Interleukin 2 Abrogates the Nonresponsive State of T Cells Expressing a Forbidden T Cell Receptor Repertoire and Induces Autoimmune Disease in Neonatally Thymectomized Mice

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Summary

Under physiological conditions, the vast majority of T cells differentiate in the thymus, an organ that provides an optimal microenvironment for T cell maturation and shapes the T cell repertoire via positive and negative selection processes. In the present report, we demonstrate that neonatal thymectomy of CBA/H mice results in a diminution of T cells in peripheral lymphoid organs (spleen, lymph nodes), but is followed by a marked transient (12 wk) increase in Thy-1$^+$ CD3$^+$ cells in the peritoneal cavity. These cells exhibit predominantly a double-negative (CD4$^-$CD8$^-$) phenotype among which products of the T cell receptor (TCR) V_β11 gene family (i.e., an I-E-reactive TCR normally deleted in I-E-bearing CBA/H mice) are selectively overexpressed. This observation suggests that, under athymic conditions, T cell differentiation and/or accumulation may occur in the peritoneal cavity. Intraperitoneal inoculation of an interleukin 2 (IL-2) vaccinia virus construct that releases high titers of human IL-2 in vivo induces conversion of these double-negative T cells to either CD4$^+$CD8$^-$ or CD4$^-$CD8$^+$ single positives, and allows in vitro stimulation of TCR V_β11-bearing cells with a clonotypic anti-V_β antibody. Since IL-2 induces autoimmune manifestations (DNA autoantibodies, rheumatoid factors, and interstitial nephritis) in thymectomized CBA/H mice, but not in sham-treated littermates, this lymphokine is likely to enhance the autoaggressive function of T cells that bear forbidden, potentially autoreactive TCR gene products and that are normally deleted in the thymus.

The mature T cell repertoire is known to be shaped by both negative and positive selection events in the thymus (1, 2). Albeit the designation "T cell" is due to the apparent thymus dependence of T lymphocyte differentiation, an increasing body of evidence indicates the possibility that T cell precursors mature under extrathymic conditions. Thus, congenitally athymic (nude or *nu/nu*) mice, i.e., mutant strains whose thymic anlage is rudimentary and lacks colonization by pro T cells, are not completely devoid of lymphocytes bearing T cell markers (3). *nu/nu* mice exhibit a numeric T cell defect and an oligoclonal T cell repertoire (4) that is rich in cells expressing products of "forbidden" TCR V_β gene families, e.g., V_β genes normally deleted intrathymically due to their autoreactivity with self minor lymphocyte stimulating (Mls)[1] antigens (V_β3 in BALB/c and C3H/HeN mice) or with self MHC-encoded $E_\alpha E_\beta$ products (V_β11 in BALB/c, C3H/HeN, and B10.D2 mice) (5–7). Similarly, neonatal thymectomy results in an adult repertoire enriched in T cells deleted in normal adult thymus (8, 9). Thus, cells expressing V_β6 that are normally deleted in the Mls-1^a-expressing DBA/2 strain and the potentially I-E-reactive V_β11 (in [C57BL/6 × A/J]F_1 and BALB/c mice) and V_β5 (in DBA/2 mice) that are normally deleted in I-E-expressing mice augment after surgical removal of the thymus at day 3 (8, 9). In neonatally thymectomized (neoTx) BALB/c and DBA/2 mice T cells that escape clonal deletion are not functional, i.e., they fail to proliferate in response to clonotypic V_β-specific antibodies (9). However, functional inactivation of cells bearing a forbidden TCR repertoire is not absolute, since both thymectomized (C57BL/6 × A/J)F_1 mice (8) and aged BALB/c *nu/nu* mice exhibit signs of autoimmunity (10). TCR-α/β^+ lymphocytes from athymic mice display functional and phenotypical abnormalities ranging from decreased TCR and CD3 density (11) to low Thy-1 expression, defec-

[1] *Abbreviations used in this paper:* h, human; IL-2-VV, interleukin 2 vaccinia virus; mls, minor lymphocyte stimulating; neoTx, neonatally thymectomized; WT-VV, wild-type vaccinia virus.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/91/06/1323/07 $2.00
Volume 173 June 1991 1323–1329

tive lymphokine production (3), and abnormally high amounts of double-negative ($CD4^-CD8^-$) cells (7).

