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SOMATIC MUTATIONS IN THE VARIABLE REGION OF THE λ2 CHAIN OF M315 INDUCE SUPPRESSION OF ANTIBODIES TO THE IDIOTYPE OF M315

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SUMMARY BALB/c mice immunized with purified BALB/c myeloma protein M315 (α, λ2) produce anti-idiotypic antibody directed predominantly to a combinational (V_H-315+V_L-315) determinant(s) of the M315 paratope (Sirisinha and Eisen, 1971; Tungkanak and Sirisinha, 1976). We examined whether the unique B cell response is influenced by pretreatment of mice with fragments or chains derived from M315 before immunization with M315. Intravenous (i.v.) injection of the Fv-315 fragment (V_H-315+V_L-315) into normal BALB/c mice seven days before immunization with M315 resulted in marked suppression of anti-M315 idiotype antibodies. Studies on the structural requirement for suppression indicated that V_L-315, but not V_H-315, is involved. Structural comparison with a defined λ2 light (L) chain suggested that three contiguous amino acid residues in the third hypervariable loop of the variable (V) domain of the L chain of M315 are important for down-regulation of production of antibodies to the M315 idiotype.

INTRODUCTION

The variable (V) region of an antibody molecule contains not only its combining site, but also antigenic determinants (called its idiotype) against which anti-idiotypic antibodies can be induced in other animals (see reviews by Bona and Pernis, 1984 and Eichman, 1978). Moreover, a number of studies have demonstrated that an animal can show immune responses against the idiotypes of its own immunoglobulins (Ig) or of Ig from other genetically identical individuals (Lynch and Milburn, 1984; Sakato et al., 1976; Sakato and Eisen, 1975; Sirisinha and Eisen, 1971). BALB/c mice, for example, can produce antibodies to the idiotypes of myeloma proteins of BALB/c origin (Sakato and Eisen, 1975; Sirisinha and Eisen, 1971). In addition, several studies have shown that an animal can produce T cells to the idiotypes of isogeneic Ig (Bona and Pernis, 1984; Janeway et al., 1975; Sakato et al., 1982). It has also been

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shown that the immune system of a single animal, after producing specific antibodies to a given antigen X, continues to make antibodies to the idiotype of anti-X antibodies (Eichmann, 1978; Schrater et al., 1979; McKearn et al., 1974). As the latter anti-idiotypic antibodies likewise display new idiotypic profiles, the immune system can be conceived as a network of idiotypic interactions (Jerne, 1974).

Although there is much evidence for the idiotypic network hypothesis (Bona and Pernis, 1984; Eichmann, 1978), information on the regulation of anti-idiotypic responses is still limited. Recently, we initiated studies on the mode of regulation of immune responses to the idiotype of isologous Ig (Sakato et al., 1982 and 1981). In this work, we explored the effects of in vivo administration of an idiotype on the humoral anti-idiotype response.

MATERIALS AND METHODS

1. *Mice*

Female BALB/cAnNCrj strain mice were obtained from Charles River, Co., Kanagawa, Japan. Mice of 7–9 weeks old were used throughout.

2. *Immunoglobulins*

The myeloma tumor MOPC-315 was obtained several years ago from M. Potter (National Institute of Health) and has been maintained by serial passage in BALB/cAnN mice. The following myeloma proteins of the indicated H and L chain isotypes were used: M315 (α, $\lambda 2$) and T952 (α, $\lambda 2$). M315 was isolated as a mildly reduced and alkylated (iodoacetamide) 7S protein from ascitic fluid or serum of mice carrying the MOPC-315 tumor as described (Inbar et al., 1971; Sirisinha and Eisen, 1971). T952, which has no known specificity, was isolated as described (Cotner et al., 1981) from ascites fluid provided by H. N. Eisen (Massachusetts Institute of Technology). Hibridomas MA5-5 (μ, $\lambda 3$) and MA5-7 (μ, $\lambda 2$), which have specificity for a hapten dinitrophenyl (DNP), were produced by fusing spleen cells from BALB/c mice immunized with 2,4-dinitrophenyl-N-(2-aminoethyl)-carbamylmethyl-Ficoll. These hybridomas were obtained and given to us by R. Zaugg in M.I.T. MA5-5 and MA5-7 moncolnal antibodies bind DNP and were purified by affinity chromatography as described (Elliott et al., 1984; Azuma et al., 1984).

