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Immunology

A small hypervariable segment in the variable domain of an immunoglobulin light chain stimulates formation of anti-idiotypic suppressor T cells

(idiotype/delayed-type hypersensitivity/monoclonal antibody/λ light chain/somatic mutation)

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ABSTRACT **The induction in BALB/c mice of suppressor T cells that block a delayed-type hypersensitivity (DTH) response to the idiotype of M315, a myeloma protein of BALB/c origin, was examined with a variety of immunoglobulin chains and fragments whose amino acid sequences are known. Normal BALB/c mice receiving either the light chain of M315 (L^{315}, λ2 isotype) or the variable (V) domain of this chain prior to sensitization with M315 showed marked suppression of DTH to the M315 idiotype. In contrast, neither the heavy chain nor the variable domain of the heavy chain of M315 affected the DTH response. Two other λ2 chains were tested and they also failed to suppress DTH to M315. Comparison of amino acid sequences in the three λ2 chains indicates that in L^{315} at most four V region amino acid substitutions (each resulting from a somatic mutation in the Vλ2 germ-line gene) determine the specificity of the T-cell suppressor pathway. One of the four is in the framework and probably of negligible importance; the other three, however, are all clustered in the third hypervariable loop of the L^{315} V domain. The tertiary structure of L^{315} may also be essential, because disruption of intrachain disulfide bonds abolished the ability of the chain to induce suppression.**

Thymus-derived lymphocytes (T cells), like antibody-producing lymphocytes (B cells), display exquisite specificity in their capacity to recognize intrinsic idiotype (1–3) as well as extrinsic antigens (4). For the B-cell compartment, it is well known that isogeneic anti-idiotypic antibodies can recognize the idiotypic determinant created by pairing the variable (V) domains of the heavy (H) and light (L) chains (V_H and V_L, respectively) (refs. 5–7; unpublished results). Information about the T-cell compartment is less extensive. Several studies have shown that animals can produce T cells to the idiotypes of Igs from other genetically identical individuals (1–3, 8–12), but the precise specificity of anti-idiotypic T cells remains unresolved. If the immune system acts as a network that consists of sets of V-region determinants (idiotypes) and complementary V-region determinants (anti-idiotypes) that interact even in the absence of extrinsic antigen, as proposed by Jerne (13), it should be of fundamental importance to elucidate the molecular basis for the fine specificity of T cells that recognize intrinsic (i.e., self) idiotypes.

In a recent study, Sakato *et al.* showed that a single intravenous (i.v.) injection of the Fv (V_H and V_L) fragment of myeloma protein M315 just prior to a sensitizing subcutaneous injection of M315 is able to induce idiotype-specific suppressor T (T_s) cells that block the delayed-type hypersensitivity (DTH) response to M315 in syngeneic (BALB/c) mice (12). To investigate the specificities of T cells that recognize the idiotypes of isologous Igs, the T_s cells involved in the M315-specific response offer the following advantages: (*i*) the amino acid sequences of V_H and V_L of M315 are known (14, 15); (*ii*) there is no need in inducing these T_s cells to modify the antigen, such as coupling it to cells (9, 16–18), and thus the possibility of introducing undesired alterations of the idiotype can be minimized; and (*iii*) for comparative purposes, the specificity of antibodies to the idiotype of M315 has already been established (5, 7, 11).

In this report, we characterize the M315 component that induces T_s cells for the DTH response to M315. The result provides direct evidence that T_s cells are specific for the light chain of M315 (L^{315}) and indicates that this specificity is dependent on three contiguous amino acid residues in the third hypervariable loop of the V domain of the light chain.

