ[a] recognizes on BALB/c IgD.

IgD allotyping by absorption of Fl-10-4.22. A fixed amount of Fl-10-4.22 was absorbed with varying numbers of spleen cells from representative strains and analyzed on BALB/c spleen cells as described above for absorption of Fl-RαMδ[a]. A, AL/N, and C.AL20 spleen cells had no specific absorptive capacity for Fl-10-4.22 since they did not differ from C.B20 spleen cells (Fig. 4*B*). In contrast, BALB/c, AKR, and NZB spleen cells had strong absorptive capacity for Fl-10-4.22 as judged by the reduced CSA the absorbed reagent yielded on BALB/c spleen cells. These results confirm the direct-binding studies that indicate that AL/N, C.AL20, and A lack the determinants detected by Fl-10-4.22.

IgD allotyping by absorption of C57BL/Ka anti-BALB/c IgD. The alloanti-δ antiserum prepared by immunizing C57BL/Ka mice with BALB/c spleen cells cannot be used to type inbred strains for IgD allotype by direct binding of fluoresceinated antibody since the serum is known to contain appreciable activity against other lymphocyte membrane antigens, including major histocompatibility complex (MHC) antigens. However, after anti-μ activity is removed by passage over an IgM affinity column, the serum specifically identifies IgD on B.C8 cells, which are derived from a C57BL/Ka congenic strain that possesses the *Igh-C*[a] gene complex. This MαMδ[a] was fluoresceinated and assayed by absorption of its capacity to bind to B.C8 cells. Spleen cells from all strains typed as positive with Fl-10-4.22 or strongly positive with RαMδ[a] completely removed the anti-δ activity from Fl-MαMδ[a] (Fig. 4*C*). These results indicate that spleen cells of each of these strains bear all the IgD determinants that Fl-MαMδ[a] recognize on spleen cells from mice of the *Igh-C*[a] type. Spleen cells from mice that typed as negative with RαMδ[a] removed only modest amounts of anti-δ-activity from Fl-MαMδ[a]. Absorption of Fl-MαMδ[a] with spleen cells from strains classed as intermediate with RαMδ[a] gave variable results. Thus, A/J spleen cells did not absorb more activity from Fl-MαMδ[a] than did C.B20 cells whereas A/HeN and C.AL20 spleen cells removed some anti-δ activity from MαMδ[a], although substantially less than that absorbed by the strongly positive strains. Although we suspect that strains of the *Igh-C*[e] type and the two *Igh-C*[d] strains, AL/N and C.AL20, will prove to express some δ-determinants recognized by MαMδ[a], the results are too uncertain to reach a decisive conclusion on this issue.

IgD allotyping by immunoprecipitation of radioiodinated NP-40 extracts with RαMδ[a], 10-4.22, and MαMδ[a]. In order to confirm the IgD allotyping of both C.AL20 and A/J strains, splenic lymphocytes from mice of these strains and from BALB/c and C.B20 mice were radioiodinated by the lactoperoxidose technique. NP-40 extracts from these cells were immunoprecipitated by incubation with each of the three anti-δ[a] reagents (RαMδ[a], 10-4.22, and MαMδ[a]), followed by the addition of *S. aureus* Cowan I microorganisms. The immunoprecipitates were dissolved in SDS, reduced, and analyzed by SDS-PAGE. Control precipitates prepared with anti-κ precipitated substantial amounts of polypeptide chains with the mobility of the δ H chain from each radioiodinated lysate (Fig. 5). RαMδ[a] precipitated no δ from the C.B20 lysate, a large amount of δ from the BALB/c lysate, and small but definite amounts of δ from both C.AL20 and A/J lysates (Fig. 5*A*). 10-4.22 precipitated a chain with SDS-PAGE mobility of δ from the BALB/c lysate, but not from the C.B20, C.AL20, or A/J lysates (Fig. 5*B*). MαMδ[a] contains, in addition to anti-δ, antibodies specific for MHC antigens and probably other membrane molecules. It

