Brief Definitive Report

T HELPER CELLS RECOGNIZE AN IDIOTOPE LOCATED ON PEPTIDE 88-114/117 OF THE LIGHT CHAIN VARIABLE DOMAIN OF AN ISOLOGOUS MYELOMA PROTEIN (315)*

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Mouse myeloma protein 315 (IgA, λ2), when mixed with complete Freund's adjuvant, elicits antibodies specific for idiotypic determinants that require assembled (light [L] plus heavy chain [H]) chains for their expression (1). Previous studies from this laboratory have revealed that the antibody response of BALB/c mice to M315 is T helper cell (Th)-dependent, and that free variable region light chain 315 (V_L-315) elicits Th that respond to a boost with the complete M315 (2). In contrast, V_H-315 does not induce Th of BALB/c mice (H-2^d) that respond to M315 (2). In fact, the responses of Th to the V_L and V_H domains are governed by H-2-linked immune response genes: the H-2^d haplotype conferred high responsiveness to V_L-315 and low responsiveness to V_H-315, while the H-2^k haplotype conferred high responsiveness to V_H-315 and low responsiveness to V_L-315 (3, 4). The aim of the present study was to elucidate the structural basis for the expression of epitopes of V_L-315 recognized by Th of BALB/c (H-2^d) mice.

Materials and Methods

Mice. BALB/c/A/Bom mice purchased from Gl. Bomholtgård, Ry, Denmark or bred at our institute were used throughout.

Antigens. Isolation of M315, and preparation of its Fab, Fv, V_H, and V_L fragments, and preparation of 4-hydroxy-3-iodo-5-nitrophenylacetyl preparation of (NIP)-Fab^{315} have been described (2).

Cyanogen Bromide (CNBr) Cleavage of V_L-315 and Isolation of Fragments. CNBr fragments were produced as described (5). Briefly, 40 mg solid CNBr was added to 20 mg fully reduced and alkylated V_L-315 (4) dissolved in 70% HCOOH. After incubation for 24 h at room temperature, the fragments were lyophilized and dissolved in 2 ml 9% HCOOH and subjected to gel filtration on Sephadex G-50 superfine (see Fig. 1). Polypeptides in Coomassie brilliant blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (6) were detected with an LKB Ultroscan laser densitometer (LKB Instruments, Inc., Gaithersburg, MD). Fractions that only contained one of the two major bands resulting from the cleavage were dissolved in 0.2 M Tris/HCl, pH 8.3, pooled, and stored at −20°C. Molecular weight standards were glucagon (mol wt 3,647) and uncleaved

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V_L (mol wt ~14,000). The protein concentration of the isolated fragments was measured by the method of Bradford (7), using uncleaved V_L as the standard.

Enzyme-linked Immunosorbent Assay (ELISA) of Anti-NIP Antibodies. 96-well Linbro microtitration plates were incubated with 200 μl NIP_5-bovine serum albumin (BSA) (5 μg/ml) overnight at room temperature. Wells were emptied and 220 μl 5% normal rabbit serum was added for 2 h. After three washes with phosphate-buffered saline (PBS) containing 0.02% Tween 20, duplicates of serum diluted 1:100 in PBSA (200 μl) were added and incubated for 2 h. After washing, the wells received 200 μl alkaline phosphatase- (Sigma Chemical Co., St. Louis, MO) conjugated affinity-purified rabbit anti-mouse γ chains antibodies. Finally, after another 2-h incubation and three washes, the wells received enzyme substrate (para-nitrophenyl-phosphate; Sigma Chemical Co.) diluted in 1 M diethanolamine, pH 9.8, 0.5×10^{-3} M $MgCl_2$ (200 μl:1 mg/ml). Absorbances at 405 nm were read after 45–60 min in a Titertek Multiscan (Flow Laboratories, Inc., McLean, VA) spectrophotometer. The absorbances were corrected for background by using normal sera. A standard curve was obtained using affinity-purified mouse anti-NIP antibodies (range, 0.8–100 μg/ml). This technic was also used for measuring antibodies to the fragments of V_L-315 (see Fig. 3).

