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Clonal deletion of specific thymocytes by an immunoglobulin idiotype

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We have investigated whether immunoglobulin can induce clonal deletion of thymocytes by employing two strains of transgenic mice. One strain is transgenic for an α/β T cell receptor (TCR) which recognizes a processed idiotypic peptide of the $\lambda 2^{315}$ light chain variable region, bound to the I-E^d class II major histocompatibility complex molecule. The other mouse strain is transgenic for the $\lambda 2^{315}$ gene. Double transgenic offspring from a TCR-transgenic ♀ mated with a $\lambda 2^{315}$ transgenic ♂ exhibit a pronounced clonal deletion of CD4$^+$CD8$^+$ thymocytes. Analysis of neonates from the reciprocal ($\lambda 2^{315}$-transgenic ♀ × TCR-transgenic ♂) cross suggests that the deletion in double transgenic offspring most likely is caused by $\lambda 2^{315}$ produced within the thymus rather than by maternally derived IgG, $\lambda 2^{315}$. Nevertheless, IgG, $\lambda 2^{315}$ can cause deletion of CD4$^+$CD8$^+$ thymocytes when injected in large amounts intraperitoneally into either adult or neonatal TCR-transgenic mice. Deletion is evident 48 and 72 h after injection, but by day 7 the thymus has already regained its normal appearance. A serum concentration of several hundred μg/ml is required for deletion to be observed. Therefore, the heterogeneous idiotypes of serum Ig are probably each of too low concentration to cause thymocyte deletion in normal animals.

Key words: clonal deletion/idiotype/self antigen/thymus/tolerance

Introduction

Immunological tolerance to self antigens is of paramount importance in the prevention of autoimmune diseases. Tolerance can be established both in the B lymphocyte (reviewed in Goodnow, 1992) and T lymphocyte (reviewed in von Boehmer and Kisielow, 1990; Miller and Morahan, 1992) compartments. A well established mechanism to achieve T cell tolerance is clonal deletion of autoreactive thymocytes (negative selection) (Kappler *et al.*, 1987, 1988; Fowlkes *et al.*, 1988; Kisielow *et al.*, 1988; MacDonald *et al.*, 1988a,b; Sha *et al.*, 1988; Pircher *et al.*, 1989). However, many self antigens are not produced in the thymus and T cell tolerance has also to be established to such antigens. This could be achieved if extrathymic antigens, soluble or bound to cells, could gain access to the thymus and be presented as processed short peptides bound to a major histocompatibility complex (MHC) molecule on antigen-presenting cells (APC). To explore this possibility, we have examined if a circulatory self antigen, immunoglobulin (Ig), can induce clonal deletion of specific thymocytes.

Ig is a class of self antigens of particular significance because their variable (V) regions have highly diversified idiotypic (Id) determinants thought to be important in immune regulation (Jerne, 1974). It has recently become clear that idiotypes can be recognized not only by antibodies, but also by MHC-restricted T cells. Thus, Ig can be endocytosed and processed by APC, followed by presentation of short Id-peptides to MHC class II molecule-restricted T cells (Bogen *et al.*, 1986a,b; Bogen and Lambris, 1989; Weiss and Bogen, 1989). In addition, a B cell can process its endogenously produced Ig and present the generated Id-peptides (Weiss and Bogen, 1989, 1991).

Owing to their diversity, processed Id-peptides could conceivably have a major influence on the T cell repertoire through negative selection in the thymus. To approach this question, we have established two strains of transgenic (TG) mice. The first TG mouse (Bogen *et al.*, 1992) expresses an α/β T cell receptor (TCR) derived from a T cell clone specific for an Id-peptide encompassing residues 91–101 of the $\lambda 2^{315}$ Ig light chain of the BALB/c myeloma MOPC315. The Id-peptide is presented by the I-E^d class II molecule on APC (Bogen *et al.*, 1986a,b; Bogen and Lambris, 1989). The transgenic TCR requires the Phe^{94} Arg^{95} Asn^{96} residues for recognition; these residues are unique to $\lambda 2^{315}$ due to somatic mutations (Bogen *et al.*, 1986b, 1992). The second TG mouse (Bogen and Weiss, 1991) is transgenic for the $\lambda 2^{315}$ gene. Thus, mice of the first TG strain have a high frequency of T cells with a receptor which recognizes a complex of $\lambda 2^{315}$ Id-peptide and I-E^d class II molecules generated in mice of the second TG strain.

Results

Double TCR/$\lambda 2^{315}$ transgenic mice delete CD4$^+$CD8$^+$ thymocytes

A premise for the breeding experiments described below is that the transgenes in both TG strains are integrated on a single autosomal chromosome and segregate in a Mendelian fashion. Furthermore, both of the TG mouse strains used in the present experiments have been backcrossed to BALB/c for more than seven generations and thus have a fairly homogeneous background (Bogen and Weiss, 1991; Bogen *et al.*, 1992).

In the first set of experiments, we analyzed the T and B cell compartments of 3- to 4-week-old offspring from heterozygous TCR-TG females which had been mated with heterozygous $\lambda 2^{315}$-TG males. In agreement with our previous report (Bogen and Weiss 1991), the single $\lambda 2^{315}$-TG offspring had a moderately hypoplastic B cell compartment. Most B cells stained with an anti-$\lambda 2$

monoclonal antibody (mAb), while κ expression was suppressed (Figure 1). Also confirming previous results (Bogen *et al.*, 1992), the single TCR-TG offspring had moderately hypoplastic thymi and lymph nodes, and almost all thymocytes and most peripheral T cells expressed the transgenic TCR α chain (α_T) detected by the GB113 mAb (Figure 1). The double-TG offspring, having both the TCR and $\lambda 2^{315}$ transgenes, exhibited dramatic changes in their T cell compartment compared to single-TCR-TG mice. The number of thymocytes was reduced to only ~2% of normal. The thymocytes which expressed the α_T were either of a $CD4^-CD8^-$ or $CD4^-CD8^{low}$ phenotype, and $CD4^+CD8^+$ thymocytes were lacking (Figure 1 and data not shown). The lymph node cell number of double-TG offspring was reduced to 10% of normal, and the lymph nodes had a reduced frequency of T cells. The T cells which expressed the α_T were almost exclusively $CD4^-CD8^-$ (Figure 1 and data not shown). In some double-TG animals, like that shown in Figure 1, a prominent $CD4^+$ population not expressing α_T could be found; this population probably had productively rearranged endogenous α-genes (Blüthmann *et al.*, 1988) and therefore escaped deletion. In contrast to the dramatic changes in the T cell compartment, the B cell compartment of double-TG offspring appeared unaffected because the $\lambda 2^{315}$ transgene was expressed on most B cells, as in single $\lambda 2^{315}$-TG mice (Figure 1).

