ALLOANTIGEN-SPECIFIC IDIOTYPE-BEARING RECEPTORS ON MOUSE T LYMPHOCYTES

I. Specificity Characterization and Genetic Association with the Heavy-Chain IgG Allotype*

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B lymphocytes express their specificity through Ig receptor molecules that are actively synthesized by the cells and subsequently released into the body fluids when the B cells have reached a certain step in their differentiation toward plasma cells (1). The genes responsible for the synthesis of the B-cell Ig receptors in the mouse seem to be situated on at least three different chromosomes (2), the heavy-chain-linkage group on chromosome 12 (2, 3), the kappa-light-chain genes on chromosome 6 (2–5), and the lambda-chain genes on chromosome 9, 13, or 16 (2).

T lymphocytes also express antigen-specific membrane-bound receptor molecules, and the range of functional specificities displayed by T cells may be similar to that of B cells (6, 7). The detailed biochemical nature of T-cell receptors (Tcr[s])[1] is, however, largely unknown; one reason being the lack of regularly and actively released specific Tcr molecules. Recent data on released helper- and suppressor-T-cell products have demonstrated the presence of antigen-binding sites, Ig-like idiotypes (Id), and Ia antigens on such molecules (8–10). T-cell lymphomas seem to synthesize products that contain Ig heavy- and (or) light-chain determinants (11–13), and some T-cell products express different combinations of the aforementioned determinants (6, 14–18). If all these products should prove to represent true Tcr determinants, then the buildup of Tcr(s) would depend on gene products from at least four different chromosomes,

* Supported by the Danish Medical Research Council grant 512-3506, 512-8729; the Swedish Cancer Society; and National Institutes of Health grant AI-CA-13485, and the UICC.

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[1] *Abbreviations used in this paper:* ADCC, antibody dependent cell-mediated cytotoxicity; Bcr, B-cell receptor; CFA, complete Freund's adjuvant; CTL, cytotoxic T lymphocyte; Con A, concanavalin A; ER, 1:1 mixture of Eagle's high amino acid medium and RPMI-1640; Fcr^-, Fc receptor negative; FCS, fetal calf serum; FIP, Ficoll-Isopaque; FITC, fluorescein isothiocyanate; GH, guanidine hydrochloride; γG, 3 times $(NH_4)_2SO_4$ precipitated γ-globulin; HSA, human serum albumin; Id, idiotype; Id^+, idiotype positive; Id^-, idiotype negative; IFF, immunofluorescence; LU, lytic unit(s); MγG, mouse γ-globulin; MHC, major histocompatibility complex; MLC, mixed lymphocyte culture; MRBC, mouse erythrocytes; NMS, normal mouse serum; NP, the hapten hydroxynitrophenacetyl; NRS, normal rabbit serum; pA, protein A; PBS, 1/15 M phosphate-buffered saline, pH 7.38; R, RPMI-1640 medium; RC′, rabbit complement; SRBC, sheep erythrocytes; Tcr, T-cell receptor.

because the Ia antigens are coded for by genes in the major histocompatibility complex (MHC) on chromosome 17 (19).

The questions emerge whether all these genes are structural genes for one and the same type of Tcr, or for two different types of receptors (20), or whether some of the genes are regulatory genes. We have initiated a study on these problems using as probes anti-Id antibodies raised against mouse anti-H-2 antibodies. Because we have shown previously (*a*) that such anti-Id antibodies can be produced in relatively large amounts, and (*b*) that they react against both Tcr and B-cell receptor (Bcr) molecules of the same specificity (21), these reagents can be used for isolation and purification of Id-positive (Id^+) Tcr(s). Studies on the structure of such purified Tcr(s) would demonstrate directly which of the aforementioned determinants were present.

The data in this paper will demonstrate that the expression of specific Id^+ receptor molecules on a panel of activated T cells is associated with the Ig heavy-chain-linkage group and not with the MHC (6, 14, 15). Furthermore, data to be published in a subsequent paper will show that our Id^+ T-cell products do not express Ia antigen[2] (14). Thus, these results suggest that structural genes for at least one type of Tcr are closely associated with the structural genes for Bcr(s).

Materials and Methods

Animals. Inbred mice were maintained in our breeding colony at the Immunobiology Laboratory, Statens Seruminstitut Copenhagen, Denmark (21, 22). They possessed the following H-2 types (H-2^b, abbreviated: b) and Ig-1 allotypes (Ig-1^a, abbreviated: a); e.g., C57BL/6 (B6) is H-2^b/Ig-1^b or, for short, b/b; B10.A (a/b); B6 or B10 (b/b); A.BY (b/e); C3H.SW (b/a^-); BALB/B (b/a^+); B10.D2 (d/b); BALB/c (d/a^+); DBA/2 (d/c); A.CA (f/e); B10.A(4R) (hi/b); CBA/J (k/a^-); B10.Br (k/b); C3H.OH (o2/a^-); DBA/1 (q/c); and A.SW (s/e). C3H.B10 (b/a^-); B10.M (f/b); C3H.OL (o1/a^-); B10.RIII (r/b); B10.S (s/b) mice, and ATL (tl/e); ATH (t2/e); CB20 (d/b); AQR (y1/e) mice were obtained from the Centre d'Immunologie INSERM-CNRS de Marseille-Luminy, Marseille, France, and the Institute for Immunology, Biomedical Center, Uppsala University, Uppsala, Sweden. Strain B65a mice were produced by backcrossing (B6 × CBA)F_1 mice to B6 mice. Selection was made for H-2^b and Ig-1^a during five generations. Then H-2^b, Ig-1^b heterozygotes were mated; and H-2^b, Ig-1^a/Ig-1^a mice were inbred by brother-sister mating. These mice were unexpectedly found Id^+ with our rabbit 5,936 anti-Id antiserum (see Results). They were therefore tested for a possible crossing-over between the V_H and C_H genes. B65a mice were immunized with chicken γ-globulin coupled with the hapten hydroxy-nitrophenacetyl (NP) and day-28 antisera and were tested for heteroclitic antibodies (23). The results showed that B65a anti-NP antibodies were heteroclitic and, therefore, these mice contain at least part of the Ig-1^b V_H genes (U. Krawinkel and B. Rubin, unpublished data).