Antisera. Six BALB/c mice were immunized two times with either the N- or the C-terminal CNBr fragments of V_L-315. The first immunization (0.2 ml total) was distributed in the rear footpads and intraperitoneally and consisted of an emulsion of an equal volume of 70 μg peptide dissolved in 0.2 M Tris/HCl, pH 8.2, and complete Freund's adjuvant containing 1 mg/ml *Mycobacterium tuberculosis* (Behringwerke). The second injection (100 μg peptide in 0.2 M Tris/HCl, pH 8.3) was given intraperitoneally 30 d later. The mice were bled 14 d after the last injection and the antisera against each fragment were pooled.

Immunization and Cell Transfer. Carrier-specific Th cells were primed by one injection in each rear footpad of an emulsion consisting of an equal volume of 50 μg of either V_L or the N- or C-terminal CNBr fragment, in complete Freund's adjuvant. 12–30 d later (see Table I), the popliteal lymph node cells were harvested and pooled with spleen cells from animals primed 8 wk before with 200 μg NIP-keyhole limpet hemocyanin (KLH) emulsified in complete Freund's adjuvant. The cells were washed once and transferred intravenously to x-irradiated (500 rad) BALB/c recipients. Each recipient was given one popliteal lymph node equivalent of carrier-primed cells plus one-half spleen equivalent of NIP-primed cells. The recipients were boosted the following day with one injection (0.2 ml i.p.) of 200 μg NIP_3-Fab^{315} in saline.

Results

CNBr Fragments of V_L-315. Cleavage of the V_L-315 fragment at its methionine residue at position 87 (5) yielded two major fragments that were separated on a Sephadex G-50 superfine (Pharmacia Fine Chemicals, Piscataway, NJ) column (Fig. 1). Fractions which by SDS-PAGE were pure with respect to the two major fragments were pooled. The protein in each pool migrated as a single band by SDS-PAGE with molecular weights of about 10,000 and 4,000 (Fig. 2), which are the expected sizes of V_L-315 fragments resulting from cleavage at Met^{87}.

The N-terminal amino acid sequence of the smallest (4,000 mol wt) fragment was Tyr-Phe-Cys (carboxymethylated)-Ala, which is identical with positions 88–91 of V_L-315 (5). This proved that the 4,000 mol wt fragment corresponded to the C-terminal part of V_L-315. However, ~10% of the peptides in this pool had N-terminal amino acid sequence Phe-Arg-Asn and thus represented fragments resulting from slight CNBr cleavage at tryptophane (8) in position 93 (5).

The N-terminal amino acid of the large (10,000 mol wt) fragment is blocked (5). On the basis of these facts and the molecular weight data, we conclude that this fragment represented the N-terminal 1–86 amino acids of V_L-315.

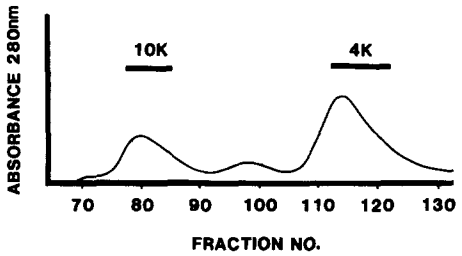

FIGURE 1. Gel filtration of the CNBr fragments of V_L-315 applied on Sephadex G-50 superfine in 9% CHOOH (180 × 2.5 cm column). Flow rate; 26 ml/h. 5.3-ml fractions were collected per tube. Horizontal bars indicate the collected fractions that contained the N-terminal (10 K) and C-terminal (4 K) fragments.

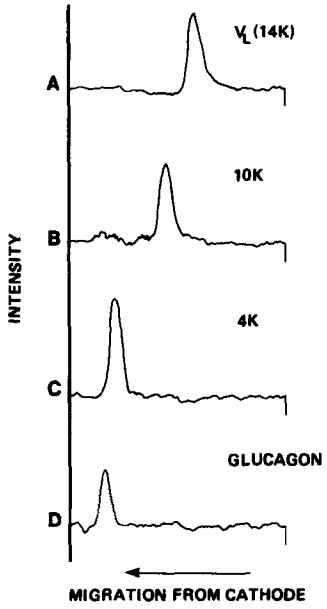

FIGURE 2. SDS-PAGE (17%). (*A*) Uncleaved V_L, ~14,000 mol wt (14 K). (*B*) N-terminal fragment of V_L, containing residues 1–86 (10 K). (*C*) C-terminal fragment of V_L, containing residues 88–114/117 (4 K). (*D*) glucagon, 3,647 mol wt.