Functional assays were in agreement with the staining results described above. As expected from a lack of $CD4^+$ $\alpha_T\beta_T$ lymph node cells, peripheral T cells from double-TG did not proliferate in response to $\lambda 2^{315}$, while T cells from TCR-TG did (Table I). As anticipated from the high frequency of B cells expressing $\lambda 2^{315}$, spleen cells from both double-TG and single $\lambda 2^{315}$-TG offspring produced large amounts of $\lambda 2^{315}$ Ig when stimulated *in vitro* by an Id-specific T cell clone (Table I). In concordance with the functional B cell data, both double-TG and single $\lambda 2^{315}$-TG mice had elevated $\lambda 2^{315}$ and reduced $\lambda 1$ and κ expression in serum Ig (Table I). In conclusion, double TCR/$\lambda 2^{315}$-TG mice appear to be tolerant to $\lambda 2^{315}$ because developing TCR-transgenic thymocytes are deleted at the $CD4^+CD8^+$

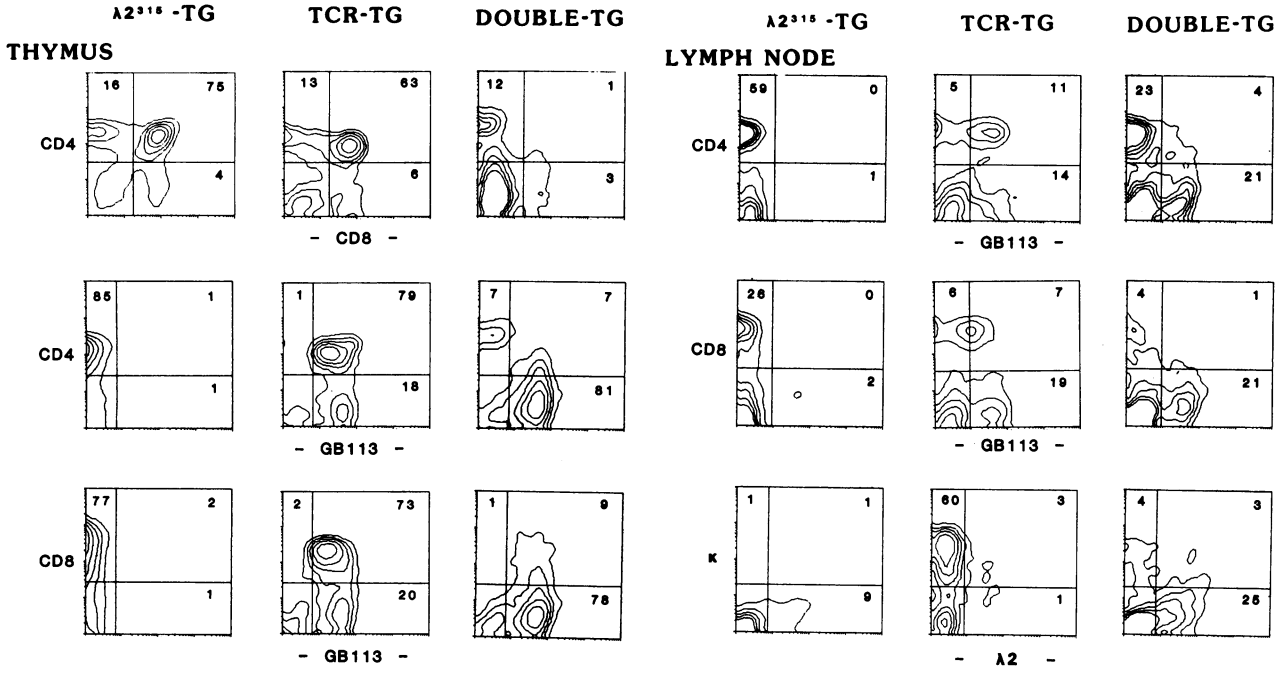

Fig. 1. Clonal deletion of Id-specific T cells in double $\lambda 2^{315}$/TCR-TG mice. Thymus and lymph node cells from 3- to 4-week old offspring from (TCR-TG♀ × $\lambda 2^{315}$-TG♂) matings were double stained as indicated. The numbers in each quadrant indicate the percentage of the total number of analyzed cells. Double stainings of non-transgenic mice have been shown previously (Bogen and Weiss, 1991; Bogen *et al.*, 1992).

Table I. Function of transgenic B and T cells in (TCR-TG♀ × $\lambda 2^{315}$♂) offspring

Genotype[a]	T cell proliferation[b]		Antibody production[c]		Serum immunoglobulin[d]		
	λ-TG APC	N-TG APC	T cells	No T cells	λ2/3	λ1	κ
λ^-TCR^-	0.5	0.7	<40	<40	26	30	2349
λ^-TCR^+	7.3	0.9	<40	<40	33	48	2376
λ^+TCR^-	1.1	1.3	10 184	256	166	12	713
λ^+TCR^+	0.4	0.2	6658	186	111	7	819

[a]The 25 analyzed offspring (four litters), aged 3–6 weeks, were genotyped by Southern blotting.
[b]Lymph node cells were stimulated with irradiated $\lambda 2^{315}$-TG or non-transgenic (N-TG) spleen APC in a proliferation assay. The arithmetic mean of animals with the same genotype is given (c.p.m. × 10^{-3}).
[c]Spleen cells were cultured either with or without cloned, Id-specific T cells (4B2A1) for 12 days and the combined λ2 and λ3 L-chain production (ng/ml) was determined in an ELISA.
[d]Immunoglobulin light chain expression (μg/ml) in serum was measured by ELISA.

