T CELL DEVELOPMENT IN NORMAL AND THYMOPENTIN-TREATED NUDE MICE*

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There has been much uncertainty regarding the presence, variety, and maturity of T cells in athymic mice. Placental passage of maternal T cells cannot account for T cells in *nu/nu* progeny of *nu/nu* parents (1, and unpublished observations), and there is good evidence against the hypothesis of a functional thymic rudiment (2).

Prothymocytes, which are present equally in athymic and normal mice, are already committed to express the thymocyte surface phenotype and can be induced to do so by many nonspecific, as well as thymus-related, agents. Thus, nonspecific induction, attributable to incidental conditions, such as endemic viral hepatitis (3), which vary from one mouse colony to another, can explain some of the inconsistencies among published reports, at least as these concern surface phenotype.

As regards function, *nu/nu* splenocytes, cultured under special conditions including provision of interleukin 2 (IL-2)[1] (4), respond to T cell mitogens and allogeneic stimulator cells (mixed leukocyte culture) and can yield cloned lines of allogeneic killer effector cells (5). It has even been suggested that lack of IL-2 could be the only primary deficiency resulting from lack of a thymus (6).

In the present study, we sought to define the T cell population of *nu/nu* mice, specific pathogen free (SPF) or germ free, in more detail, according to the criteria of diversity of Ly sets and expression of the markers TL and Qa-1, and to see how the proportions of T cells expressing these phenotypes may be affected by treatment with thymopentin (TP-5), a synthetic pentapeptide fragment of thymopoietin.

Materials and Methods

Mice. SPF BALB-*nu/nu* and *nu/+* mice were obtained at 4–5 wk of age from Gibco Animal Resources Laboratories (Madison, WI). These mice were derived from the fifth backcross generation to the BALB-Bom strain. We found no differences in expression of T cell markers in these mice and mice of the 20th backcross generation from the same source, which were not available in quantity. Mice of both genotypes, *nu/nu* and *nu/+*, were maintained under pathogen-free conditions in germ-free isolators and were tested periodically for the constancy of their bacterial flora. We also studied pathogen-free HSFS/N-*nu* mice (Tla^a:TL^+:$Qa1^+$), germ-free HSFS/N-*nu* derived by caesarian section at the Sloan-Kettering Institute for Cancer Research, and pathogen-free B6-*nu/nu* (originally from Dr. Carl T. Hansen, NIH), all main-

* Supported by grants AI-00270, CA-22241, CA-25738, and CA-09149 from the National Institutes of Health.

[1] *Abbreviations used in this paper:* IL-2, interleukin 2: SPF, specific pathogen-free; PBS, Dulbecco's phosphate-buffered saline; sIg^+, surface immunoglobulin-positive; FCS, fetal calf serum; FITC, fluoresceine isothiocyanate; PA-SRBC, *Staphylococcus aureus* protein A-coated sheep erythrocytes; TP-5, thymopentin.

tained at the Sloan-Kettering Institute.

TP-5 Treatment. The TP-5 synthetic pentapeptide fragment of thymopoietin (7) was diluted to 2 μg/ml in phosphate-buffered saline (PBS) and filter sterilized. Mice aged 5–7 wk received intraperitoneally 0.5 ml of TP-5 (1 μg) five times/wk for 3 wk. Control mice were given either saline or a randomly synthetic control pentapeptide (8). Treated mice and controls were tested 1d after cessation of treatment, when the mice were 8–10 wk of age.

Antisera. Monoclonal alloantibodies were used throughout, except for the Qa-1 antiserum, (B6 × A.TL$^-$)F_1 anti-A strain leukemia ASL1, absorbed as required according to Stanton and Boyse (9). In tests for specificity, performed on concanavalin A-induced splenic blast cells to boost sensitivity, Qa-1 antiserum was negative for B6 and positive for B6-*Tla*^{a} congenic, BALB, and BALB-*nu*. The positive reaction with BALB is controversial and is being investigated further, but for present purposes it is sufficient to note that we have seen no difference between BALB/*nu* and HSFS/N-*nu* in reactivity with Qa-1 antiserum.

