# ANTI-IDIOTYPES TO MONOCLONAL ANTI-H-2 ANTIBODIES

## II. Expression of Anti-H-2K$^k$ Idiotypes on Antibodies Induced by Anti-Idiotype or H-2K$^k$ Antigen

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Anti-idiotypic reagents (anti-Id) have been used to manipulate both idiotype (Id) expression and antigen-binding activity of immunized animals in various experimental systems (1–10). In many studies, in vivo administration of small amounts of purified anti-Id has been shown to suppress subsequent immune responses to the immunogen (2–5). In contrast, evidence for induction of Id-positive molecules and antigen-specific cells in mice treated with anti-Id antibodies in the presence (3, 6, 8, 10) or absence (1, 7, 9) of antigen has also been reported. Attempts have been made to extend these studies to the production of anti-idiotypic antibodies directed against alloantisera and/or alloimmune cell populations (11–13) and to use these anti-Id to manipulate in vivo responses (14–15). This approach has led to variable success, in part, because of difficulties in reproducibly generating anti-Id antisera by these methods, possibly because of the heterogeneity of many anti-major histocompatibility complex (MHC) responses (13). We have therefore recently developed heterologous anti-idiotypic antisera raised against monoclonal anti-H-2K$^k$ antibodies. This approach offers the advantages of reproducibility and availability of large quantities of immunogen for anti-Id production (16). These anti-Id have previously been shown (7, 16) to detect "private" determinants restricted to the monoclonal reagent used as immunogen and expressed in low, often undetectable levels in alloantisera containing antibodies with the same H-2 reactivity. We examined the effects of in vivo administration of these anti-Id. We report the induction of both Id-positive molecules and antigen-binding activity as a result of such treatment. The genetic requirements for such responses and the effects of pretreatment with anti-Id on the expressed repertoire of anti-H-2K$^k$ antibodies induced after subsequent immunization to alloantigen are also described.

## Materials and Methods

*Animals.* BALB/c mice (6–8 wk old) were purchased from either the Charles River Laboratories, Wilmington, Mass. or The Jackson Laboratory, Bar Harbor, Maine. BALB.B,

1305

BALB.K, B.C8, and C.B20 mice were kindly provided by Dr. Michael Potter, National Institutes of Health, Bethesda, Md., and BAB.14 mice were provided by Dr. Martin Weigert, Institute for Cancer Research, Philadelphia, Pa. All other strains and F$_1$ and backcross mice were bred in our own colonies.

*Monoclonal Antibodies and Sera.*    Clone 11-4.1 (IgG$_{2a}$, k) was prepared by Oi et al. (17) and obtained through The Salk Institute, San Diego, Calif. Clones 3-83P and 36-7-5S (both IgG$_{2a}$, k) were produced in our laboratory, as described previously (18). These monoclonal antibodies were generated in either BALB/c or A.TL mice and possess anti-H-2K$^k$ reactivities differing in their fine specificities, as shown in Table I. The myeloma LPC-1 (IgG$_{2a}$, k), which has no known specificity, was kindly provided by Dr. Michael Potter. Conventional alloantisera were prepared by published methods (19).

*Production and Purification of Anti-Id.*    Hybridoma antibodies were purified from culture supernates by affinity chromatography on protein A Sepharose columns (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Uppsala, Sweden). Heterologous anti-idiotypic antibodies were prepared in both miniature swine and rabbits (7). Swine were immunized with 200 µg i.m. of purified monoclonal antibodies in complete Freund's adjuvant. Rabbits were immunized in a similar manner with 100 µg Id in complete Freund's adjuvant. Anti-mouse immunoglobulin (Ig) activity was monitored by passive hemagglutination of sheep erythrocytes (SRBC)[1] coated with LPC-1 Ig, as described elsewhere (1). Once the levels of anti-Ig antibodies had plateaued, animals were exsanguinated, and pooled sera were stored at −20°C.

Immune sera from animals treated with the purified Id were extensively absorbed with LPC-1 coupled to Sepharose-4B (20) until all antinormal mouse Ig activity was removed, as detected by passive hemagglutination on LPC-1 and normal BALB/c Ig-coated SRBC. Anti-idiotypic antibodies were further purified by specific absorption to, and elution from, Sepharose columns bearing the specific hybridoma reagent, using 4 M guanidine-HCl, pH 7.0, as the eluant. Antibodies were dialyzed into phosphate-buffered saline (PBS) and stored at −20°C until use (21).

*In Vivo Treatment with Heterologous Antibodies.*    Mice were treated with injections of 20 µg i.p. of purified anti-Id or anti-Ig antibodies in saline on days 0 and 3, and were bled on day 10 and weekly thereafter. Id levels were assessed by a hemagglutination inhibition (HAI) assay, described in detail elsewhere (1). Selected mice from each group were subsequently grafted with H-2K$^k$ (C3H) tail skin.