MATERIALS AND METHODS

Immunoglobulins. Myeloma tumors were obtained several years ago from M. Potter (National Institutes of Health) or from Litton Bionetics (Baltimore, MD) and were maintained by serial passage in BALB/cAnN mice. The following myeloma proteins of the indicated H and L chain isotypes were used: M315 (α, λ2), HOPC-1 (γ2a, λ1), T952 (α, λ2), and M167 (α, κ). The proteins were isolated as described (19–21). Hybridomas 8-13 and 8-47, produced by fusing spleen cells from BALB/c mice (immunized with 2,4,6-trinitrophenyl-hemocyanin) with NS-1 myeloma cells, were generously provided by Ann Marshak-Rothstein and Malcolm Gefter of the Massachusetts Institute of Technology. Hybridoma 5-8 was produced by the same procedure except that Sp-2/0 cells were used for fusion and 2,4-dinitrophenyl-Ficoll was the immunogen (unpublished data). Monoclonal antibodies $MA^{8\text{-}13}$ (γ1, λ2), $MA^{8\text{-}47}$ (γ1, λ3), and

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Abbreviations: M315, T952, HOPC-1, and M167, purified myeloma proteins isolated from the sera of mice bearing the corresponding myeloma tumors (MOPC-315, TEPC-952, HOPC-1, and MOPC-167); H and L, heavy and light chains, respectively, of the Ig molecule; V_H^{315} and V_L^{315}, variable (V) domains of the H and L chains of M315; Fv^{315}, fragment consisting of the two V domains (V_H and V_L) of M315; L^{315}, L^{952}, and $L^{HOPC\text{-}1}$, L chains of myeloma proteins M315, T952, and HOPC-1, respectively; $L^{8\text{-}13}$ and $L^{5\text{-}8}$, L chains of monoclonal antibodies $MA^{8\text{-}13}$ and $MA^{5\text{-}8}$, respectively; H^{315} and H^{167}, H chains of M315 and M167, respectively; $H^{8\text{-}47}$, H chain of monoclonal antibody $MA^{8\text{-}47}$; L^{315}_{suc}, succinoylated L^{315}; CRA-L^{315}_{suc}, completely reduced and alkylated L^{315}_{suc}; V_λ, variable region of the λ chain; i.v., intravenous(ly); T_s, suppressor T cells; DTH, delayed-type hypersensitivity; T_h, helper T cells.

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MA^{5-8} (μ, λ3), were isolated from ascites fluid by adsorption on dinitrophenyllysyl-Sepharose 4B and elution with 2,4-dinitrophenylglycine or 2,4-dinitrophenylaminocaproic acid (19, 22, 23).

H Chains, L Chains, and Fragments. The purified Igs were partially reduced in 0.01 M dithiothreitol/0.2 M Tris·HCl, pH 8.2; they were then alkylated with iodoacetamide and subjected to gel filtration on Sephadex G-100 in 6 M urea/1 M acetic acid to separate H and L chains. Previously described methods were used to reconstitute Ig molecules from isolated H and L chains (24), to prepare the Fv fragment (Fv^{315}) of M315 by pepsin digestion, and to isolate V_L^{315} and V_H^{315} from Fv^{315} (25, 26).

Chemical Modifications. Intrachain disulfide bonds of L^{315} were reduced with 0.01 M dithiothreitol in 6 M guanidine·HCl/0.2 M Tris·HCl, pH 8.2, and the liberated SH groups were alkylated with iodoacetamide. Succinoylation of L^{315} was carried out with succinic anhydride as described (27).

Animals. Female BALB/cAnNCrj mice were purchased from Charles River, Ltd., Japan. The mice were 6–8 wk old when first immunized.

Sensitization and Elicitation of DTH. Mice were injected subcutaneously at the base of the tail with 50 μl of an emulsion of 60 μg M315 in complete Freund's adjuvant (Difco) (12, 16). Ten days later, they were challenged by injecting ear skin with 10 μg of M315 in 10 μl of 0.01 M potassium phosphate/0.15 M NaCl, pH 7.4. The difference in ear thickness before and 24 hr after challenge was measured with a Peacock dial thickness gauge H (Ozaki Mfg., Ozaki, Japan).

