IDENTIFICATION OF AN IDIOTYPIC MARKER OF A MAJOR REGULATORY T CELL OF THE IMMUNE RESPONSE IN B10.BR MICE TO FERREDOXIN

The Relationship of Idiotypic Regulation to Conventional Hapten-Carrier Effects

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Since the concept of the immune system based upon a network of B cells was first proposed, our understanding of immunoglobulin gene expression and cell interaction has expanded to a remarkable degree (1). Jerne's original hypothesis has since been extended to take into account these recent developments, particularly in the area of expression of idiotypes by T cells and T cell-derived factors (2, 3). It has been shown quite convincingly that specific T cells bear idiotypic determinants which interact with anti-idiotypic antisera raised against the major idiotypes expressed in the serum of immunized animals (4, 5). Idiotypic as well as anti-idiotypic T lymphocytes have been shown to play a dominant role in regulating the expression of specific idiotypes during normal immune responses (6).

In general, idiotypic interactions have so far dealt only with responses towards individual epitopes, whereas in classic immunology, immune responses are primarily based upon a cooperative association between clones of lymphocytes specific for different epitopes of a complex antigen molecule. Clearly, if idiotypic regulation can so markedly control immune responses towards a given epitope, then it is conceivable that the effects may also influence the outcome of responses towards other idiotypically unrelated determinants on the same molecule. It would seem unlikely that idiotypic regulation operates in isolation of regulation based upon hapten-carrier interactions. Predictable as this hypothesis may be, it has not been verified in any experimental system to date.

In our laboratory, the molecular basis of the immune response to ferredoxin (Fd)[1] has been studied. Ferredoxin is a simple antigen that consists of 55 amino acid residues. It was found to have only two antigenic determinants, one of which is located within a seven-amino acid portion of the NH_2-terminal of the protein (N epitope), and the other which consists of the five amino acids located at the COOH-terminal (C epitope) (7). The existence of these two determinants of Fd has more

[1] *Abbreviations used in this paper*: CFA, complete Freund's adjuvant; DME, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; Fd, ferredoxin; KLH, keyhole limpet hemocyanin; MHC, major histocompatibility complex; NRS, normal rabbit serum; PBS, phosphate-buffered saline; PC, phosphorylcholine.

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recently been defined through the isolation of hybridomas secreting monoclonal antibodies with specificities for either epitope (8). In mice, it has been shown that the immune response to Fd is under strict major histocompatibility complex (MHC) control (9). In view of the fact that each Fd molecule possesses only a single copy of each epitope, the restriction of an immune response to these two non-cross-reacting determinants provides a useful experimental model with which to investigate theories of immune regulation.

Recently, the idiotype of one monoclonal anti-Fd antibody has been shown (10) to be representative of a significant proportion of IgG antibodies in serum that are directed towards the N epitope. In this publication, we show that whereas this idiotype constitutes a small fraction of the total anti-Fd antibody, idiotypic interactions that regulate its expression also exert significant influence over the response towards the other, C epitope.

Materials and Methods

Details concerning the purification of Fd, derivation of the monoclonal anti-Fd antibody, Fd-1, enzyme-linked immunosorbent assay (ELISA) of anti-Fd antibody in immune serum, and the preparation and characterization of anti-idiotype serum have been given in a previous publication (10).

Animals. Female mice 6–8 wk of age of the following strains were purchased from The Jackson Laboratory, Bar Harbor, ME: B10.BR/oS$_n$, DBA/2J, C3H/J, CBA/J, SJL/J, AKR/J, SM/J, and C58/J. They were used between the ages of 10 and 20 wk.

Antigens and Immunization. Mice were immunized with 50 μg purified Fd emulsified in complete Freund's adjuvant (Difco Laboratories, Detroit, MI) by intraperitoneal injection on day 0. Primary immune sera were obtained on day 21 by retro-orbital bleeding. Secondary immunization with Fd was given on day 28 by intraperitoneal injection of 50 μg Fd emulsified in incomplete Freund's adjuvant (Difco Laboratories), and secondary immune sera were subsequently taken on days 35 and 49.

Radioimmunoassay for Idiotype. Idiotype expression in mouse serum was measured using a solid-phase ELISA inhibition (10) or a radioimmunoassay. Affinity isolated antiidiotype antibody was used to coat the wells of a vinyl 96-well microtest plate (Microtiter; Dynatech Laboratories, Alexandria, VA) at a concentration of 1 μg/ml in pH 9.6 carbonate buffer for a period of 1 h at 37°C. Subsequently, coated plates were washed with phosphate-buffered saline (PBS) -Tween buffer and incubated for 15 min with PBS-Tween buffer containing 0.1% bovine serum albumin (Sigma Chemical Co., St. Louis, MO) and normal B10.BR serum (0.25%). Plates were then washed in PBS-Tween buffer before incubation with assay samples. Test samples of immune sera at a dilution of 1:40 in PBS-Tween buffer and control samples of nonimmune sera containing added quantities of monoclonals Fd-1 antibody ranging from 1 to 1,280 ng/ml were dispensed in triplicate to the wells of prepared plates. After 60 min incubation at room temperature, the plates were washed and incubated as before with 100 μl/well of a solution of ^{125}I-labeled Fd-1 monoclonal antibody labeled by the chloramine T procedure (11) using a total of 150,000 cpm/well (sp act 2.4×10^9 cpm/mg protein). After 60 min, the plates were washed, the individual wells were cut out, and the amount of radioactivity bound per well was counted in a Beckman Biogamma radioisotope counter (Beckman Instruments, Fullerton, CA). A standard inhibition curve was constructed from counts obtained from samples containing normal B10.BR serum and known amounts of cold Fd-1 monoclonal antibody as a competitor. Percent inhibition was calculated using cpm per well in the presence of normal mouse serum as zero, and taking counts per minute in the lower plate portion of the curve as 100% inhibition. Percent inhibition of binding in the presence of test sera was then read directly from this standard curve. Values obtained were corrected for dilution and are expressed as ng equivalents of idiotype per ml of serum. The lower limit of sensitivity for this assay depends upon the detection limit of the standard curve (~1 ng/ml of idiotype), and the working dilution of serum used (1/4:0). The effective limit of sensitivity taking into account this working limit

(40 ng/ml of serum) as well as the range of variation observed between serum samples taken from nonimmune individuals establishes a statistical baseline of 50 ng/ml.