Inbred strain-2 guinea pigs and selected outbred rabbits donated complement. Pregnant rabbits were purchased from Statens Seruminstitut. Newborn rabbits were made neonatally tolerant to mouse γ-globulin (MγG) as described previously (21). Adult outbred rabbits produced test antisera as described (21, 22).

Immunization

ANTI-H-2 ANTISERA. Mice were immunized subcutaneously (s.c.) or intraperitoneally (i.p.) with 20 × 10^6 histoincompatible spleen cells with or without complete Freund's adjuvant (CFA) in 1:1 or 1:9 mixtures (21). At least six weekly immunizations were given before the first bleeding. Some mice immunized with spleen cells/CFA at a ratio of 1:9 developed ascites. These animals would produce 40–60 ml of ascites/serum.

ANTI-ALLOTYPE ANTISERA. Ig-1^a- or Ig-1^b-specific antisera were raised by immunizing B6

[2] Kimura, A., B. Rubin, B. Hertel-Wulff, and H. Wigzell. Alloantigen-specific idiotype-bearing receptors on mouse T cells. II. Partial biochemical characterization of the receptor molecules. Manuscript in preparation.

and Balb/c × CBA mice s.c. with 500 μg CBA Ig, and B6 Ig, in CFA, respectively. After 4 wk, these mice were boosted three times i.p. at weekly intervals with 0.25 ml CBA anti-B6 and B6 anti-CBA antiserum, respectively. Anti-allotype antisera were harvested 7 and 14 d after the last immunization.

ANTI-(ANTI-H-2) ANTISERA. MγG-tolerant rabbits were immunized with DEAE-cellulose-purified IgG from four individual B6 anti-CBA spleen-cell immune mice. (Two of these IgG preparations were kindly donated by Dr. H. Frieschknecht, Department of Microbiology, University of Zürich, Zürich, Switzerland.) The rabbits received 100 μg of IgG in CFA on days 0, 28, 63, 103, and 138; they were bled for 40 ml of blood 7, 14, 21, and 28 d after each immunization as described previously (21) (see Results). Out of 16 rabbits immunized, 4 rabbits produced strongly precipitating anti-(B6 anti-CBA) antibodies. Antisera from rabbit 5,936 were selected for this study.

Anti-H-2 Serology. The presence of cytotoxic antibodies was assayed by a trypan-blue-exclusion test. 0.2 ml of different antiserum dilutions were added to 0.1 ml of target cells (2×10^6 spleen cells). This mixture was incubated for 1 h at 4°C followed by centrifugation and resuspension in 0.5 ml rabbit complement of (RC′) (1:5). The incubation was continued for 1 h at 37°C. Then, trypan blue was added and the number of trypan-blue-excluding cells was counted. Control mixtures included target cells alone, target cells and normal mouse serum (NMS), and target cells and RC′. The percentage of killed cells in the latter combination was 5–20%. Antigen-binding anti-H-2 antibodies were detected as described previously (24).

Adsorption of Antisera. Anti-H-2 antisera or γ-globulin (γG) preparations (10 mg/ml) were diluted in balanced salt solution and adsorbed with 0.5 ml of packed mouse erythrocytes (MRBC) or with 0.5 ml of spleen cells for 1 h at 4°C. Adsorptions were repeated until antibody activity against adsorbing cells was negative as defined by the cytotoxicity assay.

Immunochemistry. MγG was prepared from NMS or from anti-H-2 antisera as described previously (21). IgG preparations were obtained by DEAE-Sephacel (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) fractionation. All preparations were adjusted to 10 mg/ml. Protein determinations, purity evaluations and the micro-Ouchterlony assay were described previously (21).

γG preparations of normal serum or antisera were coupled to cyanogen bromide-(CNBr) activated Sepharose 4B-CL (Pharmacia Fine Chemicals, Inc.) for 2 h at pH 8.7, and then washed with 0.1 M $NaHCO_3$ buffer, pH 9, containing 0.1 M ethanolamine, and with 1% normal rabbit serum (NRS) per 1/15 M phosphate-buffered saline (NRS/PBS) (25). The beads were stored in 1% NRS/PBS, containing 0.02 M NaN_3. Absorbed antibodies were eluted by 5 M guanidine hydrochloride (GH)/1 M NaCl, dialyzed three times against 6 liters of saline and concentrated by negative-pressure dialysis.

Detection of Id^+ T Cells. Binding of rabbit anti-Id antibodies to T blasts was assayed either by a protein A (pA) assay as described before (24) or by an indirect immunofluorescence (IIF) assay: 3×10^6 T blasts were incubated for 1 h at 4°C with either NRS or anti-Id antiserum in 0.02 M NaN_3-containing medium, washed three times at 4°C and further incubated for 1 h at 4°C with fluorescein isothiocyanate-labeled sheep IgG anti-rabbit Ig. After an additional three washes, the number of Id^+ cells was counted as membrane fluorescent cells in a Carl Zeiss/Jena fluorescence microscope (HPO 200, Carl Zeiss, Inc., New York). The porportion of specific Id^+ cells in a given cell population is calculated as the percentage of fluorescent cells counted after incubation with anti-Id antiserum minus the percentage of fluorescent cells counted after incubation with NRS (0–3%) (21).