Antibody Response of BALB/c Mice to the CNBr Fragments of V_L-315. Sera from mice immunized with the CNBr fragments were tested by ELISA. As seen in Fig. 3*A*, antibodies against the small C-terminal fragment reacted with the homologous antigen and V_L-315, but not with the large N-terminal fragment or V_H-315. Furthermore, antibodies against the large N-terminal fragment reacted with the homologous antigen and V_L-315 but not with the C-terminal fragment or V_H-315. This demonstrated that both fragments were immunogenic for BALB/c animals and further attested to the purity of the CNBr fragments.

Fragment 88–114/117 Elicits Th That Respond to NIP_3-FAB^{315}. In all four experiments, recipients that received lymph node or spleen cells from mice primed with intact V_L-315 or its C-terminal fragment responded significantly ($P < 0.005$) to a boost with NIP_3-Fab^{315} by an augmented anti-NIP antibody response (Table I) compared with mice that received cells from minimum

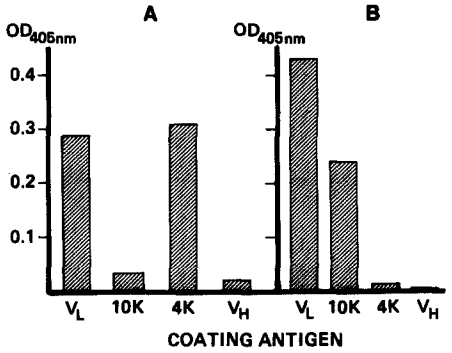

FIGURE 3. Specificity of BALB/c antisera against two fragments of V_L-315 in ELISA. (*A*) Anti-C-terminal (4 K), (*B*) anti-N-terminal (10 K). The antisera were diluted 1:20. Microtiter plates were coated with V_L-315, 10 K, 4 K, or V_H-315 (5 μg/ml).

TABLE I

V_L and its C-Terminal but not its N-Terminal Fragment Elicit M315-specific Helper Cells that Respond to a Boost with NIP_5-Fab^{315}

Group	Helper cell priming	Secondary, day 10 anti-NIP responses (μg/ml) in recipients*			
		Exp. 1	Exp. 2	Exp. 3	Exp. 4
1	MEM	<0.8	<0.8	1.3 (0.4)	<0.8
2	VL	24.9§ (2.5)*	5.6§ (1.7)	38.3§ (12.3)	9.1§ (0.8)
3	N-terminal fragment	2.1 (0.9)	<0.8	2.5 (0.5)	Not done
4	C-terminal fragment	44.1§ (2.5)	15.6§ (4.7)	36.6§ (12.9)	3.9§ (1.3)

In each experiment, the number of animals per group was five to seven. The time between priming and intravenous transfer of popliteal lymph node helper cells was 12 d in experiments 1 and 3, and 30 d in experiment 2. In experiment 4, recipients received one-half spleen equivalent of NIP-BSA and one-half spleen equivalent of carrier-primed cells harvested 6 wk after priming.

* Standard error of the mean in parentheses.

‡ Complete Freund's adjuvant mixed with minimal essential medium.

§ $P < 0.005$ when compared with group 1 or 3 (Wilcoxon two sample test).

essential medium (MEM)-primed animals. In marked contrast, the large N-terminal fragment did not elicit cells able to help the responses of NIP-primed B cells to a boost with NIP_3-Fab^{315} (Table I).