Adoptive Transfer of Anti-Idiotype-treated Spleen Cells. Spleen cells from either immune or nonimmune B10.BR mice were resuspended in anti-idiotype serum at a concentration of 12 μg equivalents/ml, or in an equal dilution of normal rabbit serum (NRS) that had been adsorbed with mouse immunoglobulin for a period of 45 min on ice. Cells were then washed three times with Dulbecco's modified Eagle's medium (DME) (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) and then resuspended in rabbit complement (Low-tox, Cedarlane Laboratories, Ontario, Canada) at a final dilution of 1:5 in DME and incubated 45 min at 37°C. In later experiments, the complement step was not carried out. Cells were then washed three times in DME, adjusted to a concentration of 2×10^8 cells/ml, and injected intraperitoneally (0.1 ml/mouse) into normal B10.BR mice that had received 500 rad whole-body irradiation from a gamma source (Gammacell 200; Atomic Energy of Canada, Ottawa, Canada) 24 h previously. Immediately after cell transfer, recipient mice were given an intraperitoneal injection of 50 μg Fd in complete Freund's adjuvant. Antibody titers of animals receiving nonimmune cells were determined from sera taken 21 d post-transfer, whereas titers of transferred immune spleen cells were determined from sera taken 7 or 21 d post-transfer. In some experiments, spleen cells were first fractionated before treatment with anti-idiotype or NRS using the techniques of nylon wool separation (12) for T cells and pretreatment with monoclonal anti-Thy-1.2 plus complement for B cell enrichment. Nylon wool-treated cells were shown to be mainly T cells, in that 98% of them were killed with anti-Thy-1 plus complement. Cell recovery was between 20 and 30%. Cell recovery in anti-Thy-1 plus complement-treated spleen cells was between 40 and 50%. After anti-idiotype plus complement treatment, equivalent numbers of T and B cells were resuspended to a concentration of 2×10^8 cells/ml for injection into irradiated recipients.

Epitope Specificity and Quantification of Antisera. The amount of anti-Fd antibody in immune sera was determined by a quantitative ELISA procedure that has been described previously (9). Briefly, affinity-isolated anti-Fd antibody was labeled with ^{125}I, and the specific activity determined. The antibody was then used in a standard ELISA. After the plates had been read, they were rinsed, individual wells were cut out, and the counts per minute were determined in a gamma counter. A standard curve was established from which the amount of specific antibody in subsequent ELISA could be calculated.

The procedure by which the relative amounts of antibody in antisera directed to either the N or C epitope of Fd was calculated has also been described in detail previously (8). Briefly, preparations of Fd were selectively degraded with either trypsin (which inactivates the N epitope by cleavage of lys at the third position) or carboxypeptidase A (which removes the terminal gln-glu of Fd and inactivates the C epitope). Antisera were assayed in ELISA with whole Fd and both fragments. Relative color development with the fragments is a function of antibody directed to either the N or C epitope and can be quantified in this way.

Results

The amino acid sequence of Fd, showing the location of its two antigenic determinants, is shown in Fig. 1. In high responder mice ($H\text{-}2^k$), antibody titers, after secondary antigen stimulus, reach ~20.0 μg/ml. This moderate level of immunogenicity, even in high responder mice, is not surprising considering the low molecular weight of Fd, and that the response is generated through only two epitopes. In other systems, anti-hapten antibody titers up to 5 mg/ml have been obtained, however, this response occurs after immunization with hapten coupled to multi-determinant carriers (13–14). Production of such large quantities of antibody presumably reflects the magnitude of T cell help provided by the carrier. In comparison, Fd is a molecule that can be viewed as two covalently linked haptenic groups. Thus, there can be only a single carrier moiety providing help for the alternate hapten. Because of the relative simplicity of this immunogen, it was believed that Fd could provide a model system

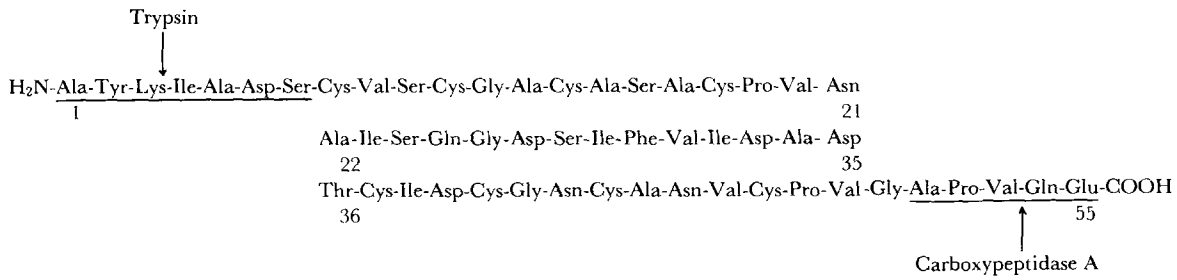

FIG. 1. The amino acid sequence of Fd. The two major epitopes (N and C) are designated, as are the two points at which selective enzyme degradation (trypsin or carboxypeptidase A) inactivates the immunologic properties of either epitope.

whereby idiotypic regulation could be examined in the context of a hapten-carrier relationship.

The approach used in this study was to generate monoclonal antibody directed to one of the two epitopes on Fd (the N epitope in this instance), identify a monoclonal antibody representing a conserved idiotype in anti-Fd antibody raised in syngeneic mice, develop anti-idiotypic antisera to the monoclonal antibody, and test its ability to influence the response of Fd immune spleen cells to either the N or C epitope using an adoptive transfer system.

The monoclonal antibody used in this study has been described previously (10), and is designated as Fd-1. Briefly, Fd-1 is an IgG_{2b} immunoglobulin that has binding specificity for the N epitope of Fd. An anti-idiotypic serum to Fd-1 (anti-Fd-1), raised in rabbits, has also been described previously (10). It was shown to be truly anti-idiotypic after appropriate absorptions by its ability to competitively inhibit binding of Fd to Fd-1. In addition, affinity-purified rabbit anti-Fd-1, when used as an immunoadsorbent, was capable of binding serum anti-Fd antibody in which it was shown that >90% of such idiotypically selected antibody demonstrated strict binding specificity for the N epitope (10).

