Immunology 1991 **73** 52–57

ADONIS 0019280591001108

Studies of immunological function in mice with defective androgen action. Distinction between alterations in immune function due to hormonal insensitivity and alterations due to other genetic factors

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Accepted for publication 24 January 1991

SUMMARY

The presence of androgen receptors in thymocytes and the well-described effects of exogenous androgens on thymus size suggest a role for androgenic hormones in thymocyte growth and maturation. Testicular feminization (Tfm/Y) mice which bear a heritable defect in the androgen receptor protein were studied to investigate how androgens might influence immune phenotype and function. These mice were compared to two types of controls; their Tabby/Y normal male littermates and male mice of the C57 Bl/6 strain from which the Tabby and Tfm mice were derived. Thymuses and spleens from Tfm/Y mice were larger than both types of controls. Phenotypic differences in thymocyte and splenocyte subpopulations identified by the T-cell markers CD3, CD4 and CD8 suggested that T-cell maturation was altered in the androgen-resistant animal. However, both Ta/Y and Tfm/Y were found to be high producers of interleukin-4 (IL-4) by both spleen and thymus cells, while cells from the C57 mice produced predominantly IL-2. These findings suggest that some immunological features of the Tfm/Y mouse may be related to its defect in androgen action, but that high levels of IL-4 production are probably related to other genetic changes in the C57 background.

INTRODUCTION

A significant body of accumulated evidence supports a role for androgenic hormones in modulation of the immune response. Thymocytes express a high affinity androgen-binding protein with properties of androgen receptors from classical target tissues of the reproductive tract.[1] We recently attempted to address the question of whether the putative thymic androgen receptors, and the hormonal effects mediated through such receptors, might play a significant role in thymocyte development. We reasoned that mice bearing a heritable defect in androgen action might exhibit alterations in immunological function that would be attributable to lack of androgen effects during development. In such a mouse strain bearing a defective androgen receptor gene (Tfm/Y) we found remarkable thymic enlargement compared to controls and an increased capacity of cultured Tfm/Y thymocytes to produce an IL-2-like cytokine under conditions of concanavalin A (Con A).[2]

An underlying assumption in studies of the functional consequences of such mutations as Tfm is that the difference between control and mutant comprises only the single gene defect under consideration. In the absence of highly inbred strains of animals the assumption of such a single gene difference may not be correct. The Tfm mutation, established on the background of the C57Bl/6 mouse, is certainly not the only gene difference between C57Bl/6 and Tfm/Y and cannot be assumed to be the only difference between Tfm/Y mice and their normal male (Ta/Y) littermates. In the present study we report further experiments designed to define more clearly which distinctive features of immune function in the Tfm/Y mouse might be attributable to defective androgen action and which might be ascribed to other heritable or acquired factors.

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MATERIALS AND METHODS

Animals

Androgen-resistant mice (designated Tfm/Y) and normal male litter mates (designated Tabby/Y or Ta/Y because of a coat color marker) were obtained at 6–8 weeks of age from Jackson Laboratories (Bar Harbor, ME). Normal C57 Bl/6 male mice (6–8 weeks of age) were also obtained from Jackson Laboratories. The C57 constitutes the genetic background on which the Tfm mutation is established. The animals were housed in the Vanderbilt University Animal Resources Center and their use in the protocols described was approved by the Vanderbilt University Animal Care Committee.

Thymocyte preparation

Thymus glands were removed as previously described.[2] Thymocytes were prepared by breaking up the tissue using the frosted

ends of two glass slides. Single cell suspensions were prepared by passing the resulting material through a 70 μM mesh. Cells were resuspended in RPMI-1640 with 10% foetal calf serum (FCS; Gibco, Grand Island, NY), 2 mM L-glutamine, 500 U/ml penicillin, 40 μg/ml gentamicin and 0·02 mM 2-mercaptoethanol. Thymocytes used in these experiments routinely showed greater than 95% positivity with fluorescein-labelled anti-Thy-1.2 (ICN ImmunoBiologicals, Lisle, IL), and no differences in the various strains used have been observed.

