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# Clonal regulation in the response to phosphorylcholine II. Heterogeneity among T15 idiotype-positive antibodies in inbred and wild mice*

Inbred stains of mice routinely respond to phosphorylcholine (PC)-containing antigens, *S. pneumoniae* and PC-keyhole limpet hemocyanin, with the production of three major families of antibodies. Two of these groups, the M 511 and M 603 idiotype (Id) families, are clearly heterogeneous, whereas the third, T15, normally exhibits much less heterogeneity and appears to be monoclonal in many strains. We report here on the occurrence of additional T15 $Id^+$ antibodies which can be detected readily by isoelectric focusing. These antibodies have been observed in 9 of 12 strains examined at an average frequency of 10%. They occur in both the $IgG_1$ and $IgG_3$ isotypes. Collectively, the antibodies comprise a heterogeneous group although cofocusing antibodies were observed in individuals of a strain or between strains. Two H-2-congenic strains, BALB.B and BALB.G, regularly displayed multiple cofocusing T15 $Id^+$ antibodies in each individual. Idiotypic analysis of these antibodies in all strains showed that each contained H, L and H-L-specific variable region determinants common to T15. A surprising finding was the consistent expression of a heterogeneous T15 Id response in two wild *Mus musculus* lines. Most of these antibodies cofocused with T15 $Id^+$ antibodies in BALB.G or C3H as well as contained strain-specific idiotypic determinants found among inbred mice. These data demonstrate that BALB/c and other inbred strains possess the genetic potential to generate a family of closely related T15 $Id^+$ antibodies. The results further suggest that the restricted response usually seen among inbred strains is not normal and exists because of a highly selective regulatory system.

## 1 Introduction

The hallmark of the response to phosphorylcholine (PC) in most inbred mouse strains is the restricted nature and apparent monoclonality of the expressed antibodies possessing idiotypic (Id) determinants of the PC-binding myeloma protein TEPC 15 (T15) [1]. This is supported by isoelectric focusing (IEF) and Id studies [2–4], from analyses of B cell precursors [5] and by recent studies on T15 $Id^+$ hybridoma antibodies [6]. By contrast, the antibody response to other defined antigens, even when idiotypy is used to select the antibodies, shows definite, though probably limited heterogeneity [7–9]. Even non-T15 $Id^+$ antibodies, *e.g.* M 511 and M 603 $Id^+$ antibodies clearly exist as families of related immunoglobulins [10, 11]. This lack of easily demonstrable heterogeneity in the T15 $Id^+$ response is even more enigmatic in view of the recent findings that T15 $V_L$ (Campbell, Stavnezer and Claflin, to be published) and a $V_H$ [12] DNA probe detect multiple genes in germ line DNA. Thus, a potential for heterogeneity among T15 $Id^+$ antibodies seems *a priori* to exist, but it is not readily manifest.

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In the present communication, we report on the occasional appearance of T15 $Id^+$ bands focusing outside the usual pattern. The frequency of appearance of these additional antibodies is high (0–20%) indicating that they do not arise as somatic mutants of germ line genes. In support of this is the finding that T15 antibodies among two BALB/c congenic strains and wild *Mus musculus* are normally quite heterogeneous. The results of these studies are reported and the implications are discussed.

## 2 Materials and methods

### 2.1 Animals and immunization

BALB/c and AKR mice were obtained from our mouse colony which was begun with breeder stock obtained from The Jackson Laboratory, Bar Harbor, ME. C3H/HeJ, C57L/J and 129/J were purchased directly from The Jackson Laboratory. BALB.B (H-$2^b$) and BALB.G (H-$2^g$) breeder stock were obtained from Dr. Frank Lilly, Albert Einstein College of Medicine (Bronx, NY) and raised in our mouse colony. The wild mouse strains, *Mus musculus musculus* (Denmark, Vegrumbro) and *M. m. m.* (Denmark, Skive) were kind gifts of Dr. Michael Potter, NCI, Bethesda, MD. All animals were immunized i.p. with two 200 μg doses of PC-KLH (keyhole limpet hemocyanin) in complete Freund's adjuvant (CFA) separated by 12–15 days. The antigen in the first injection was $PC_{15}$ KLH and the antigen in the second was $PC_{15}$KLH or $PC_{30}$KLH where the subscript refers to the estimated hapten density on the carrier [3]. Animals were bled at various times after both the primary and secondary immunizations.