Monoclonal antibody to Lyt-1.2 (clone 3-3.1) and TL (clone 79-10.4) were provided by Dr. F.-W. Shen; to Thy-1.2 (clone 937), Lyt-2.2 (clone 19/178) by Dr. U. Hämmerling; and to Lyt-3.2 by Dr. M Palladino (all of the Memorial Sloan-Kettering Cancer Center). Affinity-purified rabbit anti-mouse $F(ab)_2$ was provided by Dr. U. Hämmerling.

Staphylococcus aureus Protein A-coated Sheep Erythrocyte (PA-SRBC) Assay. This assay was performed according to Scheid and Triglia (10). Especially with antisera that require absorption of auto- or other unwanted antibody, there is an appreciable background of nonspecific rosetting. This was abolished by routine centrifugation (30 min at 130,000 *g*) of all antisera and monoclonal antibodies before use. Before being tested, all cell suspensions were incubated at 37°C for 60 min and then washed two or three times to remove cytophilic antibody.

Enrichment and Selection of T Cells. Depletion of surface immunoglobulin-positive (sIg$^+$) cells was carried out on plastic dishes coated with affinity-purified rabbit anti-mouse $F(ab)_2$ (11). 1–2 × 10^7 spleen cells in 3 ml of PBS with 5% fetal calf serum (FCS) were incubated at 4°C on antibody-coated 100 × 20-mm petri dishes (1005; Falcon Labware, Oxnard, CA). The nonadherent (T cell-enriched) fraction was harvested after 70–90 min by multiple cycles of gentle rinsing of the monolayers with PBS-5% FCS. 10–20% of the *nu/nu* spleen cells and 30–40% of the *nu/+* spleen cells were recovered in the nonadherent fraction with <2–5% sIg$^+$ cells remaining, as judged by fluorescein isothiocyanate (FITC) or PA-SRBC assay. The nonadherent portion of *nu/nu* spleen cells was 50–60% Thy-1$^+$ as compared with 80–90% Thy-1$^+$ from *nu/+* .

No detectable loss of T cells during the cell fractionation procedure was observed throughout these experiments, and <1% Thy-1$^+$ cells were found in the adherent population recovered by pipetting, as measured by direct immunofluorescence. In some experiments, further enrichment of the *nu/nu* T cell populations (up to 80–95%) was achieved by depletion of Ia$^+$ and Lyb-2.2 cells (some of which are sIg$^-$) by sensitizing the unseparated spleen population with optimal concentrations of combined Ia and Lyb-2.2 antibodies, washing twice, and exposing to anti-Ig-coated dishes as above.

Enumeration of Ly Sets. Ly sets of T cell-enriched spleen cells were estimated from PA-SRBC counts with anti-Lyt-1 antibody alone, anti-Lyt-2 antibody alone, and the two antibodies combined (equalling total Lyt$^+$ cells) (for controls and calculations see ref. 12). The data for Ly sets are expressed as percent of total Lyt$^+$ cells. Because Lyt-2 and Lyt-3 have not yet been found to be expressed independently, and anti-Lyt-2.2 and anti-Lyt-3.2 antibodies gave similar results, we generally used only the former.

Results

Proportions of TL:Qa-1:Lyt T Cell Sets in nu/nu and nu/+ Mice as a Function of Age. Despite reports that BALB/c mice are Qa-1^b (13), we found that the BALB-*nu/+* and BALB-*nu/nu* mice we used, like mice of our BALB/c colony, are Qa-1^a; this was established by the PA-SRBC assay with concanavalin A-induced blast cells as targets, and was confirmed by specific absorption.

Neither we (3) nor others (14) have previously succeeded in demonstrating significant numbers of Thy-1$^+$:Lyt$^+$ splenocytes in germ-free or SPF young *nu/nu* mice (<2

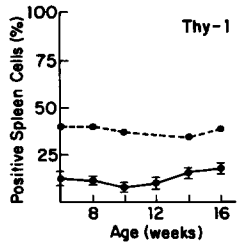

FIG. 1. Expression of Thy-1 on spleen cells from BALB/*nu/nu* (——) and *nu/+* (- - -) mice. Age- and sex-matched *nu/nu* and *nu/+* mice were assayed for Thy-1 expression. Each time point represents the average of two to eight pairs of mice.

