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EVIDENCE FOR A REGULATORY IDIOTYPIC NETWORK IN THE IN VIVO RESPONSE TO H-2 ANTIGENS

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One of the central questions in immunology concerns specific regulation of antibody responses. Jerne (1) proposed that interactions between idiotype (Id)[1] and antiidiotype (anti-Id) constitute a key element in such regulation, leading to the concept of immunoregulatory networks. Subsequently, numerous examples of idiotypic regulation have been described. The production of anti-Id antibody has been demonstrated during normal immune responses (2–4). In addition, exogenously administered anti-Id antibody has been found to regulate relevant Id-specific antibody responses (5–9) and to induce suppressor T lymphocytes (10–13). In classical systems (15), treatment of animals with anti-Id leads to the induction of antigen-nonbinding Id-positive molecules.

In our laboratory extensive studies of anti–major histocompatibility complex (MHC) Id have been performed in recent years (16–19). Among the xenogeneic anti-Id reagents that have been raised are several against anti-H-$2K^k$ monoclonal antibodies (8, 20). Treatment of mice in vivo with those xenogeneic anti-Id has been found to induce the production of serum antibodies (Id′) that bear some of the idiotopes of the original monoclonal anti-MHC antibody, but which have unknown binding specificity. In addition, some mice treated with anti-Id developed antigen-binding activity for the original MHC antigen, even though they had never been exposed to that antigen (20).

In the present report we examine the Id network involved in immune responses to MHC antigens. The effect of subsequent immunization of anti-Id–primed mice with the original MHC antigen was examined. The experiments described here demonstrate that boosting with skin grafts bearing the original H-2 antigens leads to increased levels of Id′-positive, antigen-nonbinding molecules, as well as antigen-binding molecules, in the serum of the immunized mice. In contrast, boosting with other irrelevant antigens had no effect on either Id-positive population. These findings support strongly the existence of network interactions invoked after anti-Id treatment that may be involved in the control of subsequent responses to H-2 antigens.

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; FMF, flow microfluorometry; Id, idiotype; Id′, idiotype-bearing molecules; MHC, major histocompatibility complex; NMS, normal mouse serum; NPIg, normal pig Ig; PBS, phosphate-buffered saline.

Materials and Methods

Animals. BALB/cAnN mice were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, MA). BALB.K mice were bred in our animal colonies. B10.A and B10.D2 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). F-344 rats were purchased from the NCI–Frederick Cancer Research Facility (Frederick, MD).

Monoclonal Antibodies. Clone 11-4.1 (IgG2a,κ) was raised in a BALB/c mouse immunized with CKB spleen cells by Oi et al. (21) and obtained through The Salk Institute (San Diego, CA). The antibodies of this clone are specific for the H-2K^k antigen with crossreactivities against antigens of the K^q and the H-2^{p,r} haplotypes. The myeloma LPC-1 (IgG2a,κ) was obtained from Dr. M. Potter.

Preparation of Xenogeneic Anti-Id. Miniature swine and rabbits were immunized with protein A–purified 11-4.1 antibodies in complete Freund's adjuvant by several intraperitoneal injections. Details of the immunization scheme and preparation of anti-Id have been described previously (20). Briefly, immune sera were fractionated on Sepharose 4B coupled with LPC-1 to remove antibodies to normal mouse Ig. This procedure has been shown previously to remove all anti–constant region antibodies (20). This procedure was repeated several times to ensure the removal of anti–constant region antibodies. Specific anti-Id antibodies were then obtained by affinity chromatography on Sepharose 4B coupled with 11-4.1 monoclonal antibody.

In Vivo Treatment of Mice. BALB/c mice were injected intraperitoneally with 20 μg of pig anti-Id in phosphate-buffered saline (PBS) on day 0 and 3. Control mice were immunized with 20 μg purified normal pig Ig (NPIg) on day 0 and 3. Id′ levels, assessed by enzyme-linked immunosorbent assay (ELISA), were detectable within 7 d after immunization. Skin grafting of mice was performed using tail skin from either BALB.K or B10.D2 mice or Fisher rats. Sera of skin grafted mice were usually tested 21 d after skin grafting. In some experiments skin-grafted mice were boosted by interperitoneal injection of 1×10^7 spleen cells. Sera were collected 1 wk after each immunization.