This anti-idiotypic antibody was used in a solid-phase radioimmunoassay to determine the frequency of expression of the Fd-1 idiotype in Fd-immune B10.BR and other strains of mice. A standard curve for the binding of ^{125}I-labeled Fd-1 to anti-Fd-1 (solid phase) is shown in Fig. 2. The assay for idiotype expression in Fd-immune sera was carried out by standard competitive inhibition of binding of labeled Fd-1 in the presence of 1:4.0 dilutions of Fd-immune serum. ^{125}I-labeled monoclonal Fd-1 antibody was added to the assay at a concentration sufficient to saturate 50–75% of binding sites on the solid phase.

The results of this survey for idiotype expression in immune serum from a number of different mouse strains is shown in Table I. The ratio of N/C serum antibody is characteristic of each strain, and constant in mice of the same strain. By ELISA measurement, ~80% of the antibody produced in high responder animals ($H\text{-}2^k$ haplotype) is shown to be directed to the C-epitope. Intermediate responders ($H\text{-}2^b$ and $H\text{-}2^s$ haplotype) make essentially equal amounts of antibody directed toward the N and C epitopes. As can be seen in Table I, expression of the Fd-1 idiotype is not related to high or intermediate responder status, but appears to map to allotypes coding for genes linked to the IgH gene complex and is observed in mice bearing the $Ig\text{-}1^b$ allotype. A closer examination of this linkage is hampered by the fact that the

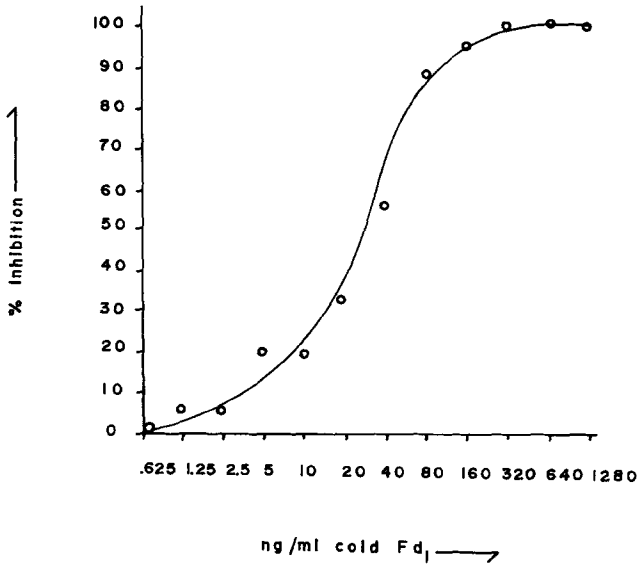

FIG. 2. Standard curve showing the cold inhibition of binding of ^{125}I-labeled Fd-1 to plates coated with affinity purified anti-Fd-1. Inhibition was carried out in 1:40 normal B10.BR serum to which varying concentrations of Fd-1 had been added.

TABLE I

Analysis of Anti-Fd Sera Taken from Mice of Various Haplotypes and Allotypes after Secondary Immunization with Fd

Strain	Haplo-type	Allotype	Anti-Fd	Anti-N*	Idiotype‡ expression	Percent idiotype expression
			μg/ml ± SEM		*ng/ml ± SEM*	
B10.BR	k	b	9.03 ± .85	1.81 ± 0.42	310 ± 37	22.9
B10.S	s	b	1.20§	0.66	88	13.3
C57BL/6	b	b	10.51 ± 1.25	2.21 ± 0.56	237 ± 77	10.8
SJL	s	b	1.35 ± 0.15	0.74 ± 0.23	250 ± 64	36.8
CE/J	k	f	13.99 ± 0.32	1.24 ± 0.20	<40	—
AKR/J	k	d	10.77 ± 0.159	1.15 ± 0.16	<40	—
ST/J	k	a	12.32 ± 0.76	2.38 ± 0.24	<40	—
RF/J	k	c	10.66 ± 0.139	0.51 ± 0.22	<40	—
C58/J	k	a	13.2 ± 0.42	1.11 ± 0.29	<40	—

All animals were bled 7 d after receiving antigen.

* Antibody directed to the N epitope as calculated in the ELISA.

‡ Antibody expressing the Fd-1 idiotype as calculated by solid-phase radioimmunoassay.

§ Between 8 and 15 mice were used in each group, with the exception of B10.S sera, in which a pool of antiserum was used.

appropriate allotype recombinant strains are derived from the H-2^d background, a haplotype which designates unresponsiveness to Fd (9).

Although a wide variation in expression of the Fd-1 idiotype in responder B10.BR mice was observed (between 0 and 100% of the total anti-N antibody produced), the median level of expression of this idiotype was 310 ng/ml (~20% of the total N epitope-specific antibody in immune serum). It was therefore recognized that the monoclonal Fd-1 antibody represented a major idiotype in B10.BR anti-Fd serum, as

it does in other mice of the IgH allotype, and one that is conserved during a secondary response.

A number of adoptive transfer experiments were carried out to determine whether the anti-Fd-1 serum would have any effects on the adoptive primary or secondary response to Fd in B10.BR mice. Splenocytes from naïve or Fd-primed B10.BR mice were treated in vitro with the anti-idiotypic serum with or without complement before