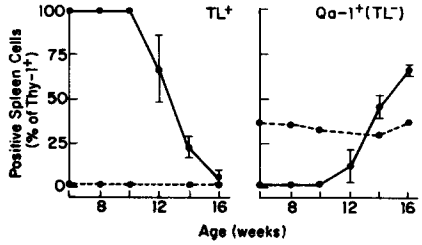

FIG. 2. Expression of TL and Qa-1 on spleen cells from BALB/*nu/nu* (——) and *nu/+* (- - -) mice (see Fig. 1). Data expressed as percent of Thy-1$^+$ cells.

mo of age). However, the use of a highly sensitive PA-SRBC rosette assay together with a preparatory T cell-enrichment procedure, not encountering significant T cell losses, allows the reliable detection of such cells in 6-wk-old mice and their characterization with respect to TL, Qa-1, and Ly phenotypes. As seen in Fig. 1, for mice of 6–16 wk of age the number of T cells in *nu/nu* spleen was only 20–50% of the number in *nu/+* spleen. Like all normal mice, *nu/+* mice have no TL$^+$ cells in spleen, (Fig. 2, left panel), whereas the Thy-1$^+$ T cells of spleen of *nu/nu* mice are TL$^+$, in proportions of ~100% TL$^+$ cells during the first 10 wk after birth, falling to 8–10% at 16 wk of age. The availability of monoclonal TL antibody makes it possible to count TL$^+$ cells and Qa-1$^+$:TL$^-$ cells separately. Previously it was necessary to use the same conventional antiserum [(B6 × A-Tla^b)anti-ASL1] for typing of both TL on thymocytes and Qa-1 on peripheral T cells. The proportion of Qa-1$^+$:TL$^-$ cells in *nu/nu* and *nu/+* spleens was deduced by subtracting the count for monoclonal TL antibody from the count for conventional TL antiserum. Clearly, this still does not allow distinction of cells of possible TL$^+$:Qa-1$^+$ and TL$^+$:Qa-1$^-$ phenotypes.

As the right-hand panel of Fig. 2 shows, the proportion of Thy-1$^+$ spleen cells that were Qa-1$^+$:TL$^-$ in *nu/+* controls remained constant (~30%) throughout the age range studied (4–16 wk), as compared with 0% in *nu/nu* mice until 10 wk of age, rising thereafter to ~70% at 16 wk.

The uniformly TL$^+$ splenic T cells of *nu/nu* aged <10 wk (Fig. 2, left panel) evidently all belong to the Ly-123 set (Fig. 3, lower panel). At 10–16 wk of age, during which the proportion of TL$^+$ cells fell to <10% of total splenic Thy-1$^+$ (Fig. 2, left panel), there was corresponding diversification into Ly1 and Ly23 sets. As Fig. 3 shows, the proportional representation of Ly-123, Ly-1, and Ly-23 sets in BALB *nu/nu* spleen at 14–16 wk of age was roughly similar to the proportions already reached by *nu/+* and other normal mice at 4 wk of age (Fig. 3, and other data not shown).

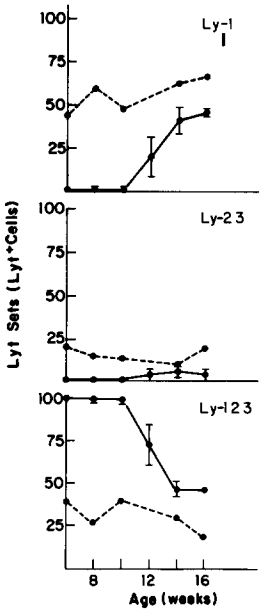

FIG. 3. Age-related Lyt set alterations in spleens from BALB/*nu/nu* (——) and *nu/+* (- - -) mice (see Fig. 1).

In summary, there are three salient differences between *nu/nu* and *nu/+* mice as regards the composition of their T cell populations during the later age period studied, 16 wk onward, and these are (*a*) a lower total number of T cells, (*b*) persistence of 5–10% TL^+ splenic T cells, and (*c*) a greatly raised proportion of Qa-1^+ T cells. All the data described above for BALB-*nu* mice have been duplicated, with no significant differences, for HSFS/N-*nu* and B6-*nu* mice, except that the latter are of Tla^b genotype and therefore cannot be scored for TL and Qa-1. A recent report from MacDonald et al. (14) also describes the presence of Thy-1^+:Lyt-1^+:2^- cells in the spleens of older but not younger *nu/nu* mice (>6 mo of age) detected by fluorescence-activated cell sorter (FACS) analysis of nylon wool-purified T cells. In contrast to our findings reported here, these investigators did not identify this or other T cell populations in younger *nu/nu* mice. This is most likely caused by the significant preparatory T cell loss (50%) encountered (15).