ELISA Inhibition Assay for Detection of Id. ELISA assays were performed as described in detail previously (9). Briefly, microELISA plates (Nunc, Roskilde, Denmark) were coated with 200 μl of protein A–purified 11-4.1 antibodies at 1 μg/ml in PBS overnight at 4°C. The ELISA assay was performed in 0.05% PBS–Tween 20 (Sigma Chemical Co., St. Louis, MO) to reduce nonspecific binding.

Test sera from either pig anti-Id′ or NPIg-immunized mice were serially diluted in 25–50 μl volumes and incubated with 25 μl of NPIg (2 μg/ml) to inhibit potential anti–constant region antibodies. Simultaneously, anti-Id preparation was diluted to the appropriate concentration and swamped with 10% normal mouse serum (NMS). Rabbit anti-Id preparation was used to avoid inhibition by anti(anti-Id) antibodies.

After these preincubations, rabbit anti-Id was added to the test sera, and the mixtures were incubated for 60 min and transferred to the 11-4.1-coated ELISA plates. Binding of rabbit anti-Id to the 11-4.1-coated plate was detected 60 min later wtih horseradish peroxidase–coupled goat anti–rabbit IgG (Cappel Laboratories, Cochranville, PA). Enzyme bound to plates was then exposed to an excess of substrate, *o*-phenylenediamine (Aldrich Chemical Co., Milwaukee, WI). The reaction was stopped by adding 8 N H_2SO_4. Absorbance at 492 nm was measured by a TiterTek Multiskan spectrophotometer (Flow Laboratories, McLean, VA). Standard errors were always <10%. Percent inhibition was calculated according to the formula: percent inhibition = [OD_{492} (uninhibited) − OD_{492} (test sample)/OD_{492} (uninhibited)] × 100.

Complement-mediated Cytotoxicity. Two stage microcytotoxicity assays were performed as described previously (22) using media 199 (Gibco Laboratories, Grand Island, NY) containing 10% fetal calf serum (FCS). 5×10^4 ^{51}Cr-labeled target cells in 25 μl were mixed with 25 μl of various antiserum dilutions in microtiter wells, incubated 15 min at 37°C, washed, and then treated for 30 min at 37°C with appropriately diluted rabbit complement (C′). Lysis was determined by counting ^{51}Cr released from the labeled target cells. Percent lysis was calculated by the formula: specific ^{51}Cr release = [experimental (cpm) − complement alone (cpm)/maximum (cpm) − complement alone (cpm)] × 100.

Fluorescence Measurement by Flow Microfluorometry (FMF). FMF was performed using a

fluorescence-activated cell sorter (FACS II; Becton Dickinson Immunocytometry Systems, Mountain View, CA). Detailed procedures for the operation of the sorter and data collected have been reported previously (23). 1×10^6 B10.A lymph node cells were incubated for 45 min at 4°C with 25 μl of a mixture of fluoresceinated goat $F(ab')_2$ anti-mouse IgG2 and $F(ab')_2$ anti-mouse IgG1 at a final dilution of 1:64 of each antibody. The suspension was incubated for 30 min at 4°C, washed, and resuspended in 0.4 ml for analysis by FMF. All staining procedures were carried out in Hanks' balanced salt solution containing 0.1% bovine serum albumin (BSA) and 0.1% sodium azide. Fluorescence data were collected using 5×10^4 viable cells as determined by forward light scatter intensity and the results were displayed as a curve with cell number plotted on the ordinate and fluorescence intensity on the abscissa. Fluorescence units were arbitrary values selected on the basis of the fluorescence intensity of positively stained cells.

Absorption Procedure. Sera from anti-Id–treated mice were absorbed with either BALB.K spleen cells or BALB/c spleen cells at 100 μl of serum per $1–3 \times 10^8$ washed, packed spleen cells. The absorption mixtures were kept 1 h at 4°C, mixed occasionally, centrifuged, and the supernates were collected.