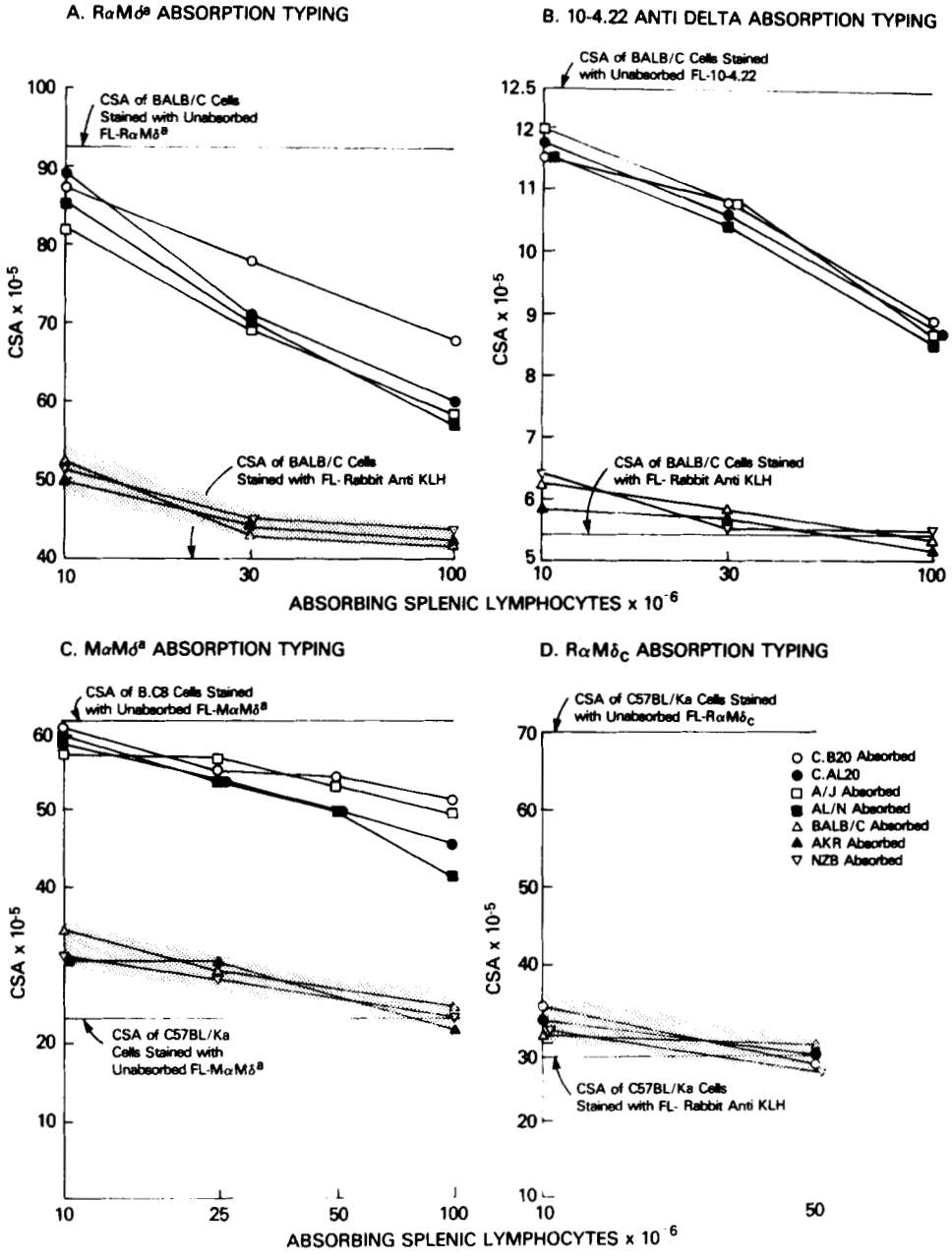

Figure 4. IgD allotyping by absorption of Fl-RαMδ^a (*A*), Fl-10-4.22 (*B*), Fl-MαMδ^a (*C*), or Fl-RαMδc (*D*). Fl-reagents were absorbed with graded numbers of spleen cells from 27 strains of mice (*A*, *C*, and *D*) or from 7 strains (*B*). Absorbed Fl-reagents were then used to bind to BALB/c (*A* and *B*), B.C8 (*C*), or C57BL/Ka (*D*) spleen cells. Shaded areas in Figure 3*A*, 3*C*, and 3*D* encompass CSAs obtained after absorption of indicated sera with the positive strains not individually shown in the figures.