Although it seems clear that selective expansion of forbidden clones occurs in athymic condition, either after surgical thymectomy or in nude mouse models, it is unknown whether extrathymic T cell maturation is triggered under these conditions. Here, we report that neonatal thymectomy leads to a surge of $V_\beta 11^+$ T cells (normally deleted due to their I-E reactivity) in the periphery of CBA/H mice and entails a numeric defect in cells bearing pan T cell markers (Thy-1, CD3) in peripheral lymphoid organs. Surprisingly, however, it results in a selective expansion of T cells bearing the immature $CD3^+CD4^-CD8^-$ phenotype and expressing products of the forbidden $V_\beta 11$ gene family in the peritoneal cavity. Using a human (h)IL-2 vaccinia virus construct as an autonomously replicating IL-2 releasing device, we were able to induce conversion of these peritoneal cells to mature $CD4^+$ or $CD8^+$ cells and to induce autoimmune manifestations in vivo. Moreover, cells from mice treated with IL-2 vaccinia virus acquired the capacity to proliferate in response to anti-$V_\beta 11$. These results suggest: (*a*) peritoneum is a site for extrathymic T cell maturation or accumulation; (*b*) the function of nonresponsive T cells may be triggered by IL-2 in vivo; and (*c*) IL-2 may precipitate autoimmune disease in athymic conditions.

Materials and Methods

Animals and Cell Suspensions. 3-d-old CBA/H mice (The Jackson Laboratory, Bar Harbor, ME) were anesthetized by hypothermia and were thymectomized by the suction technique previously described (12). Mice were killed by CO_2 inhalation at 6, 12, and 24 wk of life. Cell suspensions from bone marrow, spleen, and inguinal lymph nodes were prepared using standard techniques, and peritoneal cells were obtained by washing the cavity with 10 ml of chilled BSS-3% FCS, followed by two washings. In some experiments, mice were injected intraperitoneally with 10^7 plaque forming units of rIL-2 vaccinia virus (IL-2-VV) (13) or wild-type vaccinia virus (WT-VV) starting from 6 wk of age. Treatment was repeated three times in 2-wk intervals. At necropsy, at 12 wk of age, various organs were removed and subjected to hematoxylin eosin histology.

ELISAs. Sera were tested for the presence of anti-DNA antibodies and rheumatoid factors following standard ELISA protocols (14). Briefly, polyvinylchloride microtiter plates (Dynatech, Alexandria, VA) were coated by overnight incubation (4°C) with sheared salmon sperm DNA (5 μg/ml) or pools of IgG2a or IgG1 mAbs (10 μg/ml) in 50 mM PBS (pH 7.4), followed by blocking with PBS containing 1% gelatin. Plates were incubated at room temperature for 2 h with serial dilutions of mouse sera (in PBS + 1% gelatin), washed with peroxidase-labeled goat anti-mouse Ig, and developed using *o*-phenylendiamine as substrate. Optical density was measured at 450 nM.

Fluorocytometric Analysis. mAbs directed against CD3 (clone 145.C11), CD4 (GK1.5; Becton Dickinson & Co., Mountain View, CA), CD5 (537.3), CD8 (H02.2), Thy-1.2 (30-H12), $V_\beta 8$ (F23.1, specific for $V_\beta 8.1$, $V_\beta 8.2$, and $V_\beta 8.3$; reference 15), $V_\beta 11$ (RR3; reference 16), and $V_\beta 6$ (44.22.1; reference 17) were either FITC labeled or used as biotin-conjugated reagents and developed by means of streptavidin-PE (Becton Dickinson & Co.). Cells were stained following standard protocols and fluorescence was measured using the Epics Profile flow cytometer (Coulter Electronics, Hialeah, FL). Background values were defined by using isotype-matched irrelevant antibodies as negative controls.