*Assessment of Anti-H-2 Activity.*    Anti-H-2 antibody activity was assessed by both complement (C)-mediated cytotoxicity assays, as described previously (18) and by flow microfluorometry (FMF), using the fluorescence-activated cell sorter (FACS). For analysis by the FACS, lymph node cell suspensions from C3H (H-2K$^k$) or C3H.OH (H-2K$^d$) were prepared. Aliquots containing 10$^6$ cells were washed in Hanks' balanced salt solution containing 0.1% BSA and 0.1% Na azide (staining medium). Cells were pelleted (400 g, 5 min) and resuspended in 25 µl of test serum from individual mice. Test sera were ultracentrifuged (100,000 g, 25 min) to remove aggregates before their addition to the cells. The cells were incubated with the test sera for 45 min at 4°C, washed twice in staining medium, and resuspended in 25 µl of a mixture of fluoresceinated goat F(ab)′$_2$ anti-mouse IgG$_2$ and F(ab)′$_2$ anti-mouse IgG$_1$ at a final dilution of 1:64 of each antibody. The suspension was incubated for an additional 30 min at 4°C, washed twice, and resuspended in 0.4 ml of staining medium for analysis by FMF (FACS II; B-D FACS Systems, Becton, Dickinson & Co., Sunnyvale, Calif.), as previously described (22). Fluorescence data were collected on 5 × 10$^4$ viable cells, and the results were displayed as a curve in which cell number was plotted on the ordinate and fluorescence intensity (fluorescence units, FU) on the abscissa. FU were arbitrary values selected on the basis of the fluorescence intensity of positively stained cells above background fluorescence as determined by the intersection point of curves from genetically positive (H-2K$^k$) and genetically negative cells for each reagent tested (22).

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[1] *Abbreviations used in this paper:*    BSA, bovine serum albumin; C, complement; FACS, fluorescence-activated cell sorter; FMF, flow microfluorometry; FU, fluorescence units; HA, hemagglutination; HAI, hemagglutination inhibition; IEF, isoelectric focusing; PBS, phosphate-buffered saline; SRBC, sheep erythrocytes.

TABLE I

*Hybridoma Antibodies Used to Produce Anti-Id*

| Hybridoma cell line | Immune cells fused | Specificity | Known cross-reactions | Reference |
|---|---|---|---|---|
| 11-4.1 | BALB/c anti-CKB | $K^k$ | $K^q$,p,r | 17 |
| 3-83P | BALB/c anti-C3H | $K^k$ | $D^k$,$K^b$,p,q,r,s | 18 |
| 36-7-5S | A.TL anti-A.AL | $K^k$ | None | 18 |

### Assessment of Id Expression

(a) BINDING INHIBITION ASSAY. Anti-H-2K$^k$ antibodies from alloimmunized mice and/or anti-Id-treated mice were examined for Id expression by an antigen-binding inhibition assay. 25 μl of sera from treated mice was mixed with either 25 μl of anti-Id (250 μg/ml) or 25 μl of an irrelevant (i.e., noncross-reactive) anti-Id (250 μg/ml) prepared in the same species to another monoclonal anti-H-2K$^k$ antibody. After a 1-h preincubation of the test sera and the inhibitor, binding to cells bearing H-2K$^k$ antigen was assessed by FMF, as described above. Sera were ultracentrifuged before use to remove complexes.

(b) ISOELECTRIC FOCUSING (IEF). IEF was performed by the method of Briles and Davie (23) as modified by Claflin (24). Briefly, 5.5 inch vertical slab gels were prepared using a Plexiglass rear gel-forming plate and front glass plates, treated for polyacrylamide gel adherence by the method of Nicolotti (25). Whole serum or ascites fluid was focused and Id were detected by the method of Keck et al. (26). Anti-idiotypic antibodies were radioiodinated ($^{125}$I; New England Nuclear, Boston, Mass.) by the chloramine T method (specific activity, 0.5 mCi/mg), and 2 × 10$^7$ cpm was overlaid on the gels for 16 h. Autoradiographs were exposed for 10–14 d.

*Separation of Ig Subclasses and Id-bearing Molecules by Affinity Chromatography.* Anti-idiotypic antibodies were coupled to cyanogen bromide-activated Sepharose (Pharmacia Fine Chemicals) at a concentration of 5 mg/2 ml of beads. Goat anti-mouse IgG1 and anti-mouse IgG2 sera, previously dialyzed into 0.1 M Na bicarbonate, 0.075 M NaCl, pH 9.0, were coupled in a similar manner using 1 ml serum/4 ml of beads. 0.5 ml of immune ascites from either alloimmunized or anti-Id-treated mice was passed over either a 5-ml anti-Id column or sequentially over 10 ml of mouse anti-IgG subclass affinity columns. The pass-through (non-adherent fraction) was collected and was designated peak I. The adsorbed material (designated peak II) was eluted from the columns with 4 M guanidine-HCl in PBS, pH 7.0. Eluted antibodies were immediately dialyzed into PBS, pH 7.2, concentrated to the original volume, and stored at −20°C.