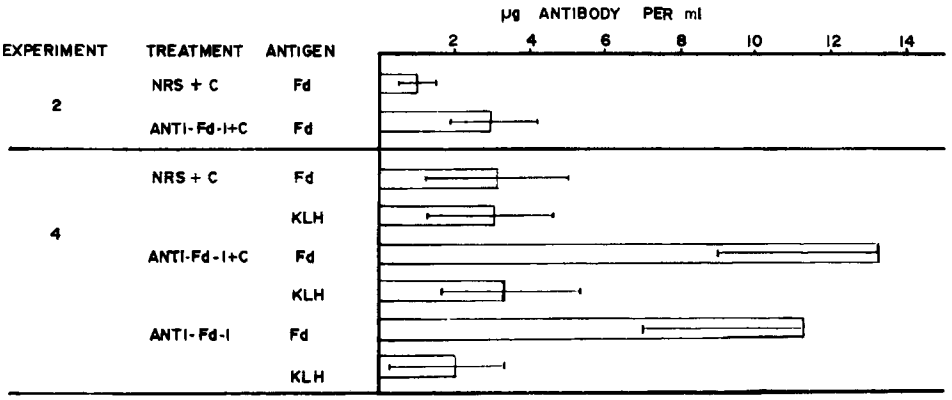

FIG. 3. Anti-Fd responses in the adoptive secondary response to Fd in B10.BR mice. Experiment 2; the anti-Fd response after the adoptive transfer of Fd-immune splenocytes treated with either anti-Fd-1 or NRS plus complement. Test bleeds were carried out 7 d after cell transfer. Experiment 4; adoptive transfer of Fd-primed splenocytes treated with either NRS or anti-Fd-1 plus complement or anti-Fd-1 alone. Recipient animals were immunized simultaneously with Fd and KLH. Test bleeds were done 21 d after adoptive transfer.

TABLE II

Influence of Anti-Fd-1 on the Adoptive Primary or Secondary Response of B10.BR Mice to Fd

Experiment number	Treatment	Time of bleed	Anti-Fd	P^* anti-N	Anti-N	Idiotype expression	P^*	Percent idiotype expression
			μg/ml ± SEM		*μg/ml ± SEM*	*ng/ml ± SEM*		
		Primary						
1‡	NRS + C§	21 d	1.82 ± 0.95		7.28 ± 0.249	60.0 ± 32		8.3
	anti-Fd-1 + C	21 d	3.89 ± 1.83	NS	1.489 ± 0.746	121.0 ± 57	NS	8.1
		Secondary						
2‡	NRS + C	7 d	1.01 ± 0.57		0.239 ± 0.212	87.2 ± 41		39.8
	anti-Fd-1 + C	7 d	2.94 ± 1.34	NS	0.644 ± 0.340	358.4 ± 297	NS	56.5
		Secondary						
3‖	NRS + C	7 d	1.07 ± 0.53		0.355 ± 0.333	ND¶		
	anti-Fd-1 + C	7 d	2.12 ± 0.9	<0.05	0.560 ± 0.286	ND		
	anti-Fd-1	7 d	2.90 ± 0.95	<0.05	0.830 ± 0.444	ND		
		Secondary						
4‖	NRS + C	21 d	3.19 ± 1.92	<0.025	0.974 ± 0.70	340 ± 192		17.4**
	anti-Fd-1 + C	21 d	13.27 ± 4.38	<0.05	4.240 ± 0.140	870 ± 4.76	NS	16.4
	anti-Fd-1	21 d	11.34 ± 4.77		2.280 ± 0.85	380 ± 144	NS	14.3

Spleen cells from Fd-immune or naïve B10.BR were treated with NRS + complement or anti-Fd-1 ± complement before injection into irradiated B10.BR recipients and bled either 7 or 21 d later. Each experient had 6 (experiments 1 and 2) or 10 (experiments 3 and 4) animals per group.

* Differences in titers between anti-Fd-1-treated mice and NRS-treated controls were analyzed by Student's *t* test to establish the significance of differences between the groups. NS, not significant ($P \geq 0.05$).

‡ Six animals per experimental group.

§ Complement.

‖ 10 animals per experimental group.

¶ Not determined.

** Percentages are based on values obtained only from sera of mice which had detectable levels of N epitope-specific antibodies and therefore do not reflect a direct percentage of numbers shown in columns 6 and 7.

injection into irradiated B10.BR recipients, which were simultaneously boosted with Fd and keyhole limpet hemocyanin (KLH) and bled 7 and/or 21 d later. Sera were tested for their reactivity to Fd, KLH, the N and C epitopes, and expression of the Fd-1 idiotype. Results of several experiments are shown in Fig. 3 and Table II. It can be seen that treatment of Fd-immune splenocytes of anti-Fd-1 anti-idiotype before adoptive transfer results in an overall increase in anti-Fd-specific antibody in adoptively transferred mice, regardless of the immune status of the transferred cells. The effect is apparent whether or not cells are treated with complement in the presence of anti-idiotype. The effect is most pronounced 3 wk after cell transfer in the adoptive secondary response.

The specificity of this effect was established by the observation that the response to KLH did not differ significantly between the three experimental groups (Fig. 3). In other experiments (data not shown) it was also observed that the adoptive secondary response to KLH in B10.BR mice was not affected by treatment of transferred cells with either NRS or anti-Fd-1. A summary of various experiments of this nature are shown in Table II. In experiment 1, unprimed cells were treated with either anti-Fd-1 or NRS, adoptively transferred to irradiated B10.BR mice, and primed with Fd. Test bleeds taken at day 21 showed that sera from the two groups differed (but not significantly) in their titers to Fd in that animals receiving cells pretreated with anti-Fd-1 appeared to be producing somewhat more antibody to Fd. When the nature of the antibody was analyzed further, it was found that the ratio directed to the N epitope or the percent of the Fd-1 idiotype expressed did not differ between the two groups. However, because the actual antibody titer was approximately doubled in the experimental group, one saw a doubling in both the amount of N epitope-specific antibody and in Fd-1 idiotype expression. In the adoptive secondary Fd response (experiments 2, 3, and 4), similar results are observed. When larger groups of animals were used the differences between NRS- and anti-Fd-1-treated groups were found to be significant. In all experiments done, anti-Fd-1 (± complement) treatment of Fd primed spleen cells resulted in increased anti-Fd responses in recipient animals. However, whereas levels of both N epitope specific antibody and Fd-1 idiotype-positive Ig were both increased in these animals, their ratio, in comparison with control animals, was not significantly changed. Two clear observations were thus made from these experiments: the treatment of cells with anti-Fd-1 was influencing positively the total anti-Fd response (80% of which was still directed to the C epitope), and that this effect was not dependent on the presence of complement.