T Cell Proliferation Assays. Flat-bottomed microtiter wells (Nunc, Kamstrup, Denmark) were incubated for 3 h at 37°C with 100 μl of PBS containing 0.2, 2, or 20 μg/ml 145.C11 (αCD3), $V_\beta 8$ (F23.1), RR3 ($\alpha V_\beta 11$), or KJ23a ($\alpha V_\beta 17a$, reference 18) and then washed three times before use. 10^5 peritoneal exudate cells or splenocytes were incubated for 3 d in 200 μl in RPMI 1640 supplemented with 10% FCS, 50 μM 2-ME, 10 mM Hepes, 200 mM L-glutamine, 10 U/ml penicillin, and 100 μg/ml streptomycin, and 10 U/ml rhIL-2 (Hoffmann-La Roche, Basel, Switzerland). Cells were harvested after an 18-h pulse label with 1 μCi of [^{3}H]thymidine (Amersham, London, UK).

Results and Discussion

As evident from Table 1, relative and absolute T cell numbers were reduced after thymectomy both in spleen and in lymph node, a phenomenon associated with a diminution of the CD4/CD8 ratio, since depletion was more pronounced among the $CD4^+$ subset. The decrease in splenic T cells was manifest at the relative level, whereas lymph nodes displayed only a mild relative T lymphopenia, but were greatly reduced in size. In contrast to the T cell depletion observed in peripheral lymphoid organs, among cells recovered from the peritoneal cavity of neoTx animals, the relative and absolute number of lymphocytes bearing the Thy-1 or CD3 pan-T cell markers was significantly increased at 6 and 12 wk of age, but returned to control values at 4 mo (Table 1). This transient elevation of peritoneal T cell numbers was accompanied by the presence of a high portion of cells exhibiting the $CD3^+CD4^-CD8^-$ phenotype, i.e., a cell type that is practically absent in the peritoneum of control mice and that is found at very low percentages (<1%) in spleens or lymph nodes of neoTx or control CBA/H mice (Table 1). This double-negative phenotype has been associated with autoimmune phenomena in mice affected by the *lpr*, *gld* (19), and *nu* mutations (7), as well as in individuals afflicted with systemic autoaggressive diseases (20), and may either represent an immature stage of T cell maturation preceding acquisition of the accessory molecules CD4 and/or CD8 (1) or, alternatively, may derive from a "loss pathway" where accessory molecules are downregulated on autoreactive T cells that have escaped negative selection (19, 21, 22), thus exhibiting an antigen-unresponsive, anergic behavior. In this context, it may be noteworthy that the pleuroperitoneal cavity has already been reported to represent the primordial differentiation site of coelomic $CD5^+$ B lymphocytes (23) and, therefore, is likely to possess a lymphocytotrophic nature.

In accordance with previous reports (5–10), thymectomy results in the expression of forbidden TCR gene products in peripheral lymphoid organs, specifically $V_\beta 11$, i.e., a V_β gene product that is clonally deleted in euthymic CBA/H mice due to its high reactivity with yet unidentified non-MHC antigens presented by class II I-E molecules (15, 24). Neonatal thymectomy has been equally reported to result in the abolition of clonal deletion of $V_\beta 11$ in (C57BL/6 × A/J)F_1 and BALB/c mice (8, 9). In contrast, the percentage

Table 1. *Effect of Neonatal Thymectomy on the Frequency of T Cells in Peripheral Lymphoid Organs*