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**Abbreviations: Id:** Idiotype(-ic) **IEF:** Isoelectric focusing **PC:** Phosphorylcholine **SRIA:** Solid-phase radioimmunoassay **T15:** TEPC 15 **KLH:** Keyhole limpet hemocyanin **CFA:** Complete Freund's adjuvant

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### 2.2 Purification of antibody

Where indicated anti-PC antibodies were purified from immune serum by affinity chromatography using PC-Sepharose [3]. Individual sera were treated separately.

### 2.3 Anti-Id antibodies

The two different anti-T15 antibody preparations used in these experiments have been described in detail elsewhere [3]. Rabbit anti-T15 and A/J anti-T15 antisera were made specific by adsorption on Sepharose-4B columns to which mouse serum and appropriate myeloma proteins were attached. By direct solid phase radioimmunoassay (SRIA) neither serum reacted at a 1:10 dilution with the structurally distinct PC-BMP, M511, M167, and M603 (all $\alpha,\kappa$) or with myelomas of any other isotype. Both antisera reacted with T15 in the indirect SRIA (inhibition) but not with other PC-BMP. The rabbit antiserum to M511 was prepared in an analogous fashion and showed comparable specificity for M511 [11].

### 2.4 Quantitation of Id

Quantitative SRIA for Id determinants were performed in flexible 96-well microtiter plates according to previously described procedures [4]. The amount of T15 Id determinants in serum or a PC-specific antibody population was determined by comparing its inhibition profile to that of a standard titration of T15. T15 was iodinated with $^{125}I$ by the chloramine-T procedure [13] to specific activities ranging from 13–24 μCi/μg (= 481–888 kBq/μg).

### 2.5 Analytic IEF

Individual antibodies or sera were examined by IEF in 5% polyacrylamide gels cross-linked with 0.75% (w/v) diallyltartardiamide or 0.14% bisacrylamide as previously described [4]. Antibodies bearing T15 Id were detected *in situ* with $^{125}I$-labeled anti-T15 antibodies. These antibodies were radioiodinated while bound to T15-Sepharose [3].

## 3 Results

### 3.1 Heterogeneity among inbred strains

Spectrotypic analysis of T15 $Id^+$ antibodies in IEF gels usually reveals a simple redundant pattern in BALB/c as well as other strains of mice. This pattern (IEF-T15) consists of three major sets of bands which correspond to $IgG_1$, $IgG_2$ and $IgG_3$ antibodies [3, 4]. Evidence for heterogeneity is routinely seen only among certain strains of the $Igh^j$ haplotype [14]. During the course of examining hundreds of mice in these previous studies we noted the occasional appearance of additional T15 $Id^+$ bands. These occurred at low frequency but they were not rare (~10%). They were most noticeable when clearly independent of the redundant pattern, but they were often seen as slight, cathodal extensions of the redundant $IgG_1$ or $IgG_3$ pattern (*e.g.* A/He, AL/N and CE in [3] and C58 and A/He in [4]). A collection of some of these instances discovered during a three-year period of investigation is shown in Fig. 1. Additional examples are reported elsewhere [4]. The redundant $IgG_1$ and $IgG_3$ pattern in IEF-T15 can be traced by the hatched bars. The extra sets of bands (IEF-T15′) can readily be seen in C57L mouse No. 7, AKR mouse No. 27 and 28, 129 mouse No. 19 and BALB/c mouse No. 13 and 7s. The latter mouse is part of a group which was neonatally suppressed with an intermediate dose of anti-T15. The IEF-T15′ bands were usually $IgG_1$ (pH 6.0–7.0) but could be found in the $IgG_3$ subclass (pH 7.5–8.0). In about 1–2% of cases both $IgG_1$ and $IgG_3$ bands were seen (*e.g.* C57L No. 7 and AKR No. 28) and less often mice contained multiple sets in the same isotype. For instance, three and possibly four sets of T15 $Id^+$ IEF bands of the $IgG_1$ isotype can be seen in C57L mouse No. 7. In no instance did all members of an experimental group display IEF-T15′ bands;