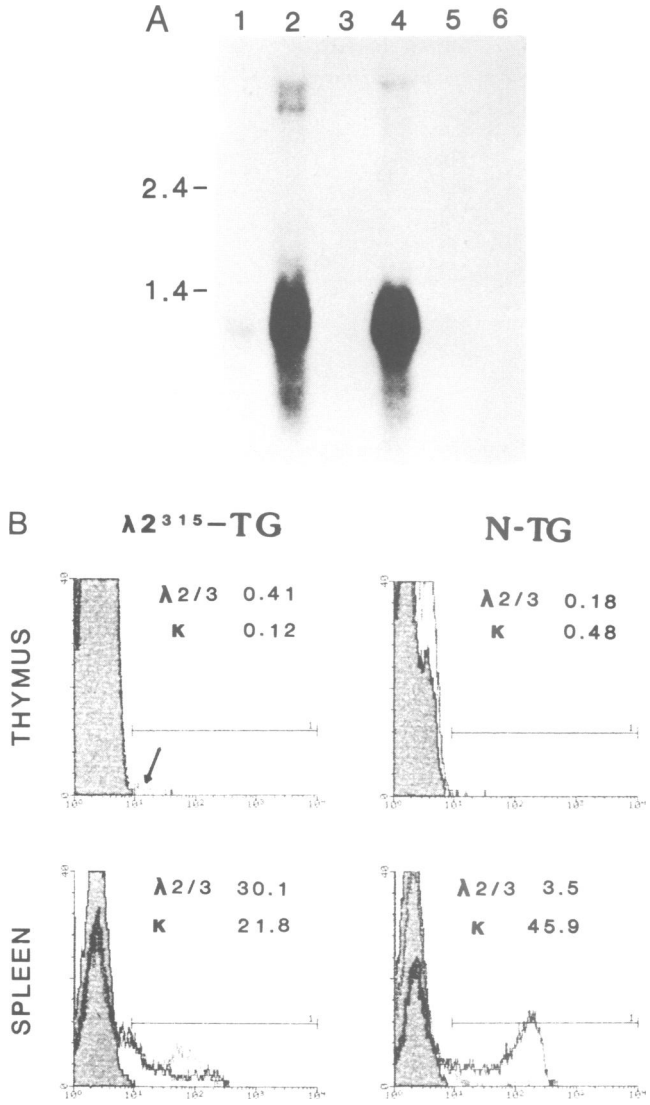

Fig. 2. Expression of the $\lambda 2^{315}$ transgene in the thymus. (**A**) Northern blot analysis of λ2 transgene expression in various tissues from adult $\lambda 2^{315}$-TG and non-transgenic (N-TG). Lane 1, N-TG spleen; lane 2, TG-thymus; lane 3, TG liver; lane 4, TG-spleen; lane 5, N-TG thymus; lane 6, N-TG liver. Size markers (kb) are indicated. (**B**) Expression of λ2 or λ3 chains, and κ chains, on the surfaces of thymocytes and splenocytes of N-TG and $\lambda 2^{315}$-TG. The dense line represents staining with biotinylated anti-κ mAb (187.1), the weak line represents staining with biotinylated anti-λ2/3 mAb (2B6) and the shaded area represents background with secondary reagent (FITC–streptavidin) alone. Given percentages are corrected for background. The arrow indicates dull-staining thymic $\lambda 2^{315}$ B cells.

stage. On the other hand, $\lambda 2^{315}$-expressing B cells in double-TG mice are spared because they are present and functional in the peripheral lymphoid organs of these mice.

Deletion in TCR/$\lambda 2^{315}$ double-TG mice may be caused by expression of $\lambda 2^{315}$ in the thymus

Two distinct mechanisms could account for the deletion of $CD4^+CD8^+$ thymocytes in double TCR/$\lambda 2^{315}$-TG mice. First, circulatory $\lambda 2^{315}$ Ig found in the 100–200 μg/ml range in the sera of 3- to 4-week-old animals (Table I) could enter the thymus and be processed and presented by thymic APC. Second, $\lambda 2^{315}$ produced *in situ* could cause thymocyte deletion because the $\lambda 2^{315}$ transgene was strongly transcribed in the thymus (Figure 2A). However, thymic B cells (Rudensky *et al.*, 1990; Inaba *et al.*, 1991; Mazda *et al.* 1991) expressing $\lambda 2^{315}$ in membrane Ig were very few (<0.4%) and stained dully (Figure 2B). Furthermore, intracellular $\lambda 2^{315}$ was not detected in cryosections of the thymus, stained with a mAb directed against native λ2 chains (data not shown). Thus, despite the strong signal in the Northern blot, we do not yet know in which thymic cells the $\lambda 2^{315}$ gene is active.

To distinguish between the two mechanisms described above, we investigated offspring from the reciprocal ($\lambda 2^{315}$-TG♀ × TCR-TG♂) cross. Offspring from such a cross have been exposed to transplacentally transferred maternal IgG bearing $\lambda 2^{315}$ light chains. To our surprise, TCR-TG neonates (<24 h) born of $\lambda 2^{315}$-TG mothers showed no signs of thymocyte deletion (Figure 3) even though the IgG,$\lambda 2^{315}$ serum concentration in some newborns was as high as 250 μg/ml. [However, because many of the maternally transmitted IgG molecules carry both $\lambda 2^{315}$ and κ chains due to incomplete isotypic exclusion in adult $\lambda 2^{315}$-TG mice (Bogen and Weiss, 1991), the given concentration could represent a maximum 2-fold overestimation of the molar $\lambda 2^{315}$ concentration.] TCR-TG offspring 4 days (serum $\lambda 2^{315}$ 90 μg/ml) and 9 days (serum $\lambda 2^{315}$ 50 μg/ml) of age also lacked signs of thymocyte deletion. In striking contrast, double TCR/$\lambda 2^{315}$-TG neonates exhibited a pronounced thymocyte deletion (Figure 3) even though their own production of $\lambda 2^{315}$ was yet undetectable (<80 ng/ml of IgM, $\lambda 2^{315}$). Thus, thymocyte deletion in neonates cannot be explained by circulatory $\lambda 2^{315}$ Ig, but rather by *in situ* production of $\lambda 2^{315}$ in the thymus.