		Percentage of cells after:					
		Thymectomy			Sham treatment		
Organ		6 wk	12 wk	24 wk	6 wk	12 wk	24 wk
Peritoneum	Cell yield ($\times 10^{-5}$)	$33^{\uparrow *}$	$29^{\uparrow}$	24	15	18	20
	Thy-1$^+$	$18^{\uparrow}$	$20^{\uparrow}$	16	4	14	15
	CD3$^+$	$17^{\uparrow}$	$18^{\uparrow}$	14	4	12	14
	CD4$^+$	4	6	7	3	6	8
	CD8$^+$	<0.5	3	7	<0.5	4	4
	CD3$^+$CD4$^-$CD8$^{-‡}$	$71^{\uparrow}$	$39^{\uparrow}$	2	<0.5	<0.5	<0.5
Spleen	Cell yield ($\times 10^{-6}$)	40	60	87	42	83	125
	Thy-1$^+$	$15^{\downarrow *}$	$10^{\downarrow}$	$26^{\downarrow}$	47	50	50
	CD3$^+$	$7^{\downarrow}$	$8^{\downarrow}$	22	45	43	45
	CD4$^+$	$3^{\downarrow}$	$4^{\downarrow}$	20	30	30	32
	CD8$^+$	$4^{\downarrow}$	$4^{\downarrow}$	10	16	16	12
	CD3$^+$CD4$^-$CD8$^-$	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Lymph node	Cell yield ($\times 10^{-6}$)	$1^{\downarrow}$	$2^{\downarrow}$	$4^{\downarrow}$	11	25	23
	Thy-1$^+$	$51^{\downarrow}$	$47^{\downarrow}$	$55^{\downarrow}$	87	85	90
	CD3$^+$	$46^{\downarrow}$	$45^{\downarrow}$	60	82	81	86
	CD4$^+$	$22^{\downarrow}$	$21^{\downarrow}$	$29^{\downarrow}$	60	57	43
	CD8$^+$	28	25	33	30	26	30
	CD3$^+$CD4$^-$CD8$^-$	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5

Cells derived from peritoneum, spleen, or lymph nodes from neoTx or sham-treated CBA/H mice were analyzed by immunocytofluorometry as described in Materials and Methods, and results were expressed as percentage of stained cells. In each case, at least six animals were analyzed. Variances were <10% for cytofluorometric data and <15% for determinations of viable cell numbers.

* Arrows indicate significant differences between thymectomized and respective control animals ($p < 0.01$, unpaired student's t test).

‡ The percentage of double negatives among CD3$^+$ cells was determined by double staining.

of cells expressing V$_\beta$11 gene products does not augment in neoTx DBA/2 mice (9), although V$_\beta$11 T cells should be as potentially self-reactive as in the other I-E-expressing strains studied, a fact that is not at present understood. In neoTx CBA/H mice, V$_\beta$11 attained a level of 25.1 ± 4.7% (mean ± SEM; n = 6) among CD3$^+$ cells in the spleen and 42.2 ± 7.8% in the peritoneum, i.e., 8–10-fold higher than in sham-treated controls, and remained constant throughout the period examined (6–24 wk of age), whereas control V$_\beta$ products (V$_\beta$6, V$_\beta$8) are expressed at near to normal levels (Table 2). We have no explanation for the twofold increase in V$_\beta$6$^+$ cells among splenic CD3$^+$ cells, which was not observed in peritoneum. In spite of the high expression of a potentially autoreactive (V$_\beta$11$^+$) TCR, neoTx CBA/H mice did not display any clinical (arthritis, proteinuria, cutaneous ulcera), serological (rheumatoid factor, anti-DNA antibodies), or histopathological signs of autoimmune disease (vide infra). This absence of autoaggression in spite of failure of clonal deletion is thought to be due to the preservation of autotolerance by clonal anergy, since nondeleted V$_\beta$11$^+$ cells from neoTx I-E-bearing mice fail to proliferate in response to I-E$^+$ stimulator cells or immobilized anti-V$_\beta$11 (9).