Results

In Vivo Treatment of Mice with Anti-Id. We have previously reported (8, 20) that BALB/c mice treated with pig anti-11.4.1 anti-Id produced Id′-bearing molecules capable of inhibiting the binding of anti-11-4.1 anti-Id to 11-4.1-coated ELISA plates. A time course study (Fig. 1) demonstrated that, after injection of 20 μg of pig anti-11-4.1 on days 0 and 3, significant serum levels of Id′-bearing molecules, which inhibited the binding of anti-Id to 11-4.1-coated plates, were detected as early as day 7 and were present at the same high level at least 3 wk after treatment. At week 5, in a high proportion of the in vivo immunized mice, the level of Id′ serum reactivity began to decrease. At week 10, only a low level of Id′ molecules could still be detected in the immune sera (Fig. 1). As has been previously reported (25) only 20% of such anti-Id–immunized mice developed detectable anti-H-$2K^k$ activity, as assessed by cell-bound fluorescence quantified by FMF on the FACS (not shown).

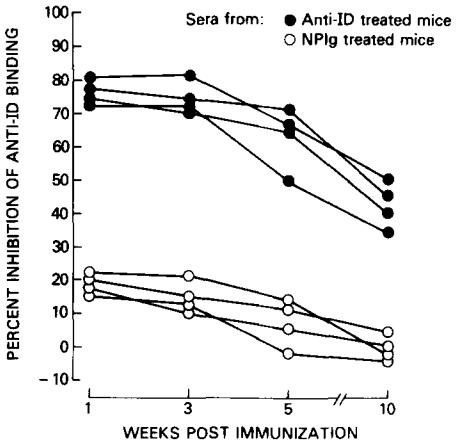

FIGURE 1. Induction of Id-positive molecules by in vivo treatment. Individual BALB/c mice were treated with either 20 μg of pig anti-Id (●) or 20 μg of NPIg (○) on day 0 and 3 and their sera subsequently assayed for their ability to inhibit the binding of rabbit anti-Id to Id-coated ELISA plates, as described in Materials and Methods.

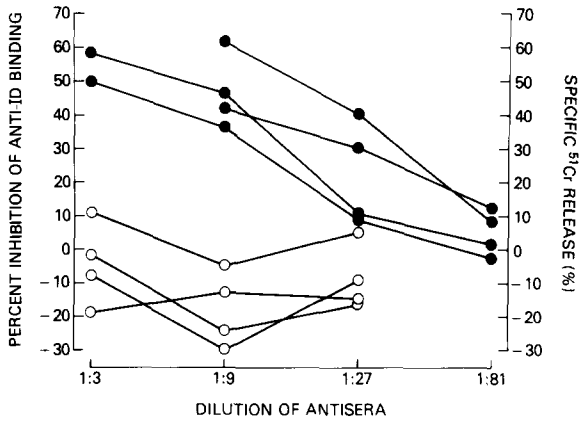

FIGURE 2. The effect of alloimmunization on the induction of Id-positive molecules and cytotoxic antibodies. BALB/c mice were skin grafted with BALB.K tail skin. 3 wk later sera from individual mice were tested for their ability to either inhibit the binding of rabbit anti-Id to Id-coated ELISA plates (○) or to lyse B10.A spleen cells (●) in the presence of complement.

In contrast to anti-Id immunization, treatment of BALB/c mice with 20 μg NPIg did not induce any significant level of Id′ molecules (Fig. 1) above the inhibition seen by NMS. None of the mice so treated developed detectable anti-H-2K^k activity.

Treatment of Naive BALB/c Mice with H-2K^k. It was of interest to examine whether alloimmunization with H-2K^k antigen would have any effect on the induction of Id′-bearing molecules. Alloantisera to H-2K^k antigen, obtained from BALB/c mice that were skin grafted with BALB.K tail skin, were tested for their ability to inhibit the binding of xenogeneic anti-11-4.1 anti-Id to 11-4.1-coated ELISA plates. Inhibition of Id–anti-Id interaction in ELISA was not detectable with any of the alloantisera tested (Fig. 2). In contrast, all of the sera tested showed a significant anti-H-2K^k reactivity when assayed by microcytotoxicity analysis (Fig. 2) or by direct cell-bound fluorescence quantified by FMF (not shown). These results suggested that the majority of anti-H-2K^k antibodies induced in BALB/c mice by BALB.K skin grafts do not express the 11-4.1 idiotype.