Circulatory $\lambda 2^{315}$ Ig can cause deletion of $CD4^+CD8^+$ thymocytes

The failure of maternally transmitted IgG,$\lambda 2^{315}$ to delete thymocytes in newborn TCR-TG mice prompted us to

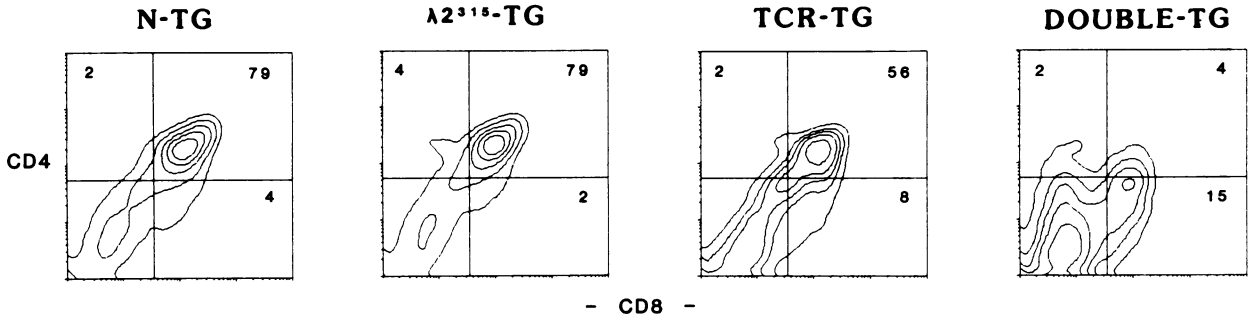

Fig. 3. In neonates, $\lambda 2^{315}$ produced *in situ* in the thymus, rather than circulatory $\lambda 2^{315}$, is likely to cause clonal deletion. Thymus of offspring (<24 h) from ($\lambda 2^{315}$-TG♀ × TCR-TG♂) matings was double stained as indicated. The serum concentration of maternally transferred IgG, $\lambda 2^{315}$ was 250 μg/ml while IgM,$\lambda 2^{315}$, produced by the offspring themselves, was undetectable (<80 ng/ml).

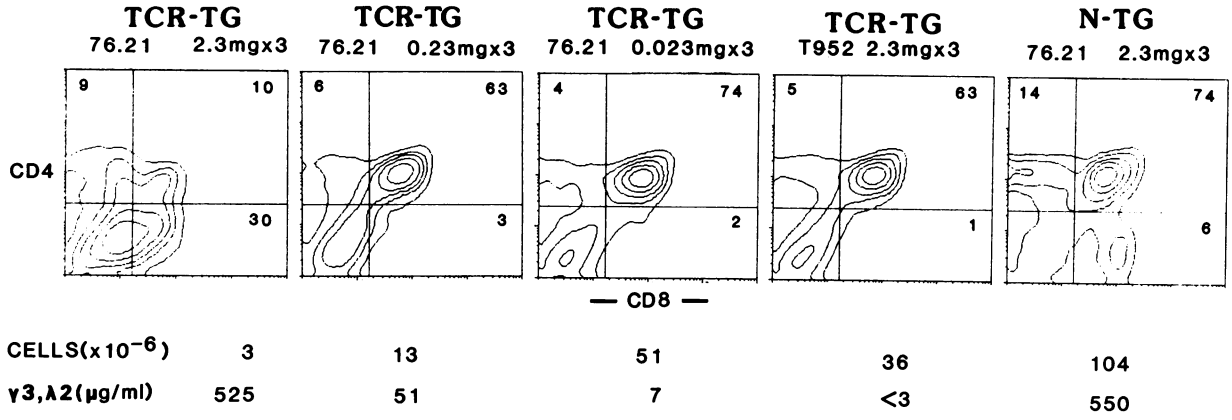

Fig. 4. Circulatory IgG3 can cause clonal deletion of thymocytes. Thymus of adult TCR-TG or N-TG was analyzed on day 4, after three daily i.p. injections of Ig. Above each staining, the genotype of the animal as well as the type and amount of injected Ig is indicated. Below each staining, the number of thymocytes and the serum $\gamma3,\lambda2$ concentration at the time of killing is given. Injected Ig was ammonium sulfate-precipitated ascitic fluid of an IgG3,$\lambda2^{315}$ mAb (76.21), which expresses Id (Bogen and Weiss, 1991) and an IgA$\lambda2$ myeloma protein (T952), which does not express Id, as a specificity control (Bogen *et al.*, 1986a).

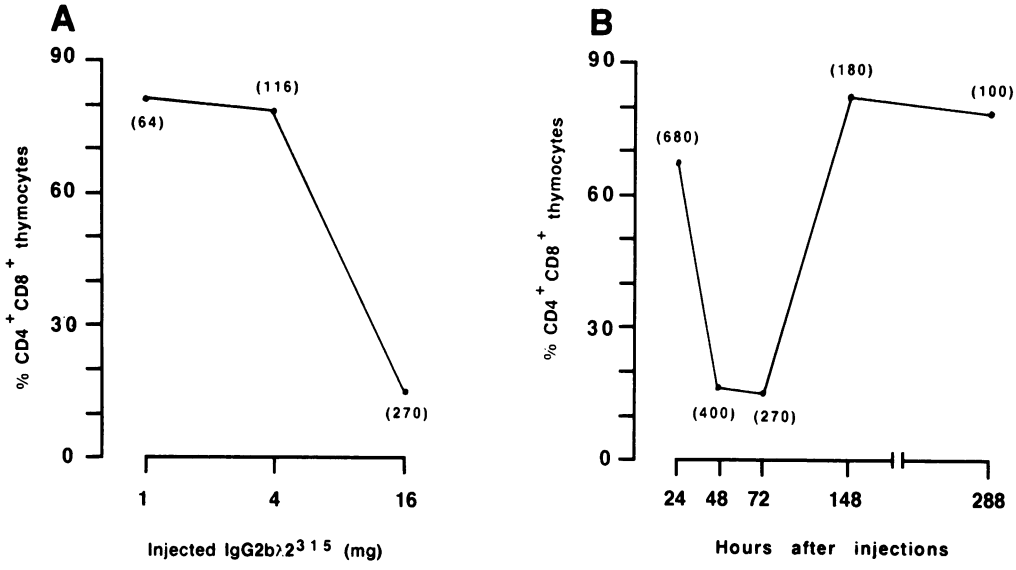

Fig. 5. Dose–response curve (**A**) and kinetics (**B**) of deletion of $CD4^+CD8^+$ transgenic thymocytes after injection of an IgG2b, $\lambda2^{315}$ mAb (136.4). In A, TCR-TG adult mice were injected i.p. with the indicated amounts of mAb (ammonium sulfate-precipitated ascitic fluid) and analyzed 72 h later. The numbers in parentheses represent $\lambda2^{315}$ in serum (μg/ml) at the point of killing. As a control, a non-transgenic littermate injected with 16 mg IgG2b,$\lambda2^{315}$ had a $\lambda2^{315}$ serum concentration of 340 μg/ml and 60% $CD4^+CD8^+$ thymocytes. In B, TCR-TG adult mice were injected with 16 mg IgG2b,$\lambda2^{315}$ mAb and analyzed at the indicated time points. The numbers in parentheses represent $\lambda2^{315}$ in serum (μg/ml) at the point of killing. As controls, non-transgenic littermates were injected with the same volumes of phosphate-buffered saline (PBS) (analyzed 24 and 48 h later) and IgG2b,$\lambda2^{315}$ (analyzed 72 h later). These mice had 53, 63 and 60% of $CD4^+CD8^+$ thymocytes, respectively.

investigate if injection of IgG carrying $\lambda2^{315}$ L chains could induce clonal deletion of thymocytes in TCR-TG mice.