3. *H chains, L chains and fragments*

Purified immunoglobulins were partially reduced in 0.2 M Tris HCl, pH 8.2, containing 0.01 M dithiothreitol; they were then alkylated with iodoacetamide and subjected to gel filtration on Sephadex G-100 in 6 M urea containing 1 M acetic acid to separate H and L chains (Sakato et al., 1982). The chains were rechromatographed on Sephadex G-100. Previously described methods were used 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 (Inbar et al., 1971; Hochman et al., 1973).

4. *Immunization*

Female BALB/c mice were given two, or sometime three injections at 2-week intervals of purified myeloma protein in intraperitoneal (i.p.) and subcutaneous (s.c.) sites. The protein was mixed with complete Freund's adjuvant (CFA, Difco Labs, Detroit, MI, USA, *Mycobacterium butylicum*) for the injection, with incomplete Freund's adjuvant (IFA, Difco Labs.) for the second, and with phosphate buffered saline (PBS) for the third injection. In the first immunization, a total of 150 μg of protein was injected i.p. (50 μl) and into the base of the tail (50 μl). In the second and third immunizations, totals of 100 μg of protein (100 μl) were injected s.c. into 4 sites.

5. *Induction of suppression*

Volumes of 200 μl of PBS containing 200 μg of purified immunoglobulins or fragments or sometimes chains were injected intravenously (i.v.) into normal BALB/c mice seven days before immunization with M315 or T952. Blood was taken from individual mice seven days after the 2nd and 3rd immunizations and sera were separated and stored at −80 C.

6. *Measurement of anti-idiotype antibodies*

In one assay, a double antibody radioimmunoassay was used as described (Sakato and Eisen, 1975). In most cases, a solid phase radioimmunoassay was performed using polyvinyl microtiter plates (Dynatech Labs. Inc., No. 001-010-2101) as described

previously (Sakato et al., 1980). Individual wells were coated with 150 μl of M315 (or T952)-PBS solution (100 μg/ml) at room temperature. The plates were stood for 18 h, and then the solution was replaced by a 1% solution of bovine serum albumin (BSA) in PBS-Az (PBS containing 0.04% sodium azide). The plates were stood for 1 h at room temperature, and then the wells were washed four times with PBS-Az containing 0.1% BSA (PBS-BSA-Az). Appropriately diluted antisera were inoculated into M315-coated individual wells, and stood overnight at room temperature. Then the wells were washed four times with PBS-BSA-Az, 25 ng of ^{125}I-anti-Ig was added and the wells were again stood overnight at room temperature. The wells were again washed, and cut out and their radioactivity was determined. Titration of antibodies to the T952 idiotype was performed in the same way. Anti-M315 antibodies raised in BALB/c mice were isolated by affinity chromatography on a Sepharose 4B column bearing M315, and these were used as standards in quantitation of anti-idiotypic antibodies. It is noteworthy that antibodies made against normal mouse Ig in rabbits had to be adsorbed on a Sepharose 4B column bearing M315 protein to minimize the background.

7. *Labelling of immunoglobulins with* ^{125}I

Purified immunoglobulins were iodinated by a slight modification of the chloramine-T method (Sonoda and Schlamowitz, 1970). Their specific activity was usually 5×10^{5} c.p.m./μg.

RESULTS

1. *Suppressive effect of Fv-315 on antibody production to the idiotype of M315 in vivo*

We examined the effect of a single i.v. injection of Fv-315 into normal BALB/c mice on subsequent antibody production directed to the M315 idiotype measured as serum antibody. Normal mice that had received 200 μg of Fv-315 in buffered-saline solution (PBS) 7 days before immunizations with M315 in CFA had a much lower serum level of anti-idiotype (Fig. 1-B) than control mice (Fig. 1-A). The amount of Fv-315 needed for significant suppression was 10 μg, but so far 100% suppression has not been observed.