## Results

*Specificity of Anti-Idiotypic Reagents.* Anti-idiotypic antibodies were purified from the sera of either individual miniature swine or rabbits immunized with purified monoclonal anti-H-2K$^k$ antibodies, as described in Materials and Methods. Because both 3-83P and 11-4.1 monoclonal antibodies were derived from BALB/c mice and were of the IgG2a, k subclass, the BALB/c myeloma protein LPC-1 was used to remove anti-isotypic and anti-allotypic antibodies from the immune sera. Table II shows representative results after sequential affinity purification of the anti-Id. Anti-Id antibodies prepared in this manner were found to react strongly with the immunogen, as evidenced by the agglutination of Id-coupled SRBC but did not react significantly above background levels (i.e., 2$^4$ on FCS-coupled SRBC) with either LPC-1-coupled or other anti-H-2K$^k$-coupled SRBC. Furthermore, analysis of these anti-idiotypic antibodies by an HAI assay on a series of anti-H-2 antibodies showed no detectable cross-reactive Id on numerous other monoclonal anti-H-2 antibodies, including several with identical serological specificities (18), nor on anti-H-2 antibodies produced by alloimmunization of BALB/c mice (16). More recently, however, shared Id have been detected on a small percentage of alloantibodies using the very sensitive FMF antigen-

TABLE II

*Specificity of Anti-Idiotypic Antibodies Purified from Pig and Rabbit Antisera*

| Sample* | Indicator cells (HA titer log₂) | | | |
|---|---|---|---|---|
| | 3-83P-SRBC | 11-4.1-SRBC | 36-7-5S-SRBC | LPC-1-SRBC |
| Purified pig anti-11-4.1 | 3 | 17 | 1 | 2 |
| Purified rabbit anti-11-4.1 | 2 | 19 | <1 | 2 |
| Purified pig anti-3-83P | 21 | 4‡ | 2 | 2 |
| Purified rabbit anti-3-83P | 18 | 3 | 2 | 1 |
| Purified pig anti-BALB/c Ig | 14 | 16 | 14 | 17 |

* All samples were adjusted to concentrations ranging from 0.5 to 1 mg/ml for analysis.
‡ The low level agglutination of inappropriate Id-coupled cells was less than control agglutination (FCS-SRBC) and, unlike the specific agglutination, could not be inhibited by the appropriate Id.
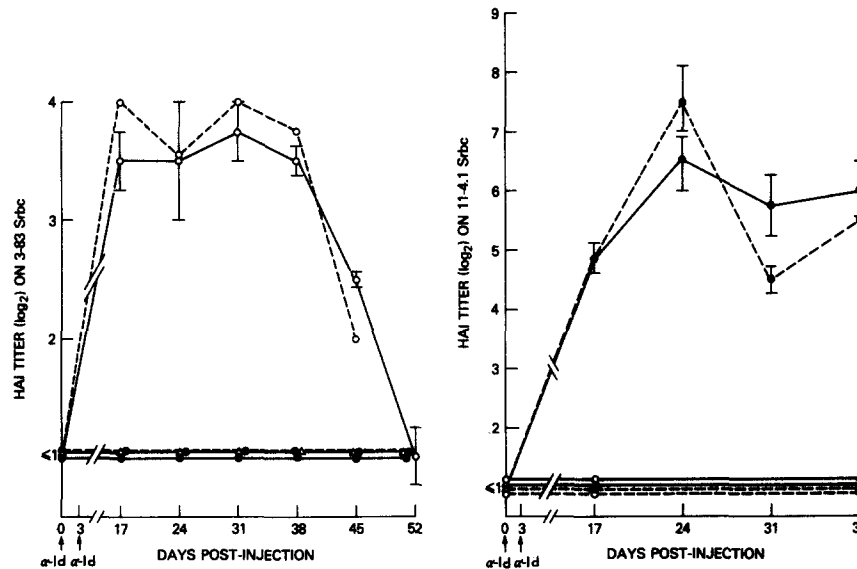


FIG. 1. Clone 3-83P Id and clone 11-4.1 Id levels in sera of anti-Id-treated BALB/c mice. Sera from BALB/c mice treated with anti-Id or anti-nl mouse Ig were analyzed in an HAI assay for Id expression. The last dilution of serum causing inhibition of agglutination was taken as the HAI titer. Sera from mice treated with pig anti-Id were assessed for Id with rabbit anti-Id and vice versa to obviate the need for absorption of antispecies antibodies. In vivo treatment groups of eight mice: (●———●), pig anti-11-4.1 Id; (●- - -●), rabbit anti-11-4.1 Id; (○———○), pig anti-3-83 Id; (○- - -○), rabbit anti-3-83 Id; (△———△), pig anti-nl mouse Ig.

binding inhibition assay described above (J. A. Bluestone, H. Auchincloss, S. E. Epstein, and D. H. Sachs, manuscript in preparation).

*In Vivo Treatment of Mice with Anti-Id*

(a) ASSESSMENT OF ID IN SERA OF TREATED MICE. We have previously reported (7) that BALB/c mice treated with either anti-11-4.1 or anti-3-83P anti-Id antibodies produced Id as assayed by an HAI assay. A time-course study (Fig. 1) demonstrated that after injection of 20 μg of pig or rabbit anti-Id in saline on day 0 and 3, significant serum levels of Id were detected as early as day 17 and were present, in the case of the 11-4.1 Id, for at least 2 mo post-treatment. The Id-positive molecules induced in mice

treated with the pig anti-Id were detected by both rabbit and pig anti-Id agglutinators, as were similar Id$^+$ molecules induced by in vivo treatment with rabbit anti-Id. Treatment with rabbit or pig anti-mouse Ig antibodies did not lead to detectable Id. These findings suggested that the inhibition of hemagglutination by immune sera was due to induced Id (designated Id$'$) (1, 27) and not to an antispecies response. The Id$'$ were also found to be detected only by the homologous anti-Id and were not cross-reactive with anti-Id directed against other anti-H-2 hybridoma antibodies, as reported previously (16). The difference in HAI titers between anti-3-83P- and anti-11-4.1-treated mice was due to sensitivity differences between the indicator cells.