As IgG,$\lambda2^{315}$ we used IgG3 (76.21) and IgG2b (136.4) mAbs derived from a $\lambda2^{315}$-TG mouse (Bogen and Weiss, 1991). The mAbs were prepared as ammonium sulfate-precipitated ascitic fluid and injected intraperitoneally (i.p.). Deletion of $CD4^+CD8^+$ thymocytes in adult animals was observed at an IgG3,$\lambda2^{315}$ serum concentration of 525 μg/ml at the day of killing. However, at 51 μg/ml no deletion was observed (Figure 4). Correspondingly, the required serum IgG2b, $\lambda2^{315}$ concentration for deletion was <270 μg/ml, but >180 μg/ml (Figure 5).

A kinetic study (Figure 5B) demonstrated that a >24 h post-injection lapse was needed for deletion to be evident; at 48 and 72 h the deletion was pronounced, while at 7 and 12 days no signs of deletion were found.

In newborn TCR-TG mice, the IgG2b,$\lambda2^{315}$ serum concentration required for deletion was <410 μg/ml, but >111 μg/ml. In 19-day-old TCR-TG mice, a serum IgG2b, $\lambda2^{315}$ concentration of 244 μg/ml resulted in partial deletion of $CD4^+CD8^+$ thymocytes (Table II). These data, together with those of Figures 4 and 5, indicate that the serum concentration requirement for deletion is surprisingly high and roughly independent of age.

Repetitious i.p. injections of mice could cause a non-specific deletion of $CD4^+CD8^+$ thymocytes due to stress. However, this appears not to be the explanation in our experiments because the pronounced deletion (only 10–20% $CD4^+CD8^+$ cells left) was both Id-specific (Figure 4) and transgenic TCR-specific (Figure 4, legend of Figure 5, Table II). In any of these experiments, the lowest percentage of $CD4^+CD8^+$ cells in the control mice was 53%.

Table II. Deletion of $CD4^+CD8^+$ thymocytes in newborn TCR-transgenic mice by circulatory IgG2b,$\lambda 2^{315}$

Age (days) when analyzed	Received IgG2b,$\lambda 2^{315}$[a] (mg)	Genotype of littermates[d]	Serum $\lambda 2^{315}$ (μg/ml)[e]	% $CD4^+CD8^+$ thymocytes[g]
3	2.4[b]	7 TG 2 N-TG	410[f]	17 (11) 60 (4)
3	0.8[c]	3 N-TG	85 (23)	67 (5)
5	0.8[c]	4 TG 2 N-TG	111 (8) 110 (0)	54 (5) 69 (2)
16	0.8[c]	3 TG 5 N-TG	100 (5) 86 (13)	75 (3) 78 (5)
19	4[c]	5 TG 1 N-TG	244 (38) 260	34 (14) 54

[a]Whole litters from (BALB/c ♀ × TCR-TG♂) crosses were injected i.p. with the indicated amounts of an IgG2b,$\lambda 2^{315}$ mAb (136.4; Bogen and Weiss, 1991), prepared as ammonium sulfate-precipitated ascitic fluid.
[b]Mice received 0.4 mg (day 0, day of birth), 0.6 mg (day 1, morning), 0.6 mg (day 1, afternoon) and 0.8 mg (day 2) i.p.
[c]Mice were injected with a single dose i.p. 3 days prior to analysis.
[d]Transgenic (TG) and non-transgenic (N-TG) genotype was determined by PCR.
[e]Serum $\lambda 2^{315}$ was measured in the $\lambda 2/\lambda 3$-specific ELISA. The arithmetic mean (SD) is given.
[f]Serum from a pool of blood from the littermates was analyzed.
[g]Staining of thymocytes was performed as in Figure 1. The arithmetic mean (SD) is given.

Discussion

We report that an immunoglobulin idiotype can specifically delete thymocytes transgenic for an Id-peptide/MHC class II molecule-specific receptor. Our observation that clonal deletion occurs at the $CD4^+CD8^+$ stage of thymocyte differentiation is in agreement with findings in other TCR-transgenic models for MHC class I-restricted (Kisielow *et al.*, 1988; Sha *et al.*, 1988; Pircher *et al.*, 1989) and class II-restricted (Murphy *et al.*, 1990; Spain and Berg, 1992; Vasquez *et al.*, 1992) antigens. In the class II-restricted TCR-transgenic models, studies have been performed *in vivo* (Murphy *et al.*, 1990), in thymocyte suspension culture (Vasquez *et al.*, 1992) and thymus organ culture (Spain and Berg, 1992), but all three studies have been performed with synthetic peptides (not requiring processing) derived from non-self antigens like ovalbumin and cytochrome C. Thus, the novelty of our observations is that: (i) a self antigen, which requires processing, can cause thymocyte deletion *in vivo*; (ii) self antigen produced within the thymus, as well as circulatory self antigen, can cause deletion; (iii) under physiological conditions, the highly heterogeneous immunoglobulin idiotypes are unlikely to influence the TCR repertoire through clonal deletion.