Anti-idiotype treatment of adoptively transferred Fd-primed cells did not appear to be eliminating idiotype-positive B cells as unaltered idiotype expression was observed in treated cells and it was therefore considered possible that the treatment was affecting a T cell population. Fd-immune splenocytes were fractionated using nylon wool or anti-Thy-1.2 plus complement. Enriched T or B cell populations were treated with anti-Fd-1 or NRS before mixing and transfer to irradiated B10.BR mice, which were tested for their adoptive secondary response to Fd. The results are shown in Fig. 4. In experiment 1, it can be seen that only in those animals in which the transferred T cells have been treated with anti-idiotype is the anticipated increase in the anti-Fd titer observed. In contrast, treatment of B cells in this way has no effects on overall anti-Fd titer observed. In contrast, treatment of B cells in this way has no effect on overall anti-Fd titers. A second adoptive transfer of primed B10.BR T and

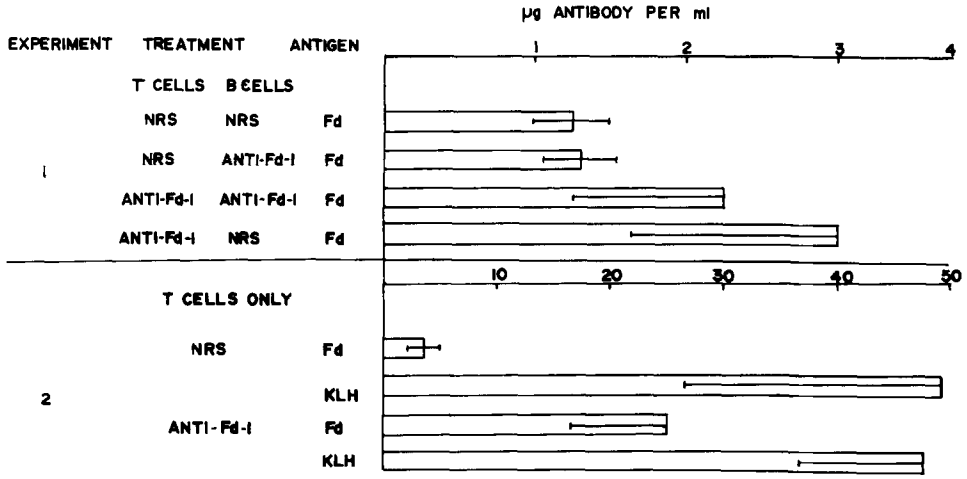

FIG. 4. Anti-Fd responses in the adoptive secondary Fd response in B10.BR mice. Experiment 1: Fd-primed B10.BR B or T cell enriched populations were treated with either anti-Fd-1 or NRS before mixing and transfer to irradiated recipients. Animals were bled 7 d after cell transfer and antigenic stimulus. There were six animals per group. Experiment 2: Fd-primed T cell enriched populations were treated with anti-Fd-1 or NRS before mixing with equal number of Fd-primed B cell enriched populations and transfer to irradiated recipients. Recipients were immunized with both Fd and KLH at the time of cell transfer and bled 21 d later. There were 12 animals per group.

TABLE III

Influence of Anti-Fd-1 on the Adoptive Secondary Response of B10.BR Mice to Fd

Experiment number	Treatment		Time of bleed	Anti-Fd	*P**	Anti-N-epitope	Fd-1 idiotype expression	Percent idiotype expression
	T cells	B cells	*day*	*µg/ml ± SEM*		*µg/ml ± SEM*	*ng/ml ± SEM*	
1	NRS	NRS	7	1.22 ± 0.88		ND‡		
	NRS	Anti-Fd-1	7	1.26 ± 0.72	NS	ND		
	Anti-Fd-1	NRS	7	3.05 ± 2.83	NS	ND		
	Anti-Fd-1	Anti-Fd-1	7	2.25 ± 2.2	NS	ND		
2	NRS		7	1.4 ± 0.55		ND		
	Anti-Fd-1		7	2.8 ± 0.8	NS	ND		
	NRS		21	3.93 ± 1.27		1.30 ± 0.8	627 ± 484	37.2
	Anti-Fd-1		21	23.63 ± 12.0	<0.005	3.32 ± 1.1	555 ± 264	16.7

Spleen cells from Fd-primed B10.BR mice were separated by nylon wool or anti-Thy-1 + complement to yield T or B cell-enriched populations. These populations were treated with either anti-Fd-1 or NRS before transfer into irradiated recipients. In experiment 2, recipients were immunized with both Fd and KLH. Animals were bled 7 and 21 d later. There were 6 animals per group in experiment 1 and 12 per group in experiment 2.

* Differences in titers between anti-Fd-1-treated and NRS-treated and NRS-treated cells were analyzed by Students' *t* test to establish significance of differences between groups. NS, not significant ($P > 0.05$).

‡ Not done.

B cells were carried out. In this case, only T cells were treated with anti-Fd-1. Animals were boosted with both Fd and KLH. The 21-d response data are shown in Fig. 4 (experiment 2) and Table III. In this experiment, as in the whole cell treatment studies, the major increase in antibody titer was attributable to an increase in C-

specific antibody, since the ratio of N/C specific antibody remains unchanged in anti-Fd-1-treated cells. Idiotype levels do not differ significantly between the two groups. That the effect is specific is demonstrated by the observation that the KLH response is not significantly different between the two treated groups.