Induction of Suppression. Two hundred microliters of phosphate-buffered saline containing 100 μg of M315, one of its fragments or chains, various other λ chains, or reconstituted Igs were injected i.v. into normal BALB/c mice 7 days prior to sensitization (12). Induction of suppression was revealed by the results of the skin test for DTH, performed 17 days later (i.e., 10 days after sensitization with M315).

RESULTS

As shown previously (12), prior i.v. administration of a buffered saline solution of Fv^{315}, without using adjuvants or coupling the fragment to spleen cells (10, 16–18), suppresses the DTH response to myeloma protein M315. The amount of Fv^{315} needed for significant suppression was less than 10 μg and almost 100% suppression was achieved with 100 μg (Fig. 1). To determine whether the idiotypic determinant formed by the combination

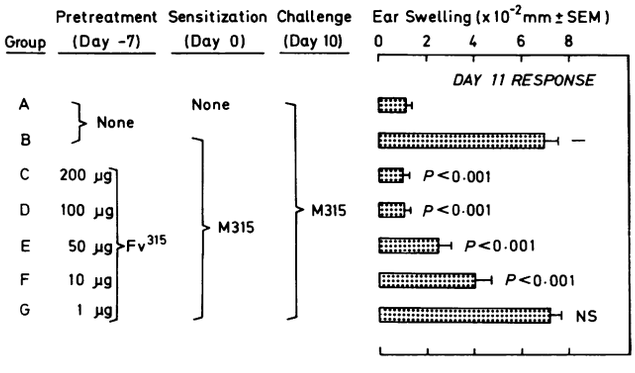

FIG. 1. Suppression of M315-idiotype-specific DTH response in BALB/c mice by a single i.v. administration of soluble Fv^{315}. Groups of seven mice that had received different doses of Fv^{315} 7 days earlier were sensitized subcutaneously at the base of the tail on day 0 with 60 μg of M315. On day 10, they were ear challenged with 10 μg of M315 and ear thickness was measured 24 hr later. Results represent mean ear swelling response ± SEM. *P* values were calculated by Student's two-tailed *t* test. NS, not significant.

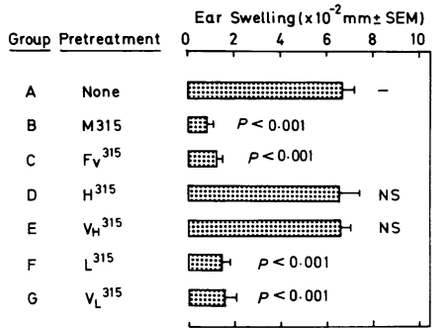

FIG. 2. In mice previously treated with V_L^{315} and L^{315}, but not in those previously treated with V_H^{315} and H^{315}, the DTH response to the idiotype of M315 was suppressed. Groups of seven mice that had received 100 μg of M315-derived chains or fragments 7 days earlier were sensitized and challenged with M315 as in Fig. 1. NS, not significantly different ($P > 0.05$) from positive controls (group A).

of V_H^{315} and V_L^{315} was essential, we examined the effects of the individual chains and V fragments of M315. Strong suppression was elicited by a single i.v. injection of L^{315} or V_L^{315}, but no detectable suppression was evoked by H^{315} or V_H^{315} (Fig. 2). Because the effect elicited by V_L^{315} was virtually complete, it appears that the constant domain of L^{315} made no contribution.

It was shown previously that the suppression elicited by the Fv fragment depends on the generation of T_s cells (12). As shown in Fig. 3, the suppression induced by L^{315} (actually by V_L^{315}) also appears to depend on the induction of T_s cells. Thus, suppression of the DTH response to M315 could be transferred to normal syngeneic (BALB/c) recipients by spleen cells, but not by serum, from donors that had been injected with L^{315} 7 days earlier, and treatment of these spleen cells *in vitro* with anti-Thy 1.2 antiserum and complement abrogated their ability to transfer the effect.