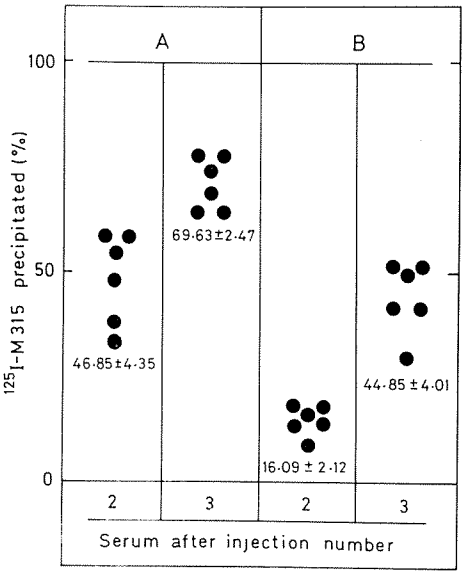

FIGURE 1. Suppression of antibody to the isologous idiotype of M315 by Fv-315. A group of six female BALB/c mice were treated i.v. with 200 μg of Fv-315 in PBS 7 days before immunization on day 0 with 150 μg of M315 emulsified in 100 μl of CFA; 50 μl at the base of the tail and 50 μl i.p. They were boosted on day 14 with 100 μg of M315 in IFA s.c. in 4 sites. On day 28, animals were treated s.c. with 100 μg of M315 without adjuvant. Individual sera were obtained 7 days after the 2nd and 3rd immunizations, i.e. on days 21 and 35. The immunization schedules in A and B were the same except that in A Fv-315 was not injected previously. The antiidiotype was determined by a double antibody radioimmunoassay as described (Sakato and Eisen, 1975) using 4 μl of antiserum and 50 ng of ^{125}I-M315. Points represent values of individual test sera and means ± SEM are shown below the points.

2. *Idiotypic determinant located on V_L-315 causes suppression*

Having revealed that the Fv region of the M315 molecule is involved in the suppression of anti-idiotypic antibody formation (Fig. 1), we next examined whether (i) the idiotypic determinant created by a combination of V_H-315 and V_L-315 is needed or (ii) the idiotype carried on either chain is sufficient for sup-

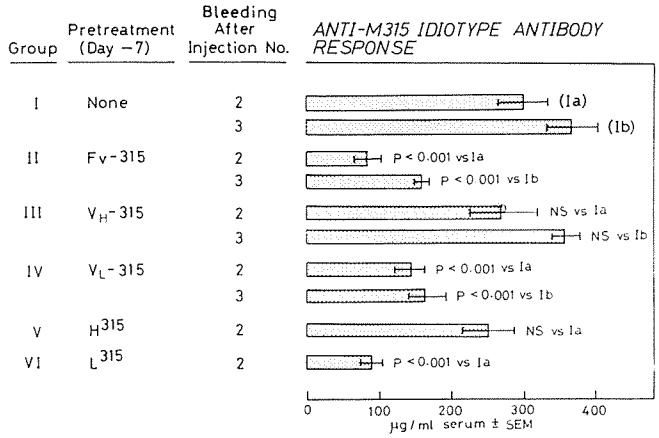

FIGURE 2. Suppression of the anti-idiotype antibody in mice treated with V_L-315 and L^{315}, but not with V_H-315 and H^{315}. Groups of seven mice that had received 200 μg of chains of fragments 7 days earlier were immunized with M315 as for Fig. 1. Individual sera was taken as for Fig. 1, and antibodies were quantitated by solid-phase radioimmunoassay as described under Materials and Methods. Columns and bars show means±SEM. P values were calculated by Student's two-tailed *t* test. NS, not significant.

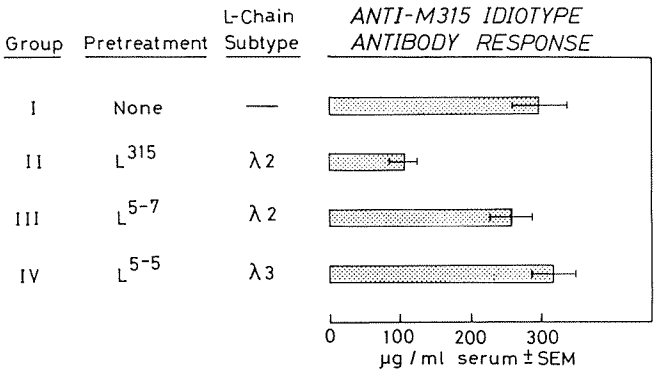

FIGURE 3. Effect of pretreatment with structurally similar λ chains on the antibody response to the M315 idiotype. Groups of seven normal BALB/c mice treated i.v. with 200 μg of L chains 7 days previously, were immunized twice as for Fig. 1, and sera obtained 7 days after the 2nd immunization were assayed by solid-phase radioimmunoassay as indicated under Materials and Methods. The amino acid sequences of L^{315} and L^{5-7} are described under Discussion.

pression. To this end, we tested the effects of individual chains and fragments of M315. Marked suppression was induced by a single i.v. injection of L^{315} (Fig. 2, Gr. VI) or V_L-315 (Fig. 2., Gr. IV), whereas no significant suppression was evoked by H^{315} or V_H-315 (Gr. III and V). Because the effects elicited by V_L-315 and L^{315} were essentially similar, the constant domain of L^{315} apparently makes no contribution to the effect. The amount of V_L-315 needed for significant suppression was 10 μg.