Intraperitoneal inoculation of a rhIL-2 vaccinia virus construct, which induces production of high levels (up to 20 U/ml in serum) of hIL-2 around day 10 after infection (13), resulted in appearance of accessory molecules (CD4 or CD8) on the peritoneal T cell population (Fig. 1) without inducing significant changes in the TCR repertoire expressed by these cells. The decrease in CD3$^+$CD4$^-$CD8$^-$ cells is not due to an outgrowth of pre-existing single-positive T cells proliferating with IL-2, since no significant increase of single-positive cells were found in sham-operated CBA/H mice treated with hIL-2-VV (data not shown). It remains elusive whether IL-2 stimulates de novo expression of CD4 and/or CD8 on an immature double-negative population or induces restitutio ad integrum of cells that have lost their accessory molecules during an anergization process. A daily follow-up of the phenotype of peritoneal cells changing their antigenic make-up after the application of hIL-2-VV failed to detect double-positive cells (CD4$^+$CD8$^+$; Fig. 1 and data not shown), thus

Table 2. *Effect of Neonatal Thymectomy on the TCR Repertoire Expressed by Peripheral T Cells*

Organ	Vβ gene family	Percentage (n = 6) among $CD3^+$ cells after: Thymectomy			Sham treatment		
		6 wk	12 wk	24 wk	6 wk	12 wk	24 wk
				± *SEM*			
Peritoneum	$V_\beta 6$	9.8 ± 1.7	9.3 ± 1.6	8.7 ± 1.5	14.9 ± 1.6	13.5 ± 1.5	14.7 ± 1.3
	$V_\beta 8$	35.2 ± 6.2	33.1 ± 6.3	34.7 ± 6.7	29.7 ± 3.5	28.4 ± 3.2	29.3 ± 3.5
	$V_\beta 11$	42.2 ± 7.8†*	40.5 ± 7.2†	37.2 ± 6.7†	5.1 ± 0.5	4.7 ± 0.5	4.3 ± 0.4
Spleen	$V_\beta 6$	15.2 ± 3.0†	14.7 ± 3.1†	15.1 ± 2.9†	6.5 ± 0.6	7.2 ± 0.5	6.8 ± 0.6
	$V_\beta 8$	37.6 ± 7.3	35.3 ± 6.8	35.7 ± 7.1	38.1 ± 3.2	37.5 ± 3.1	38.3 ± 3.5
	$V_\beta 11$	24.7 ± 4.5†	25.1 ± 4.7†	24.3 ± 4.3†	2.6 ± 0.3	3.1 ± 0.8	2.3 ± 0.2

Cells derived from peritoneum or spleen from neoTx or sham-treated CBA/H mice were subjected to two-color cytofluorometric analysis, and results were expressed as percentage of cells expressing products of a particular TCR V_β gene family (biotin-labeled mAbs F23.1, RR3, and 44.22.1 specific for products of the $V_{\beta 8}$, $V_{\beta 6}$, and $V_{\beta 11}$ gene families, respectively) among CD3 (FITC-labeled) cells (Table 1).

* Arrows indicate significant differences between thymectomized and respective control animals ($p < 0.01$, unpaired student's t test). In no instance were significant differences in the median fluorescence intensity detected for V_β gene expression.