**Table 1.** Frequency of IEF-T15′ antibodies among inbred and wild mice

| Strain | No. of expts. | No. of mice | Isotype distribution $\gamma_1$ | Isotype distribution $\gamma_3$ | Total mice positive | Frequency[b] (%) |
|---|---|---|---|---|---|---|
| BALB/c | 11 | 251 | 17 | 3 | 18 | |
| B.C8 | 2 | 15 | 2 | 0 | 2 | |
| C57L | 3 | 22 | 1 | 1 | 1 | |
| C57Br | 1 | 8 | 0 | 0 | 0 | |
| C58 | 3 | 63 | 12 | 10 | 12 | |
| SEC | 1 | 5 | 0 | 0 | 0 | |
| 129 | 1 | 10 | 1 | 0 | 1 | |
| DBA/2 | 1 | 6 | 0 | 0 | 0 | |
| AKR | 2 | 11 | 3 | 1 | 3 | |
| AL/N | 1 | 6 | 0 | 1 | 1 | |
| A/He | 3 | 18 | 3 | 1 | 3 | |
| CE | 2 | 14 | 1 | 2 | 2 | 10.0 |
| | (31)[a] | (429)[a] | | | (43)[a] | |
| BALB.B | 2 | 9 | 8 | 5 | 8 | 89 |
| BALB.G | 5 | 51 | 47 | 23 | 49 | 96 |
| *M.m.m.* (Vegrumbro) | 1 | 10 | 9 | 1 | 9 | 90[c] |
| *M.m.m.* (Skive) | 1 | 5 | 3 | 1 | 3 | 60 |

a) Totals among inbred strains.
b) Percent of all mice tested showing IEF-T15′ antibodies in either $\gamma_1$ or $\gamma_3$ isotpye.
c) One animal failed to respond.

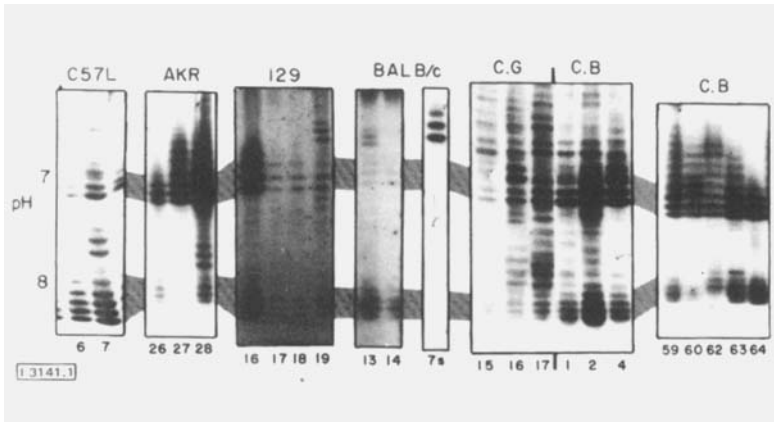


*Figure 1.* IEF patterns of T15 $Id^+$ antibodies. Portions of independent IEF gels are compiled, each developed with $^{125}I$ -labeled rabbit anti-T15. Immune sera were obtained 5–8 days after a secondary immunization with PC-KLH. The positions of the redundant patterns of T15 $Id^+$ $IgG_1$ (pH 6.9–7.2) and $IgG_3$ (pH 7.9–8.4) are indicated by the hatched bars.

the highest frequency was observed in one group of AKR mice (Fig. 1) in which two of eight mice were positive. A summary of all the results obtained from screening sera in our collection is given in Table 1. A total of 429 mice from 31 experiments conducted over a four-year period were analyzed. No one strain, except for C58, appeared to exhibit a higher frequency of IEF-T15′ antibodies than another. Some strains did exhibit IEF-T15′, but this could be attributed to the small sample size.