Because L^{315} is a λ2 chain (14), we next asked whether other λ2 chains, having similar amino acid sequences in their V_λ re-

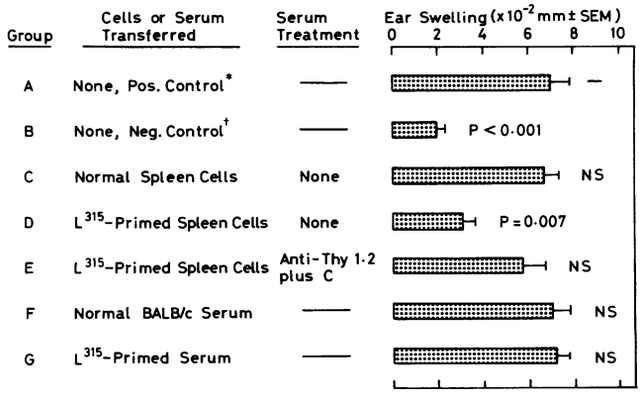

FIG. 3. Suppression of DTH response to M315 by transfer of T lymphocytes from L^{315}-treated donors. BALB/c mice were sensitized with M315 on day 0. On the same day, recipient mice were injected with 55 × 10^6 spleen cells obtained from either normal (group C) or L^{315}-primed mice (group D); the latter had been injected 7 days earlier with 100 μg of L^{315}. The donor spleen cells were either treated (group E) or not treated (group D) with anti-Thy 1.2 serum and complement before transfer. In groups F and G, recipient mice were injected i.v. with 0.1 ml of serum from normal or L^{315}-primed mice. All recipient mice were sensitized with M315 within 1 hr after transfer of cells or serum; they were ear challenged and the ear thickness was measured as in Fig. 1.

*Positive control: normal mice sensitized and challenged with M315 as in Fig. 1.

†Negative control: mice that had received 100 μg of L^{315} i.v. 7 days earlier were sensitized and challenged with M315. NS, not significant.

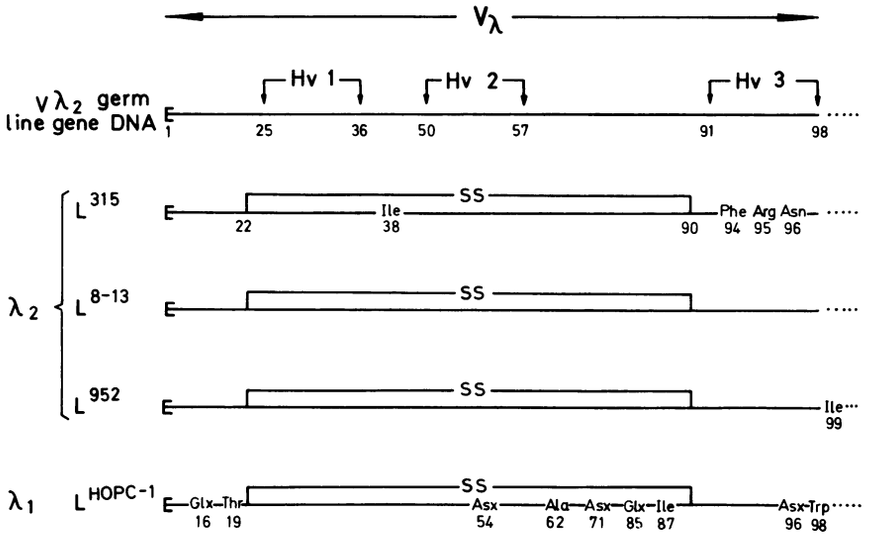

FIG. 4. Comparison of the amino acid sequences of the V regions (positions 1–110) of three λ2 chains. Included is a comparison with the germline DNA encoding sequences for λ2 chains (the Vλ2 gene for positions 1–97 or 98 and the Jλ2 gene segment for positions 98 or 99–110). Shown for comparison is the amino acid sequence for positions 1–98 of a λ1 chain (L^{HOPC-1}). The diagram is based on sequences from the following sources: DNA (28–30), L^{315} (14), L^{952} and L^{8-13} (31, 32), L^{HOPC-1} (33).

gions (Fig. 4), can also suppress the DTH to M315. As shown in Fig. 5, both L^{8-13} and L^{952} failed to do so. As anticipated, neither λ1 (L^{HOPC-1}) nor λ3 (L^{5-8}) chains were effective.