(b) GENETIC CONTROL OF IDIOTYPE INDUCTION. The anti-idiotypic reagents used in this study were raised against monoclonal BALB/c anti-H-2K$^k$ antibodies. After treatment with anti-Id, BALB/c mice but not B10 mice produced Id$'$ (data not shown), as assayed by HAI. Thus, the induction of Id$'$ molecules in in vivo treated BALB/c animals might be genetically controlled, for example, by genes linked to constant Ig region genes. We therefore investigated the genetic control of induction of Id$'$, using both backcross analysis and allotype congenic recombinant mice. As seen in Table III, only backcross mice that bore the a allotype genes from the BALB/c parent produced Id$'$ after anti-Id administration. Similarly (Table IV), analyses of sera produced by allotype congenic recombinant mice inoculated with pig anti-11-4.1 indicated that, in general, only a allotype mice could make the Id. In one case

TABLE III

Genetic Analysis of Id Expression in (BALB/c × C57BL/6)F$_1$ × C57BL/6 Backcross Mice Treated with Pig Anti-11-4.1

| Sample | Allotype | Id (HAI titer log$_2$) |
|---|---|---|
| BALB/c serum pool | aa | 4–5 |
| C57BL/6 serum pool | bb | <1 |
| (BALB/c × C57BL/6)F$_1$ | ab | 4 |
| F$_1$ × C57BL/6 backcross | | |
| BC$_1$ | ab | 7 |
| BC$_2$ | ab | 6 |
| BC$_4$ | ab | 5 |
| BC$_5$ | ab | 7 |
| BC$_6$ | ab | 8 |
| BC$_{11}$ | ab | 6 |
| BC$_{13}$ | ab | 3–4 |
| BC$_3$ | bb | <1 |
| BC$_7$ | bb | <1 |
| BC$_8$ | bb | <1 |
| BC$_9$ | bb | <1 |
| BC$_{10}$ | bb | <1 |
| BC$_{12}$ | bb | <1 |
| BC$_{14}$ | bb | <1 |
| BC$_{15}$ | bb | 1 |
| BC$_{16}$ | bb | <1 |
| BC$_{17}$ | bb | 1 |
| BC$_{18}$ | bb | <1 |
| BC$_{19}$ | bb | <1 |
| BALB/c (prebleed) | aa | <1 |
| C57BL/6 (prebleed) | bb | <1 |

TABLE IV

Genetic Analysis of Id Expression in Allotype Congenic Mice Treated with Pig
Anti-11-4.1 Anti-Id

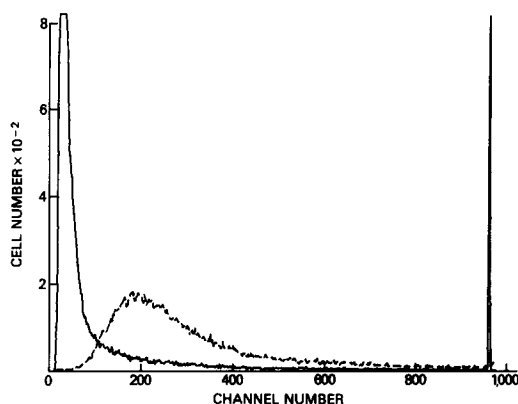| Sample (serum pool) | Allotype | | H-2 type | Id (HAI titer log$_2$) |
|---|---|---|---|---|
| | V$_H$ | C$_H$ | | |
| BALB/c | a | a | d | 5–7 |
| BAB.14 | a | b | d | 5 |
| BC.8 | a | a | b | 5–6 |
| BALB.B | a | a | b | 7 |
| BALB.K | a | a | k | 5 |
| C.B20 | b | b | d | <1 |
| C57BL/6 | b | b | b | <1 |
| A.TL | e | e | d | <1 |



FIG. 2. Antigen specificity of anti-H-2 antibodies induced by anti-Id. Anti-H-2K$^k$ activity was assessed by FMF using H-2 congenic lymph node cells stained with test sera from an anti-Id-treated mouse and counter stained with a mixture of fluoresceinated anti-mouse IgG antibodies. Staining of lymph node cells of the inappropriate H-2 haplotype was not significantly greater than the fluorescence of the cells in the absence of sera. —, B10.MBR (bkkq); – – –, B10.AKM (kkkq).

(BAB.14), expression of BALB/c V$_H$ genes alone was sufficient for Id induction despite b allotype constant region genes. These results suggested that the gene(s) for Id expression was linked to V$_H$-encoded genes.