Several mechanisms can explain the deletion caused by $\lambda 2^{315}$ produced *in situ* in the thymus. First, even though thymic $\lambda 2^{315}$ B cells were few ($<0.4\%$), they could process and present their endogenous $\lambda 2^{315}$ (Weiss and Bogen, 1991) and cause deletion of thymocytes, as previously described for the Mls-1[a] superantigen (Inaba *et al.*, 1991; Mazda *et al.*, 1991). Second, thymic dendritic cells or macrophages could endocytose, process and present $\lambda 2^{315}$ locally secreted by thymic B cells (Bogen *et al.*, 1986a; Weiss and Bogen, 1989). Third, owing to the Ig H-chain enhancer used in the $\lambda 2^{315}$ gene construct (Grosschedl *et al.*, 1984; Hagman *et al.*, 1989), thymocytes could produce and rapidly degrade $\lambda 2^{315}$. Thymocytes undergoing apoptosis could release $\lambda 2^{315}$, or fragments thereof, and sensitize thymic APC. If true, class II-restricted tolerance to most intracellular antigens could normally be achieved in this way.

The mechanism for the thymocyte clonal deletion caused by injected IgG,$\lambda 2^{315}$ also remains unknown. Possibly, IgG, $\lambda 2^{315}$, or fragments thereof, extravasate in the thymic cortex. Alternatively, if a blood–thymus barrier exists, IgG could instead diffuse across the thymic capsule (reviewed in Niewenhuis *et al.*, 1988). Regardless of entrance, the IgG is presumably processed and presented to $CD4^+CD8^+$ thymocytes by cortical APC. A quite different possibility is that IgG, $\lambda 2^{315}$ is processed and presented by peripheral APC which later migrate to the thymus and cause deletion. Indeed, spleen APC (Swat *et al.*, 1991) and even fibroblasts transfected with class II genes (Vasquez *et al.*, 1992) can induce thymocyte deletion, at least *in vitro*.

Induction of deletion by injected IgG,$\lambda 2^{315}$ has a longer latency than that observed in *in vitro* thymocyte culture experiments (Swat *et al.*, 1991; Spain and Berg, 1992; Vasques *et al.*, 1992); this may reflect that additional time is required for access to the thymus, and processing of the Ig, *in vivo*. With diminishing serum concentrations of IgG,$\lambda 2^{315}$, signs of clonal deletion have already disappeared by day 7 post-injection. Presumably, $CD4^+CD8^+$ cells are rapidly regenerated from precursor cells.

A high concentration of circulatory IgG,$\lambda 2^{315}$ (~ 300 μg/ml, or 2 μM) was needed to induce a pronounced deletion of thymocytes in TCR-TG mice. This contrasts with the low amounts of cytochrome *c* synthetic peptide ($\sim$ 0.1 μM) required for deletion of TCR-transgenic $CD4^+CD8^+$ thymocytes in suspension culture (Vasquez *et al.*, 1992) and organ culture (Spain and Berg, 1992) *in vitro*. In the experiments of Murphy *et al.* (1990), the serum concentration of synthetic ovalbumin peptide causing deletion is unknown, but could have been as high as 3 μM. The high concentration requirement in our experiment could have several explanations. First, entry of Ig into an intact thymus *in vivo* could be poor (see above). Second,

Id-peptides have been shown to be relatively cryptic in complete serum Ig molecules, perhaps due to resistance to processing (Bogen *et al.*, 1986a).

Regardless of the explanation, concentration considerations suggest that for the extremely diversified Ig molecules (Tonegawa, 1983), perhaps only the constant regions can cause deletion. Id-peptides encoded by the several hundred V_H and V_L germline gene segments are each probably of too low molar concentration (<0.3 μM) in serum Ig to effect deletion. The Id-peptides corresponding to the CDR3 regions, which depend on unique V-D-J recombinations and N-region sequences, should certainly be much too rare (Sanz, 1991) to cause deletion. However, this generalization might be an oversimplification for a number of reasons. First, Ig isotypes other than IgG2b and IgG3 could be relatively more efficient in gaining access to the thymus, or be more susceptible to processing. Second, free Ig chains or fragments, if occurring *in vivo*, could cause a more efficient clonal deletion than the complete Ig we have used. Third, other Id-specific TCR could have a higher affinity than the transgenic TCR we have employed and therefore require a lower concentration for deletion. Fourth, idiotypes produced *in situ* in the thymus (see above) could possibly cause deletion much more efficiently than serum idiotypes.

If processed idiotypes fail to induce clonal deletion of thymocytes in normal animals, there are a number of other options to obtain tolerance. Id-peptides could induce clonal anergy of thymocytes (reviewed in Ramsdell and Fowlkes, 1990), clonal deletion (Jones *et al.*, 1990; Webb *et al.*, 1990) or clonal anergy of peripheral T cells (reviewed in Burkly *et al.*, 1990; Schwartz, 1990; Sprent *et al.*, 1990), downregulation of TCR and accessory (CD8) molecules (Rocha and von Boehmer, 1991; Schönrich *et al.*, 1991) or simply be ignored (Lassila *et al.*, 1988; Ohashi *et al.*, 1991). Alternatively, peripheral T cells might actually never be rendered tolerant to Id-peptides and could play a role in an Id-dependent network type of interaction between T and B cells (Bogen *et al.*, 1986a; Bogen and Weiss, 1989).

Previous findings (Winchester *et al.*, 1984; Bogen *et al.*, 1986a; Lin and Stockinger, 1989; Lorenz and Allen, 1989) have shown that APC, including thymic APC (Lin and Stockinger, 1989; Lorenz and Allen, 1989), do not distinguish between foreign and self proteins because both are processed and presented by MHC class II molecules. We have now extended this to show that an extrathymic self protein, Ig, can cause a specific deletion of Id-specific $CD4^+CD8^+$ thymocytes. If other circulatory self proteins produced extrathymically require as high as 2 μM serum concentration to cause deletion, T cell tolerance to the multitude of less abundant serum proteins may be induced by other means than by clonal deletion in the thymus. Relevant to this, transgenic mice expressing small amounts of hen egg white lysozyme (0.5 and >10 ng/ml serum, respectively) (Adelstein *et al.*, 1991; Cibotti *et al.*, 1992) or physiological amounts of human insulin (Whiteley *et al.*, 1990) exhibited T cell tolerance. In contrast, a soluble $H\text{-}2K^k$ molecule expressed at 200 ng/ml of serum induced no tolerance (Arnold *et al.*, 1988). However, in all these cases only functional assays were performed, leaving open whether clonal deletion had occurred or not.

Materials and methods

Transgenic animals and molecular biology techniques

Mice transgenic for the TCR α and β genes of the 4B2A1 T cell clone have been described previously (Bogen *et al.*, 1992), as have mice transgenic for the $\lambda 2^{315}$ gene with the H-chain enhancer (Bogen and Weiss, 1991). Offspring were genotyped by Southern blot analysis or polymerase chain reaction (PCR).