The frequency of Id^+ T cells was also determined by an anti-Id antiserum and RC′ cytotoxicity assay: 2×10^6 H-2-activated T cells from day 4–5 mixed lymphocyte cultures (MLC), purified on a (Ficoll-Isopaque) (FIP) (Ficoll, Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J., Isopaque, Winthrop Laboratories, New York) gradient as described below, were mixed with 0.2 ml of rabbit anti-human serum albumin (HSA), rabbit anti-thymocyte antiserum, or rabbit anti-(anti-H-2) antiserum diluted 1:5, 1:25, or 1:125 in RPMI-1640 (R) medium, containing 0.02 M NaN_3. The mixture was incubated for 1 h on ice, followed by centrifugation. The supernates were removed and the cells were resuspended in 0.5 ml RC′, diluted 1:5 in R (without NaN_3). Incubation was continued for 1 h at 37°C (waterbath). The reaction was terminated by adding 0.5 ml ice-cold R, containing NaN_3; and the number

of trypan-blue-excluding cells was counted. The percentage of killed cells in the presence of anti-HSA and RC′ was 5–10%.

Preparation and Test of Lymphocyte Cultures. Spleen and lymph-node cells were prepared as described (26, 27). MLC were set up in 3013 Falcon Flasks (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) containing 25×10^6 purified Fc-receptor-negative (FcR^-) T-responder cells (carbonyl-iron- and magnet-treated, followed by passage through Ig-anti-Ig columns) and 10×10^6 anti-Thy-1.2-and-complement-treated parental or F_1 hybrid stimulator cells (21, 26). The medium consisted of 5% fetal calf serum (FCS) in a 1:1 mixture of Eagle's high amino acid medium (28) and R medium (ER); the culture time was 4–5 d. Cells were harvested and further purified using FIP (26). Such activated T cells were 30–95% blasts, >98% Thy-1.2, <1% Ig^+, <0.5% Fc-receptor-positive, and without antibody-dependent cell-mediated cytotoxic (ADCC) potential (22, 23, 26, 27). They were tested for the percentage of Id^+ cells by the methods outlined above, and for specific cytotoxic T-cell (CTL) activity against ^{51}Cr-labeled target cells (L cells: C3H fibroblast cell line, H-2^k; RBL-5: Rauscher virus-induced lymphoma in B6 mice, H-2^b; L1210: DBA/2 leukemia, H-2^d). One lytic unit (LU) is defined as the number of effector cells leading to a 25% ^{51}Cr release from 3×10^4 target cells during a 4-h assay.

Trypsin Treatment and Reculture. T blasts were adjusted to 20×10^6 cells/ml in R and added to an equal volume of trypsin solution (5 mg/ml of trypure [crystalline trypsin], Novo Research Institute, Copenhagen, Denmark). The mixture was incubated for 30 min at 37°C and then washed three times in 10% FCS/R. Some trypsin-treated cells were tested for the number of Id^+ cells and for CTL activity, and the rest of the cells were adjusted to 5×10^5/ml in 5% FCS/ER and incubated for 18 h at 37°C, 5% CO_2. They were then washed three times in 5% FCS/ER and tested for the percentage of Id^+ cells and for CTL activity. Recovery after trypsin treatment was 40–60% of the input; recovery of recultured cells after reculture was 50–70% of the input.

Results

The Dominant Antibody Specificity of the B6 Anti-CBA IgG Imunogen Is Directed against H-2K- and I-Region Antigens. B6 anti-CBA (anti-H-2) antisera produced as described above are assumed to contain anti-H-2 antibodies, anti-allotype (IgD and [or] IgM) antibodies, and anti-non-H-2 antibodies (e.g., antibodies against viral and minor histocompatibility antigens) (29). Previous characterization of the B6 anti-CBA antibodies selected as the immunogen in these studies has shown clearly dominant IgG clones, as judged by isoelectric focusing (H. Frischkneckt, unpublished data). Additionally, the CFA immunization protocol has allowed large quantities of specific anti-H-2 antibody to be obtained (40–60 ml ascites plus serum per mouse) to fulfill the requirements for specificity analysis, anti-Id antiserum production, and characterization.

We have analyzed the range of serological specificities present in the immunizing B6 anti-CBA IgG preparation through the use of antibody and RC′ serology, quantitative adsorption studies and pA radioimmunoassay of the various preparations on a panel of H-2^k recombinant mice. As seen in Table I, B6 anti-CBA antibodies raised by these immunization protocols are equally effective in recognizing determinants present in the original CBA immunizing strain as well as on the B10 congenic B10.BR (H-2^k) strain. Using B10.A and B10.A (4R) as recombinants, equal lysis can be achieved, thus further dissecting the major activity to the H-2K and IA regions of the H-2^k complex. Reported cross-reactivities of the serological specificities detected by the B6 anti-CBA antisera are seen in the lysis of B10.D2, DBA/1 and C3H.OH haplotypes, although no detectable complement-fixing auto-antibodies against B10 were found in this sera. The reactivity against C3H.OH and B10.D2 cells can be

TABLE I

Specificity of B6 Anti-CBA Antiserum, Adsorbed or Nonadsorbed with C3H.OH Cells

Target cells	Percentage of killed spleen cells (target cells)*							
	B6 anti-CBA‡				B6 anti-CBA (adsorbed)§			
	1:10	1:30	1:90	1:270	1:10	1:30	1:90	1:270
B10	6	3	—	—	1	3	—	—
CBA	96	91	93	51	93	89	65	24
B10.BR	98	95	91	37	96	85	70	19
B10.A(4R)	98	94	92	54	98	91	83	35
B10.A	96	95	94	50	93	92	56	6
B10.D2	85	80	73	21	61	21	6	0
C3H.OH	83	74	60	30	39	1	2	0
DBA/1	80	56	32	7	54	27	13	3

* The percentage of spleen cells (target cells) killed in presence of antiserum and RC′, diluted 1:5. The percentage killed in the presence of RC′ was 17% in this experiment.