Splenocyte preparation

Spleens were removed and weighed. Cell suspensions were prepared by breaking up the organ with frosted glass slides as described above or with a ground-glass homogenizer. Erythrocytes were lysed with buffered ammonium chloride solution. Cells were resuspended in RPMI culture medium with supplements as listed above. The viability of such cell preparations, as determined by trypan blue dye exclusion, was routinely greater than 95%.

Cell cultures for production of IL-2 and IL-4

Thymocytes or splenocytes were cultured in flat-bottomed microwells (CoStar, Cambridge, MA) at 5×10^6 cells/ml in RPMI-1640 with 10% FCS and supplements listed above. Cultures were made with and without addition of Con A (Sigma, St Louis, MO) at 10 μg/ml or phorbol myristate acetate (Sigma) at 1 ng/ml and calcium ionophore A23187 (Boehringer Mannheim, Indianapolis, IN) at 300 ng/ml. After 48 hr of incubation, supernatants were harvested and stored at −70° until assayed for IL-2 and IL-4 activity.

IL-2 and IL-4 assay

Measurement of IL-2 activity was done using the IL-2-dependent mouse T-cell line CTLL-2.[3] These cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD) and were maintained in continuous culture with 10% supernatant of the IL-2-secreting gibbon lymphoma line MLA-144 (ATCC). For use in the assay, CTLL cells were harvested after 4–7 days of growth, washed and incubated for 30 min at 37° to elute bound cytokines, then cultured in flat-bottomed microwells at 25,000 cells per well. Serial dilutions of test supernatants from cell cultures were added in triplicate. The total culture volume was 0·10 ml per well. A standard curve prepared from mouse recombinant IL-2 (Genzyme, Boston, MA) was included with each assay. Cultures were continued for 48 hr including a terminal 6-hr pulse with the tetrazolium salt MTT.[4] At the end of the incubation, wells were acidified with acid-isopropanol and absorbance measured in a Titertek Multiskan PLUS (Flow Laboratories, McLean, VA). The difference between OD values at 570 and 630 nm was determined and used to calculate IL-2 activity. Results were expressed as units of IL-2 per ml.

Since the CTLL-2 cell line is responsive to IL-4 as well as IL-2,[5] determination of whether the stimulatory activity observed was due to IL-2 or IL-4 or both was done by immunoneutralization with the monoclonal antibody 11B.11, specific for murine IL-4.[6] The 11B.11 antibody was obtained from the Biological Response Modifiers Program, National Cancer Institute, Frederick, MD, and also grown from hybridoma stock obtained from the ATCC. The amount of 11B.11 added blocked 95% of the stimulatory activity of an optimum concentration of recombinant murine IL-4 (Genzyme). Supernatants were tested with and without the addition of 11B.11 to determine relative amounts of each cytokine.

Proliferation assays

Cells isolated from spleens and thymuses were cultured at 1×10^6 cells per ml in flat-bottomed microwells. Cultures were done with and without Con A at 10 μg/ml or, for splenocytes, with lipopolysaccharide (LPS; Sigma) at 25 μg/ml. Cultures were harvested at various times from 24 to 96 hr, including a terminal 6-hr pulse with [^{3}H]thymidine (New England Nuclear, Boston, MA) at 1 μCi per well. Cells were harvested onto glass-fiber filter strips and radioactivity on individual filter discs was quantified by liquid scintillation counting.

Antibody production and assay

Production of antibody by spleen cells was in round-bottomed microwell cultures containing 1×10^6 cells/ml. Cultures were done with and without addition of LPS at 25 μg/ml. Supernatants were harvested after 7 days and stored at −70° until assayed. Measurement of IgG, IgA and IgM was done by ELISA using class-specific antibodies (Southern Biotechnology Associates, Inc., Birmingham, AL). The wells of a 96-well plate were coated with goat anti-mouse Ig diluted in borate-buffered saline (pH 8·2) and incubated overnight at 4°. The plate was washed and blocked with 1% bovine serum albumin (BSA). Supernatant samples were then added in various dilutions, and incubated for at least 4 hr at room temperature. After a second washing, the alkaline phosphatase-labelled class-specific antibodies (anti-IgG, anti-IgA, anti-IgM) were added and plates incubated at 4° overnight. Wells were emptied, and the p-nitrophenyl phosphate substrate (Sigma), prepared at 1 mg/ml in pH 9·8 buffer, was added. OD values at 405 nm were recorded using the Titertek Multiskan. A standard curve for each immunoglobulin class was constructed from purified mouse antibodies included with each assay. Results were expressed as ng/ml.

