DEFINITION OF T CELL IDIOTYPES USING ANTI-IDIOTYPIC ANTISERA PRODUCED BY IMMUNIZATION WITH T CELL CLONES

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The concept of idiotypy of antigen-binding receptors suggests that in addition to possessing a particular binding site for antigen, each receptor has a set of serologic determinants (idiotypes) which distinguish it from other receptors. The finding of idiotypy of antibody molecules (1) eventually led to a proposal for a network theory of the immune response (2). The network theory postulates that idiotypic determinants on antibodies are recognition sites for other, anti-idiotypic antibodies within an individual's own immunoglobulin repertoire. The interplay of these idiotypic-bearing and idiotypic-recognizing antibodies is thought to be a major factor in regulating an immune response. Evidence for the occurrence of idiotypy (Id)¹ on immunoglobulin (antibody) molecules is by now abundant and considerable data has accumulated suggesting that the regulation of a given immune response involves the interaction of antigen-specific, Id-bearing antibody molecules and anti-idiotypic antibodies. In particular, it can be shown that: (a) xeno- and allogeneic anti-idiotypic antibodies can induce specific immune responses in the absence of "antigen" (3-5), and (b) that auto-anti-idiotypic antibodies (6) can be detected in vivo.

Idiotypic interactions at the T cell level have also been defined both directly and indirectly. The induction of Id-bearing helper and suppressor T cells in the antibody response to the group A streptococcal antigen has been studied using anti-idiotypic reagents (7). In these studies a striking finding was that when guinea pig anti-Id was fractionated into IgG₁ and IgG₂ components, the IgG₁ αId-induced specific T helper cells, whereas the IgG₂ fraction induced suppressor T cells. In the immune response to lysozyme, suppressor T cells share idiotypy with antibodies (8). Alloreactive T cells (9, 10) and antigen-specific helper T cells (11) can also be shown to possess idiotypic determinants. In the case of alloreactive T cells, anti-idiotypic antibodies can elicit specific immune reactions in the absence of antigen; they stimulate the production of alloantibody in vivo and the induction of cytotoxic T lymphocytes (CTL) in vitro (9). The majority of the above studies relied on anti-Id produced against specific antibodies

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¹ Abbreviations used in this paper: APC, antigen-presenting cell; CTL, cytotoxic T lymphocyte; [³H]TdR, tritiated thymidine; Id, idiotypy; IL-1, interleukin 1, IL-2, interleukin 2, T cell growth factor; MLR, mixed lymphocyte reaction; T_H, T helper cell.

to study T cell Id. Recently, however, direct production of anti-Id by immunization with virus immune CTL has been reported (12). The ability of anti-idiotypic reagents to deliver specific activating signals to appropriate clones of cells strongly suggests that such reagents interact with cellular receptors bearing the Id in question. If the anti-idiotypic reagents can allow the simplification of experimental systems used for the study of T cells, they may yield useful information on the activation of T cells.

We have shown previously that it is possible to derive clones of strain A/J (A) T cells reactive with alloantigens on (C57BL/6 $\times$ A/J) F_1 [(B6A) F_1] cells (13). These clones are designated A α (B6A) F_1 T cell clones. In addition to clones reactive with strain C57BL/6(B6) alloantigens, shown to be I-A antigens of the configuration A $_{\alpha}^b$ A $_{\beta}^b$, clones were also found that reacted with F_1 -specific alloantigens (i.e., A $_{\alpha}^b$ A $_{\beta}^k$ and A $_{\alpha}^k$ A $_{\beta}^b$ [14]). The clones that were isolated have many properties in common with the helper T cell subset, including the ability to proliferate in response to the specific antigen and to induce alloantibody in vivo (Fathman, unpublished observation). We reasoned that it should be possible to study T cell idiotype by raising antisera against these T cell clones. Theoretically, the only difference between two A α (B6A) F_1 clones, one of which recognizes B6 and the other (B6A) F_1 -specific alloantigens, should be idiotypic determinants on their respective receptors for alloantigen.

By using an F_1 anti-(parent anti- F_1) immunization procedure (15) we have developed a series of antisera which fulfill criteria for anti-idiotypic antisera. The antisera distinguish between A α (B6A) F_1 T cell clones with different reactivity patterns. In addition, by simplifying our assay system, we could show that cloned alloreactive T cells produce interleukin 2 (IL-2) upon specific activation by anti-idiotypic antisera. In this situation, anti-T-cell anti-idiotypic antisera appear to bypass the need for T cell-macrophage interaction.