Total cellular RNA from various tissues was purified and 20 μg electrophoresed and blotted as described previously (Øyen *et al.*, 1987). The filter was hybridized with a Cλ2 probe (Weiss and Wu, 1987) and rehybridized with a β-actin probe (Cleveland *et al.*, 1980) to check for efficient transfer of RNA.

Antibodies, ELISA, flow cytometry and immunofluorescence

The GB113 mouse mAb is specific for the 4B2A1 TCR transgenic α chain (Bogen *et al.*, 1992). The 2B6 rat mAb reacts with the C domains of λ2 and λ3 chains, while the 9A8 rat mAb reacts with the V domains of both λ1 and λ2 chains (Bogen, 1989). The L22.18.2 rat anti-mouse λ1 mAb has been described previously (Weiss *et al.*, 1983). The B cell hybridomas 76.21 (IgG3,$\lambda 2^{315}$), 45.14 (IgG3,$\lambda 2^{315}$) and 136.4 (IgG2b,$\lambda 2^{315}$) were derived from a $\lambda 2^{315}$-TG mouse (Bogen and Weiss, 1991). The Bet 2 anti-μ (Kung *et al.*, 1981), 187.1 anti-κ (Yelton *et al.*, 1981), HB128 anti-mouse γ3 and TIB95 anti $H\text{-}2K^k$ (IgG2a,κ) hybridomas were from the American Type Culture Collection (Rockville, MD). mAbs were affinity-purified on Protein G–Sepharose and biotin- and fluorescein isothiocyanate (FITC)-conjugated as described previously (Bogen, 1989). The A3-14 (μ, λ2) and 1.69 (μ,κ) mouse mAbs, and the M315 (α,$\lambda 2^{315}$), J558(α,λ1) and M104E (μ,λ1) mouse myeloma proteins, were prepared as described previously (Bogen *et al.*, 1986a; Bogen and Weiss, 1991). 76.21, 136.4 and T952 (α, λ2; Litton Bionetics, Kensington, MD) for injections were prepared from ascitic fluid by ammonium sulfate precipitation. Phycoerythrin (PE)-conjugated anti-CD4, PE–streptavidin and FITC–anti-CD8 (Becton Dickinson, Mountain View, CA), PE–anti-CD8 (Boehringer Mannheim, Mannheim, Germany) and FITC–streptavidin (Amersham International, Amersham, UK) were purchased.

Sandwich ELISA was performed as described by Bogen and Weiss (1991). The following combinations of [capturing mAb; standard; biotinylated mAb] were used to measure: total λ2 and λ3 chains [9A8; M315; 2B6]; total λ1 chains [9A8; J558; L22.18.2]; IgM with λ2 or λ3 chains [Bet 2; A3-14; 2B6]; γ3 with λ2 or λ3 chains [HB128; 45.14; 2B6]; IgM with λ1 chains [Bet 2; M104E; L22.18.2]; IgM with κ chains [Bet 2; 1.69; 187.1]. Total κ chains were measured in a sandwich ELISA with non-conjugated and alkaline phosphatase-conjugated affinity-purified rabbit anti-mouse κ antibodies (own production) and TIB95 as standard. Because total λ2 and λ3 (λ2/3) and IgG3,λ2/3 in normal mouse sera were <30 and 3 μg/ml, respectively, the large amounts of λ2/3 and IgG3λ2/3 found after injection were assumed to be $\lambda 2^{315}$ (see Results).

Double staining of cells was performed as described by Bogen and Weiss (1991). Cells ($1-3 \times 10^4$) were run on a FACScan (Becton Dickinson) and lymphoid cells gated for analysis by forward and side scatter parameters. The data are presented as 20% probability contour plots (Lysys program, BD). After single staining, 3×10^4 cells were run on the FACScan and propidium iodide-negative lymphoid cells were gated (FL2/FL3 channels and forward and side scatter channels, respectively) for histogram analysis (Lysys program). Cryosections of thymus and spleen were stained with biotinylated 2B6 mAb, followed by FITC–streptavidin.

Cellular assays

The proliferation assays were essentially performed as described previously (Bogen *et al.*, 1986a). Lymph node cells (1×10^5/well) were stimulated with 800 Rad irradiated spleen cells (2.5×10^5/well) and 1 μCi [^{3}H]TdR (2 μCi/mmol, Amersham) was added for the last 16 h of a 65 h culture. In the antibody production assay, spleen cells (1×10^5/well) were cultured with 2×10^4/well of the cloned 4B2A1 Id-specific T cells (Bogen *et al.*, 1986a), from which the transgenic TCR was isolated. On day 12, supernatant (SN) was withdrawn and total λ2 and λ3 chains determined in ELISA.

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References

Arnold,B., Dill,O., Küblbeck,G., Jatsch,L., Simon,M.M., Tucker,J. and Hämmerling,G.J. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 2269–2273.

Adelstein,S., Pritchard-Briscoe,H., Anderson,T.A., Crosbie,J., Gammon,G., Loblay,R.H., Basten,A. and Goodnow,C.C. (1991) *Science*, **251**, 1223–1225.

Blüthmann,H., Kisielow,P., Uematsu,Y., Malissen,M., Krimpenfort,P., Berns,A., von Boehmer,H. and Steinmetz,M. (1988) *Nature*, **334**, 156–159.

Bogen,B. (1989) *Scand. J. Immunol.*, **29**, 273–279.

Bogen,B. and Lambris,J. (1989) *EMBO J.*, **8**, 1947–1952.

Bogen,B. and Weiss,S. (1991) *Eur. J. Immunol.*, **21**, 2391–2395.

Bogen,B., Malissen,B. and Haas,W. (1986a) *Eur. J. Immunol.*, **16**, 1373–1378.

Bogen,B., Snodgrass,R., Briand,J.-P. and Hannestad,K. (1986b) *Eur. J. Immunol.*, **16**, 1379–1384.

Bogen,B., Gleditsch,L., Weiss,S. and Dembic,Z. (1992) *Eur. J. Immunol.*, **22**, 703–709.

Burkly,L.C., Lo,D. and Flavell,R.A. (1990) *Science*, **248**, 1364–1368.