Flow cytometry

Expression of surface components of the T-cell receptor on thymocytes was measured using the monoclonal antibody 145-2C11, which is specific for the epsilon chain of the murine CD3 complex[7] and which was used as a fluorescein conjugate (Boehringer Mannheim). Thymocytes were analysed as previously described[2] in cold PBS with 2% BSA and 0·01% sodium azide. Each sample of 10^6 cells was incubated with the appropriate monoclonal antibody for 30 min and washed once by centrifugation through a cushion of FCS. Cells were kept on ice and analysed the same day in the Vanderbilt Flow Cytometry Laboratory. Flow cytometry gates were set using an unstained sample of each preparation to establish background autofluorescence.

Spleen cells and thymocytes were also analysed by flow cytometry for expression of the major T-cell subsets defined by the surface markers CD4 and CD8. Double direct immunofluorescence was performed with fluorescein-conjugated anti-Lyt-2 (CD8; Becton-Dickinson, Mountain View, CA) and phycoerythrin-conjugated anti-L3T4 (CD4; Becton-Dickinson). Cells to be stained were washed as described above, then incubated with the two monoclonal antibodies for 30 min and washed once by centrifugation through FCS. Flow cytometry

gates were set so that over 99% of an unstained sample was in the double-negative quadrant.

Northern blotting

Total RNA was extracted from thymocytes cultured under basal, Con A-stimulated, or PMA/ionophore-stimulated conditions by lysis of the cells in buffer containing 10 mM vanadyl ribonucleoside complexes.[8] A total of 10 μg RNA was loaded into each lane of a 1·2% agarose/formaldehyde gel. Samples were electrophoresed at 50 V and transferred by capillary blotting to a nylon membrane. The blot was probed with a 760 basepair HindIII–Acc I fragment of the plasmid pcD-IL-2[9] obtained from the ATCC. The probe was radiolabelled with [^{32}P] dATP by the random primers method.[10,11] The membrane was pre-hybridized for 24 hr at 42°, probed for 24 hr at the same temperature, washed twice in 1 × SSC, 0·1% SDS at 50° and the blot was exposed to Kodak XAR-5 film for 48 hr.

Statistics

Results were analysed using Student's *t*-test, with a *P* value of less than 0·05 considered significant. For the flow cytometry experiments, pairs of mice which were analysed on the same day were compared using a paired *t*-test to account for small daily variations in the flow cytometry settings and staining efficiency.

RESULTS

Phenotypic analysis of thymocyte subsets in Tfm/Y and Ta/Y mice

As previously observed,[2] we found that the mean thymus weight in Tfm/Y mice (95 ± 5 mg) was significantly and consistently greater than that of Ta/Y littermate males (51 ± 5 mg; $P < 0.001$) and was also larger than thymuses from C57 Bl/6 male mice of comparable age (38 ± 3 mg; $P < 0.001$). Thymocyte yields averaged 247 × 10^6 cells for Tfm/Y mice compared to 125 × 10^6 cells for Ta/Y mice ($P \leq 0.001$) and 108 × 10^6 cells for C57 mice ($P \leq 0.001$). It should also be noted that the mean body weight of C57 Bl/6 mice (25·7 ± 1·14 g) was significantly greater than either Tfm/Y (18·1 ± 0·8 g) or Ta/Y mice (18·8 ± 1·2 g; $P \leq 0.002$ for each comparison).