Examination of H-2 and Igh-congenic strains (BALB/c background) revealed two strains which, surprisingly, exhibited consistent expression of IEF-T15′ bands. Virtually every BALB.B ($K^bI^bS^bD^b$) and BALB.G ($K^dI^dS^dD^b$) showed multiple T15 $Id^+$ antibodies (Fig. 1). These appeared regularly in both the $IgG_1$ and $IgG_3$ isotypes, but most prominently in the former. Moreover, band broups in different mice cofocused suggesting a lack of random variation among the IEF-T15′ antibodies. Quantitation of T15 Id determinants in 10 BALB/c and 10 BALB.G gave values of $112 \pm 15$ μg/ml and $235 \pm 52$ μg/ml, respectively.

We then focused all the samples we had available as well as BALB.B and BALB.G immune sera to determine if there were spectrotypic similarities among the various antibodies. The results, shown in Fig. 2, indicate that there are both similarities and differences. For instance, neither the $IgG_1$ nor $IgG_3$ IEF-T15′ bands in C57L No. 7 and AKR No. 28 cofocused. However, many individual bands in the two H-2 congenics cofocused with each other. Moreover, bands corresponding closely with those in C57L and AKR can be found in BALB.G and BALB.B.

Fig. 2 also shows that the IEF-T15′ antibodies are equally well detected by an A/J anti-T15 serum. We have found similar results using an AKR anti-T15L chain antiserum (data not shown). Thus, the antibodies in the IEF-T15′ pattern contain multiple Id determinants, some associated with the H chain (A/J anti-T15), others associated with the L chain (AKR anti-T15) and still others associated with the whole molecule (rabbit anti-T15).

Fig. 3 demonstrates that the antibodies appearing in the IEF-T15′ pattern are a consistent and significant component of the response. These five animals were selected from a group of 30 BALB.G simply to show differences in their response patterns. It is evident that the antibodies persist over at least a 22-day period during the primary and secondary response. It is also apparent that individual animals may differ quantitatively in the IEF-T15′ antibodies it produces, but within the strain there appears to be no qualitative differences.

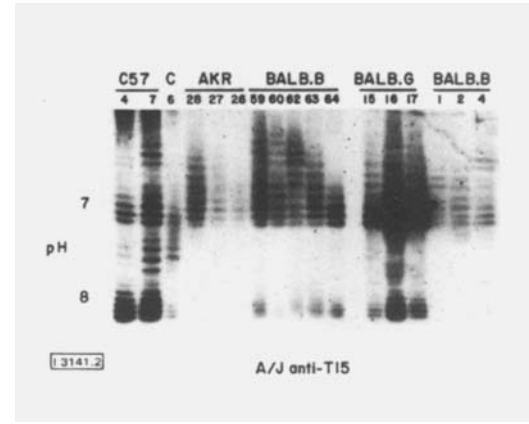


*Figure 2.* Spectrotypic comparisons of T15 $Id^+$ antibodies from different inbred strains. Antibodies from some of the same samples shown in Fig. 1 plus additional sera were focused in the same gel and developed with $^{125}I$-labeled A/J anti-T15. Serum in slots 2–5, 7–10, 13–18 contain IEF bands in addition to the redundant pattern.

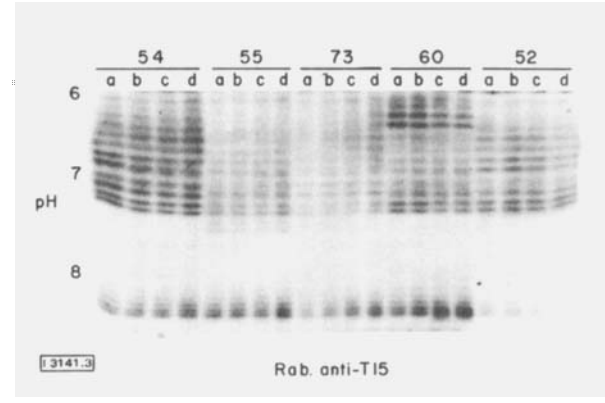


*Figure 3.* IEF patterns of T15 $Id^+$ antibodies from individual BALB.G mice. Sera from five individual animals were tested 11 (a) and 14 (b) days after the first injection of PC-KLH and 5 (c) and 8 (d) days after the second injection.