‡ DEAE-Sephacel IgG fraction of a B6 anti-CBA antiserum pool (10 mg/ml).

§ The same IgG preparation, but adsorbed three times with C3H.OH spleen cells. Both IgG preparations were Id^+ in immunodiffusion against rabbit 5,936 antiserum.

readily adsorbed out with C3H.OH spleen cells while only minimally affecting the reactivity against CBA, B10.BR, and B10.A (4R).

Essentially identical results were obtained by radioimmunoassay of the binding of these adsorbed antibody preparations to cells from a panel of H-2 recombinant mice (data not shown). These experiments have shown very similar levels of antibody binding to B10.A (4R) and AQR. As these two mouse strains share only the IA^k region, the present results suggest that the dominating antibody activity associated with these B6 anti-CBA preparations is directed against the $H\text{-}2K^k$ and IA^k regions of the H-2 complex.

Production and Characteristics of the Rabbit 5,936 anti-Id serum. Anti-Id antibodies were produced by immunizing MγG-tolerant rabbits with purified B6 anti-CBA IgG preparations in CFA (21). Individual bleedings of rabbit 5,936 were obtained 7, 14, 21, and 28 d after each immunization, and in cases where specificity and titers were comparable, serum pools were made. The various preparations of rabbit 5,936 were found to contain agglutinating antibodies against MRBC, precipitating antibodies against a so-far unidentified component in NMS, and strongly precipitating antibodies against B6 anti-CBA IgG preparations. Accordingly, for the present study, these antiserum preparations were adsorbed with B10 MRBC and passed through either B6 × CBA-NMS or B6 × CBA-IgG-Sepharose-4B-CL affinity adsorbents to remove these activities. Eluted material from B6 × CBA-IgG-Separose-4B-CL columns could be identified as anti-Ig antibodies that were present in quantities too low to be detected in the immunodiffusion assay. Therefore, subsequent analysis of the reactivity of the rabbit 5,936 preparations were done after such adsorption procedures followed by an $(NH_4)_2SO_4$ precipitation and fractionation into the γG-enriched fraction on Sephadex G 200 or DEAE-Sephacel. In each preparation the anti-Id activity was exclusively found in the IgG-enriched fraction.

TABLE II

Id^+ B6 Anti-CBA Molecules Have Preferential Reactivity against the IA Region of the H-2^k Complex

IgG of antiserum*	Adsorption with‡	Cytotoxic titer against B10.BR cells§	Reactivity against rabbit 5,936 antiserum‖
B6 anti-CBA	B10-MRBC	1:2,430	+
B6 anti-CBA	B10-spleen cells	1:2,430	+
B6 anti-CBA	CBA-MRBC	1:270	+
B6 anti-CBA	CBA-spleen cells	<1:10	−
B6 anti-CBA	B10.A(4R)-MRBC	1:270	+
B6 anti-CBA	B10.A(4R)-spleen cells	1:30	−
B6 anti-CBA	B10.A-spleen cells	1:90	−
B6 anti-CBA	C3H.OH-spleen cells	1:270	+
B6 anti-CBA	BALB/c-spleen cells	1:810	+
B6 anti-CBA	Anti-Ig (nonadherent)	<1:10	−
B6 anti-CBA	Anti-Ig (eluted)	1:810	+
B6 anti-CBA	5936-Sepharose (nonadherent)	1:270	−
B6 anti-CBA	5936-Sepharose (eluted)	1:90	+

* See Table I.

‡ Adsorptions performed either with MRBC, spleen cells, or on Sepharose columns coated with anti-Ig or with rabbit 5,936 antiserum.

§ Cytotoxicity assay performed in presence of 1:200 diluted, anti-Ig antibody. Titer is defined as the final dilution of anti-CBA IgG which gives a 50% kill of spleen cells. In case of adsorption with CBA or B10.A(4R) MRBC, the maximal cytotoxic activity of the IgG preparation did not exceed 55%.

‖ +, precipitation band against rabbit 5,936 antiserum at protein concentrations 5, 1, and 0.2 mg/ml. −, no precipitation against rabbit 5,936 antiserum at protein concentration 5 mg/ml.

Evidence That the Primary Reactivity of Rabbit 5,936 Anti-Id Antiserum Is Directed Against Anti-IA^k Alloantibodies. As shown above, the antibody activity of the anti-H-2^k IgG preparations used for the production of the anti-Id antisera could be shown to react strongly against H-$2K^k$ and IA^k serological specificities. It would therefore be expected that adsorption of the B6 anti-CBA IgG preparations with cells carrying the relevant antigens (H-$2K^k$ and [or] IA^k) would render them non-reactive with rabbit 5,936 antisera. The results shown in Table II do in fact support that contention. Here the only cell populations effective in rendering the B6 anti-CBA antisera nonreactive with rabbit 5,936 antiserum were from CBA, B10.A, and B10.A (4R) spleens. Extensive adsorption of the B6 anti-CBA antisera with MRBC from the same strains was inefficient as immunoadsorbants of anti-H-2^k antibody molecules reactive with rabbit 5,936 antiserum, further indicating that the dominating antibody molecules reactive with rabbit 5,936 antiserum in B6 anti-CBA IgG preparations are directed against the IA^k antigens (see also Table III). As seen in Table II, filtration of B6 anti-CBA γG preparations through either anti-Ig or rabbit 5,936-Sepharose-4B-CL affinity columns removes the reactivity detected by the rabbit 5,936 antisera. Interestingly, although the anti-Ig column completely removed the cytotoxic activity against B10.BR cells, the rabbit 5,936 column only partially reduced the total cytotoxic activity, suggesting that only a portion of the cytotoxic anti-H-2^k antibodies bear the idiotypic determinants reactive with rabbit 5,936. Such an interpretation would then clarify the resulting cytotoxic titers of the various Id negative (Id^-) preparations of adsorbed B6 anti-CBA antibodies and the high but incomplete frequency of 76%