Since a previous analysis of thymocyte subpopulations only compared Tfm/Y mice to C57Bl/6 males,[2] we re-examined the relative numbers of the four major thymocyte subpopulations defined by the surface markers CD4 and CD8 in nine pairs of Tfm/Y and Ta/Y littermates (Table 1). In each of the nine pairs, Tfm/Y mice had relatively fewer 'double-negative' thymocytes that carried neither CD4 nor CD8 surface markers (4·6 ± 0·8%) than did Ta/Y mice (8·6 ± 1·1%; $P \leq 0.001$ by paired *t*-test). A compensatory increase in double-positive thymocytes, which labelled for both CD4 and CD8, was seen in Tfm/Y mice (76·5 ± 1·9%) compared to Ta/Y mice (68·0 ± 2·9%; $P = 0.018$ by paired *t*-test). Relative numbers of single-positive CD4 and CD8 thymocytes were not significantly different in the two types of mice. Expression of CD3 on the surface of thymocytes from Tfm/Y and Ta/Y mice was analysed in four pairs of mice (Table 2). The percentage of cells bearing the epsilon chain of CD3 was higher in Ta/Y mice (43·7 ± 9·1%) than in Tfm/Y mice (22·4 ± 2·8%; $P = 0.05$).

Table 1. Thymocyte subsets in Ta/Y and Tfm/Y mice

Subset	Ta/Y	Tfm/Y	*P**
$CD4^-CD8^-$	8·58 ± 1·1	4·61 ± 0·8	≤0·001
$CD4^+CD8^+$	68·0 ± 2·9	76·5 ± 1·9	0·018
$CD8^+$	6·6 ± 1·0	5·4 ± 0·9	0·142
$CD4^+$	16·6 ± 2·1	13·5 ± 0·7	0·154

Values represent mean % ± SEM in nine pairs of mice.

* *P* values determined by paired *t*-test.

Table 2. CD3 expression on thymocytes from Tfm/Y and Ta/Y mice

Experiment	Ta/Y	Tfm/Y
1	69·9*	28·2
2	41·4	26·2
3	34·7	18·9
4	29·0	16·5
Mean values ± SEM	43·7** ± 9·1	22·4** ± 2·8

* Values represent percentage positive cells.

** $P = 0.05$ comparing Ta/Y and Tfm/Y.

Functional analysis of thymocytes from Tfm/Y and Ta/Y mice

Our previous experiments showed significantly greater production of CTLL-2-stimulating activity *in vitro* by Tfm/Y thymocytes compared to a control group comprising both Ta/Y and C57Bl/6 mice. Analysis of larger numbers of animals revealed that both Tfm/Y and Ta/Y mice produce CTLL-2-stimulating factors under Con A stimulation *in vitro*. In the present series of experiments, the mean CTLL-2-stimulating level for Ta/Y mice was 27 ± 8 U/ml and for Tfm/Y mice was 45 ± 15 U/ml ($P > 0.3$). Thymocytes from C57Bl/6 mice produced a mean of only 4 ± 2 U/ml of CTLL-2-stimulating activity, which was significantly less than both Ta/Y and Tfm/Y mice ($P = 0.02$ and $P = 0.04$, respectively).

The nature of the cytokine produced by Ta/Y and Tfm/Y thymocytes was examined in immuno-neutralization experiments. Addition of the monoclonal anti-IL-4 antibody 11B.11 to test supernatants from Con A-stimulated Tfm/Y or Ta/Y thymocyte supernatants abolished the cytokine bioactivity (Fig. 1). The mean values for Ta/Y and Tfm/Y supernatants without anti-IL-4 (27 and 45 U/ml, respectively) were decreased to less than 0·9 U/ml for each with addition of anti-IL-4 ($P < 0.012$), indicating that virtually all of the CTLL-2-stimulating activity produced was IL-4. Levels of CTLL-2-stimulatory activity produced by C57 thymocytes in response to Con A were too low to assess effects of immuno-neutralization with 11B.11. In contrast, thymocyte supernatants from all three types of mice which were produced with phorbol ester and calcium ionophore contained a cytokine that stimulated CTLL-2 cells that was not bio-inactivated by the anti-IL-4 antibody (data not shown).