Materials and Methods

Mice. Mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or bred in our animal facility at the Mayo Clinic, Rochester, MN. Strains utilized were A/J (A) C57BL/6 (B6) and (A/J $\times$ B6) F_1 [designated (B6A) F_1]. Mice of either sex, aged 6–20 wk were used.

Cell Cultures. Complete culture medium consisted of RPMI 1640 (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) supplemented with 10% fetal calf serum (Microbiological Associates, Walkersville, MD), 3×10^{-5} M 2-mercaptoethanol, 12 mM Hepes, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 2×10^{-3} M L-glutamine. In vitro primary mixed lymphocyte reactions (MLR) were done in which responding cells were strain A/J lymph node cells stimulated with 3,300 rad-irradiated (B6A) F_1 spleen cells. Responding cells were continuously cultured by serial restimulation every 10–14 d as described (16). Cultures were done in plastic culture flasks (3024, Falcon Labware, Div., Becton, Dickinson & Co., Oxnard, CA) in a humidified 37°C incubator with a 95.5% air/4.5% CO₂ atmosphere.

Isolation of T Cell Clones. Alloreactive T cell clones were produced and characterized as described previously (13). Briefly, soft agar colonies were selected from an in vitro MLR derived from strain A (responder) lymph node cells and irradiated (B6A) F_1 (stimulator) spleen cells. The colonies isolated from soft agar plates were subcloned by limiting dilution in microtiter well cultures. Clones of cells were maintained in long-term culture by restimulating every 10–14 d with irradiated stimulator-strain spleen cells in fresh media.

Assay of T Cell Proliferation. 1.5×10^4 T cells were placed in 0.2-ml microtiter wells with 10^6 irradiated stimulator cells. Proliferation was measured by adding 1 μ Ci tritiated thymidine ($[^3\text{H}]\text{TdR}$) on day 2, harvesting cells onto filter paper 16 h later, and measuring $[^3\text{H}]\text{TdR}$ incorporation by standard liquid scintillation counting. To assess the effects of anti-idiotypic antisera, 1.5×10^4 T cells were cultured with 10^6 irradiated syngeneic spleen cells and varying amounts of antisera. Proliferation was measured as above.

Assay of IL-2 Production. Production of IL-2 (T cell growth factor) was assayed by the ability of test samples to support the proliferation of a cloned CTL line (as measured by [^3H]TdR incorporation), as described previously (17). Standardization was done by comparison to purified rat IL-2. Samples were treated with a monoclonal antibody to mouse IL-2 to test specificity of the growth-supporting activity (18).

To test T cell clones for the production of IL-2, 1.5×10^4 cloned alloreactive cells were cultured in microculture wells with anti-idiotypic antisera. Cloned cells were used 10–14 d after restimulation with alloantigen and were separated from residual stimulator cells by Ficoll-hypaque density gradient centrifugation.

Production of Anti-Idiotypic Antisera. Cloned, alloreactive [A α (B6A)F $_1$] T cells were injected into (B6A)F $_1$ recipient mice. Three intraperitoneal injections, 10–14 d apart, each with 1×10^6 live cloned T cells, were performed without adjuvant. Antisera were collected 10–14 d after the last injection. Activity of each antiserum was measured by adding varying amounts to a T cell proliferation assay. Mice whose sera showed anti-idiotypic activity were given an Ehrlich's ascites tumor, and ascitic fluid was collected. Anti-idiotypic antibody was obtained by purifying immune ascites on a column of protein A Sepharose and eluting as described (19).

Results

Specificity of Selected Alloreactive T Cell Clones. Four alloreactive A α (B6A)F $_1$ T cell clones were used in this study in an attempt to raise anti-idiotypic antisera. As described above, clones of different specificities were used to define idiotypes in the context of antigen specificity. As shown in Table I, two clones, 6-4 and 6-59, were specifically reactive to "parental" I-A b alloantigens. Two other clones, 6-23 and 1-8, recognized as "hybrid" I-A $^{b/k}$ determinant(s) present only on (B6A)F $_1$ stimulator cells. Clones 6-4, 6-23, and 6-59 were isolated from the same cloning experiment; clone 1-8 was isolated from a distinct MLR. Thus, there should exist at least two different alloantigen receptors among these four clones.