Some of the anti-idiotype antibodies elicited against an immunoglobulin, say Ig-X, react with native IgX or recombinant immunoglobulin molecules made up of the H and L chains of X (H^xL^x) but not with recombinants in which H^x or L^x is replaced by a H or L chain from another Ig molecule (refs. 5, 34, 35; unpublished results). To determine whether anti-idiotypic T_s cells have similar specificity requirements, we prepared three reconstituted Igs ($H^{167}L^{315}$, $H^{8-47}L^{315}$, and $H^{315}L^{952}$) in which H^{315} and L^{315} were combined with heterologous H and L chains. H^{167} (an α chain) was derived from a myeloma protein (M167) having antiphosphorylcholine activity and H^{8-47} (a γ1 chain) was from a monoclonal Ig (MA^{8-47}) having antitrinitrophenyl activity. Both $H^{8-47}L^{315}$ and $H^{167}L^{315}$ elicited significant suppression (Fig. 6), whereas $H^{315}L^{952}$ did not.

To determine whether the ability of L^{315} to induce suppression depends on the conformation of the chain, we examined the effect of reducing its intrachain S—S bonds. Because reductive cleavage of these bonds diminished the solubility of the chain it was necessary to increase the solubility by introducing negative charges (by succinoylation). As shown in Fig. 7, the succinoylated, completely reduced, and alkylated chain ($CRA-L^{315}_{suc}$) was unable to elicit suppression. As a control, succinoylated L^{315} having intact intrachain S—S bonds (L^{315}_{suc}) was also tested. L^{315}_{suc} was less effective than L^{315}, but it could still cause detectable suppression of the DTH reaction (Fig. 7).

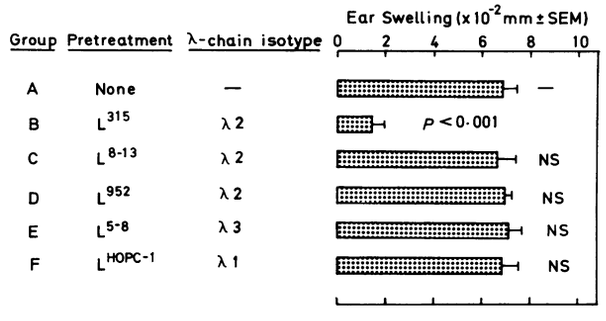

FIG. 5. Effect of previous treatment with various λ chains on DTH response to the M315 idiotype. Normal BALB/c mice, injected i.v. with various L chains in phosphate-buffered saline 7 days earlier, were sensitized and challenged with M315, and ear swelling was measured as in Fig. 1. NS, not significant.

DISCUSSION

An earlier study showed that i.v. injection of a buffered saline solution of M315 or its Fv^{315} fragment stimulates the development of T_s cells that suppress a DTH response to the M315 idiotype (12). The simplicity of this procedure led us, in this study, to test various chains and fragments from M315 for their ability to similarly suppress the DTH response to M315. Our results show that the suppressive activity was duplicated by L^{315} and V_L^{315}, a fragment that corresponds to the variable domain of L^{315}. Other components of the M315 molecule, including H^{315} and V_H^{315}, were ineffective, and other findings (see results with L^{8-13} and L^{952} below) indicate that the constant region of L^{315} also lacked activity. In accord with the ability of isolated L^{315} and V_L^{315} to induce suppression, Ig molecules reconstituted by pairing L^{315} with heterologous H chains ($H^{167}L^{315}$ and