Treatment of Anti-11-4.1 Anti-Id–treated BALB/c Mice with H-2K^k Antigen. The effect of alloimmunization on the expression of Id-bearing molecules in anti-Id–treated mice was next examined. Both anti-Id–primed mice and NPIg-treated mice were grafted with BALB.K skin. Skin grafting was performed 7 wk or more after Id′ molecules decreased. Sera from anti-Id–primed BALB/c mice contained levels of Id′ serum reactivity after skin grafting with BALB.K skin, which were as high as the level of Id′ detected immediately after immunization with anti-Id (Figs. 3*A* and 5). In contrast, NPIg-primed mice that were skin grafted with BALB.K skin did not show any significant amount of Id′ serum reactivity when tested by ELISA inhibition assay (Fig. 3*B*).

The increased serum levels of Id′ molecules in sera of anti-Id–primed mice was first evident after skin grafts were fully rejected, and remained at the same high level for at least 3 wk. The amount of Id′ molecules continued to decrease

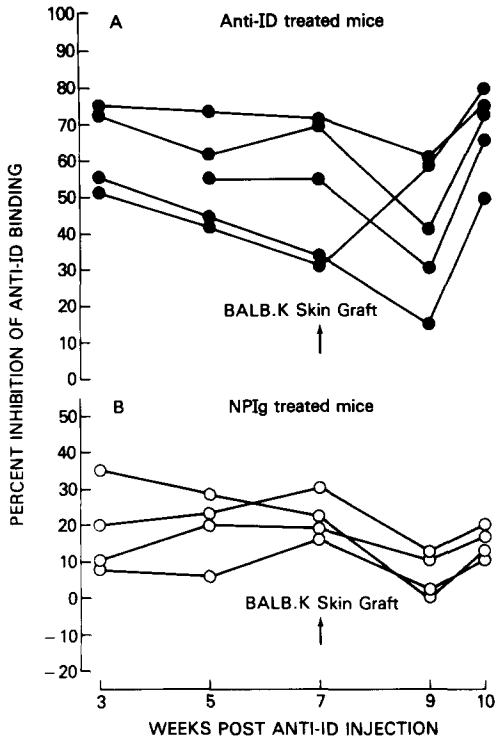

FIGURE 3. The effect of subsequent anti-H-2K^k immunization on Id-bearing molecules. BALB/c mice were first treated with either 20 μg of pig anti-Id (*A*) or 20 μg NPIg (*B*) on day 0 and 3. Skin grafting of the mice was performed 7 wk later using tail skin of BALB.K. The ability of sera from individual mice to inhibit the binding of rabbit anti-Id to Id-coated ELISA plates was tested at different time intervals before and after skin grafting.

until skin grafts were fully rejected, but increased thereafter. These findings suggested that the increased level in Id′ molecules after skin grafting was the result of an antigenic stimulation. Absorption studies were performed to determine whether the increased level of Id′-binding molecules that could now be detected in the sera of anti-Id–primed mice by ELISA represented an increase in the amount of anti-H-2K^k Id-positive antibodies or antigen-nonbinding molecules. In all sera tested, anti-H-2K^k reactivity, as detected by a microcytotoxicity assay, was fully removed by absorption with BALB.K spleen cells (Fig. 4, Table I). In two of four individual sera, such absorption (which removes all anti-H-2^k activity, data not shown) did not have any effect on the capacity of the sera to inhibit the Id–anti-Id interaction in the ELISA (Fig. 4, Table I). In sera from mice 1279 and 1284, absorption with BALB.K spleen cells partially removed the inhibitory capacity of the sera. However, the level of Id′ was still greater than that observed before skin grafting. These findings suggest that the level of antigen-nonbinding Id′ increased after exposure to the H-2K^k antigen.