Effects of Anti-Idiotypic Sera. Putative anti-idiotypic sera were raised against each of the four clones shown in Table I. Each was raised in a (B6A)F $_1$ α [A α (B6A)F $_1$] protocol. To test the effects of the "anti-clone" antisera, they were first added to microtiter well cultures containing various A α (B6A)F $_1$ clones and B6A stimulator cells. The antisera had no discernible effect on these cultures compared with control cultures without antisera (data not shown).

We then added the antisera to cultures containing cloned alloreactive T cells and syngeneic (i.e., strain A) irradiated spleen cells as "filler cells". These cultures produced the responses shown in Table II. The responses in the presence of nonimmune, syngeneic mouse serum or (B6A)F $_1$ stimulator cells are included as negative and

TABLE I
MLR Responses of Selected Clones of A α B6A T Cells

Stimulators	MHC						Clone			
	K	A α	A β	Ae	E α	D	6-4	6-59	6-23	1-8
A/J	k	k	k	k	k	d	1,014	1,150	976	768
B6	b	b	b	b	b	b	52,332	53,466	1,146	911
(B6A)F $_1$	b/k	b/k	b/k	b/k	b/k	b/d	60,554	45,672	60,412	42,136
B10.A(5R)	b	b	b	b	k	d	56,042	50,264	1,306	827
[B10.A(4R) $\times$ B6]	k/b	k/b	k/b	k/b	b/b	b/b	55,724	41,429	59,348	46,123
B10.A(4R)	k	k	k	k	b	b	488	663	240	318

MLR responses of selected clones of A α (B6A)F $_1$ T cells. Triplicate cultures of 1.5×10^4 responder cells and 10^6 irradiated spleen cells (stimulators) were set up and assayed for [^3H]TdR incorporation on day 2.

TABLE II
Idiotypic Specificity of Anti-Clone Antisera

Antibody added	T cell clone			
	6-4	6-59	6-23	1-8
Normal mouse serum	2,158	1,169	1,762	1,486
$\alpha 4$	42,681	26,883	1,287	1,501
$\alpha 23$	2,016	1,216	28,724	1,103
$\alpha 59$	17,211	11,986	1,302	1,276
B6A cells	39,402	24,655	39,743	36,724

Idiotypic specificity of anti-clone antisera. Proliferation of clones 6-4, 6-59, 6-23, and 1-8 was measured in cultures containing 1.5×10^4 T cells, 10^6 irradiated syngeneic (strain A) spleen cells, and 1 μ l of normal mouse serum or antisera raised against clone 6-4($\alpha 4$), 6-59($\alpha 59$), and 6-23($\alpha 23$). Proliferation in the presence of 10^6 (B6A)F₁ stimulator cells (without syngeneic spleen cells) is shown as a control.

TABLE III
Ig Anti-Id-induced Proliferation

Clone Number	Normal mouse serum	$\alpha 4$ Ascites	Protein-A effluent	Protein-A bound fraction
1-8	194	2,182	1,757	485
6-4	1,253	12,064	5,226	14,507
6-59	1,006	11,677	1,094	13,949
$\alpha 59$ Ascites				
1-8	194	1,456	1,243	424
6-4	1,253	10,699	3,828	9,643
6-59	1,006	13,744	691	11,682

Ig anti-Id-induced proliferation. Cultures were performed as for Table II. In addition, immune ascites to clone 6-4($\alpha 4$) and to clone 6-59($\alpha 59$) were fractionated into effluent and bound fractions on protein-A Sepharose, reconstituted to original volume, and retested.

positive controls, respectively. Antisera raised against clones 6-4, 6-59, or 6-23 stimulated the homologous clones to proliferate. Antisera to clones 6-4 or 6-59, which had identical MLR reactivities, caused stimulation of both clones but not of clone 6-23. Conversely, antiserum to clone 6-23, which differed in MLR specificity from clones 6-4 and 6-59, caused proliferation of clone 6-23 only. Antisera to clones 6-4, 6-59, or 6-23 did not stimulate clone 1-8 to proliferate. Likewise, antisera raised to clone 1-8 have been without stimulatory activity, even to the immunizing clone.

To show that the stimulatory activity of the anti-clone antisera resided solely in the immunoglobulin fraction, we passed the antisera over protein A-Sepharose columns and collected the bound and unbound fractions. Stimulatory activity to clone 6-4 resided in both recovered fractions; activity to clone 6-59 was found only in the protein-A bound fractions (Table III). On 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the protein A-bound fraction of antiserum to clone 6-4 showed only two bands, corresponding in molecular weight to γ and L chains of IgG (not shown).