(c) NATURE OF INDUCED ID-BEARING MOLECULES. We have previously shown (7) by FMF that some of the Id-positive molecules induced in anti-Id-treated mice bind H-2K$^k$ antigen specifically (Fig. 2). However, these same sera, when assayed for reactivity against H-2K$^k$ spleen cells by microcytotoxicity analysis, revealed either no lysis or minimal lysis of target cells. Because certain subclasses of mouse Ig fix C poorly and are thus poor cytotoxic antibodies, we examined the subclass distribution of anti-H-2K$^k$ antibodies induced in anti-Id-treated mice. By FACS analysis, the majority (>75%) of anti-H-2K$^k$ antibodies induced in anti-Id-treated mice were of the IgG$_1$ subclass, with a smaller proportion being IgG$_2$ (Fig. 3). Serum from one anti-Id-treated mouse (1529) was passed successively over both an anti-IgG$_2$ and an anti-IgG$_1$ antibody column, and the bound fractions were eluted. These fractions were then examined for cytotoxicity by C-mediated cytotoxicity (Table V). Removal of IgG$_1$ antibodies from this immune serum resulted in residual high titered (1:128-256)
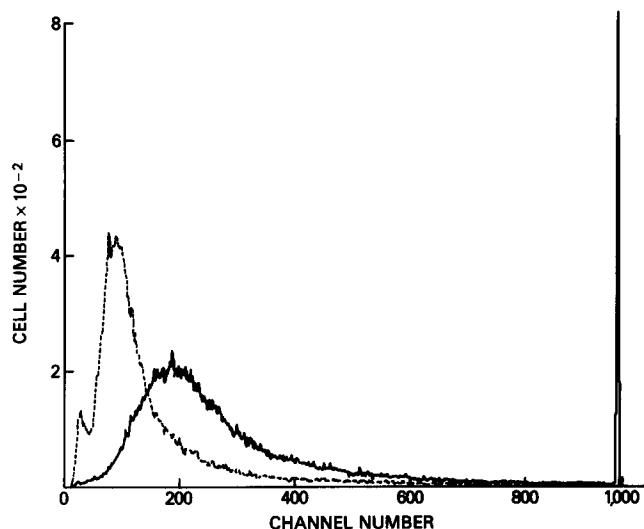
FIG. 3. Subclass distribution of anti-H-2K$^k$ antibodies induced in anti-Id-treated mice. Subclass distribution of the anti-H-2K$^k$ antibodies was determined using fluoresceinated anti-IgG subclass specific reagents as in Fig. 2. FU analysis of staining showed that anti-IgG$_1$ stained 100% of cells with a mean fluorescence of 380 FU and that anti-IgG$_2$ stained 100% of cells with a mean fluorescence of 246 FU. 1529 ascites was used in a dilution of 1:2. —, anti-IgG$_1$ fluorescent reagent; - - - -, anti-IgG$_2$ fluorescent reagent.

TABLE V

*Anti-H-2 Cytotoxic Activity of IgG$_1$ and IgG$_2$ Antibodies from an Anti-3-83P-treated mouse*

| Sample | Cytotoxic titer$^{-1}$ | |
|---|---|---|
| | B10.BR (H-2K$^k$) | B10.D2 (H-2$^d$) |
| 1529* | 4-8 | <2 |
| 1529 (IgG$_1$)$_{II}$‡ | <2 | <2 |
| 1529 (IgG$_1$)$_I$ (IgG$_2$)$_{II}$ | 128-256 | <2 |
| 1529 (IgG$_1$)$_I$ (IgG$_2$)$_I$ | <2 | <2 |

* Ascites (1:2) from mouse 1529 treated with rabbit anti-3-83P.

‡ For the affinity purification column, bound antigen is in parentheses, and Roman numerals I and II represent unbound and bound fractions, respectively, after a sequential purification on the Ig-coupled columns.

cytotoxic anti-H-2K$^k$ antibody activity, whereas the IgG$_1$ antibodies recovered were essentially devoid of cytotoxic activity. Thus, the IgG$_1$ anti-H-2K$^k$, which does not fix C, was apparently masking the cytotoxic activity of the smaller popuation of C-fixing IgG$_2$ antibodies by competitive binding to the H-2K$^k$ cells.

These results demonstrate subclass differences between some of the anti-H-2K$^k$-induced antibodies (IgG$_1$ subclass) and the original monoclonal antibody, 3-83P (IgG$_{2a}$, k), used to prepare the anti-Id. Other differences between the monoclonal immunogen and the Id′ molecules were also detected. First, not all of the Id′ molecules induced by anti-Id treatment were antigen binding. Only ~20% of mice injected with anti-Id expressed antigen-binding Id′ molecules, although 100% of treated mice produced Id′. Table VI illustrates the HAI activity of Id$^+$ sera absorbed with H-2K$^k$

TABLE VI

*Absorption Analysis of Anti-3-83P-induced Id'*

| Antibody source | Number of absorbing spleen cells | Fluorescence binding on B10.A lymph node cells | HAI titer |
|---|---|---|---|
| | | FU* | log$_2$ |
| Ascites from | | | |
| Rabbit anti-3-83P | 0 | 282 | 8 |
| Treated mouse (1529) | 2 × 10$^7$ C3H (H-2K$^k$) | 19 | 7–8 |
| | 4 × 10$^7$ C3H (H-2K$^k$) | 16 | 7–8 |
| | 4 × 10$^7$ C3H.OH (H-2K$^d$) | 239 | 8 |
| Pig anti-11-4.1 | 0 | 103 | 6 |
| Treated mouse (1505) | 2 × 10$^7$ C3H | 8 | 6–7 |
| | 4 × 10$^7$ C3H | 13 | 5–6 |
| | 4 × 10$^7$ C3H.OH | 91 | 6 |
| Normal mouse | 0 | 22 | <1 |