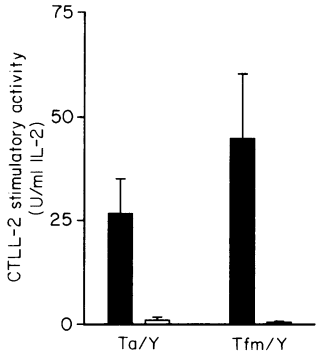

Figure 1. *In vitro* production of CTTL-2 stimulatory activity by Con A-stimulated Ta/Y and Tfm/Y murine thymocytes. Thymocytes were cultured for 48 hr in the presence of 10 μg/ml Con A and the supernatants assayed for CTLL-2 stimulatory activity with (□) or without (■) the monoclonal anti-IL-4 antibody 11B.11.

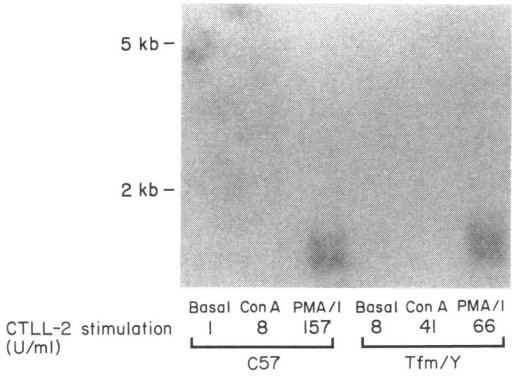

Figure 2. Northern blot of C57 and Tfm/Y thymocyte RNA probed for IL-2 mRNA. Total RNA (10 micrograms) was isolated from C57 or Tfm/Y thymocytes cultured under basal conditions or with added Con A or PMA and calcium ionophore. The RNA was electrophoresed on an agarose/formaldehyde gel and probed with a 760 bp fragment of IL-2 cDNA, as described. Supernatant medium from each cell culture was assayed for IL-2 bioactivity.

The immuno-neutralization data are supported by the observations of steady-state levels of IL-2 mRNA in cultured thymocytes stimulated with Con A. After PMA/ionophore stimulation, IL-2 transcripts are detectable in Tfm/Y and C57 thymocytes but IL-2 mRNA is not detectable in Con A-stimulated Tfm/Y cells that produce similar amounts of CTLL-2-stimulating bioactivity (Fig. 2).

Quantitative and phenotypic analysis of splenocytes from Tfm/Y and Ta/Y mice

Spleen weights were significantly greater in Tfm/Y mice than in Ta/Y mice (93·5±8·3 g versus 59·6±5·1 g; $P=0·001$). Corresponding cell yields were also greater in Tfm/Y than in Ta/Y mice (83·3±12·1 × 10^6 cells versus 48·3±6·1 × 10^6 cells; $P=0·015$). However, Tfm/Y spleen weights and cell yields were not significantly different from those observed in C57Bl/6 male animals (data not shown). Immunofluorescent staining and flow cytometry in six pairs of Tfm/Y and Ta/Y mice revealed a statistically significant decrease in the relative proportion of mature T cells in Tfm/Y mice, with an increase in the proportion of unlabelled cells (Table 3). The mean CD4:CD8 ratios for Tab/Y and Tfm/Y mice were 3·0 and 3·4, respectively. In contrast, the mean CD4:CD8 ratio for spleen cells from three C57 mice was 1·7.

Functional analysis of splenocytes from Tfm/Y and Ta/Y mice

Proliferation of splenocytes in culture in response to Con A and LPS was assessed by [^{3}H]thymidine uptake. No significant difference was seen between Tfm/Y and Ta/Y splenocytes, despite the observed difference in spleen size and cell yields *in vivo*. At 72 hr of culture, the proliferative response of Tfm/Y spleen cells to LPS (93,551±9080 c.p.m.) was significantly greater than the corresponding C57 response (56,500±8560 c.p.m. $P=0·015$). However, Con A responses were not significantly different. In addition, unstimulated responses and responses to mitogens at other times from 24 to 96 hr did not show significant differences (data not shown).