TABLE III

Reactivity of Rabbit 5,936 Antiserum Against Anti-H-2 IgG Preparations Directed toward Different H-2 Haplotypes or Recombinant H-2 Types

Antiserum*	Anti-H-2 reactivity‡	Reactivity against	
		B10.BR§	Rabbit 5,936 antiserum‖
B6 anti-CBA	*k kkkkk kkk*	>1:270	+
B6 anti-P815	*d* ddddd dd*d*	1:270	–
B10 anti-B10.D2	*d ddddd ddd*	1:270	(+)
B6 anti-B10.A	*k kkkkd ddd*	>1:270	+
B6 anti-B10.A(4R)	*k k*bbbb bbb	>1:270	+
B6 anti-C3H.OH	*d ddddd ddk*	1:270	(+)
B6 anti-DBA/1	*q qqqqq qqq*	1:30	–
B6 anti-SRBC	?	ND	–
B6 anti-ovalbumin	?	ND	–
A.BY anti-CBA	*k kkkkk kkk*	>1:270	+
ATH anti-ATL	s *kkkkk kk*d	1:90	+
B10.S(7R) anti-B10.HTT	s sss*kk kk*d	1:30	–
B10.A anti-B10.BR	k kkkk*k kkk*	1:30	–
CBA anti-B6	*b bbbbb bbb*	1:810 (B10)	–

* IgG preparations of the mentioned antisera at a concentration of 5 mg/ml.

‡ Serological reactivities of a given antiserum is visualized by italics.

§ 50% cytotoxicity titer against B10.BR spleen cells and RC′, except the latter antiserum is B10 spleen cells and RC′; B6 anti-SRBC and anti-ovalbumin had $\log_2$ hemagglutination titers against SRBC and ovalbumin-SRBC of 12 and 14, respectively.

‖ DEAE-Sephacel Ig preparations of different anti-CBA antisera were tested against rabbit 5,936 antiserum, the Ig concentrations being 5, 1, and 0.2 mg/ml. +, precipitation bands at all three concentrations; (+), precipitation band only at 5 mg/ml; –, no precipitation at 5 mg/ml.

(based on 41) of the individually tested B6 anti-CBA preparations that were found to be Id^+. Although greatly enriched for IgG, we cannot rule out the possibility that a low amount of the residual cytotoxic titers may be due to contaminating IgM which has been consistently found to be Id^- (data not shown). Finally, analysis of the antibodies eluted from both the rabbit 5,936 and anti-Ig affinity adsorbents showed them to be cytotoxic and Id^+.

Analysis of the Specificity and Strain Distribution of the Id(s) Defined by Rabbit 5,936 Antiserum. Different anti-H-2 IgG preparations at concentrations 5, 1, and 0.2 mg/ml; and 40 μg/ml were tested in immunodiffusion against rabbit 5,936 antiserum. Ig preparations that gave no precipitation bands even at 5 mg/ml will be designated negative (– in Tables II–IV), Ig preparations that gave a weak precipitation band only at 5 mg/ml will be designated: (+), whereas Ig preparations that gave precipitation bands at 5, 1, and 0.2 mg/ml is designated Id^+ (+ in Tables II–IV).

IgG preparations from NMS of all the mouse strains tested failed to react with rabbit 5,936 antiserum, providing evidence against strictly an anti-allotype reactivity of the anti-Id antisera. Furthermore, preliminary experiments have shown that $F(ab)_2$ fragments of B6 anti-CBA IgG are as reactive against rabbit 5,936 antiserum as are

TABLE IV

Reactivity of Anti-H-2^k Ig Preparations with Rabbit 5,936 Antiserum in Immunodiffusion

Mouse strain	H-2 type	Ig-1 allotype	Reactivity against		Cytotoxic titer against B10.BR cells§	Number of Ig preparations‖
			Rabbit 5,936 antiserum*	Anti-light-chain antiserum‡		
B6/B10	b	b	+	+	≥1:90	41
A.BY	b	e	+	+	≥1:90	3
C57/L	b	a^+	−	+	≥1:90	1
BALB/B	b	a^+	−	+	≥1:90	2
C3H.SW	b	a^-	−	+	≥1:90	1
C3H.B10	b	a^-	−	+	≥1:90	2
B65a	b	a^-	+	+	≥1:90	3
B10.D2	d	b	+	+	≥1:270	8
CB 20	d	b	(+)	+	≥1:90	2
BALB/c	d	a^+	−	+	≥1:270	8
DBA/2	d	c	(+)	+	≥1:810	2
C3H.OL	o1	a^-	−	+	≥1:90	2
C3H.OH	o2	a^-	−	+	≥1:90	5
A.CA	f	e	(+)	+	≥1:90	2
DBA/1	q	c	(+)	+	≥1:810	6
A.SW	s	e	(+)	+	≥1:90	2

* See Table III.

‡ Ig preparations tested at protein concentrations of 5, 1, and 0. 2 mg/ml. +, precipitation bands at 0.2 mg/ml against a rabbit anti-light-chain antiserum.

§ Final dilution of anti-CBA Ig which gave a 50% kill of B10.BR cells in presence of RC′.

‖ In all cases except DBA/2, A.CA, and A.SW, there is at least one serum that is directed against B10.BR rather than CBA. The results obtained are identical.

nondigested B6 anti-CBA IgG (O. Nordfang and B. Rubin. Manuscript in preparation).