3. *Other λ chains do not cause suppression*

The effects of two purified λ chains, L^{5-7} and L^{5-5}, with similar amino acid sequences

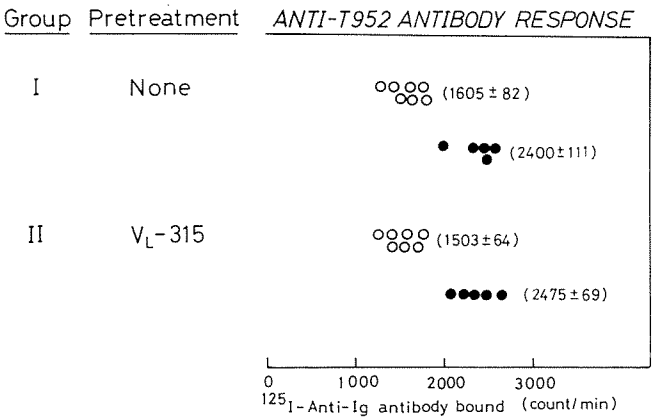

FIGURE 4. Absence of suppression of the antibody response to the idiotype of an isologous myeloma protein T952 by V_L-315. Groups of seven mice received 200 μg of V_L-315 i.v. and 7 days later were immunized twice with T952 as for Fig. 1. Sera obtained 7 days after the 2nd immunization, i.e. on day 21, were titrated by solid-phase radioimmunoassay using ^{125}I-anti-Ig as described under Materials and Methods. Samples of 20 μl (open circles) or 50 μl (closed circles) of diluted antiserum (1 : 2000 dil.) were assayed. Values in parentheses are means ± SEM for bound radioactivity.

to that of L^{315} on anti-M315 idiotype antibody production were examined. In sharp contrast to the suppressive effect of L^{315}, neither L^{5-7} (λ2) nor L^{5-5} (λ3) blocked the production of anti-M315 idiotype antibodies (Fig. 3).

4. *V_L-315 specifically suppresses the production of anti-M315 antibodies*

We examined the specificity of the effect of V_L-315. T952 is also a BALB/c myeloma protein with α chains and λ2 chains like M315. As shown in Fig. 4, production of antibody to the idiotype of T952 was not affected by a single i.v. injection of V_L-315 before immunization of BALB/c mice with purified T952. Thus previous i.v. exposure of normal BALB/c mice to the V_L-315 domain specifically suppressed production of antibodies to the M315 idiotype.

DISCUSSION

There have been only few studies on down-regulation of the specific immune response to the idiotype of isologous Ig (Sakato et al., 1981, 1982). The present results demonstrate that injection of Fv-315, L^{315} or V_L-315 solution without adjuvant has a marked suppressive effect on the antibody response to the M315 idiotype. Because the same inhibition profiles were observed after a single i.v. injection of Fv-315, L^{315} or V_L-315, but not of H^{315} or V_H-315, we conclude that the structure responsible for suppression is located on the V_L-315 sequence. V_L-315 causes highly reproducible suppression of antibody production to the M315 idiotype in CFA. Our data also demonstrate that the suppression is idiotype-specific, because i.v. injection of V_L-315 did not suppress the production of antibodies to T952 (α, λ2), which differs from V_L-315 in five amino acids at positions 38, 94, 95, 96 and 99. The use of M315 as a model idiotype has the following advantages. First, the entire amino acid sequences of V_H and V_L are known (Dugan et al., 1973; Francis et al., 1974), so that the precise structure of the idiotype that regulates the immune responses to the idiotype can be determined. Second, the specificity of isologous anti-M315 antibodies (i.e. those produced in the syngeneic strain) has already been established:, i.e. speci-

fic for a combinational (V_L-315 plus V_H-315) determinant of the M315 paratope (Sirishinha and Eisen, 1971; Tungkanak and Sirisinha, 1976; Lynch and Milburn, 1984).