### 3.2 Relationship to M511 $Id^+$ antibodies

Since the IEF-T15′ antibodies of the $IgG_1$ and $IgG_3$ class focus in the same regions as the $IgG_1$ and $IgG_3$ M511 $Id^+$ antibodies, respectively [11], it was important to determine if these two Id populations might be related. To test this possibility two identical sets of antibodies from the same individuals were run side by side in the same IEF gel. The sets were separated after focusing as described previously [11]. One set was exposed to $^{125}I$-labeled rabbit anti-T15 and the other to $^{125}I$-labeled rabbit anti-M511. Typical results are shown in Fig. 4. There is no evidence that the T15′ or M511 antibodies cofocus or that they share idiotypic determinants, at least as defined with these anti-idiotypic sera.

### 3.3 Heterogeneity among wild *Mus*

Four colony-bred wild mouse strains were obtained from Dr. M. Potter and immunized with PC-KLH. Two of these gave a significant anti-PC response, the Vegrumbro and Skive *Mus*. The spectrotypes of T15 $Id^+$ antibodies of these mice identified by rabbit anti-T15 antibodies is shown in Fig. 5. All were identified in separate *in situ* assays as $IgG_1$ anti-PC antibodies (data not shown). They can be compared with T15 $Id^+$ antibodies from two strains, C3H and BALB/c whose IEF-T15 patterns are slightly shifted relative to each other [4, 14]. Moreover, C3H clearly exhibit heterogeneity in their T15 antibodies [14]. Both groups of wild mice display a striking diversity in their T15 $Id^+$ antibodies. The $IgG_1$ IEF-T15 span a pH range from 5.8 to 7.4 which is as broad as that noted for BALB.B and BALB.G. Sets of bands (individual clonal products?) do not cofocus with those of BALB/c but some do cofocus with those of C3H (compare C3H No. 8 and Vegrumbro mouse No. 1). Others cofocus with those in BALB.G, *e.g.* No. 22 and 2. The wild mice clearly express additional T15 $Id^+$ antibodies not seen in the BALB congenics.

Because some of the bands in many individual Vegrumbro mice cofocused with bands in C3H the possibility existed that the T15 antibodies in wild mice might be more related to C3H (a pattern IV animal [14]) than to BALB/c (a pattern I animal [4]). To test this possibility we examined their antibodies with A/J anti-T15 which reacts with T15 $Id^+$ antibodies in BALB/c but not C3H. The results, shown in Fig. 6, provide two important pieces of information. First, they demonstrate that wild *Mus* contain two types of T15 $Id^+$ antibodies. Animals No. 1, 4 and 58 like C3H, are unreactive with the A/J antisera, but animals 22 and 2 are definitely reactive. The A/J anti-T15 does not divide T15 $Id^+$ antibodies by isotype or within an isotype. Thus it must recognize $V_H$ regions that are part of a haplotype or, alternatively, all T15 $Id^+$ antibodies in the spectrotype share the same or a very similar $V_H$ region.

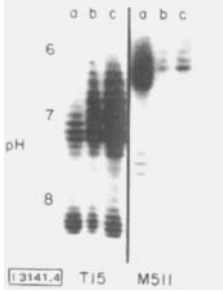


*Figure 4.* Comparison of IEF patterns of T15 and M511 $Id^+$ antibodies. Samples a–c were run in duplicate in the same gel; each set was developed separately, one with $^{125}I$-labeled rabbit anti-T15 and the other with $^{125}I$-labeled rabbit anti-M511 [11]. Sample a = typical redundant pattern, sample b and c are 5 and 10 µl, respectively, of a BALB.G pool.

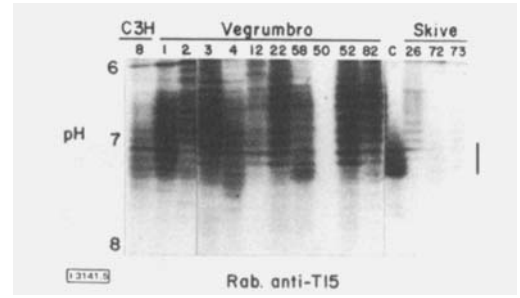


*Figure 5.* Comparison of IEF patterns of T15 $Id^+$ antibodies in inbred and wild *Mus*. Inbred mice were C3H No. 8 and a BALB/c pool (C). Only the pH range encompassing $IgG_1$ antibodies is shown. The position of the redundant T15 $Id^+$ $IgG_1$ antibodies is indicated on the right by a vertical bar.