Analysis of the reactivity of rabbit 5,936 antiserum with a variety of anti-CBA IgG preparations from mice with different H-2 and Ig-1 allotypes have shown the highly reproducible and significant associations seen in Table IV. Here, anti-CBA IgG preparations from H-2^b mice were Id$^+$ if the mice possessed the Ig-1^b or Ig-1^e allotype, and Id$^-$ if the mice possessed the Ig-1^a allotype. A further dissection of the Ig-1^b association for the expression of the rabbit 5,936 Id(s) comes from preliminary experiments with B65a mice, that expressed the Ig-1^a C_H-linked allotype and the Ig-1^b-V_H-associated NP heteroclitic marker (U. Krawinkel and B. Rubin, unpublished data). B65a anti-CBA antibodies were found to react with rabbit 5,936 antisera as well as B6 anti-CBA antibodies (Table IV).

All other mice tested that possess the Ig-1^b or Ig-1^e allotype produced Id$^+$ anti-CBA antibodies, irrespective of the H-2 haplotype as seen with B10.D2, CB 20, A.CA, and A.SW. Despite the fact that the quantity of Id$^+$ anti-CBA antibodies from these mice were judged at least 10-fold lower than B10 mice by dilution analysis in gel diffusion, a clearly significant reaction can be seen (Table III). DBA/1 and DBA/2 anti-CBA

TABLE V

Strain Distribution of MLC Blasts Bearing the Rabbit-5,936-Antiserum-Defined Id

MLC combination	Genotypes of responding cells		Detection of the rabbit 5,936 Id	
	H-2	Ig-1	IIF*	pA‡
B6 anti-CBA	b	b	38 (18–60)	+ + + +
B10 anti-B10.BR	b	b	41 (25–58)	+ + + +
A.BY anti-CBA	b	e	20 (18–26)	+ + + +
C57/1 anti-CBA	b	a^+	ND	–
BALB/B anti-CBA	b	a^+	<5§	ND
C3H.SW anti-CBA	b	a^-	2	ND
C3H.B10 anti-CBA	b	a^-	<5§	ND
B65a anti-CBA	b	a^-	25 (18–30)	ND
B10.D2 anti-CBA	d	b	11 (9–19)	+ +
CB 20 anti-CBA	d	b	ND	+
BALB/c anti-CBA	d	a^+	3 (0–7)	–
C3H.OL anti-CBA	o1	a^-	<5§	ND
C3H.OH anti-CBA	o2	a^-	1 (0–5)	ND
A.SW anti-CBA	s	e	18§	ND
DBA/1 anti-CBA	q	c	5 (0–10)	ND
CBA anti-B6	k	a^-	1 (0–5)	–

ND, not done.

* Mean values of 3–28 experiments are given together with the range of results.

‡ Arbitrary values of amount of ^{125}I-labeled pA bound to 10^6 T blast cells incubated with rabbit 5,936 antisera. + + + +, ≥10-fold; + +, ≥5-fold; +, ≥2.5-fold over background; –, no significant binding.

§ Value determined by antiserum and RC′ cytotoxic activity.

preparations, which displayed the highest cytotoxic titers among the strains tested, can also be shown to produce significant levels of Id^+ antibodies, suggesting the presence of a cross-reactive Id(s) in mice bearing the Ig-1^c allotype as well.

The contention that the above reactions reflect truly idiotypic differences in the expression of anti-CBA antibodies is substantiated by the lack of reactivity of non-anti-H-2^k Ig preparation (Table III). Thus, B6 anti-P815, B6 anti-DBA/1, B6 anti-sheep erythrocytes (SRBC), B6 anti-OA and CBA anti-B6 Ig preparations have consistently been shown to be Id^-. Also, B10.A anti-B10.BR and B10.S(7R) anti-B10.HTT Ig preparations are Id^-, a result which further demonstrates that the Id^+ anti-H-2^k antibody molecules are directed mainly against the IA^k serological specificities. A cross-reaction between IA^k antigens and IA^d antigens (B10 anti-B10.D2 and B6 anti-C3H.OH Ig preparations are Id^+) is to be expected, in fact the only non-cross-reacting IA antigens as recognized by H-2^b mice are IA^k and IA^q (19).

Expression of the B6 Anti-CBA Id(s) on MLC-Activated T Blasts. Purified FcR^- T cells from different strains of mice were activated in MLC against H-2^k alloantigens. The choice and care used in this method of in vitro activation was based on extensive analysis of the resulting cell populations (22, 23, 26, 27), which could be shown to >98% Thy-1.2^+, <0.5% FcR^-, <1% surface Ig^+, and without ADCC activity. These characteristics are of special importance in the evaluation of anti-Id-antibody-binding studies.

TABLE VI

Percentage of Id^+ T Cells among T Blasts Activated against Different H-2 Haplotypes or Recombinant H-2 Types

MLC combination*	Percentage of Id^+ cells	
	IIF‡	Cytotoxicity§
B10 anti-B10.BR	41	35 (96)
B10 anti-B10.D2	8	<5 (97)
B10 anti-B10.A	27	25 (94)
B10 anti-C3H.OH	8	<5 (98)
B10 anti-B10.A(4R)	ND	27 (97)
B6 anti-DBA/1	7	<5 (96)
B10 Con A blasts	ND	7 (87)
CBA Con A blasts	ND	<5 (98)
B10.A(4R) blasts	ND	8 (83)

* MLC cells harvested on day 5, Con A cells harvested on day 3 (treated twice with 0.1 MαMM).

‡ Percentage of Id^+ cells determined by IIF. ND, not done. Values given are means of at least two experiments.

§ Percentage of Id^+ cells were determined by rabbit 5,936 antiserum and RC′ cytotoxicity assay as described in Materials and Methods. The numbers in parentheses are the percentage of killed effector cells in presence of an anti-thymocyte antiserum and RC′. These numbers represent the lysability of the cells. A rabbit anti-HSA antiserum served as negative control with a percentage of cytotoxicity of 5%.