Table 3. Splenocyte subsets in Ta/Y and Tfm/Y mice

Subset	Ta/Y	Tfm/Y	*P**
$CD4^-CD8^-$	55·9±3·9	67·7±2·3	0·026
$CD4^+CD8^+$	0·9±0·2	0·6±0·1	0·015
$CD8^+$	11·1±1·3	7·2±0·5	0·029
$CD4^+$	32·1±2·6	24·4±1·7	0·027

Values represent mean percent±SEM in six pairs of mice.

* *P* values determined by paired *t*-test.

Cytokine production by cultured splenocytes was assessed by the CTLL-2 assay with and without added anti-IL-4. In response to Con A, C57 Bl/6 spleen cells produced a mean of 15±4 units/ml of CTLL-2 stimulating activity. With addition of anti-IL-4, the mean stimulating activity was 13±9 U/ml of IL-2 standard ($P>0·3$), indicating that IL-2 was the predominant cytokine produced. Spleen cells from Ta/Y and Tfm/Y mice produced mean CTLL-2 stimulatory levels of 62±14 U/ml and 53±22 U/ml, respectively. In the presence of anti-IL-4, activity decreased to mean values of 6 U/ml or less for both Ta/Y and Tfm/Y ($P=0·015$), indicating that the major cytokine produced by these spleen cells was IL-4 rather than IL-2.

Production of antibodies by cultured splenocytes was determined by immunoglobulin class-specific ELISAs (Table 4). Both Ta/Y and Tfm/Y splenocytes produced significantly greater quantities of all classes of antibodies than C57Bl/6 cells under basal as well as LPS-stimulated conditions. Although the increased levels of antibody synthesis may be related to the presence of increased relative numbers of CD4 cells and in turn to IL-4 production in these spleens, a possible role for decreased

Table 4. Antibody production by spleen cells from Tfm/Y, Ta/Y and C57 Bl/6 mice

	C57	Ta/Y	Tfm/Y	P_1**	P_2**	P_3**
IgA						
Control	312±182*	330±233	1758±693	>0·3	0·083	>0·3
LPS	402±284	1400±76	7465±1889	0·044	0·004	0·126
IgG						
Control	71±35	731±307	2345±371	0·003	<0·001	0·051
LPS	50±16	10,311±4477	8753±906	0·001	0·001	>0·3
IgM						
Control	33±12	708±290	10,817±3401	0·001	0·011	0·158
LPS	2136±561	66,091±35,213	151,683±33,999	0·006	0·001	0·238

*Values shown are mean ng/ml±SEM.
** P_1 compares C57 versus Ta/Y.
P_2 compares C57 versus Tfm/Y.
P_3 compares Ta/Y versus Tfm/Y.
Numbers of mice in each group: nine C57, three Ta/Y and 11 Tfm/Y.

suppressor function should also be considered. Preliminary data suggest that Con A-preactived spleen cells from Ta/Y and Tfm/Y mice are less efficient than C57 spleen cells in suppressing LPS-induced proliferation of normal C57 spleen cells (data not shown).

DISCUSSION

The androgen-resistant mouse (Tfm/Y, testicular feminization) was studied to determine whether specific alterations in immunological function due to defective androgen action might be discerned. Tfm is an X-linked mutation that has now been shown to be due to a nonsense mutation in the murine androgen receptor gene.[12] This mutation is established on the background of the C57Bl/6 mouse. The Tabby gene (Ta) is a marker for the normal X chromosome of the mother; Tfm is carried on the other X. We have compared Tfm/Y mouse immunological function to that of Ta/Y littermates as well as to age-matched C57Bl/6 males. The data indicate that many of the changes in immune function between C57Bl/6 and Tfm are not attributable to mutation at the Tfm locus, and are thus not due to lack of effect of androgenic hormones during development.