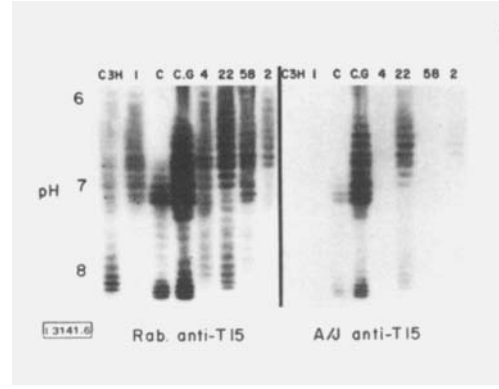


*Figure 6.* Idiotypic variation of T15 $Id^+$ antibodies in inbred and wild *Mus*. Two identical sets of immune sera were run and separately processed in the same fashion as described in Fig. 4. The C3H and BALB/c sera are the same as listed in Fig. 5. C. G. is a pool of secondary sera from four BALB.G mice.

The second feature of the data results from comparing the pI of the different bands. Wild mice expressing antibodies which cofocus with those in C3H, particularly between pH 6.5 to 7.4, do not react with A/J anti-T15. The converse also appears to be true. For instance, compare BALB.G and Vegrumbro mice No. 22 and 2.

Thus, these wild mice contain T15 $Id^+$ antibodies that are very similar to inbred strains, but quite unlike their usual laboratory counterpart, the antibodies expressed are regularly heterogeneous.

## 4 Discussion

Up until recently, it had been anticipated that an Id determinant marked one or at most a few clones of antibody-producing cells, especially when the Id represents a binding site determinant found on a myeloma protein or a restricted antibody population. However, direct examination of individual $Id^+$ antibodies (hybridoma proteins) has clearly demonstrated that an Id marks a family of antibodies [7–9, 15], even in cases where specificity is highly restricted. In some instances the $Id^+$

family appears restricted in size [7], but in others it is clearly large [8, 9]. Among anti-PC antibodies, three independent Id groups have been identified. Each was initially detected with highly specific anti-Id antisera of homologous and heterologous origin. Two of these groups, the M511 and M603 Id families, are clearly heterogeneous [10, 11], whereas the third, the T15, normally exhibits much less heterogeneity and initially appeared monoclonal in many strains [3–5]. In the present report we have demonstrated that BALB/c clearly has the potential to generate a rather diverse family of T15 $Id^+$ antibodies, but for reasons that are not entirely clear it is not normally expressed. By inference and because a relatively high frequency of "extra" T15 $Id^+$ antibodies can be demonstrated, it is quite probable that all inbred strains carry the genetic potential for making a diverse T15 response.

Gearhart et al. [5] in an analysis of PC-specific precursors in BALB/c found that between 0.9 and 6% of the B cells expressed Id determinants cross-reactive with T15, 72% were Id identical. These T15 Id cross-reactive clones may give rise to the IEF-T15′ antibodies that we observed. Unfortunately, the antisera Gearhart et al. used exhibited cross-reactivity with M511 and we know that this family is definitely heterogeneous. However, it is probable that at least some of the clones were members of the T15 antibody family.

Why is the T15 response normally so restricted? Multiple possibilities can be invoked but the most likely seems to be specific regulation at the B cell level. Two rather diverse antigens PC-KLH (T-dependent) and *S. pneumococcus* R36A (T-independent) in CFA stimulate the same restricted response. The dose of PC-KLH or degree of substitution of hapten have been found to alter the quantity but not the quality of the response (Pease and Claflin, in preparation). The frequency of B cells expressing different T15 antibodies could account for the differences, but this does not easily explain the relatively high frequency of their appearance nor the magnitude of the response of IEF-T15′ antibodies when they appear. IEF-T15′ antibodies may be somatic mutational descendents of IEF-T15 antibodies. This seems unlikely since they appear at too high a frequency in inbred mice. Moreover, the BALB/c congenics regularly express IEF-T15′ antibodies and many of these antibodies cofocus between individuals and with T15 $Id^+$ antibodies in other strains. The data is more consistent with the hypothesis that there is a specific clonal regulatory mechanism, positive or negative, which normally operates in BALB/c to select one member of a family of closely related antibodies. The mechanism is not precise and thus additional members of T15 Id family occasionally gain expression. In the two BALB/c congenics there is an absence of this regulatory mechanism and the T15 response is thus regularly heterogeneous. Of particular interest is that the mechanism displays a high degree of specificity since it can readily distinguish between idiotypically related molecules. Nisonoff and colleagues have demonstrated that suppression induced by $Id^+$ serum antibodies or hybridoma proteins conjugated to thymocytes or anti-Id antiserum suppresses all members of the Id family [16, 17]. Our initial thoughts were that the major histocompatibility complex may be directly responsible for the specific regulation, but subsequent investigation has ruled that out.