precipitated polypeptides with apparent m.w. of ~33,000 and ~25,000 daltons from all four lysates; most likely, these molecules are Ia antigens. In addition, MαMδ^a precipitates a molecule with the mobility of δ-chain from BALB/c. Small amounts of radioactivity associated with molecules with the mobility of δ-chain were observed in the C.AL20 lysate; however, this was no greater than that found in the C.B20 lysate. This strongly suggests that MαMδ^a contains very little anti-δ that cross-reacts with δ from A/J and C.AL20 donors. Thus MαMδ^a resembles 10-4.22 more than RαMδ^a in this respect.

Strain distribution of the common IgD specificity present in RαMδc. RαMδc contains the anti-IgD specificities present in RαMδ^a in addition to anti-IgD antibodies that do not distinguish between BALB/c and C57BL/6 IgD. The latter can be detected by direct binding of Fl-RαMδc to C57BL/6 spleen cells. The fluorescence profiles displayed by C57BL/6 spleens incubated with Fl-RαMδc are similar to those that RαMδ^a produces on BALB/c spleen cells (Fig. 2). The capacity of Fl-RαMδc to bind to C57BL/6 spleen cells is not affected by prior capping of membrane IgM or by preincubation of the reagent with TEPC-183, a BALB/c IgM κ-myeloma protein. Preincubation of RαMδc with TEPC-1017, a BALB/c IgD κ myeloma protein, completely blocks binding to spleen cells. These results strongly suggest the RαMδc is specific for IgD determinants. To examine the strain distribution of the antigenic determinants identified on C57BL/6 spleen cells by Fl-RαMδc, a fixed amount of the reagent was absorbed with varying numbers of spleen cells from each of the 27 inbred strains of mice used previously; the resulting absorbed reagent was then used in binding to C57BL/6 spleen cells. As shown in Figure 4*D*, cells from each of the strains tested were able to completely absorb the RαMδc activity. This indicates that the common antigenic determinant(s) expressed on C57BL/6 IgD is also found on IgD of each of the other tested strains.

Cross-blocking studies with RαMδ^a, MαMδ^a, 10-4.22, and RαMδc. The anti-δ antibodies in RαMδ^a, MαMδ^a, 10-4.22, and RαMδc all bind to the mIgD of spleen cells from mice of the *Igh-C^a* haplotype group. To further study the IgD determinants

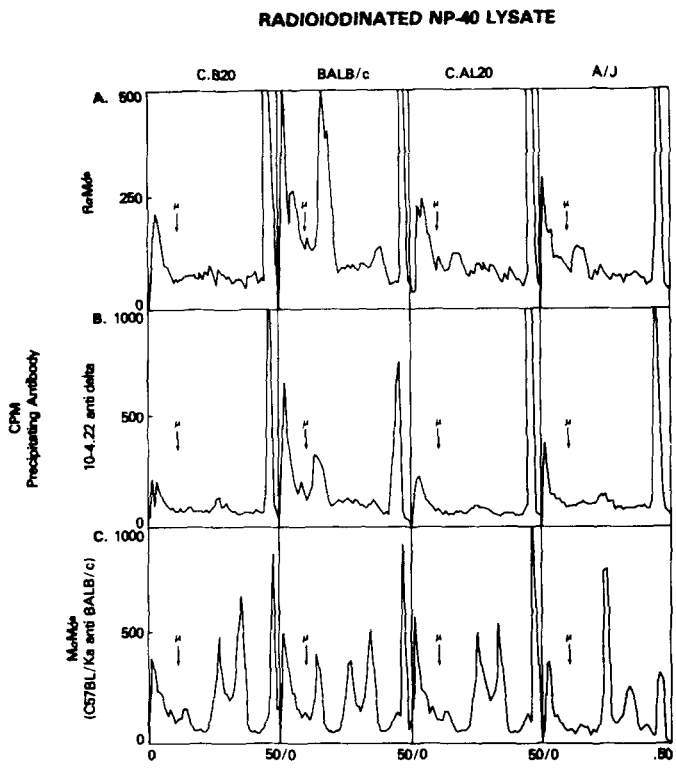

Figure 5. SDS-PAGE analysis of immunoprecipitates of ^{125}I-labeled lysates of C.B20, BALB/c, C.AL20, and A/J spleen cells. RαMδ^a, 10-4.22, and MαMδ^a were used for immunoprecipitation.