It would appear that anti-Fd-1 antiserum is capable of influencing a population of immunoregulatory T cells, possibly suppressor cells, that bear the Fd-1 idiotype (are idiotype positive). The most profound increase in antibody titers as a result of this treatment is in antibodies directed to the C epitope, as they comprise 80% of the anti-

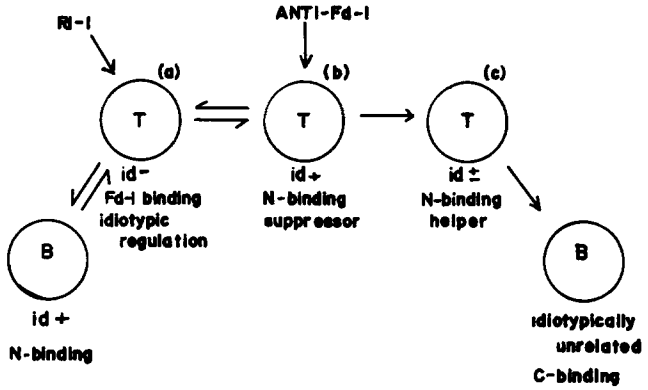

FIG. 5. Model for idiotypic interaction in the immune response to Fd. A major id^+ regulatory T cell (cell b) controls the level of help generated by other N-epitope binding T cells (cell c), which may or may not be idiotypically related but control the level of antibody produced by B cells reactive with the C-epitope. Elimination of this cell (cell b) by anti-Fd-1 increases the response to the C epitope. Because the responses to the N epitope is also increased in this process, a second, idiotype-negative regulatory T cell is also postulated (this population [a] would be bound by the Fd-1 monoclonal, because they are anti-idiotypic). Elimination of either of these cell populations destroys this level of network regulation.

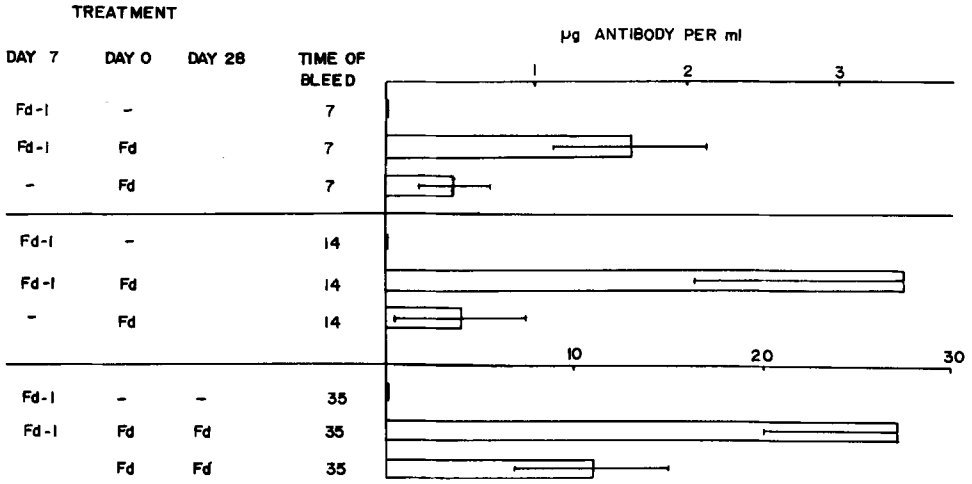

FIG. 6. Anti-Fd response in B10.BR mice treated before immunization with 10 μg Fd-1 monoclonal antibody 7 d before immunization. Animals were bled 7 and 14 d after immunization, reimmunized on day 28, and bled again on day 35. In all instances, mice treated with Fd-1 alone showed no anti-Fd titer, and mice treated with Fd-1 and Fd had significantly higher titers of anti-Fd antibody than did controls. There were 12 animals in each experimental group.

TABLE IV

Influence of the Monoclonal Fd-1 on the Immune Response to Fd in B10.BR Mice

Day 7	Day 0	Day 28	Time of bleed	Anti-Fd or anti-KLH	*P**	Anti-N	Idiotype expression	Percent idiotype expression
			day	*μg/ml ± SEM*		*μg/ml ± SEM*	*ng/ml ± SEM*	
Fd-1	—	—	7			ND‡	182 ± 65	
Fd-1	Fd	—	7	1.646 ± 0.64	<005	0.338 ± 0.87	112 ± 53	
Fd-1	KLH	—	7	0.923 ± 0.157§		ND	133 ± 64	
—	Fd	—	7	0.485 ± 0.259		0.152 ± 0.018	104 ± 61	
—	KLH	—	7	1.05 ± 0.254§		ND	0	
Fd-1	0	—	14	0				
Fd-1	Fd		14	3.330 ± 1.3	<0.005	ND		
—	Fd	—	14	0.500 ± 0.4				
Fd-1			35	0			40	
Fd-1	Fd	Fd	35	27.18 ± 7.98	<0.05	10.450 ± 3.78	386 ± 156	3.7
—	Fd	Fd	35	11.38 ± 4.63		5.300 ± 2.20	2450 ± 1644	46.2

* Differences between Fd-1 treated immunized mice and untreated controls were analysed by Students' *t* test to establish significant differences between the two groups.

‡ Not determined.

§ Titers were calculated to KLH in these instances. Differences are not significant between the two groups.

Fd response. It is of interest to note that neither the ratio of N:C epitope-specific antibody nor the percent of idiotype expression is altered significantly. It was considered possible that the idiotype-positive T immunoregulatory cells have a dual function: regulation of N-specific help, and regulation of idiotype expression by way of an anti-idiotypic population of, presumably, T cells (see Fig. 5). To test this possibility, the effects of administration of the monoclonal Fd-1 antibody (i.e., Ab-1) to nonimmune B10.BR mice was tested. Mice were injected intraperitoneally with 10 μg of the monoclonal Fd-1 7 d before immunization with Fd. The results are shown in Fig. 6 and Table IV. The treatment of mice with idiotype-positive monoclonal antibody results in a marked increase in the anti-Fd response to both epitopes; the N/C ratio remains constant, thus, a large net increase in the response to the C epitope is observed. These mice were reimmunized, and titers of secondary antibody were measured. The results are shown in Table IV, in which it can be seen that the trend is essentially the same; Fd-1-treated animals maintain elevated levels of anti-Fd antibody, the major part (70–80%) of which is directed to the C epitope. The specificity of this effect was demonstrated by the lack of significant difference between Fd-1-treated or untreated KLH-immunized animals (Table IV). After secondary immunization it was noted that treatment of animals with the Fd-1 effectively reduced expression of the Fd-1 idiotype in immune serum. Measurements of idiotype in primary serum at day 7 could not be evaluated in terms of the response to Fd-1 idiotype in their serum (Table IV) because idiotype was still detectable (180 ng/ml) in control Fd-1-treated animals. However, these levels in Fd-1-only treated animals dropped to below detectable levels after 14 d, indicating that clearance of significant quantities of the monoclonal had occurred within this 21-d period.