Cibotti,R., Kanellopoulos,J.M., Cabaniols,J.-P., Halle-Panenko,O., Kosmatopoulos,K., Sercarz,E. and Kourilsky,P. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 416–420.

Cleveland,D.W., Lopata,M.A., MacDonald,R.J., Cowan,N.J., Rutter,W.J. and Kirschner,M.W. (1980) *Cell*, **20**, 95–105.

Fowlkes,B.J., Schwartz,R.H. and Pardoll,D.M. (1988) *Nature*, **334**, 620–623.

Goodnow,C.C. (1992) *Annu. Rev. Immunol.*, **10**, 489–518.

Grosschedl,R., Weaver,D., Baltimore,D. and Constantini,F. (1984) *Cell*, **38**, 647–658.

Hagman,J., Lo,D., Doglio,L.T., Hackett,J., Rudin,C.M., Haasch,D., Brinster,R. and Storb,U. (1989) *J. Exp. Med.*, **169**, 1911–1929.

Inaba,M., Inaba,K., Hosono,M., Kumamoto,T., Ishida,T., Muramatsu,S., Masuda,T. and Ikehara,S. (1991) *J. Exp. Med.*, **173**, 549–559.

Jerne,N.K. (1974) *Ann. Immunol. (Inst. Pasteur)*, **125c**, 373–389.

Jones,LA., Chin,T., Longo,D.L. and Kruisbeek,A.M. (1990) *Science*, **250**, 1726–1729.

Kappler,J.W., Roehm,N. and Marrack,P. (1987) *Cell*, **49**, 273–280.

Kappler,J.W., Staerz,U., White,J. and Marrack,P.C. (1988) *Nature*, **332**, 35–40.

Kisielow,P., Blüthman,H., Staerz,U.D., Steinmetz,M. and von Boehmer,H. (1988) *Nature*, **333**, 742–746.

Kung,J.T., Sharrow,S.O., Sieckmann,D.G., Lieberman,R. and Paul,W.E. (1981) *J. Immunol.*, **127**, 873–876.

Lassila,O., Vainio,O. and Matzinger,P. (1988) *Nature*, **334**, 253–255.

Lin,R.H. and Stockinger,B. (1989) *Eur. J. Immunol.*, **19**, 105–110.

Lorenz,R.G. and Allen,P.M. (1989) *Nature*, **337**, 560–563.

MacDonald,H.R., Schneider,R., Lees,R.K., Howe,R.C., Acha-Orbea,H., Festenstein,H., Zinkernagel,R.M. and Hengartner,H. (1988a) *Nature*, **332**, 40–45.

MacDonald,H.R., Hengartner,H. and Pedrazzini,T. (1988b) *Nature*, **335**, 174–176.

Mazda,O., Watanabe,Y., Gyotoku,J.-I. and Katsura,Y.J. (1991) *J. Exp. Med.*, **173**, 539–547.

Miller,J.F.A.P. and Morahan,G. (1992) *Annu. Rev. Immunol.*, **10**, 51–69.

Murphy,K.M., Heimberger,A.B. and Loh,D.Y. (1990) *Science*, **250**, 1720–1723.

Niewenhuis,P., Stet,R.J.M., Wagenaar,J.P.A., Wubbena,A.S., Kampinga,J. and Karrenbeld,A. (1988) *Immunol. Today*, **9**, 372–375.

Ohashi,P.S., Oehen,S., Buerki,K., Pircher,H., Ohashi,C.T., Odermatt,B., Malissen,B., Zinkernagel,R.M. and Hengartner,H. (1991) *Cell*, **65**, 305–317.

Øyen,O., Frøysa,A., Sandberg,M., Eskild,W., Joseph,D., Hansson,V. and Jahnsen,T. (1987) *Biol. Reprod.*, **37**, 947–956.

Pircher,H.-P., Bürki,K., Lang,R., Hengartner,H. and Zinkernagel,R.M. (1989) *Nature*, **342**, 559–561.

Ramsdell,F. and Fowlkes,B.J. (1990) *Science*, **248**, 1342–1348.

Rocha,B. and von Boehmer,H. (1991) *Science*, **251**, 1225–1228.

Rudensky,A.Y., Mazel,S.M. and Yurin,V.L. (1990) *Eur. J. Immunol.*, **20**, 2235–2239.

Sanz,I. (1991) *J. Immunol.*, **147**, 1720–1729.

Schönrich,G., Kalinke,U., Momburg,F., Malissen,M., Schmitt-Verhulst,A.M., Malissen,B., Hämmerling,G.J. and Arnold,B. (1991) *Cell*, **65**, 293–304.

Schwartz,R.H. (1990) *Science*, **248**, 1349–1356.

Sha,W.C., Nelson,C.A., Newberry,R.D., Kranz,D.M., Russell,J.H. and Loh,D.Y. (1988) *Nature*, **336**, 73–76.

Spain,L.M. and Berg,L.J. (1992) *J. Exp. Med.*, **176**, 213–223.

Sprent,J., Gao,E.-K. and Webb,S.R. (1990) *Science*, **248**, 1357–1363.

Swat,W., Ignatowicz,L., von Boehmer,H. and Kisielow,P. (1991) *Nature*, **351**, 150–153.

Tonegawa,S. (1983) *Nature*, **302**, 575–581.

Vasquez,N.J., Kaye,J. and Hedrick,S.M. (1992) *J. Exp. Med.*, **175**, 1307–1316.

von Boehmer,H. and Kisielow,P. (1990) *Science*, **248**, 1369–1373.

Webb,S., Morris,C. and Sprent,J. (1990) *Cell*, **63**, 1249–1256.

Weiss,S. and Bogen,B. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 282–287.

Weiss,S. and Bogen,B. (1991) *Cell*, **64**, 767–776.

Weiss,S. and Wu,G.E. (1987) *EMBO J.*, **6**, 927–932.

Weiss,S., Lehmann,K. and Cohn,M. (1983) *Hybridoma*, **2**, 49–53.

Whiteley,P.J., Poindexter,N.J., Landon,C. and Kapp,J.A. (1990) *J. Immunol.*, **145**, 1376–1381.

Winchester,G., Sunshine,G.H., Nardi,N. and Mitchison,N.A. (1984) *Immunogenetics*, **19**, 487–491.

Yelton,D.E., Desaymard,C. and Scharff,M.D. (1981) *Hybridoma*, **1**, 5–11.

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