reactive with each of these antibodies, the capacity of each reagent to block the binding by the other anti-δ antibodies was determined. Spleen cells from the *Igh-C*^{a} C57BL/Ka congenic strain B.C8 were used as target cells in these studies in order to permit binding experiments with MαMδ^a. Target lymphocytes were incubated with either unfluoresceinated RαMδ^a, MαMδ^a, 10-4.22, or RαMδ_c for 2 hr at 4°C, in the presence of 0.2% NaN_3, at a concentration sufficient to completely inhibit subsequent binding of fluorescein conjugates of the same anti-δ with which the cells had been preincubated. Aliquots of these pretreated spleen cells were incubated with fluorescein conjugates of each of the anti-δ reagents, again in the cold and in the presence of NaN_3. In addition, an excess of the unfluoresceinated anti-δ that had been used in the pretreatment step was present.

To control for nonspecific blocking, lymphocytes pretreated with 10-4.22 were compared to lymphocytes pretreated with CBPC-101; both are IgG_{2a} proteins of the *b* allotype. Lymphocytes pretreated with MαMδ^a were compared to those pretreated with normal mouse serum. RαMδ^a and RαMδ_c pretreated lymphocytes were compared to lymphocytes pretreated with normal rabbit serum. Inhibition of binding as a result of pretreatment was calculated from reduction in CSA as indicated in *Materials and Methods*.

It was observed that RαMδ^a failed to inhibit the binding of Fl-10-4.22 to spleen cells (Table II). This indicates that 10-4.22 and RαMδ^a react with independent determinants on IgD of the *a* allotype. In keeping with previous work (11, 13), we designate the determinant recognized by RαMδ^a as IgD.36 in the Lieberman notation (Ig5.1 in the Herzenberg notation); the determinant recognized by 10-4.22 is designated IgD.43 (Ig5.4) (Table III).

RαMδ_c blocks the binding of Fl-RαMδ^a to B.C8 spleen cells. This is not surprising, since by the method of its preparation RαMδ_c should contain appreciable amounts of RαMδ^a antibody. However, RαMδ_c also blocks the binding of Fl-10-4.22 to B.C8 spleen cells. The antibody responsible for this blocking may either be specific for determinant 43 or may be directed at a non-allotypic antigenic structure sufficiently near determinant 43 so that steric hinderance prevents the simultaneous binding of RαMδ_c and Fl-10-4.22 to IgD.

MαMδ^a pretreatment blocks the binding of both Fl-10.4.22 and Fl-RαMδ^a to B.C8 spleen cells suggesting that MαMδ^a contains both anti-36 and anti-43 antibodies. Surprisingly, pretreatment with either RαMδ^a or RαMδ_c fails to cause any detectable inhibition of binding of MαMδ^a. Since the pretreatment with the two rabbit reagents should prevent binding to determinants 36 and 43, this result suggests that the bulk of the anti-δ antibodies in MαMδ^a are directed at determinants other