Effect of Treatment with TP-5 on T Cell Set Composition of nu/nu Mice. TP-5 is a synthetic pentapeptide fragment of thymopoietin. To test its effect on the T cell set composition of *nu/nu* mice, TP-5 was administered repeatedly to pathogen-free BALB *nu/nu* and *nu/+* mice, beginning at 5–7 wk of age and ending at 7–10 wk of age. The data shown are for the BALB-*nu* strain, and were duplicated with no significant differences for HSFS/N-*nu* mice. The lower part of Table I shows that treatment with TP-5 had no significant effect on the T cell set composition of *nu/+* mice. The upper part of Table I shows the effects of TP-5 in *nu/nu* mice. First, the total number of T cells, classified as Thy-1^+ and Lyt^+, rose from <9% of splenocytes in untreated mice to >20% in TP-5-treated mice, but did not reach the >35% of *nu/+* mice. Second, the proportion of TL^+ splenic T cells fell from 97% to 26%, but not to the 0% recorded for TP-5-treated *nu/+* mice. Third, the proportion of Qa-1^+ T cells rose from ~3% to

TABLE I

*Effect of TP-5 on the Splenic T Cell Population of nu/nu and nu/+ Mice**

Genotype of cell donor	Treatment	Phenotypes				
		Unselected splenocytes		B cell-depleted splenocytes	Thy-1$^+$ splenocytes	
		Thy-1$^+$	Lyt$^+$‡	Thy-1$^+$	TL$^+$	TL$^-$:Qa-1$^+$
				% ± *SEM*		
nu/nu	TP-5	21.7 ± 1.4	21.0 ± 1.1	57.8 ± 5.1	26.2 ± 3.9	33.8 ± 10.6
	Control§	8.8 ± 1.8	8.6 ± 0.8	51.0 ± 4.3	97.0 ± 1.5	3.1 ± 0.7
nu/+	TP-5	38.3 ± 1.4	38.6 ± 2.0	85.8 ± 2.7	0	36.6 ± 3.7
	Control§	36.5 ± 1.3	37.5 ± 1.5	84.1 ± 1.3	0	33.3 ± 2.6

* In the study shown, TP-5 treatment was begun at 5 wk of age and continued until testing (as shown) at 8 wk of age. Eight mice in each group matched for age and sex were tested as single pairs to reduce technical variation. (The Lyt set analysis for these mice is given in Fig. 4.)

‡ Positive reactivity with α Lyt-1 and α Lyt-2 sera combined.

§ Controls received either the TP-5 solvent medium alone or a random-sequence pentapeptide in place of TP-5; these control data are combined because there was no significant difference.

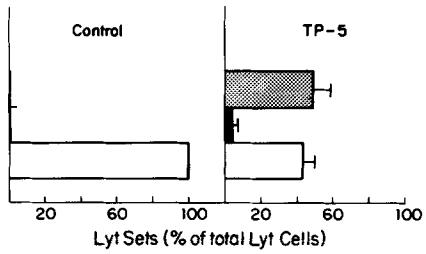

FIG. 4. Diversification of Lyt subsets (Ly-123 (□); Ly-23 (■); Ly-1 (▨) induced by TP-5 in BALB/*nu/nu* mice (see legend to Table I).

~34%, which is within the normal range for *nu/+* and other mice. As regards diversification, Fig. 4 shows extensive differentiation into Lyt sets (roughly 45% Ly-123, 5% Ly-23, 50% Ly-1) in response to TP-5.

Discussion

The main features of splenic T cell development in SPF and germ-free nude mice, according to the criteria of surface phenotypes, reported here, can be summarized as follows: The total number of T cells is diminished to ~25% rising to 50% of normal. Until 10 wk of age, all splenic T cells are TL$^+$, and there is little or no diversification into Ly-1 and Ly-23 sets—all are Ly-123. After 10 wk of age, the proportion of TL$^+$ T cells declines, diversification into Ly-1 and Ly-23 cell sets begins, and there is an increasingly excessive ratio of Qa-1$^+$ to Qa-1$^-$ cells.