Discussion

Idiotypic interactions provide a direct mechanism by which T and B lymphocytes sharing identical antigen specificity can communicate. Considerable evidence for the

participation of network interactions in the control of immune responses has been described through laboratory studies involving haptens (13, 14), polymeric antigens (15, 16), and proteins of defined structure (17–21). In addition to these model systems, network phenomena have also been implicated as pathologic factors in state of abnormal immune regulation (22, 23). Each of these examples, idiotypic in nature, are therefore consistent with the central principle of network theory: control of immune responses through idiotypic interactions. Equally prominent in immune regulation, however, are interactions between lymphocytes of differing antigenic specificity (24). With respect to the average antigen molecule, one must propose either that T and B lymphocytes act through separate repertoires, or that both modes of regulation can co-exist. It would seem reasonable that these two avenues of cell interaction might be integrated, an association that would necessarily show some degree of functional interdependence. This prediction can be experimentally tested provided a very simple defined antigen molecule is available.

We have described results of studies on a particular idiotype of the anti-Fd response in B10.BR mice. This choice of experimental antigen is singular in several respects: because it is a foreign bacterial protein obtained from a soil anaerobe, it is therefore unlikely that mice would have been in contact with this antigen through environmental exposure; it is a low molecular weight antigen consisting of two distinct epitopes separated by a nonimmunogenic peptide spacer; responses to Fd are under strict immune response gene control; antibody responses to Fd are restricted in terms of both the magnitude and heterogeneity (9). Because they are limited to these two epitopes, responses need not be analyzed under limiting immunization conditions nor conjugated with heterologous carriers to be made immunogenic.

By selecting a small defined protein as experimental antigen and then using a monoclonal antibody, it has been possible to define a specific idiotype of the anti-Fd response and then examine its relationship to the overall repertoire. Because this molecule contains only two antigenic determinants, and quantification of the response to either determinant can be carried out, it has been possible to account for the influence of anti-idiotypic antiserum on the anti-Fd response at three levels: on the overall response, on the expression of the Fd-1 idiotype, and on the relative amounts of antibody formed directed to the N or C epitope in the molecule.

The specific idiotype examined in this paper, Fd-1, is present as part of the normal repertoire of B10.BR anti-Fd antibodies. B10.BR mice ($H\text{-}2^k$) are high responders to Fd, as are all $H\text{-}2^k$ strains, and this responsiveness maps to the I-A region of the MHC (9). The Fd-1 idiotype is expressed in a significant percentage of individual B10.BR immune sera, and is also present in a number of other strains bearing the $Ig\text{-}1^b$ allele of the heavy chain allotype linkage group (Table I).

When T cells or whole spleen populations were treated with anti-idiotype and transferred, the adoptive anti-Fd response of recipients was uniformly higher than that of control animals. These results imply that this idiotype is expressed on subpopulations of T cells, which possibly play a regulatory role in the immune response. This increase in anti-Fd antibody was observed when either Fd-primed or unprimed cells were treated with anti-Fd-1 + complement. This implies that the Fd-1 idiotype is expressed not only on a population of Fd-primed regulatory (suppressor) cells, but also on a population of suppressor cells present representing part of the germ line repertoire. These cells may play a significant role maintaining a steady

state in the network. The existence of such regulatory T cells has been demonstrated previously in a system involving the T-independent response to TNP and its regulation by a population of T cells exhibiting the MOPC 460 idiotype (25). When sera from recipient animals were examined, it was observed that although there was an increase in the total amount of antibody to Fd, the N/C ratio was unchanged; as 80% of the response is C epitope directed, any ratio-conservative increase will reflect a large C-specific increase. On the basis of the N-specific antibody increase, the idiotype-specific increase did not reflect a departure from the percentage of idiotype expression. There is a similarity in this observation to the findings of Reth et al. (26) who found that regulation by a given anti-idiotype antibody need not be restricted to the expression of the corresponding idiotype but could extend to other idiotypes as well. There are other similar observations that have been reported earlier, and a number of possible explanations for these results have been suggested. In some instances, it has been suggested that antibodies directed to two individual antigenic determinants may share idiotypes. That this may be the case in some systems was demonstrated by Bona and co-workers (27) with anti-allotypic sera from a variety of mouse strains. This has also been observed with anti-idiotypes raised against monoclonal antibodies directed to distinct determinants on influenza virus (28), and with anti-idiotype directed to antibodies raised to synthetic polypeptides with differing determinant specificities (29). Whether or not this is the case with the Fd system remains to be established. At this time anti-Fd-1 antiserum has not shown reactivity with any monoclonal antibodies isolated which have specificity for the C epitope. This, of course, does not preclude the possibility of cross-reactive idiotypes on cells with specificity for the C epitope.

A second possibility by which the effect of anti-Fd-1 on the anti-C epitope response could be explained in the network is that regulatory idiotypes may be present on clones which secrete antibodies of differing specificities but which are recognized and/or suppressed by the same set of regulatory T cells. This has been suggested as a mechanism to explain control and regulation of network responses to a monoclonal levan-binding antibody (A48 or Ab_1) in which Ab_3 and Ab_1 share a cross-reactive idiotype, in that both are bound by Ab_2 and Ab_4 (30). The model presented suggests the presence or absence of regulatory idiotypes in regulation of the expression of given idiotypes. More recently (31), it was shown that T helper cells that were anti-idiotypic for antibody to phosphorylcholine (PC) were able to help in the anti-PC response, whether the hapten (PC) was conjugated to either T15 or M167 (indicating that the T helper cells recognized a shared idiotypic determinant on these two idiotypically different myeloma proteins). The possible regulatory function of such a recognition site was discussed in terms of clonal dominance and in cyclical appearance of idiotypic and anti-idiotypic cells during the immune response.