* Fluorescence units (FU) are defined as an arbitrary fluorescence value based on the percentage of total cells with immunofluorescence above a lower limit determined by the intersection point of curves from lymph node cells stained with fluoresceinated anti-Ig in the presence or absence of test sera.

antigen. Although all anti-H-2K$^k$ reactivity detected by FMF cell binding was removed by the absorption, significant amounts of residual nonantigen-binding Id' molecules remained. These results suggested that V region differences may exist between the original monoclonal anti-H-2K$^k$ antibody and the Id'. Additional evidence for V region differences was obtained by IEF. Fig. 4 depicts the IEF pattern of sera from two mice, 1529 and 1532, treated with rabbit anti-3-83P. The focused sera were overlaid with pig anti-Id labeled with $^{125}$I to avoid the effect of anti-rabbit reactivity in the test sera. The autoradiograph illustrated in Fig. 4 shows that the Id' molecules expressed were of limited heterogeneity. In addition, different animals did not express identical bands nor a set of bands corresponding to the 3-83P protein itself. Serum absorptions with spleen cells were carried out to examine the antigen-binding and nonantigen-binding Id' antibodies. Absorption of sera from mouse 1532 with C3H or C3H.OH spleen cells did not change the IEF patterns. These results support the FACS analysis, which suggested that sera from mouse 1532, although Id$^+$, had no anti-H-2 activity. Mouse 1529, however, had two predominant IgG bands that were removed by H-2K$^k$ but not H-2K$^d$ spleen cells. When the spectrotype of antibodies eluted from the anti-IgG$_1$ column were examined, several sets of bands were evident. These results suggested that the bands represented the expression of multiple V-region genes because mobility differences could not have been caused by subclass heterogeneity. All of these findings suggest that the differences that exist between 3-83P and Id' are V-region related.

*Response of Anti-Id-treated Mice to Alloantigen.* Although anti-idiotypic antibodies did not readily detect shared Id in alloantisera, their injection into naive BALB/c mice had a profound effect on the expressed anti-H-2K$^k$ antibody repertoire after skin grafting. As shown above, injection of anti-idiotypic antibodies induced anti-H-2K$^k$ antibodies in the absence of H-2K$^k$ antigen, but this effect was observed in only ~20% of mice tested. Several of the mice that expressed no detectable anti-H-2K$^k$ antibodies by cytotoxicity or FMF were skin grafted with tail skin from C3H (H-2$^k$) mice.
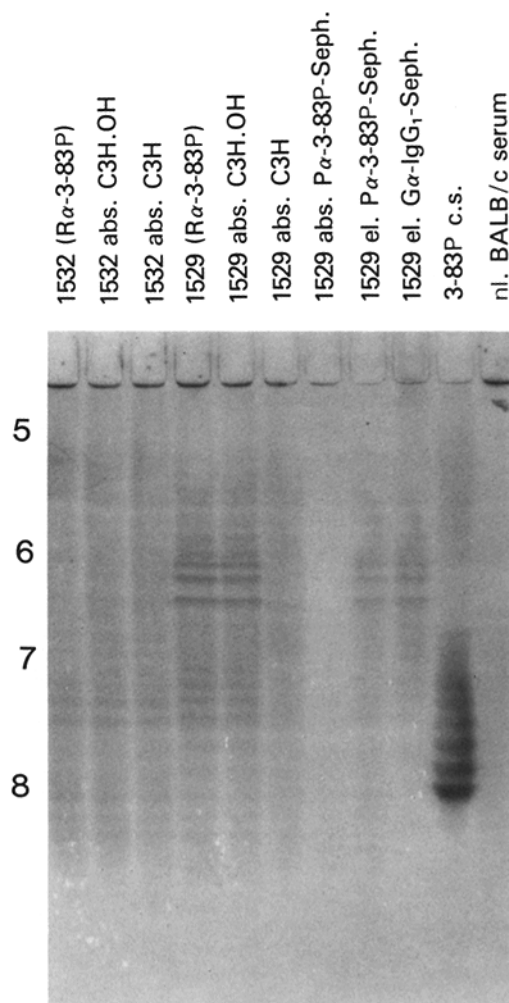
Fig. 4.   IEF of sera from mice treated with rabbit anti-3-83P anti-Id overlaid with $^{125}$I-pig anti-3-83P. $^{125}$I-labeled pig anti-3-83P anti-Id was overlaid onto isoelectric focused antibodies from rabbit anti-3-83P-treated BALB/c mice. The pH gradient is shown at the left. In some cases, aliquots of test sera were passed through Sepharose columns bearing either anti-Id or anti-IgG$_1$, as indicated, to fractionate the Id' molecules.