Only since monoclonal TL antibodies became available has it been possible to distinguish with certainty between TL$^+$ and Qa-1$^+$ cells. The finding of TL$^+$ cells in *nu/nu* (but not in *nu/+*) spleen is a striking abnormality that we are studying further. The only previously reported occurrence of extrathymic TL$^+$ cells, excepting leukemia, concerned heavily irradiated mice (16).

Treatment with TP-5 during the 5th–8th wk of age rectifies, at least partially, some of the abnormalities noted. Thus the number of splenic T cells rises, the number of TL^+ cells falls, and diversification into Ly-1 and Ly-23 sets is initiated; these changes towards the normal also occur in older mice not treated with TP-5. It remains to be seen whether other regimens of TP-5 therapy are more effective in this regard, and whether such treatment may counter the excessive Qa-1^+:Qa-1^- cell ratio, which is not grossly evident under 16 wk of age. The possibility that the Qa-1 system is compound and the lack of monoclonal Qa-1 antibodies are obstacles to the precise interpretation of Qa-1^+ counts and ratios. This is unfortunate, particularly because Qa-1 expression is associated with generation of help and suppression (17) and perhaps because Qa-1 expression is enhanced or initiated on mitogen-stimulated cells, which resemble *nu/nu* cells in terms of their blastoid appearance and light scatter (18).

At present, little can be said about the mode of action of TP-5 in nude mice. In vitro, TP-5 induces prothymocytes to express the TL^+:Ly-123 phenotype typical of early thymocytes, and prothymocytes are present in normal numbers in nude mice (7). But it seems unlikely that induction of prothymocytes can be the only effect of TP-5 because nude mice already have appreciable numbers of TL^+:Ly-123 cells. It may be that TP-5, over a period longer than the standard 2-h induction assay, drives the differentiative sequence further to yield the TL^- sets Ly-123, Ly-1, and Ly-23. This would be in accord with the fact that TP-5 reduces the absolute and relative number of TL^+ cells while increasing the total number of T cells, but such a direct effect has yet to be demonstrated. Alternatively, there is a precedent for more complex action in the observation that CR^+ splenic cell numbers are increased in *nu/nu* mice by treatment with TP-5, although TP-5 inhibits the differentiative induction of pro-CR^+ cells in vitro (7).

It is too early to report on tests of immune function of cells from TP-5-treated *nu/nu* mice. With respect to weak homograft reactivity (H-Y), TP-5 therapy alters the responses of aged mice and mice thymectomized in young adult life, tending to restore the rejection pattern (19) and T cell population structure typical of normal young adults. At the moment, we cannot point to a cell of any known surface phenotype that is lacking in athymic *nu/nu* mice, which favors the view that the thymus functions in maintaining the numbers and homeostatic balance of T cell sets, rather than directing differentiative steps that cannot otherwise occur.

Summary

The extent and diversity of T cell differentiation in nude athymic mice are matters of dispute. In this study, we examined the splenic T cell population of pathogen-free and germ-free *nu/nu* mice, treated or not treated with the pentapeptide analogue of thymopoietin (TP-5), in terms of TL, Qa-1, and Lyt phenotypes.

At all ages, 50–60% of *nu/nu* splenocytes, enriched for T lymphocytes by removal of sIg^+ cells, expressed T markers, as compared with >85% in normal mice. At 2 mo of age, all *nu/nu* splenic T cells expressed the surface phenotype TL^+:Thy-1^+:Ly-123. This is abnormal in two respects: first, because expression of TL is normally confined to thymocytes; and second, because there was no evidence of the usual diversification into the subsets Ly-1 and Ly-23.

From 10 wk of age onwards, diversification into Ly subsets was evident in *nu/nu* spleen, although the usual predominance of Ly-1 over Ly-123 cells was not attained,

and some TL^+ cells persisted. Also, the ratio of Qa-1^+ to Qa-1^- cells rose progressively to as high as 4:1 at 4–6 mo, in contrast to the usual ratio of ~1:1, regardless of age. In the spleens of *nu/nu* mice treated with TP-5 from 5–8 weeks of age and tested 1 wk later, the proportion of T cells was raised, though not to normal levels, the number of TL^+ cells was reduced, and there was diversification into Ly sets.

Received for publication 21 June 1982.

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