Among the alterations in the immune system observable in both Tfm/Y and Ta/Y mice is the unusual ease with which thymocytes and spleen cells can be induced to produce IL-4. These findings are of interest in view of the fact that IL-4-producing cells are difficult to demonstrate in most mouse strains as well as in human cells. Stimulation of murine splenocytes and thymocytes with nominal antigen, alloantigens or mitogens failed to elicit significant IL-4 production in several strains of mice reported in one study.[13] Using *in situ* hybridization, the frequency of IL-4-producing spleen cells has been estimated at 1/300 or less, with the C57 BL/6 strain showing even lower frequencies.[14] Double-negative thymocytes from both adult[15,16] and foetal[16] mice are capable of producing IL-4, but this population generally constitutes less than 10% of thymocytes and thus overall production of IL-4 by the thymus would be predicted by these results to be low. Furthermore, in humans, production of IL-4 is detected in less than 2% of unfractionated T cells and less than 5% of activated CD4 cells, while IL-2 is produced by 60% of activated CD4 cells.[17] Thus, it appears that Ta/Y and Tfm/Y mice are unusual in expressing high levels of IL-4. These mice may be useful for studies of the genetic, molecular and cellular mechanisms controlling IL-4 production.

The present data establish that high IL-4 production cannot, however, be a direct or indirect result of the mutation in the androgen receptor since thymocytes from littermate males with a normal androgen receptor gene also exhibit the phenomenon. Such Con A-stimulated IL-4 production has been observed in all litters of Tfm/Y and Ta/Y mice tested, and it is therefore unlikely to be due to some acquired or environmental condition, but probably represents variation in some gene(s) other than the androgen receptor. The nature of these other genetic differences underlying alterations in immune function is not known.

A similar analysis applies to the proliferative capacity and antibody secretory capacity of Tfm/Y and Ta/Y mice compared to C57Bl/6 males. The functional attributes of Tfm/Y splenocytes are not significantly different from those of splenocytes from Ta/Y littermates, although both are increased over C57 males. Nevertheless, the significant increases in spleen size and weight observed in Tfm/Y mice indicate that total amounts of antibodies and lymphokines produced are most likely increased over levels produced by Ta/Y mice *in vivo*. The potential impact of these increased numbers of immune effector molecules in the Tfm/Y could be significant.

The increase in thymic size and thymocyte numbers and the decrease in the proportion of $CD4^-CD8^-$ thymocytes in the Tfm/Y mouse are statistically significant differences whether Tfm animals are compared to the C57 male mouse or to their Ta/Y male littermates. A corresponding compensatory increase in double-positive thymocytes was observed in the Tfm/Y mice. Furthermore, in all four pairs studied, relative numbers of CD3-positive thymocytes were lower in Tfm/Y than in Ta/Y mice. Taken together, these findings may indicate that cell traffic patterns and maturational sequences in the thymus are altered in the absence of androgen effects. Also consistent with this interpretation is the significant decrease in mature T-cell phenotypes in the Tfm/Y spleen compared to the Ta/Y. The experiments described here, however, cannot definitively estab-

lish whether these differences in Tfm/Y and Ta/Y mice are specifically due to the heritable defect in androgen action or are due to other genetic differences.

Our initial observation of increased production of CTLL-2-stimulatory activity by Tfm/Y thymocytes suggested that the thymic expansion seen in this mutation might be a direct or indirect consequence of lack of androgen action on the regulation of the relevant cytokines IL-2 or IL-4. The data reported here show that such a mutation is not necessary for the observed IL-4 production, as isolated Ta/Y cells produce nearly equal amounts of IL-4 in response to Con A *in vitro* as Tfm/Y cells, but the Ta/Y thymus gland is significantly smaller than the Tfm/Y thymus *in vivo*. These findings indicate that IL-4 is not responsible for the observed differences in thymic size. We have also been unable to implicate either IL-2 or IL-4 in the thymic expansion that occurs after castration of normal male mice (N. J. Olsen and W. J. Kovacs, unpublished observation). The factors responsible for thymic enlargement under conditions of androgen deficiency or resistance to the actions of these hormones thus remain undefined. The potential role of other cytokines, growth factors and proto-oncogenes is currently under investigation.

ACKNOWLEDGMENTS

The technical assistance of Maxine K. Turney is much appreciated. Dr Jeffrey Bluestone of the University of Chicago Ben May Laboratories kindly contributed T cell antibodies which were used in this work. We are thankful to Dr T. Mosmann, DNAX, who released the IL-2 probe for our use. This work was supported by USPHS grant DK-41053 (to N. J. Olsen). W. J. Kovacs is the recipient of a Research Career Development Award (Research Associate) from the United States Department of Veteran's Affairs.