Although no significant alteration in skin graft rejection time was observed (data not presented), treatment with anti-Id before skin grafting had a profound effect on the expressed anti-H-2K$^k$ repertoire in these mice. Small aliquots of ascites from normal alloimmunized or anti-Id-treated alloimmunized mice were fractionated on an anti-Id column. The various fractions were then assessed for anti-H-2 activity (Fig. 5) by FMF and for idiotypy by the inhibition of anti-H-2 activity using anti-Id. The activity of conventional alloantisera could not be inhibited by either anti-11-4.1 or anti-3-83P anti-Id before fractionation (data not shown). In addition, only ~5% of the anti-H-2 activity bound to the anti-Id column, none of which was inhibitable by anti-Id. When the bound antibodies were repassed over the column, only a small percentage rebound,
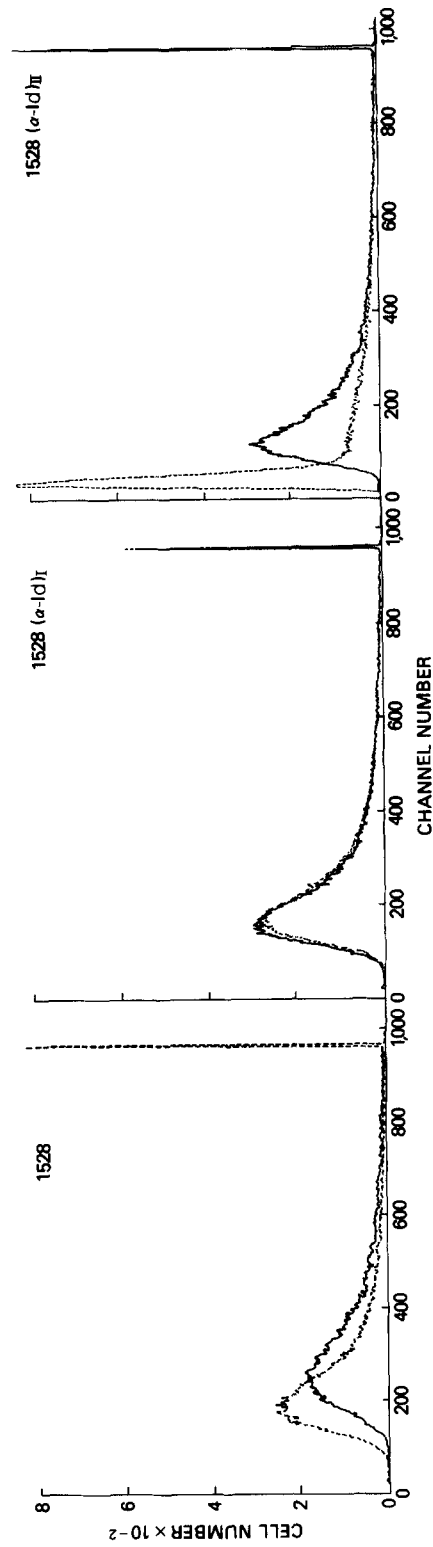
FIG. 5. Assessment of Id on anti-H-2K$^k$ antibodies induced by H-2K$^k$ antigen with either anti-Id or saline and assayed for anti-H-2K$^k$ binding activity as in Fig. after anti-Id treatment. Anti-H-2K$^k$ antibodies from an anti-Id-treated skin-graft 3. Fractionated sera were reconstituted to their initial volumes. —, no inhibitor; mouse were examined for Id expression by a binding inhibition assay before and — —, anti-3-83P. after fractionation over an anti-Id affinity column. Test sera were preincubated

suggesting that the original binding was nonspecific. In contrast, Fig. 5 illustrates that anti-H-2 antibodies from a mouse (1528) treated with anti-Id before grafting were significantly inhibited by anti-3-83P anti-Id. The pass-through fraction (peak I) from the anti-Id column could not be inhibited by anti-Id. However, the eluate (peak II) bound H-2K$^k$ specifically to a titer of 1:250 and was significantly inhibited by anti-3-83P anti-Id. In fact, if the mixture of anti-H-2K$^k$ antibodies and anti-Id were centrifuged (100,000 g, 25 min) before incubation with the lymph node cells, then the binding was totally inhibited. In this and other animals, the Id$^+$ anti-H-2K$^k$ antibodies represented between 40 and 65% of the total anti-H-2K$^k$ activity, as based on FU values. These findings suggest that anti-Id treatment before immunization with alloantigen exerts a profound effect on the repertoire of anti-H-2K$^k$ antibodies expressed in the response.