TABLE II

Blocking of immunofluorescence by preincubation with unlabeled anti-δ

Blocking Antibody	Fl-Antibody	% Inhibition of Immunofluorescence[a]	Comments
RαMδ^a	10-4.22	2%, 11%	10-4.22 is arbitrarily defined as being specific for determinant 43. RαMδ^a lacks anti-43 specificities as it does not block 10-4.22 binding.
MαMδ^a	10-4.22	99%, 97%	MαMδ^a contains anti-43 specificities as it blocks 10-4.22 binding.
RαMδ_c	10-4.22	99%, 61%	RαMδ_c contains antibodies capable of blocking binding to 43 as it blocks 10-4.22 staining.
MαMδ^a	RαMδ^a	98%, 54%	Since MαMδ^a blocks binding of RαMδ^a, MαMδ^a, and RαMδ^a share anti-δ antibodies specific for a determinant other than determinant 43. This new determinant is arbitrarily defined as 36.
RαMδ_c	RαMδ^a	100%, 50%	RαMδ_c has antibody directed at 36 as well as antibody capable of blocking binding to 43.
RαMδ^a	RαMδ_c	41%, 33%	Partial blocking is probably due to presence of anti-36 in both antisera.
MαMδ^a	RαMδ_c	53%, 34%	Partial blocking may represent presence in MαMδ^a and RαMδ_c of antibodies specific for 36 and 43.
RαMδ^a	MαMδ^a	0%, 8%	Failure to block indicates that RαMδ^a lacks antibody to a principal specificity recognized by MαMδ^a.
RαMδ_c	MαMδ^a	0%, 3%	Failure to block indicates that RαMδ_c lacks antibody to a principal specificity recognized by MαMδ^a. Since RαMδ_c can block binding to 36 and 43, this suggests that MαMδ^a recognizes a new IgD determinant in addition to 36 and 43.

[a] Results of 2 separate experiments.

TABLE III

Definition of IgD^a allotypic determinants

Reagent	Positive Haplotypes	Comments	Determinants Recognized	
			Lieberman notation	Herzenberg notation
RαMδ^a	*a, c, d, f, g, h, j, n*	1) Reactivity blocked by preincubation of cells with MαMδ^a. 2) Present on IgD Fab (19).	IgD.36	Ig5.1
RαMδ^a	*e, o*; possibly *a, c, d, f, g, h, j, n*	1) "Weak" reactivity of RαMδ^a. 2) May be an independent antigen recognized by a 2nd antibody within RαMδ^a or an antigen cross reactive with IgD.36	IgD.45	Ig5.5
10-4.22	*a, c, d, f, g, h, j, n*	1) Reactivity blocked by preincubation of cells with MαMδ^a but not RαMδ^a. 2) Present on IgD Fc (19).	IgD.43	Ig5.4
MαMδ^a (C57BL/Ka anti-BALB/c)	*a, c, d, f, g, h, j, n*	1) Preincubation of cells with MαMδ^a blocks subsequent reactivity of RαMδ^a and 10-4.22. 2) Reactivity with A, AL/N, and C.AL20 uncertain. 3) Not blocked by preincubation of cells with RαMδ^a or RαMδ_c.	IgD.36 IgD.43 ?IgD.45 ?A major additional determinant	Ig5.1 Ig5.4 ?Ig5.5

than 36 and 43.

Pretreatment with 10-4.22 fails to inhibit subsequent binding of Fl-RαMδ^a, Fl-RαMδ_c, or Fl-RαMδ^a. These results are consistent with the analysis developed thus far. However, 10-4.22 appears to be easily washed off lymphocytes, probably because it binds to IgD with a relatively low affinity. The failure of 10-4.22 to block might be due to displacement of low affinity 10-4.22 antibodies by higher affinity anti-δ antibodies directed at the same determinant. Consequently, 10-4.22 blocking data must be interpreted with caution. However, we do not believe this consideration alters our initial conclusion that RαMδ^a and 10-4.22 are directed at separate determinants, particularly since Kessler et al. (17) has recently shown that the determinants recognized by the two reagents are on physically separable parts of the IgD molecule.