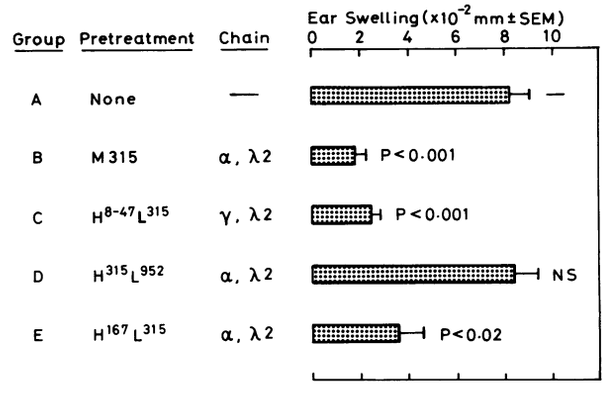

FIG. 6. Effect of previous treatment of BALB/c mice with a single i.v. injection of various reconstituted Igs on the M315 idiotype-specific DTH. Groups of seven mice that had received 100 μg of reconstituted Ig 7 days earlier were sensitized and challenged with M315, and ear thickness was measured as in Fig. 1. NS, not significant.

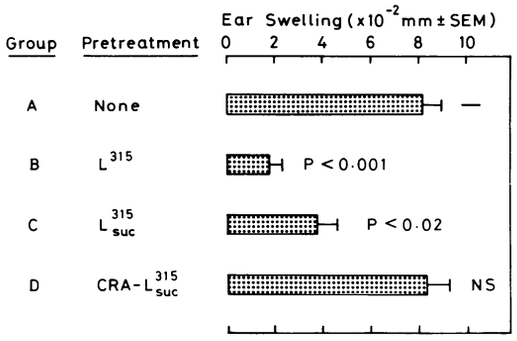

FIG. 7. Effect of succinoylation and cleavage of intrachain disulfide bonds of L^{315} on suppression of DTH responses to M315. Groups of six mice received 100 μg of L^{315}, L^{315}_{suc}, or CRA-L^{315}_{suc}; 7 days later they were sensitized and challenged, and ear thickness was measured as in Fig. 1. NS, not significant.

$H^{8\text{-}47}L^{315}$) were effective but $H^{315}L^{952}$, a reciprocal "hybrid" molecule, was not.

Perhaps our most significant finding is that the anti-M315 suppression induced by L^{315} (a λ2 chain) was not induced by two other λ2 chains, $L^{8\text{-}13}$ and L^{952}. As shown in the comparison of the V region amino acid sequences of these chains (Fig. 4), L^{315} differs at four positions from $L^{8\text{-}13}$ and at five from L^{952}. The implication of these differences is apparent from x-ray diffraction studies of Ig fragments (36, 37). According to these studies, the V_L (also the V_H) region can be viewed as having three hypervariable loops attached to a rigid framework. One of the differences between L^{315} and the other λ2 chains is in the framework (isoleucine-38 in L^{315} vs. valine-38 in the others) and is probably not significant. The other differences, however, are all clustered in the third hypervariable loop. Thus, in responding to myeloma protein M315, it appears that suppressor T_s^{315} cells (or their precursors) recognize only the third hypervariable loop of the light chain of M315 and possibly only the distinctive triplet Phe^{94}-Arg^{95}-Asn^{96} (Fig. 4).

Comparison of the sequences in the germ-line Vλ2 and Jλ2 genes (28–30) with V-region amino acid sequences (positions 1–110) of λ2 chains (14, 31, 32) shows that in $L^{8\text{-}13}$ the amino acid sequence corresponds precisely to the germ-line genes, whereas in L^{952} there is one somatic mutation ($Val^{99} \rightarrow Ile^{99}$) and in L^{315} there are four somatic mutations ($Val^{38} \rightarrow Ile^{38}$ and Tyr^{94}-Ser^{95}-$Thr^{96} \rightarrow Phe^{94}$-$Arg^{95}$-$Asn^{96}$). Whether T_s cells can be raised against $L^{8\text{-}13}$ and L^{952} has not yet been determined.