REFERENCES

1. Kovacs W.J. & Olsen N.J. (1987) Androgen receptors in human thymocytes. *J. Immunol.* **139,** 490.
2. Olsen N.J. & Kovacs W.J. (1989) Increased thymic size and thymocyte interleukin 2 production in androgen-resistant mice. *Scand. J. Immunol.* **29,** 733.
3. Gilles S. & Smith K.A. (1977) Long term culture of tumor-specific cytotoxic T cells. *Nature*, **268,** 154.
4. Mosmann T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. immunol. Meth.* **65,** 55.
5. Grabstein K., Eisenman J., Machizcki D., Shanebeck K., Conlon P., Hopp T., March C. & Gillis S. (1986) Purification to homogeneity of B cell stimulatory factor. A molecule that stimulates proliferation of multiple lymphokine-dependent cell lines. *J. exp. Med.* **163,** 1405.
6. Ohara J. & Paul W.J. (1985) Production of a monoclonal antibody to and molecular characterization of B-cell stimulatory factor-1. *Nature*, **315,** 333.
7. Bluestone J.A., Pardoll D., Sharrow S.O. & Fowlkes B.J. (1987). Characterization of murine thymocytes with CD3-associated T-cell receptor structures. *Nature*, **326,** 82.
8. Pearse M., Gallagher P., Wilson A., Wu L., Fisciaro N., Miller J.F., Scollar R. & Shortman K. (1988) Molecular characterization of T-cell antigen receptor expression by subsets of $CD4^-CD8^-$ murine thymocytes. *Proc. natl. Acad. Sci. U.S.A.* **85,** 6082.
9. Yokota T., Arai N., Lee F., Rennick D., Mosmann T. & Arai K.-I. (1985) Use of a cDNA expression vector for isolation of mouse interleukin 2 cDNA clones: expression of T-cell growth-factor activity after transfection of monkey cells. *Proc. natl. Acad. Sci. U.S.A.* **82,** 68.
10. Feinberg A.P. & Vogelstein B. (1983) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132,** 6.
11. Feinberg A.P. & Vogelstein B. (1984) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Addendum. *Anal. Biochem.* **137,** 266.
12. He W.W., Young C.Y.F. & Tindall D.J. (1990) The molecular basis of the mouse testicular feminization (Tfm) mutation: a frameshift mutation. In: *Program of the Endocrine Society*, 72nd Annual Meeting, Atlanta, Georgia, June 1990; Abstract 862, p. 240.
13. Powers G.D., Abbas A.K. & Miller R.A. (1988) Frequencies of IL-2- and IL-4-secreting T cells in naive and antigen-stimulated lymphocyte populations. *J. Immunol.* **140,** 3352.
14. Sideras P., Funa K., Zalcberg-Quintana I., Xantgioiykas K.G., Kisielow P. & Palacios R. (1988) Analysis by *in situ* hybridization of cells expressing mRNA for interleukin 4 in the developing thymus and in peripheral lymphocytes from mice. *Proc. natl. Acad. Sci. U.S.A.* **85,** 218.
15. Zlotnik A., Ransom J., Frank G., Fisher M. & Howard M. (1987) Interleukin 4 is a growth factor for activated thymocytes: Possible role in T cell ontogeny. *Proc. natl. Acad. Sci. U.S.A.* **84,** 3856.
16. Ransom J., Fisher M., Mosmann T., Yokota T., Deluca D., Schumacher J. & Zlotnik A. (1987) Interferon-gamma is produced by activated immature mouse thymocytes and inhibits the interleukin-4-induced proliferation of immature thymocytes. *J. Immunol.* **139,** 4102.
17. Lewis D.B., Prickett K.S., Larsen A., Grabstein K., Weaver M. & Wilson C.B. (1988) Restricted production of interleukin 4 by activated human T cells. *Proc. natl. Acad. Sci. U.S.A.* **85,** 9743.