Previous studies (Sakato et al., 1981 and 1982) demonstrated that in the BALB/c strain, Fv-315, L^{315} or V_L-315 induced suppression of the delayed-type hypersensitivity (DTH) response, provided that they are injected i.v. in a soluble form before sensitization with M315 in CFA. We also found that the suppressive effect of V_L-315 on M315-DTH involved the generation of suppressor T cells (Sakato et al., 1982). Our experimental protocol is useful, because there is no need to induce suppression to modify the idiotype, such as by coupling the idiotype to the cells (Moser et al., 1983), and thus there is little likelihood of undesired alterations of the idiotype. An important unresolved question is whether the B cell response to the isologous M315 idiotype is also regulated by the V_L-315. As mentioned above, the B cell response to M315 is focused on the idiotypic determinant(s) formed by both V_H and V_L. Therefore, if B cell tolerance predominates, presumably only the Fv fragment will be suppressive. However our data indicate that V_L alone is also suppressive (Fig. 2, Gr. IV). As for the specificity of T cells that regulate the immune response to the M315 idiotype, we demonstrated that V_L-315 specific suppressor T cells regulate M315-DTH (Sakato et al., 1982), and others reported that V_L-specific helper T cells involve in the production of antibodies (Jörgensen and Hannested, 1979). From these studies, helper T cell tolerance and/or the suppressor T cell mechanism can explain the suppressive effect of V_L-315 on the anti-idiotype antibody response.

Our most significant finding was that L^{315} induced suppression, whereas L^{5-7} did not (Fig. 3). The sequence of L^{5-7} differs at one position (tyrosine-98) from the $\lambda 2$ germ-line sequence (Elliott et al., 1984). L^{315} differs at four positions (isoleucine-38, phenylalanine-94, arginine-95 and asparagine-96) from the $\lambda 2$ germ-line sequence (Dugan et al., 1973; Elliott et al., 1984). Therefore, four amino acid substitutions (each resulting from a somatic mutation in the V$\lambda 2$ germ-line gene) in the $\lambda 2$ light chain of M315 determine the suppressive activity in the production of antibodies to the M315 idiotype. Because isoleucine at position 38 is in the framework and probably of negligible importance (Sakato et al., 1982), we conclude that the three amino acids at positions 94, 95 and 96 are important.

As has been pointed out (Sakato et al., 1982), it is conceivable that any lymphocytes or antibodies bearing the V_L-315 idiotype could regulate the complementary (i.e. anti-idiotypic) sets of the immune system. The V_L-315 idiotype-positive elements can be considered as nonspecific parallel sets (Jerne, 1974), because they express the regulatory idiotype located on L^{315}. Although the molecular basis of this type of regulatory idiotype is poorly understood, clustered somatic mutations, as evident in L^{315}, may be important. This hypothesis is based on the following findings. First, neither L^{952}, which differs from the germ-line V$\lambda 2$ sequence in one amino acid at position 99, nor H^{952} alone suppressed the T952-DTH. Second, we also found that a DTH response to the idiotype of BALB/c myeloma protein M167 (α, κ) could not be suppressed by pretreatment with its L chain, whose amino acid sequence differs from the germ-line sequence of Vκ in two distantly separated positions 54 and 73 (Sakato et al., unpublished observation). In both cases, L chains joined to their homologous H chains, i.e. $L^{952}H^{952}$ and $L^{167}H^{167}$, are required for regulation of the idiotype-specific DTH response to the corresponding Ig. The problem of whether antibody responses to the idiotypes of T952 and M167 are also regulated only by their own idiotype formed by a combination of V_H and V_L, and not by the nonspecific parallel sets, $L^{952}H^{x}$ and $L^{167}H^{y}$, where x and y represent unrelated H chains, remains unresolved.

A number of important questions are raised by the studies presented here and elsewhere (Sakato et al., 1982; Jörgensen and Hannestad, 1979; Lynch and Milburn, 1984). What is the relationship between the various L^{315}-specific regulatory effects? What is the mechanism of the suppressive effect of V_L-315 on the anti-M315 response? Do V_L-315 specific suppressor T cells for M315-DTH (Sakato et al., 1982) also function in the M315-specific B cell response? These questions must be studied to obtain new and useful information about the nature of immunoregulatory mechanisms.

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