Effect of Irrelevant Antigen Immunization on Id′-bearing Molecules. The above described experiments could be the result of either specific Id–anti-Id interactions or of a nonspecific effect of antigen immunization. To distinguish among these possibilities, anti-Id–primed mice were skin grafted with either B10.D2

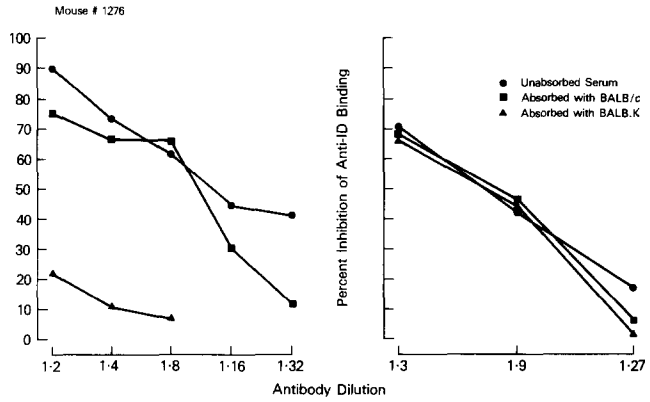

FIGURE 4. The effect of absorption of anti-H-2K^k activity on the level of Id$^+$ molecules and cytotoxic antibodies. Mouse 1276 was first immunized with pig anti-Id and then skin grafted with BALB.K tail skin as described in Fig. 3. Three weeks after skin grafting, serum from mouse 1276 was absorbed with either BALB/c spleen cells or BALB.K spleen cells, and thereafter the absorbed sera were tested for their ability to either inhibit the Id–anti-Id interaction in ELISA (*right*) or to lyse in the presence of complement and B10.A target cells (*left*); unabsorbed serum (●), serum absorbed with BALB/c spleen cells (■), serum absorbed with BALB.K spleen cells (▲).

TABLE I

Effect of Absorption of Anti-H-2K^k Activity on the Level of Id′ Molecules

Mouse No.	Percent inhibition of anti-Id binding			
	Before skin graft	After skin graft		
	Unabsorbed serum	Unabsorbed serum	Absorbed with BALB/c	Absorbed with BALB.K
1284	43	65	58	50
1276	NT	72	68	66
1985	27	46	43	40
1979	23	58	60	41

BALB/c mice were first immunized on day 0 and 3 with pig anti-Id and then skin grafted on week 7 with BALB.K tail skin. Sera from individual mice were collected before and after skin grafting, absorbed with either BALB/c spleen cells or BALB.K spleen cells, and tested for their ability to inhibit the binding of rabbit anti-Id to Id-coated ELISA plates.

(i.e., a source for a non-H-2 antigen) or rat skin (a source for a xenogeneic antigen), and the sera of the skin-grafted mice were tested for their capacity to inhibit Id–anti-Id binding in ELISA. Figs. 5–7 describe the results of such experiments. In contrast to BALB.K skin, which induced a high serum level of Id′ molecules (Fig. 5), neither B10.D2 skin (Fig. 6) nor rat skin (Fig. 7) had any effect on the level of Id′ reactivity. When the various sera were tested in a microcytotoxicity assay for the presence of antibody activity against the appropriate spleen cells, although both BALB.K and rat skin induced a significant level of cytotoxic antibodies (100% killing of the appropriate spleen cells with

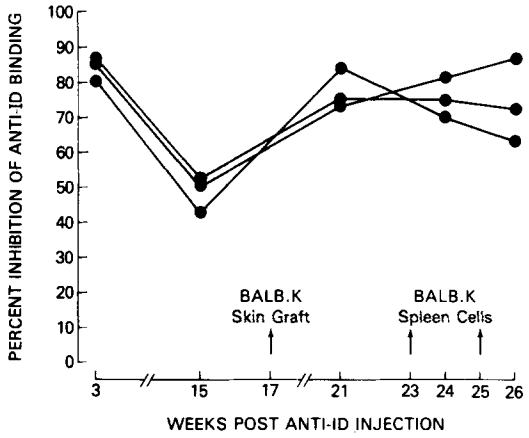

FIGURE 5. The effect of subsequent immunization with BALB.K tissues on Id′-bearing molecules. BALB/c mice first immunized with pig anti-Id were skin grafted 17 wk after anti-Id injection with BALB.K tail skin. Boosting with BALB.K spleen cells were performed at weeks 23 and 25. Sera from individual mice were assayed for their ability to inhibit Id–anti-Id interaction in ELISA at different time intervals during the immunization schedule.