The failure of fully reduced and S-alkylated (and succinoylated) L^{315} (CRA-L^{315}_{suc}) to induce the suppressor response suggests that the responding T_s^{315} cells (or their precursors) recognize the tertiary structure of V_L^{315}, not simply the key amino acid triplet in the third hypervariable loop. We have not ruled out the possibility that CRA-L^{315}_{suc} is inactive because it may be more rapidly degraded *in vivo* than L^{315} (or L^{315}_{suc}), but it is notable that Endres and Gray (38) have also found that T_s cells recognize a difference in structure between native and denatured forms of ovalbumin.

Comparison of the results of this and earlier studies of the immune responses of BALB/c mice to the BALB/c myeloma protein M315 suggest that there is a systematic difference in antigen recognition by the T and B cells that respond to the idiotype of this isologous (in the BALB/c mouse) Ig. The antibodies to M315 (presumably, therefore, the corresponding B cells) react only with the intact M315 molecule or its Fab or Fv fragments, not with isolated H^{315} or L^{315} or with reconstituted Ig molecules in which H^{315} is replaced by another H chain or L^{315} is replaced by another L chain, including λ chains of the same or other subtype (ref. 5; unpublished work). Evidently, these isologous anti-idiotypic antibodies, and the corresponding B cells, recognize V_L^{315} only in association with V_H^{315} (and vice versa). [The anti-idiotypic antibodies raised against M315 and other myeloma proteins in allogeneic mouse strains and in rabbits differ: though these antibodies react best with the associated $V_H \cdot V_L$ pair of the immunogen, they also react to a considerable extent with many reconstituted Ig molecules in which the L or H chain of the immunogen is replaced by a heterologous complementary chain (refs. 34, 35; unpublished work).]

In contrast to the isologous anti-idiotypic B cells, the BALB/c helper T (T_h) cells that are elicited against M315 can also be evoked by L^{315} (11) and, as we show here, anti-M315 T_s cells can be elicited by L^{315} or V_L^{315} but not by H^{315} or V_H^{315}. Similarly, the specific resistance to MOPC-315 tumor cells that is induced by immunizing BALB/c mice with M315 (39) can be induced by isolated L^{315} or V_L^{315} but not by V_H^{315} (40). This resistance is probably also due to T cells, perhaps those responsible for the M315-specific DTH (12), because when it is elicited by L^{315} the only antibodies that appear (anti-L^{315}) do not react with intact M315 molecules and are not adsorbed by MOPC-315 cells (40).

To elicit all of the foregoing immune responses in BALB/c mice, the animals had to be injected subcutaneously or intraperitoneally with M315 or its component chains or fragments in various adjuvants. However, in eliciting the T_s^{315} cells, M315 and its chains or fragments can be injected i.v., simply in solution in buffered saline. Nonetheless, the resulting T_s cells appear to recognize the V region of L^{315}, like T_h^{315} cells and the T cells responsible for M315-specific DTH and probably those that mediate resistance to the corresponding myeloma tumor cells. Whether the recognition of M315 by these other T cells is also determined by just the third hypervariable loop of L^{315} has not yet been established.

The evidence presented here that regulator T cells recognize only a small segment of one V domain (e.g., V_L^{315}) independently of the complementary V domain of the intact Ig molecule has special significance for Jerne's idiotype/anti-idiotype network theory (13). According to the theory, an anti-idiotype (e.g., T_s^{315}) can influence the expression of various sets of idiotype-bearing cells, not just those that are specific for a particular epitope. In this context, $H^{315}L^{315}$ (i.e., M315) would represent the idiotype with anti-epitope (dinitrophenyl) activity, while $H^{167}L^{315}$ (which does not bind dinitrophenyl—but may well bind some other ligand specifically) would represent "nonspecific" parallel sets of idiotypes. This notion provides a rational explanation for the frequent finding that immunization with a particular antigen can evoke a response not only by clones of lymphocytes that bind that antigen but also by clones that are incapable of binding it (e.g., 41).

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