Although these mechanisms may explain the regulatory effects that anti-Fd-1 antibodies exert on the response of B10.BR mice to both the N and C epitope, it is also possible that mechanisms outside the immunologic network may be responsible for the observed effect; i.e., the classical hapten-carrier effects. Previous studies on Fd have shown that there are only two immunologically reactive regions present and that these two epitopes are active at both the B and T cell level (32). Thus, in terms of hapten carrier interactions, it may be assumed that during the response to Fd, one epitope provides the help necessary to generate a response to the other. The idiotype-positive regulatory T cell influenced by anti-idiotype in these experiments is specific

for the N epitope by definition. Because N epitope binding T cells may also provide the help or suppression of the C-specific response, it is possible that the idiotype-positive cell affects the function of other N-binding T helper cells that may or may not be idiotypically related. It is impossible at this time to define more precisely the nature of the cells involved in generating the increased help for the C epitope response, as treatment of primed T cells with anti-idiotype may preferentially eliminate one idiotype population over another. That this may be the case is borne out by the observation that treatment of whole spleen cells with anti-idiotype plus or minus complement before adoptive transfer still results in a proportionate increase in idiotype expression in treated mice, indicating that the idiotype-positive B cells were not eliminated by this treatment. It was also observed that treatment of mice with small amounts (10 μg) of the Fd-1 idiotype resulted in a significant increase in both N and C antibody (i.e., a similar effect), so it may be suggested that both idiotype-positive and -negative cells interact in the same regulatory circuit. Recent studies using the levan-binding myeloma protein A48 showed that neonatal mice injected with 0.1–10 μg of the A48 monoclonal antibody and subsequently immunized with bacterial levan (BL) produced anti-BL antibody dominated by the A48 idiotype (33). The expansion of this clone was found to be attributable to the stimulation of a population of idiotype-negative T helper cells. These workers thus have demonstrated an idiotype-induced idiotype response mediated by idiotype-specific helper cells. The results presented here are essentially diametrically opposed to these observations, in that Fd-1-treated Fd-immunized animals, while demonstrating an overall increase in anti-Fd antibody, showed a marked decrease in the expression of the Fd-1 idiotype (Table IV). It would thus be compatible to propose that the putative idiotype-specific anti-Fd-1-bearing cells (those affected by Fd-1), rather than being helper cells, in terms of interaction with Fd-1-bearing B cells, may represent cells that act as helpers in the suppression circuit (already described) which is dominated by suppressor T cells bearing the Fd-1 idiotype.

A possible model by which these observations can be explained by network interactions is shown in Fig. 5. In this model, the B_N^{Id+} cell represents the idiotype-bearing/antigen-binding B cell, which may be stimulated by traditional T helper cells with specificity for the C-epitope and acting via the hapten carrier effect (these are not shown). Because anti-Fd-1 has a stimulatory effect on B_N^{Id+} expression indirectly through T cells, it is necessary to introduce a second T cell population (the *a* population), which bears the Fd-1 idiotype and is influenced by the presence of anti-Fd-1 because of its interaction with the T_N^{Id+} regulatory cells (population *b*). It is this population also (the T_N^{Id+}) that could function in regulating the help required to increase the production of C-epitope specific antibody. The N-binding T helper cells under control of the T_N^{Id+} regulatory cell (population *c*) may or may not be idiotype-positive. The finding that animals receiving cells treated with the idiotype (Fd-1) exhibited similar effects in their response to Fd, as did those receiving anti-idiotype-treated cells, making it possible to suggest that the T^{Id+} and T_N^{Id+} regulatory cells affected may be members of the same circuit and that interference with either population with either idiotype or anti-idiotype disrupts the regulatory (suppressor) circuit. This model has been kept simple, and we do not deny the possibility that there may be a number of functionally distinct idiotypically linked T cells (helpers,

suppressors) that may be preferentially affected by the treatments (anti-Fd-1 or Fd-1) used here.

Most of the work done using anti-idiotypic antisera directed to monoclonal antibodies specific for a given epitope has shown that this kind of perturbation results in either an enhanced or suppressed response to that epitope and specific idiotype expression, depending on the way in which the experiments have been carried out. In the experiments discussed here, we have shown that the anti-idiotypic sera act indirectly via T cells, but that they do not appear to enhance significantly the expression of idiotype-positive B cells. What perhaps makes these experiments of some interest in network studies is that we present clear evidence that those idiotype-positive T cells affected bring about amplification of responses to the other apparently unrelated epitope on the Fd molecule. This gives evidence for the existence of connectance between epitopically unrelated compartments of the immune system mediated through idiotypic as well as antigen-bridging interactions. The model proposed here, and for which the data presented give support, suggests connectance via idiotype-positive and -negative T cells that regulate the response to both epitopes on the molecule through both idiotypic interactions and conventional hapten-carrier interactions. Although this aspect of network has been predicted, to our knowledge, these observations constitute the first direct evidence for such an occurrence. Ongoing research in this laboratory is being directed to more definitive analysis of the idiotype-positive and -negative T cell populations involved in this regulatory circuit, both in terms of their phenotype and in terms of the mechanism of their interaction.

Summary

An anti-idiotypic antiserum was raised in rabbits to a monoclonal antibody (Fd-1) with specificity for one (the N epitope) of the two antigenic epitopes found on the ferredoxin (Fd) molecule. The anti-idiotypic antiserum (anti-Fd-1) was used to demonstrate that the Fd-1 idiotype was expressed at significant levels in most anti-Fd antisera raised in B10.BR mice. Examination of antisera raised in other mouse strains demonstrated that expression of this idiotype mapped to the IgH gene complex and was found in the antisera of all mouse strains examined with the Ig-1^b allotype. When splenocytes from Fd-immune B10.BR mice were treated with anti-Fd-1 and transferred to irradiated syngeneic recipients, the adoptive secondary response was significantly higher in animals receiving treated cells as opposed to control animals, which received normal rabbit serum-treated cells. This response produced a net increase in antibody to both determinants, and the relative amount of Fd-1 idiotype was not significantly altered. Further studies with separated cell populations showed that the overall increase of anti-Fd antibody produced was attributable to the effects of the anti-idiotypic serum on a population(s) of T cells. Treatment of mice with the Fd-1 monoclonal antibody (which should react with anti-idiotypic cells) had an analogous effect to that of the anti-idiotype, in that mice so treated produced higher concentrations of anti-Fd antibodies when they were immunized and these antibodies exhibited net increases to both determinants. A model is presented to explain these results.

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