TABLE VII

Regeneration of Id^+ T Blasts after Trypsin Treatment and Reculture

Effector cells*	Trypsin‡	Assay time			
		0 h		18 h	
		Number of LU§	Percentage of Id^+ cells‖	Number of LU	Percentage of Id^+ cells
		LU/10^7 cells	%	*LU/10^7 cells*	%
B6 anti-CBA	−	196	32	204	29
B6 anti-CBA	+	0	2	23	26
B10.D2 anti-CBA	−	174	9	204	10
B10.D2 anti-CBA	+	0	1	30	11
BALB/c anti-CBA	−	53	4	21	3

Cell recovery, see Materials and Methods.

* Effector cells were obtained 4 d after initiation of MLC.

‡ +, treatment with trypsin; −, not trypsin-treated cells.

§ LU per 10^7 T-blasts cells.

‖ Determined by IIF assay.

From the results presented in Table V it can be seen that 10–60% of T cells from the very same mouse strain combinations that produced Id^+ anti-$H\text{-}2^k$ antibodies were Id^+ upon in vitro immunization with $H\text{-}2^k$ alloantigens. Again, the importance

of the Ig-1^b or Ig-1^e allotype association is clearly seen, regardless of the H-2 type. The quantitation of Id^+ T blasts detected by IFF showed a striking correlation with the relative values obtained by the pA radioimmunoassay among the strain combinations analyzed. Normal B6 T cells were found to contain 3–6% Id^+ cells, while normal CBA or CBA anti-B6 T blasts contained <2% Id^+ cells (21) (indicating the background "noise" levels in our immunofluorescent assay). Furthermore, a significant correlation between the percentage of Id^+ T blasts detected by IIF and the percentage of T blasts killed by rabbit 5,936 antiserum and RC′ was also found (Table VI). These data show that B6 T cells are stimulated to develop Id^+ T blasts upon stimulation with B10.BR, B10.A, and B10.A (4R) cells, while B10.D2, C3H.OH, DBA/1 cells, and concanavalin A (Con A) stimulate the development far fewer numbers of Id^+ T cells. Again, these results stress that the idiotypic specificity of T-cell products exhibit specificity for the H-$2K^k$ and (or) IA^k region antigens.

Evidence for Active Synthesis of Id^+ Receptors by T Cells. The use of FcR^- T cells as responders in our MLC render the possibility of passively acquired Id^+ molecules on T blasts unlikely. The results in Table VII show that B6 anti-CBA as well as B10.D2 anti-CBA T cells do indeed regenerate their Id^+ membrane molecules after trypsin treatment and overnight reculture.

Discussion

This report describes the qualitative reaction patterns of a xenogeneic rabbit anti-(B6 anti-CBA) antiserum with mouse anti-$H2^k$ antibodies and anti-H-2^k immune T cells produced by different strains of mice. The data may be discussed in relation to the following three principal points of view: (*a*) the true anti-Id nature of rabbit 5,936 antiserum, (*b*) the specificity of the reaction between rabbit 5,936 antiserum and mouse anti-H-2^k antibodies, and (*c*) the genetic association of structural genes that code for mouse T-cell receptors reactive against alloantigen.

The anti-Id antiserum was produced in a rabbit (5,936) tolerant against three times-$(NH_4)_2SO_4$-precipitated NMS, designated: MγG. Thus, it may be assumed that MγG-tolerant rabbits are nonresponsive to constant parts of Ig and to very frequent idiotypic determinants on both Tcr(s) and Bcr(s) (30). Rabbit 5,936 antibodies reacted strongly against the immunogen B6 anti-CBA Ig, but failed to precipitate Ig preparations from NMS of any mouse strain analyzed; providing evidence against a strictly allotype-associated reactivity of the rabbit 5,936 antiserum. Additional evidence for the anti-Id reactivity of rabbit 5,936 antibodies comes from the following: (*i*) B6 anti-CBA γG preparations adsorbed onto anti-Ig Sepharose affinity columns did not react against rabbit 5,936 antiserum, whereas adsorbed and subsequent eluted molecules from such γG preparations did react with rabbit 5,936 antisera (Table II); (*ii*) rabbit 5,936 antiserum reactive molecules in anti-CBA γG preparations were removed by adsorption with CBA spleen cells but not with B6 spleen cells (Table II); (*iii*) $F(ab)_2$ fragments of B6 anti-CBA antibodies reacted as well with rabbit 5,936 antiserum as did non-pepsin-digested B6 anti-CBA antibodies (O. Nordfang and B. Rubin. Manuscript in preparation); (*iv*) B65a (H-2^b) mice that have the Ig-1^a C_H region genes (from CBA) and, seemingly at least parts of the Ig-1^b V_H region genes (from B6) (U. Krawinkel and B. Rubin, unpublished data) produce rabbit 5,936 antiserum reactive anti-CBA antibodies in quantities similar to B6 mice (Table IV); (*v*) the rabbit 5,936 antiserum reactive anti-CBA antibodies are of the 7S IgG class and ≅75% of

individually tested B6 mice produce rabbit 5,936 antiserum reactive anti-CBA antibodies of the 7S IgG classes that fix complement (31) (data not shown); (*vi*) anti-H-2^d or anti-H-2^q antibodies react only weakly, or not at all, respectively, with rabbit 5,936 antiserum (Table III) and B6 anti-SRBC or anti-ovalbumin IgG preparations are also nonreactive against rabbit 5,936 antiserum.

From these we conclude that the rabbit 5,936 antiserum satisfies the criteria of a true anti-Id antiserum.