Assignment of determinants on IgD of the a allotypic form. The results reported here indicate the existence of at least two and probably three separate allotypic determinants recognized by RαMδ^a. These are the determinants recognized by 10-4.22 and the determinant or determinants recognized by RαMδ^a. As noted above, 10-4.22 recognizes IgD.43 (Ig5.4); the strain distribution of this determinant is given in Table III. RαMδ^a recognized IgD.36 (Ig5.1); in addition, it appears to contain antibodies directed at a second determinant found on BALB/c spleen cells and on spleen cells from mouse strains of the *Igh-C^e* type and on C.AL20 and AL/N spleen cells. An alternative interpretation of our results is that *Igh-C^e*, AL/N, and C.AL20 cells contain a cross-reactive determinant, similar to IgD.36 and reactive with all antibodies in RαMδ^a, although with lower affinity. It does not appear likely that the poor binding and absorption expressed by these strains can be due to the presence of fewer IgD molecules since they absorb Fl-RαMδ_c as efficiently as BALB/c spleen cells. Consequently, we recognize a new δ determinant expressed on cells of *Igh-C^e* mice, C.AL20, and AL/N. This determinant is designated IgD.45 (Ig5.5). Whether this determinant is cross-reactive with IgD.36 or whether BALB/c mice and other mice of the δ^a type express both IgD.36 and IgD.45 is not resolved.

Our allotyping results confirm the prior removal of NZB and NZW mice from the *e* haplotype and their assignment to the *n* haplotype based on the findings that NZB and NZW mice express the *a* form of IgD (13, 25, 26), whereas mice of the *e* haplotype express a distinctive (*e*) form of IgD. Furthermore, our results indicate that the *Igh-C^d* haplotype should now be subdivided into two haplotypes, based on differences at the *Igh-5*(δ) locus of AKR mice, which express the *a* allelic form of δ and C.AL20 and AL/N mice, which express the *e* allelic form. We retain the designation *Igh-C^d* for the *Igh-C* haplotype of AKR mice and designate C.AL20 and AL/N mice as possessing the *Igh-C^o* haplotype.

DISCUSSION

The results presented here describe the distribution of three allotypic IgD determinants in a wide variety of inbred mouse strains representing each of the pricipal *Igh-C* haplotypes. Each of these determinants is expressed by IgD of the BALB/c (a) type. Two of the determinants, that recognized by 10-4.22 and the principle determinant recognized by RαMδ^a, have been previously recognized (11, 12, 19, 24), although the RαMδ^a reagent described here provides a more precise reagent for the definition of IgD.36 (Ig5.1) than the mouse reagent formerly used for this purpose because RαMδ^a lacks antibodies to the determinant recognized by 10-4.22 (IgD.43; Ig5.4).

In this regard, it was somewhat surprising to us that allospecific RαMδ^a was so simple to prepare. Even without absorption, early bleeds of the immunized rabbits were largely allospecific. Such allospecificity in heteroimmunization is not without precedent, however. It is quite common for goat and rabbit antibodies prepared against BALB/c (a) form of IgG_{2a} to react with the immunogen but not with the C57BL (b) form of the same proteins. Very recently, Finkelman *et al* have utilized purified mouse serum IgD (23) or a mouse IgD myeloma protein (27) to prepare anti-δ allotype antibodies in goats and rabbits; thus, heteroantibodies specific for IgD.36 (Ig5.1) should be quite readily available.

One of the new determinants described in this paper, IgD.45, is defined by antibodies within the RαMδ^a. This determinant is of substantial interest because its strain distribution is different from both IgD.36 and IgD.43 in that mice of the *e Igh-C* group

and AL/N and C.AL20 mice express this determinant, whereas they fail to express either 36 or 43. Furthermore, our results suggest that MαMδ^a recognizes additional, yet undefined, allotypic determinants since neither RαMδ^a or RαMδ_c cause any substantial block of the binding of MαMδ^a to B.C8 spleen cells.

Finally, our results on the characterization of IgD allotypes lead to several interesting speculations about recombination of genes within the *Igh-C* region, which will be more fully considered in the discussion to the companion paper (13). It should be pointed out here that two *Igh-C* haplotypes, *n*, represented by NZB and NZW, and the newly defined *o* type, represented by AL/N and C.AL20, could be considered as having arisen by recombination events between the gene for δ (*Igh-5*) and the genes for the other H chain constant regions. Furthermore, since AL/N and C.AL20 mice express many idiotypic determinants that are characteristic of antibodies of the A (*Igh-C*^{a}) but not AKR (*Igh-C*^{d}) strains (28), these results suggest that the *Igh-5*(δ) gene is more closely linked to the *Igh-V* genes than are the *Igh-1*(γ_{2a}) or *Igh-3*(γ_{2b}) genes.

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