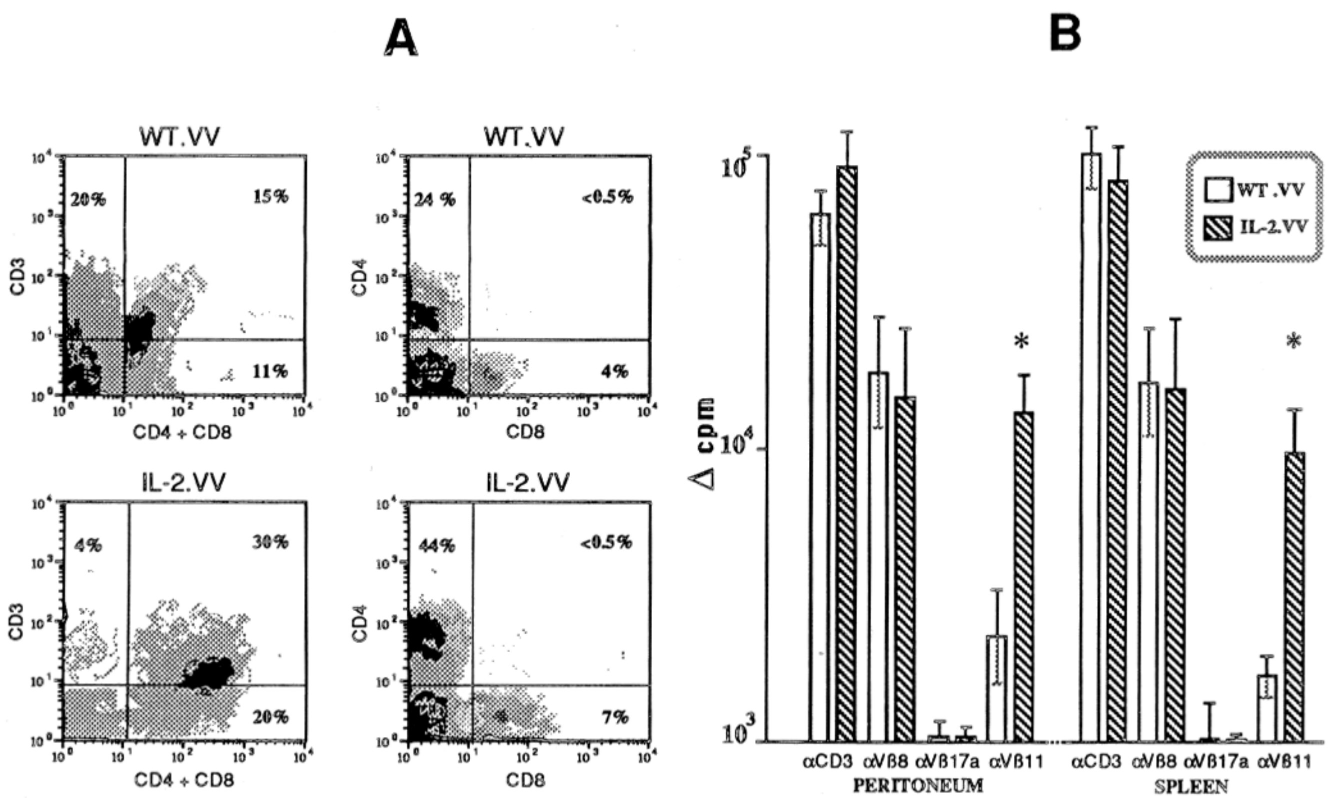

Figure 1. Effect of rhIL-2 vaccinia virus on peripheral T cells of neoTx CBA/H mice. NeoTx CBA/H mice were treated by intraperitoneal injection of rIL-2-VV or WT-VV, as described in Materials and Methods, followed by assessment of the frequency of $CD3^+CD4^-CD8^-$ (double negatives) and $CD3^+CD4^+/CD8^+$ cells in the peritoneum (*A*). The ratio between $CD4^+CD8^-$ and $CD4^-CD8^+$ cells did not change significantly upon IL-2-VV treatment. Moreover, the proliferation of peritoneal and splenic T cells in response to αCD3, $\alpha V_\beta 8$, $\alpha V_\beta 11$, and $\alpha V_\beta 17a$ was assessed (*B*). Values are as mean values ± SEM, and background values (<2 × 10^3 cpm) were subtracted. Asterisks mark significant differences ($p < 0.01$; unpaired student's t test) between the two groups of animals. $\alpha V_\beta 17a$ may be considered as negative control since CBA/H mice express the $V_\beta 17b$ allele (18). Results are shown for an antibody concentration of 20 μg/ml used for coating microtiter plates, and analogous results were obtained with lower antibody concentrations (data not shown). The percentage of $V_\beta 8^+$ and $V_\beta 11^+$ cells among $CD3^+$ T cells did not vary significantly between WT-VV (28 ± 6% $V_\beta 8^+$ and 35 ± 7% $V_\beta 11^+$ in peritoneum; 34 ± 6% $V_\beta 8^+$ and 22 ± 5% $V_\beta 11^+$ in spleen) and IL-2-VV-treated animals (27 ± 4% $V_\beta 8^+$ and 39 ± 5% $V_\beta 11^+$ in peritoneum; 32 ± 5 $V_\beta 8^+$; 26 ± 4 $V_\beta 11^+$ T cells in spleen).