Discussion

The CNBr fragment of V_L-315 that contained the epitope in question was detected by an experimental system described by Mitchison (9; and Materials and Methods). It was found that lymphocytes from animals primed with isolated V_L-315 or its C-terminal fragment 88–114/117, the latter consisting of the third hypervariable region, the J-region, and 5–7 amino acids of the C region (10), permitted the development of significant anti-NIP antibody responses in recipients boosted with NIP-Fab^{315}, indicating that these cells contained helper cells that recognized the assembled V_L of Fab^{315}. By contrast, the large N-terminal fragment 1–86, which contained the first and second hypervariable region and framework regions, did not elicit Th that recognized Fab^{315}. Using this assay for Th activity, we have in several previous experiments (2, 11) shown that anti-Thy-1.2 plus C′ treatment of the lymphocytes primed with V_L^{315} or L^{315} abolished or greatly diminished the adoptive anti-NIP responses. The assay therefore undoubtedly detects the effects of Th on anti-NIP antibody-producing B cells.

The results should be considered in light of the fact that the V region of $\lambda 2^{315}$ expresses four somatic mutations of the germline λ2 gene: $Val^{38} \rightarrow Ile^{38}$ and $Tyr^{94}Ser^{95}Thr^{96} \rightarrow Phe^{94}Arg^{95}Asn^{96}$ (12). An attractive possibility is therefore that one or more amino acids of the mutated triplet in the third hypervariable region are essential for the expression of the antigenic site recognized by Th in our experiments.

Interestingly, Sakato et al. (13) recently demonstrated that a single intravenous injection of M315 in complete Freund's adjuvant elicited T suppressor cells that suppressed the delayed-type hypersensitivity responses of the M315 idiotype. However, suppression was not induced by two other λ2 chains (L^{952} and L^{8-13}). Since the amino acid sequence of L^{8-13} is identical with the germline λ2 V gene (11), these results (13) implied that the distinctive mutated triplet of the third hypervariable region of V_L-315 was most likely responsible for expression of the epitope recognized by T suppressor cells. However, the precise submolecular localization of this antigenic site is uncertain because it is possible that the mutated amino acids could give rise to distant conformational effects. Since the Th of the present study do recognize unfolded V_L-315 (4), in contrast to the T suppressor cells studied by Sakato and his colleagues, it should be possible to determine the precise structural basis of the V_L-315 region epitope recognized by BALB/c Th.

It is notable that both the N-terminal and C-terminal CNBr fragments of V_L-315 elicited antibody responses in BALB/c mice. Since it is highly likely that these antibody responses were Th dependent, it follows that also fragment 1–86 is recognized by Th. Why were these putative Th not detected in our helper assay? One possible explanation is that the epitope of the N-terminal, in contrast to the C-terminal fragment, is structurally hidden or functionally quenched when the V_L domain interacts with the V_H domain in Fab^{315}, which was used for challenge of the 1–86 peptide-primed Th. This phenomenon has been described by Bogen et al. (14) for the λ1 L chain of myeloma protein J558. In this instance, L^{J558}-primed Th responded to a challenge with the isolated L^{J558} but not to the complete myeloma protein J558.

As discussed previously (4), it is possible that the Th of the present study function in the idiotype network (15). If we consider M315 as "antibody 1" and the Th as an analogue of antiidiotypic "antibody 2," the Th could elicit nonspecific parallel sets of clones, i.e., clones bearing antigenic sites that resemble that of the C-terminal fragment of V_L-315 but with V_H unrelated to V_H-315.

Summary

Isolated variable region light chain 315 (V_L-315), the V_L domain of a myeloma protein of BALB/c origin, induces T cells of BALB/c ($H\text{-}2^d$) mice that help the adoptive secondary anti-4-hydroxy-3-iodo-5-nitrophenylacetyl (NIP) antibody response to $NIP\text{-}Fab^{315}$. The location of the epitope recognized by helper cells was examined with two fragments of V_L-315, obtained by cleavage with cyanogen bromide at Met^{87}. Both N-terminal fragment 1–86 and C-terminal fragment 88–114/117 elicited BALB/c antibodies that bound to the respective fragments and to V_L-315. By contrast, only fragment 88–114/117, which consists of the third hypervariable region, J region, and 5–7 amino acids of the C region, induced helper cells that augmented the anti-NIP response to $NIP\text{-}Fab^{315}$.

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