Production of IL-2 by Triggering of T Cell Clones with Anti-Idiotypic Antisera. To investigate in more detail the mechanism of action of the antisera produced above,

we incubated clone 6-4 with its homologous, protein A-bound antiserum fraction in the absence of filler cells. This procedure yielded significant proliferation of clone 6-4. [^3H]TdR incorporation was more variable than when syngeneic filler cells were present, but in certain experiments the degree of proliferation induced by anti-Id with or without filler cells was indistinguishable.

We took supernatants from microcultures of clone 6-4 and its anti-clone antisera (in the absence of filler cells) and assayed for the presence of IL-2 (Table IV). The supernates contained as much as 0.47 U/ml of IL-2 activity, which could be completely removed by precipitation with a monoclonal antibody against IL-2. Control supernates from clone and antiserum combinations that did not result in T cell proliferation were devoid of IL-2 activity.

Discussion

Idiotypes have been most clearly defined on specific antibody molecules, including monoclonal hybridoma products (8, 20). Although usually associated with antibody specificity, individual idiotopes may actually be present on antibodies with apparently unrelated specificity (8, 21). Thus, certain anti-idiotypic interactions may be involved in immunoregulatory roles, rather than strictly associated with a specific antibody-combining site.

The presence of shared idiotopes between Ig and T cell receptors could be inferred from several studies. Anti-idiotypic antibodies (anti-Id) to staphylococcal nuclease-specific antibodies were given to normal or nude mice. The intact mice could produce Id-bearing Ig in response to anti-Id, whereas the nude mice could not. Moreover, anti-Id could induce nuclease-specific helper T cells (T_H). These T_H cells were able to be lysed by anti-Id and complement (5). In a separate series of experiments, lysis of lysozyme-specific, Id-bearing suppressor T cells by anti-Id and complement could also

TABLE IV
IL-2 Production by Cloned T Cells Stimulated with Anti-Idiotypic Antisera

Sample	Precipitated with*	CTL cell [^3H]TdR incorporation					
		Reciprocal of supernate dilution					IL-2
		2	4	8	16	32	
		<i>cpm</i>	<i>cpm</i>	<i>cpm</i>	<i>cpm</i>	<i>cpm</i>	<i>U/ml</i>
Rat IL-2	—	6,350	4,165	2,163	1,165	481	1.7
Rat IL-2	αgp70 IgG	5,852	3,965	1,997	1,252	684	1.6
Rat IL-2	$\alpha\text{IL-2}$ IgG	569	181	90	85	111	0.1
Clone 6-4 + $\alpha 4$	—	1,256	874	636	422	86	0.47
Clone 6-4 + $\alpha 4$	αgp70 IgG	1,121	913	582	399	123	0.41
Clone 6-4 + $\alpha 4$	$\alpha\text{IL-2}$ IgG	110	55	86	90	36	<0.05

IL-2 production by cloned T cells stimulated with anti-idiotypic antisera. An IL-2-dependent CTL line was incubated with purified rat IL-2 or supernatants of 24-h cultures of clone 6-4 and its homologous antiserum ($\alpha 4$). 1 U of IL-2 activity has been defined as that amount of rat IL-2 needed to cause 50% maximal proliferation when IL-2 is saturating at a dilution of 1:4 (17). Activity of culture supernatant (U/ml) was calculated by comparison with rat IL-2. Specificity was analyzed by pretreating test samples with monoclonal antibodies against either gp70 or IL-2 before adding to CTL cell cultures.

* Immune precipitations were conducted by room temperature incubation of 200 μl of sample with 50 μl of IgG (10 $\mu\text{g/ml}$). After 2 h, 200 μl of IgG-sorb (The Enzyme Center, Boston, MA; lyophilized *Staphylococcus aureus* 1:160 in tissue culture medium) was added and incubation continued for two additional hours. The slurry was then pelleted (1,000 g for 10 min), and the supernate tested for residual IL-2 activity.

be demonstrated (8). This anti-Id reagent was also made by immunization with antibodies to the specific antigen. For alloreactive T cells, anti-idiotypic antisera proved useful not only for probing regulation of immune responses (9, 10), but also for studying the biochemistry of Id-bearing T cell surface molecules (22).

The above studies suggest that Id-bearing molecules on T cells may play important roles in a variety of immune responses involving both humoral and cellular responses. We sought to produce anti-idiotypic reagents using cloned T cells because (a) the use of cloned cells for immunization should allow us to maximize the production of antibodies to idiotype determinants, and (b) cloned cells should be a unique source of biochemical and genetic material for studies on Id-bearing structures.