The appearance of a heterogeneous T15 response in wild *Mus* is exciting and its similarities to the response in the congenic strains is intriguing. Vegrumbro (and Skive) mice, which belong to the same species as inbred *Mus*, express T15 antibodies which focus in positions very similar to those seen in inbred mice. Some of these antibodies cofocus with BALB.G antibodies ($Igh^a$ pattern I), others cofocus with C3H antibodies ($Igh^j$ pattern IV). Moreover, two different T15 Id variations are observed, one which is A/J positive, and another which is A/J negative. Thus wild mice contain T15 $Id^+$ antibodies that share isofocusing and serologic characteristics with antibodies found in inbred strains. Since they are regularly heterogeneous, it indicates that the restricted response usually seen in inbred strains is not a "normal" situation. The findings in $Igh^j$ and the BALB H-2-congenic mice thus may actually represent the normal response. We are presently exploring this possibility to see if CBA contains a gene(s) which permits expression of the T15 family. The fact that most inbred strains give a restricted response suggests that multiple unlinked genes are involved.

The existence of heterogeneity among T15 $Id^+$ antibodies raises specific questions about the nature and genetic origin of this antibody family. As shown here, all of the antibodies share Id determinants found throughout the V regions of the molecule. Thus, they must exhibit structural similarities with T15 itself. Despite the appearance of discrete spectrotypes shown in Fig. 4, it is possible that they all are identical to T15 $V_H/V_L$ and the spectrotypic heterogeneity may reflect carbohydrate differences. It is also possible that the observed heterogeneity might be accounted for by subdivisions of the $IgG_1$ (and $IgG_3$) isotypes, but these have not been described. A third possibility is that each of the individual Id recognized may not have a precise structure. Thus, the T15 antibodies may comprise a family of closely related structures each showing only minor differences from the others. This seems to be the most likely possibility, and it is supported by recent observations of variable cross-reactivity among T15 $Id^+$ hybridoma antibodies (unpublished). One could account for the variations by random mutations in the germline $V_H$ and/or $V_L$ for T15 although the conservative and limited amount of variation in the response argues against this possibility. Another possibility has been raised by recent studies on the structure and organization of V genes in the mouse. V region probes which hybridize with the T15 $V_H$ region [12] or T15 $V_K$ region (Campbell, Stavnezer and Claflin, unpublished) each identify gene families. Thus, anti-PC antibodies of the T15 Id may arise from different permutations of V genes from two different gene families. Alternatively, VJ (L) or VDJ (H) junctional variation or J or D region differences may give rise to the limited heterogeneity [18–21]. Information to separate these hypotheses is presently lacking, although constraints on J segment substitution may exist since the L chain of T15, M511 and M603 all utilize the same $J_K$ ([19, 22], Rudikoff, personal communication). Through the use of anti-PC hybridomas and recombinant DNA technology we hope to resolve these issues.

Regardless of the outcome, the expression of T15 Id-related antibodies seems to be unlike the response occurring in the other anti-PC Id and apparently in other antigen systems as well. This may explain why the CBA/N mouse shows a differential expression of T15 *vs.* M511 and M603 Id [23]. It may also account for the interesting temporal pattern of appearance of T15 $Id^+$ precursors in the fetal liver and the newborn spleen [24]. Continued exploration of wild *Mus*, the H-2 congenics and immunodeficent CBA/N mice should provide an opportunity to decipher the control mechanisms.

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