## Discussion

In the present studies, we demonstrated that administration of anti-H-2K$^k$ anti-Id to animals in the absence of antigen leads to the induction of Id$^+$ molecules, as shown by an HAI assay. Two types of antibodies in sera from anti-Id-treated mice could have inhibited hemagglutination of Id-coated SRBC by anti-Id. First, the inhibitory activity might have been due to the induction of anti-anti-Id antibodies. These molecules, although perhaps present in the sera, probably do not represent a significant portion of the inhibiting molecules for several reasons: (a) a small but significant percentage of Id' reacted with H-2K$^k$ antigen, as did the original monoclonal Ab₁ antibody. It seems quite unlikely that anti-anti-Id would fortuitously react with these same determinants; (b) unless the binding site was highly conserved, anti-anti-Id would not be expected to react with anti-Id made in two different species, i.e., pig or rabbit, (c) the protocol used for triggering Id', namely, the injection of one or two small doses of anti-Id in saline, was considered a poor immunogenic regimen; and (d) the induction of Id$^+$ molecules was found to be linked to V$_H$ genes. It seems unlikely that the immune response to anti-Id would be linked in this manner. In fact, our previous findings[2] that the Id detected on the original monoclonal immunogen was localized to the H chains and evidence in other systems for the expression of V$_H$-encoded determinants on T and B cells (13) support the alternative hypothesis that the anti-Id in some way triggers cells to produce antibodies (Id') that express combining-site related shared idiotopes similar in nature to those of the original immunogen. This hypothesis is consistent with the cascade of Id-anti-Id reaction referred to as Ab1-Ab2-Ab3, etc., as described by Cazenave (28) and by Urbain and co-workers (29). The mechanism for this triggering phenomenon is currently under investigation. However, the fact that most of the Id' is of the IgG₁ subclass suggests a role for T cells in this phenomenon (30). In addition, preliminary studies indicate that BALB/c athymic nude mice do not make Id' after anti-Id treatment (data not shown).

In previous studies (16), we demonstrated that these anti-Id detect relatively "private" specificities on the hybridomas that were used as immunogens. However, IEF and antibody specificity data suggested that, although of limited heterogeneity,

[2] Bluestone, J. A., J.-J. Metzger, M. C. Knode, K. Ozato, and D. H. Sachs. Anti-idiotypes to monoclonal antibodies. I. Contribution of isolated heavy and light chains to idiotype expression. Manuscript submitted for publication.

the Id′ induced in individual BALB/c mice was not a single clonal species of antibody. First, multiple subclasses, including IgG$_1$, IgG$_2$, and probably other antibody subclasses (on the basis of pI) were induced. In addition, the IgG$_1$ response consisted of at least two clones in one animal (1529), as could be determined by band spacing. After studying a small number of Id′ animals using IEF techniques, we found that although a few clones are shared between Id′ antibodies induced in individual anti-Id-treated mice, many differ from other Id′ and from the original monoclonal antibody against which the anti-Id was raised. In fact, the fine specificities of the anti-H-2 antibodies induced by anti-Id-treated mice were different than the specificities of the monoclonal antibodies. For instance, the anti-H-2K$^k$ antibodies induced in BALB.B mice treated with anti-Id cross-reacted with H-2K$^d$ antigen. Although the expression of Id is under genetic control, the clones expressed could be the result of somatic diversification of members of a restricted family of anti-H-2 antibodies. Amino acid analysis of Id′ monoclonal antibodies is currently under way to address this question.

Finally, we found that within the family of Id′ there exists a small percentage of antigen-binding molecules, rarely expressed in an allo-immunization. The ability to detect antigen-binding activity in 20% of mice treated with anti-Id in the absence of antigen, in contrast to results in certain other studies (1), might be due to a larger precursor frequency of alloreactive cells. In addition, by triggering antigen-binding Id′-producing cells before alloimmunization, we were able to manipulate the subsequent response to the H-2K$^k$ antigen. In fact, after antigen administration, a significant percentage, as much as 65% of the alloantibodies, are Id positive.

As Jerne's (31) network theory might predict, perturbation of the idiotypic/anti-idiotypic network could lead to profound effects on subsequent immune responses. Indeed, such a network might exist before immunization and might help to regulate the immune responses. We demonstrated that immune responses to major histocompatibility antigens (H-2 in the mouse) can be perturbed by treatment with anti-idiotypic antibodies directed against monoclonal anti-H-2 antibodies. These results might reflect possible physiologic significance of Id and anti-Id in anti-H-2 responses and might also contribute to the manipulation of transplantation immunity.

## Summary

Anti-idiotypic antibodies were prepared against two monoclonal anti-H-2K$^k$ antibodies, 11-4.1 and 3-83P. These reagents were used to examine idiotype (Id) expression on anti-H-2K$^k$ antibodies induced by the in vivo administration of the anti-idiotypic antibodies and/or H-2K$^k$ antigen. Treatment of BALB/c mice with anti-Id induced both antigen-binding and nonantigen-binding Id-positive molecules in the absence of antigen. The level of production of anti-Id-induced Id (Id′) has been shown to be linked to V$_H$ genes using allotype congenic mice and backcross analyses. The idiotopes expressed on the Id′ induced in anti-Id-treated mice were closely related or identical to those of the original monoclonal anti-H-2K$^k$ antibody. However, the idiotopes were present on immunoglobulins of different subclasses and in some cases were not all expressed on the same molecules, as reflected by differences in their antigen specificities and isoelectric focusing patterns. In vivo administration of anti-Id had a marked influence on the subsequent humoral response to immunization with H-2 antigen.

Although conventional alloantisera lack the major idiotope(s), up to 65% of anti-H-$2K^k$ antibodies induced by antigen in anti-Id-treated mice were $Id^+$.

*Note added in proof:* Using an ELISA inhibition assay (21), which permits detection of partial inhibition, we have recently found low levels of Id′ in anti-Id-treated mice of the bb allotype that were not detectable by the HAI assay. This included both the backcross and C.B20 sera reported in this paper. However, in contrast to sera from a allotype mice, no anti-H-$2K^k$ activity was detected among induced Id′ of those animals.

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