The original immunizing B6 anti-CBA IgG preparation was shown to react preferentially against H-$2K^k$- and (or) IA^k-associated antigens (Tables I, II). Preliminary characterization of B6 anti-CBA antibodies produced following the CFA immunizing protocol used in this paper has shown clearly dominant IgG clones, as judged by isoelectric focusing (H. Frieschknecht, unpublished data). On the basis of these data it is not surprising that the rabbit 5,936 anti-(B6 anti-CBA) antibodies showed a relatively restricted specificity towards the 7S IgG antibodies directed against H-$2K^k$- and (or) IA^k-region antigens. This preferential reactivity was shown by quantitative adsorption (Table II) or by producing anti-H-2^k antisera of restricted specificity (Table III).

Inherited Id(s) studied so far have all shown close linkage to the heavy-chain allotype genes (32). However, despite this exclusive linkage to heavy-chain allotype genes, light chains are known to play an important role in the tertiary structure of the idiotypic determinants (4, 6, 23, 33). The involvement of light chain in the presently described Id(s) is not known, but our study has clearly established a strong association of the rabbit-5,936-antiserum-defined Id present on 7S IgG anti-CBA antibodies to the Ig-1^b or Ig-1^e heavy-chain allotype genes (Table IV). Preliminary experiments have shown that Id associated with Ig-1^b and with Ig-1^e are serologically different (O. Nordfang and B. Rubin. Manuscript in preparation).

With these considerations in mind, we can conclude that our present studies with the rabbit 5,936 anti-Id antiserum have demonstrated that T-cell Id(s) (actively synthesized by these cells themselves [Table VII]) specific for H-$2K^k$ and (or) IA^k region antigens are coded for by genes closely associated with the Ig heavy-chain-linkage group. Thus, anti-CBA activated T cells from the following congenic pairs of mice were found Id^+: B10 (b/b) and B10.D2 (d/b); A.BY (b/e) and A.SW (s/e), whereas C3H.SW (b/a^-) and C3H.OH (o/a^-); BALB/b (b/a^+) and BALB/c (d/a^+) were found to be Id^- (Table V) (the subdivision of the Ig-1^a group is according to [34]). Furthermore, FcR^- T cells from CB 20 mice which are BALB/c mice with the Ig-1^b allotype, and from B65a mice (H-2^b) with Ig-1^b V_H genes and Ig-1^a C_H genes, develop into Id^+ cells upon in vitro stimulation with CBA or B10.BR spleen cells (Table V). Therefore, the structural genes coding for T-cell Id(s) specific for at least H-$2K^k$ and (or) IA^k antigens are closely associated with the Ig heavy-chain-linkage group (6, 14, 15).

Rabbit-5,936-antibody-Sepharose columns have been found to specifically bind H-2^k-specific receptor molecules from Nonidet P-40- (Shell Chemical Co., New York) solubilized T cell membranes and from B6 anti-CBA MLC supernates. The biochemical characterization of these molecules will be described in a subsequent paper in this series.[2] Here it suffices to mention that the physical characteristics of the Id^+ molecules behave identically with those described by Binz and Wigzell (14). If such Tcr(s) are injected into MγG-tolerant rabbits an anti-Tcr antiserum is produced that specifically

reacts against Tcr material but not against B6 anti-CBA IgG preparations. Anti-Tcr antibody-Sepharose columns appear to react with the same Ig^-, 72,000–75,000 mol wt polypeptide chain as does rabbit 5,936 antiserum, indicating that neither of the two antisera react with Ig light chain equivalent structures on Tcr (B. Rubin, unpublished data). Thus, in conclusion, our results have demonstrated that mouse T cells have alloantigen-specific receptor molecules which carry idiotypic determinants that are recognized by an anti-Id antiserum directed against Id(s) on B6 anti-CBA IgG molecules. Genetic analyses have shown close association of the structural genes for the Tcr with the Ig-heavy-chain-linkage group. Further studies will demonstrate whether or not the present Id's on anti-CBA antibodies and on anti-CBA T cells are identical.

Summary

The present study describes the qualitative reactions of a xenogeneic anti-idiotype (Id) antiserum produced in a mouse-γ-globulin-tolerant rabbit (5,936) against B6 anti-CBA IgG antibodies. The results showed that such an anti-Id antiserum reacts specifically against anti-H-2^k antibodies and against H-2^k alloantigen-activated T cells from the following pairs of congenic mice: B10 (H-2^b) and B10.D2 (H-2^d); and A.BY (H-2^b) and A.SW (H-2^s), but not against C3H.SW (H-2^b) and C3H.OH (H-2^o); and BALB/b (H-2^b) and BALB/c (H-2^d). CB 20 (BALB/c mice with the Ig-1^b allotype) anti-CBA T blasts also express idiotypic determinants that react with rabbit 5,936 antiserum. Thus, positive reactions are obtained between rabbit 5,936 anti-Id antiserum and anti-H-2^k IgG preparations and T blasts from mice carrying the Ig-1^b or Ig-1^e allotype, but not from mice carrying the Ig-1^a allotype. These reactions are qualitatively independent of the H-2 genotype of the Id-producing mice.

Such a finding strongly suggests that the Id-bearing receptor molecules on mouse T cells are coded for by genes that are associated with the Ig heavy-chain-linkage group and not to the mouse histocompatibility complex. Furthermore, the anti-Id antibodies studied react preferentially against anti-H-2^k antibodies or T cells with specificity toward the IA^k-region-associated serological specificities. Thus, genes associated with the Ig heavy-chain-linkage group seem to be structural genes for at least T-cell receptors with specificity for IA-region-coded membrane antigens.

The skillful technical assistance of J. Lieberkind, B. Olsson, and B. Rumler is gratefully acknowledged. The authors wish to thank Prof. Hans Wigzell for his support and encouragement during these studies. The animal department of Statens Seruminstitut is given special thanks for their excellent care of the many mouse strains used as well as of pregnant and newborn rabbits. The secretarial assistance of Mrs. Colette Kourilsky at the Centre d'Immunologie de Marseille-Luminy is gratefully acknowledged.

Received for publication 12 February 1979.

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