The antisera we produced appear to satisfy definitions of anti-idiotypic reagents. First, these antisera distinguish between alloreactive T cell clones (6-4 and 6-59 vs. 6-23), which should be identical except for their antigen receptors. The finding that clones 6-4 and 6-59 are both triggered by antisera to either clone suggests that they may be derived from the same clonal precursor, i.e., they may have identical antigen receptors. Alternatively, in light of results discussed above for antilysozyme antibodies, they may have specific binding sites for two different determinants on $A_a^b A_b^b$ molecules, but possess a shared idiotope. In this regard, it is interesting that clone 6-59 is stimulated only by protein A-binding Ig (Table III), presumably IgG, whereas clone 6-4 is stimulated by both bound and unbound material. This suggests that clones 6-4 and 6-59 may not have identical alloantigen receptors. Second, the antisera simulate the effect of specific antigen, i.e., they cause proliferation of the appropriate clone. Third, purified antibody fractions alone produce the same result as whole antisera, suggesting that nonspecific mitogens or growth factors in the antisera do not play a role in anti-idiotypic activation.

T cell activation by specific signals (i.e., antigen) is thought to result in the induction of a state of T cell responsiveness to IL-2 (T cell growth factor); proliferation then proceeds in an IL-2-dependent, antigen-independent step (23). Using cell populations purified by conventional techniques, it appeared that although T cells were the source of IL-2, interaction with Ia-bearing antigen-presenting cells (APC) was necessary for IL-2 elaboration (24). Presumably, the activated T cells induced the APC to secrete a distinct lymphokine (interleukin 1 [IL-1]), which caused T cells to release IL-2. Using cloned T cells it might be possible to (a) definitively show that T cells indeed produce and release IL-2, (b) define T cell subpopulations according to their abilities to respond to and/or produce IL-2, and (c) determine the role of APC in T cell activation by lymphokines.

By directly inducing proliferation of cloned T cells with anti-idiotypic antibodies in the absence of stimulator or filler cells (Table IV), we can conclude that the T cells themselves produce significant amounts of IL-2. This production of IL-2 is concurrent with T cell proliferation. Because in our hands, as well as others, these inducer T cells can be stimulated to proliferate by supernates of concanavalin A-activated rat spleen cells, a powerful source of IL-2 and other lymphokines, it may be that these cells form a closed positive feedback loop. Alternatively, cells may proliferate in response to IL-2 at one stage of differentiation, but produce IL-2 only at a stage in which they are unable to be stimulated by it (possibly as a consequence of receptor regulation by IL-2 itself). Separate stages of IL-2 production and IL-2 responsiveness might be important in the control of immune responses. Since the Ficoll-hypaque-separated T

cells used in the experiments shown in Table IV are presumably devoid of IL-1-producing accessory cells, there are two possible conclusions concerning the role of IL-1 in this assay of T cell activation. Either IL-1 is unnecessary in this system, or an IL-1 signal is delivered by the anticlone serum itself. We are currently attempting to gather evidence to decide between these two alternatives. It appears possible that anti-Id may deliver two or more specific T cell signals, one mimicking antigen and another perhaps simulating IL-1.

It is apparent that to provide both specific (anti-idiotypic) and nonspecific (lymphokine-like) signals for T cell proliferation, the antisera we have produced must contain specificities not predicted by an F₁ anti-(parent anti-F₁) immunization protocol. This is not unexpected, however, since even syngeneic immunizations with T cells can produce anti-idiotypic sera (12, 25). Antigen dose and route of immunization may induce antibodies to syngeneic determinants, particularly antigens on activated T cells, which are normally expressed in a very restricted manner in vivo.

Summary

Alloreactive T cell clones with distinct specificities were used to raise anti-idiotypic antisera via an F₁ anti-(parent anti-F₁) protocol. Antisera were raised that could stimulate the proliferation of the appropriate T cell clone, but not other clones. The active fraction of the antisera for T cell proliferation was immunoglobulin. In addition to proliferation, an anti-idiotypic antiserum could induce the appropriate T cell clone to secrete substantial amounts of interleukin 2 (IL-2). Production of IL-2 appeared independent of the involvement of accessory cells. These accessory cells may be unnecessary for IL-2 production in our assay, or their effect may be produced by anti-idiotypic. Thus, anti-idiotypic may provide two or more specific T cell signals.

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