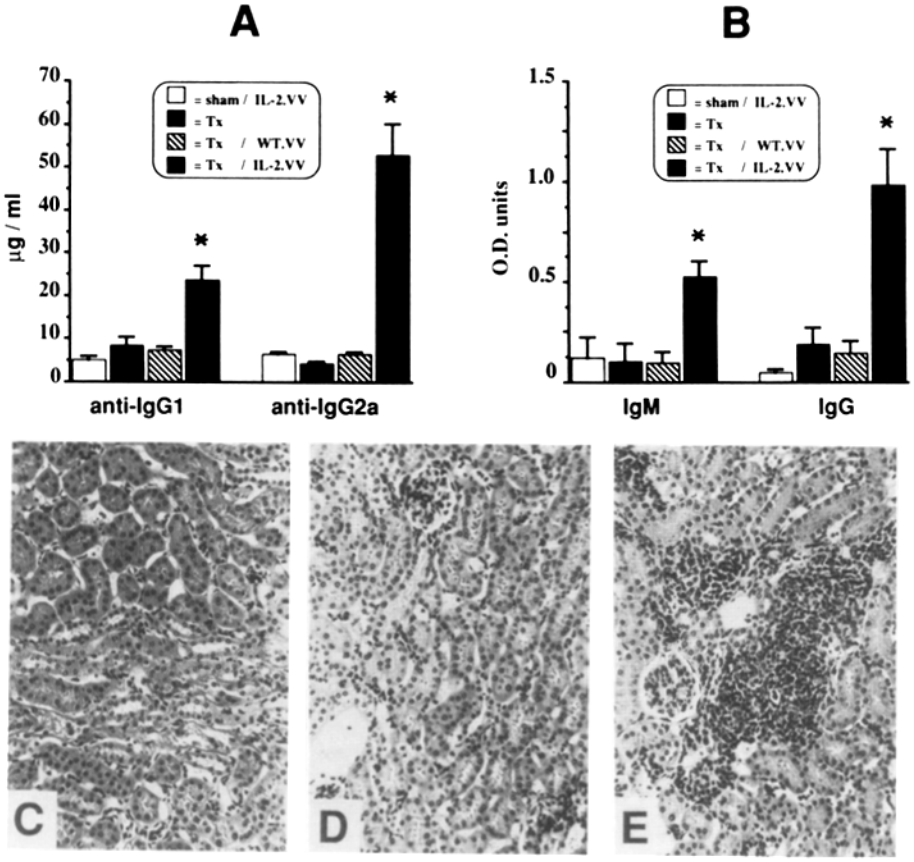

Figure 2. Autoimmune manifestations induced by IL-2 in neoTx CBA/H mice. CBA/H mice, either sham treated or thymectomized (Tx) 3 d after birth, were treated by intraperitoneal injection of 10^7 plaque forming units of rIL-2-VV or WT-VV starting from 6 wk of age. Treatment was repeated three times in 2-wk intervals followed by necropsy and assessment of serum autoantibody concentrations at 12 wk of age (*A*). Concentrations of rheumatoid factors directed against IgG1 or IgG2a (mean values ± SEM; $n = 6$ for each group). (*B*) Serum concentrations of anti-dsDNA of the IgM or IgG isotypes as determined by ELISA at a 200-fold dilution of sera. Asterisks indicate significant differences between Tx/IL-2-VV treated animals and control groups ($p < 0.01$; Student's *t* test). Total immunoglobulin levels were not affected by IL-2-VV or WT-VV treatment. (*C*) HE staining of a kidney from sham-treated CBA/H mice treated with IL-2-VV; (*D*) or from a neoTx individual treated with wild-type virus. (*E*) Representative sample from neoTx animals treated with IL-2-VV. Note the interstitial mononuclear infiltrate visible only in *E*.