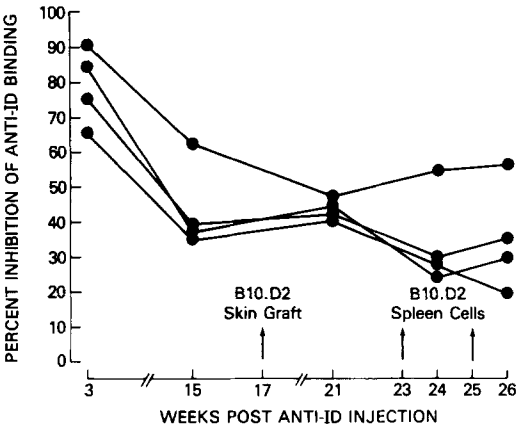

FIGURE 6. The effect of subsequent immunization with B10.D2 tissues on Id′-bearing molecules. BALB/c mice first immunized with pig anti-Id were skin grafted 17 wk after anti-Id injection with B10.D2 tail skin. Boosting with B10.D2 spleen cells were performed at weeks 23 and 25. Sera from individual mice were assayed for their ability to inhibit Id–anti-Id interaction in ELISA at different time intervals during the immunization schedule.

titers of 1:27 to 1:81), B10.D2 skin did not induce detectable antibody activity (cytotoxic titers, <2). Additional boosting of the mice with B10.D2 spleen cells led to increased levels of cytotoxic antibodies (titers 1:16 to 1:32 after the second injection), but did not have any significant effect on the level of Id′ reactivity in the immune sera (Fig. 6). Boosting of the mice with BALB.K spleen cells led to increased cytotoxic titers (titers 1:81 to 1:243 after the second injection) and persistent high levels of Id′ reactivity in the immune sera tested (Fig. 5). These results indicated that only the original H-2K^k antigen affected the production of Id$^+$ antigen-nonbinding molecules.

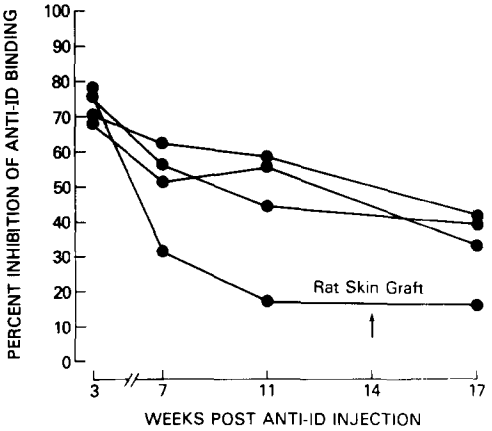

FIGURE 7. The effect of subsequent immunization with rat skin on Id-bearing molecules. BALB/c mice first immunized with pig anti-Id were skin grafted 14 wk after anti-Id injection with rat tail skin. The ability of sera from individual mice to inhibit the binding of rabbit anti-Id to Id-coated ELISA plates was tested at different time intervals before and after skin grafting.

Discussion

The experiments described in the present study demonstrate the ability of H-2K^k antigen to affect specifically the product of Ig molecules bearing the same idiotypic determinants as 11-4.1, but without detectable reactivity with the immunizing H-2K^k antigen.