providing indirect evidence in favor of the second possibility. Intermediate affinity IL-2 receptors (p75, heavy chain) have been detected both on immature thymocytes (25) as well as on abnormal T cells of MRL/Mp-*lpr*/*lpr* mice (26), suggesting that IL-2 may exert direct effects on double-negative T cells. IL-2 has been shown to trigger the maturation of pre- and intrathymic T cell precursors in vitro where it drives double-negative cells into the double-positive stage (25). On the other hand, IL-2 restores the capacity of anergic T lymphocytes, i.e., cells that have not been clonally deleted but functionally paralyzed, to proliferate in response to antigenic stimuli (24, 27–29). Accordingly, nondeleted $V_\beta 11^+$ cells of IL-2-VV-treated animals acquire the capacity of proliferating with anti-$V_\beta 11$ antibody, whereas cells from control animals fail to mount a significant response (Fig. 1). This is in contrast with the finding by Jones et al. (9) that nondeleted $V_\beta 11^+$ cells from neoTx I-E-bearing mice fail to proliferate in response to anti-$V_\beta 11$ or appropriate stimulator cells, even when exposed during a short term (3 d) culture to IL-2. Nonetheless, the very different experimental design (in vitro vs. in vivo) and the different duration of IL-2 treatment (6 wk vs. 3 d) may explain this discrepancy.

Both $CD4^+$ and $CD8^+$ T cells expressing $V_\beta 11$ receptors are known to respond to I-E antigens in vivo (30), and induction of CD4 on double-negative splenic T cells by in vitro culture with Con A has been known to induce autoreactive behavior of T cells that provide help to autoimmune B cells (31). It therefore is tempting to speculate that the induction of phenotypically mature ($CD4^+$/$CD8^+$) and functionally competent T cells bearing products of this potentially self-reactive TCR V_β gene family may be functionally linked to the development of autoimmune phenomena. IL-2-VV, not WT-VV, provokes the production of DNA autoantibodies and rheumatoid factors, as well as an interstitial nephritis, in neoTx CBA/H mice, but fails to do so in euthymic controls (Fig. 2). This observation supports the notion that IL-2 may precipitate autoimmune diseases (32). Accordingly, application of IL-2 receptor antibodies prevents the development of virtually any autoimmune disease investigated in this respect, and rIL-2 frequently induces autoimmune lesions in patients (33). Inoculation of the hIL-2-VV construct used in this report induces overt autoimmunity in neoTx CBA/H animals, as well as in young BALB/c *nu*/*nu* and C57Bl/6 *nu*/*nu* mice (Gutierrez-Ramos, Moreno de Alborán, and Martínez-A, manuscript in preparation). No effect is observed in CBA/H mice thymectomized at 3 mo of age. In contrast, the same treatment ameliorates systemic lupus erythematosus developing in MRL/Mp *lpr*/*lpr* mice (13). This discrepancy either reflects a pleiotropic involvement of the IL-2/IL-2 receptor system in the cascade of events leading to autoimmune disease or, alternatively, may be ascribed to the very different etiology of autoaggression in distinct animal models. Thus, athymia with the consequent perturbations in T lymphocyte repertoire, imbalance in distinct T cell populations, suppressor/

helper ratios, and deficient "lymphokine sink" (34) could predispose to IL-2-induced autoimmunity, whereas presence of a thymus may preclude such an IL-2 effect. In this context, it is worthwhile to mention that IL-2 actually induces thymic hyperplasia in MRL/Mp *lpr/lpr* mice, which normally are characterized by thymic atrophia (13). Future studies will address the cellular mechanism of IL-2-induced autoimmune disease and will evaluate the position occupied by the IL-2/IL-2 receptor system in the sequence of pathogenetic events leading to manifest autoimmunity.

We thank E. Leonardo and J. A. Gonzalo for skilled technical assistance, and K. M. Sweeting for editorial help. Moreover, we gratefully acknowledge the comments of the unknown referee that contributed to improve the data presentation in Table 2.

J. L. Andreu-Sánchez and A. Sánchez-Movilla were supported by INSALUD-Madrid. This work was supported in part by grants from Fondo de Investigación Sanitaria, European Community, and Comisión Interministral de Ciencia y Tecnología.

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Received for publication 30 November 1990 and in revised form 27 February 1991.

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