The generation of antigen-nonbinding Id'-positive molecules after anti-Id immunization has previously been reported in several experimental systems (14, 15, 26, 19) including the anti-H-2K^k system studied here (8, 20, 24, 25, 30). This phenomenon suggests that anti-Id in some way triggers the expansion of B cell clones bearing similar idiotypic determinants to those toward which the anti-Id is directed. The actual mechanism involved in the regulation of such idiotype-positive B cell clones is still unknown. Bluestone et al. (16) found that F(ab')$_2$ fragments of rabbit anti-11-4.1 did not lead to the production of Id'-positive molecules. On the basis of this finding, it was suggested that the expansion of those B cell clones is, in part, dependent on T cell helper factors probably generated by recognition of Fc molecules as carrier determinants (14). Our present findings, that only BALB.K skin bearing the H-2K^k antigen but not B10.D2 or rat skin boosted the serum level of antigen-nonbinding Id' molecules, could be interpreted as indicating that H-2K^k antigen triggered in a specific way B cell clones secreting low affinity antigen-binding antibodies that could not be detected by binding to test cells. Alternatively, H-2K^k antigen, like anti-Id, could trigger antigen-nonbinding idiotype-positive B cell clones. If the latter explanation is correct, our findings suggest strongly the existence of Id–anti-Id regulatory networks in the response to H-2K^k antigen. Thus, the administration of H-2K^k antigen may induce in mice a syngeneic anti-Id antibody or T cell response which, like xenogeneic anti-Id, restimulates those B cell clones bearing the same idiotypic determinants. Indeed, it has previously been reported (24) that synge-

neic anti-Id antibodies against monoclonal anti-H-2 antibodies recognize idiotypes on antibodies induced by xenogeneic anti-Id reagents.

To demonstrate the existence of such an Id–anti-Id regulatory mechanism in the response to H-2K^k antigen, it was necessary to use anti-Id–primed mice. Skin grafting of normal BALB/c mice with BALB.K skin did not generate any detectable idiotypic activity when tested in ELISA, probably because the 11-4.1 idiotype, used to generate the anti-Id reagent, constitutes a very minor proportion of the humoral response to H-2K^k antigen, and therefore could not be detected in ELISA due to the sensitivity of the assay. In manipulated mice, however, Id that ordinarily constitute only a minor proportion had been induced by anti-Idiotype to represent a very large proportion of that same response. This provides an experimental model for studying the concept of immunoregulatory network.

Using another experimental system and the cell-bound fluorescence inhibition assay (20), in some conventional anti-H-2K^k alloantisera that did not show any idiotypic activity in ELISA, a fraction of the antigen-binding molecules were found to possess the 11-4.1 Id (unpublished data). This finding is different from one previously reported (25) and may suggest that the 11-4.1 Id is more public than previously thought.

The actual cellular pathway involved in B cell stimulation by anti-Id is still unknown. Experiments done in nu/nu mice both in the antinuclease system (29) and anti-H-2K^k system (25) indicate that B cell stimulation by anti-Id requires functional T cells. The experiments reported here do not directly address the issue of T cell involvement in the expression of anti-H-2 B cell Id. However, taking into consideration the fact that B cell surface receptors are identical to their secreted antibodies, it is unlikely that H-2K^k antigen which restimulated the secretion of antigen-nonbinding Id′ molecules had bound directly to those B cells that secreted non–H-2K^k-binding antibodies. Thus, intermediate pathways, possibly involving T cells, may operate to produce this effect. The exact nature of such pathways remains uncertain. The simplest explanation may be that H-2K^k antigen acts directly on Id-bearing, antigen-specific B cells. Subsequently, such B cells or their product might induce anti-Id helper T cells (or antibodies) which, in turn, activate Id′-binding and -nonbinding B cell clones.

Summary

Treatment of BALB/c mice with purified pig antiidiotype to 11-4.1 (anti-H-2K^k) monoclonal antibody has been found previously to induce the appearance of idiotype-bearing molecules (Id′) in the serum of these mice, in the absence of detectable antigen-binding activity. In the present study we examined the effect of subsequent immunization of such antiidiotype-primed mice with the original H-2K^k antigen. Skin grafting of virgin BALB/c mice with BALB.K skin did not generate any detectable Id′ antibodies when tested by enzyme-linked immunosorbent assay (ELISA). In contrast, grafting of antiidiotype-primed mice with BALB.K skin specifically boosted the serum level of Id′ molecules. Challenge of antiidiotype-primed mice with either B10.D2 or rat skin had no effect on the production of such Id′ molecules. Absorption studies demonstrated that the majority of Id′ molecules induced by H-2K^k antigenic stimulus and detected in

ELISA are antigen-nonbinding molecules, thus indicating specific restimulation by the original H-2K^k antigen of nonbinding idiotype-positive B cell clones. The relevance of these findings to the existence of network interactions in the immune response to H-2 antigens is discussed.

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