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# Unresponsiveness to Mls$^a$ induced in newborn Mls$^b$ mice by maternal preimmunization

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## SUMMARY

Female BALB/c (H-2$^d$, Mls$^b$) mice alloimmunized prior to and during syngeneic pregnancy with DBA/2 (H-2$^d$, Mls$^a$) splenocytes gave rise to offspring with severely reduced responsiveness in adult life to DBA/2 stimulation in *in vitro* mixed lymphocyte cultures. The offspring of the hyperimmunized mothers were also tolerant to neonatal challenge with large numbers of DBA/2 splenocytes, which resulted in runting disease of control neonatal BALB/c mice. Both challenged and unchallenged offspring of the immunized BALB/c mothers were hyporesponsive to DBA/2 but both stimulated and responded to normal BALB/c lymphocytes, indicating alteration in their T-cell repertoire. There was no reduction in the V$\beta$6-positive thymocyte subpopulation in the challenged or unchallenged offspring of the alloimmunized BALB/c mothers compared to normal controls, suggesting that hyporesponsiveness to DBA/2 is not due to thymic deletion of Mls$^a$-responsive clones. Examination of the T-cell subset composition of the hydrocortisone-resistant thymocytes and peripheral lymphocytes of the challenged and unchallenged groups of experimental mice revealed large increases in the percentage of Lyt-2$^+$ T cells, sometimes accompanied by a decrease in the L3T4$^+$ T-cell subset compared to age-matched control BALB/c. Lymphocytes from the hyporesponsive mice specifically suppressed the proliferative responses of control BALB/c to DBA/2 but not to AKR. The data indicate that maternal hyperimmunization can induce tolerance by a mechanism involving intrathymic selection of suppressor cells which can be combined with a negative selection of helper cells.

## INTRODUCTION

Unresponsiveness or tolerance to self or foreign antigens has in recent years been associated with deletion of T-cell clones bearing identified variable domains of the beta chain of the T-cell receptor (TcR).

Expression of major histocompatibility complex (MHC) class II molecules and Mls determinants are the major known modifiers of the T-cell repertoire.[1–6] T cells are positively selected in the thymus to recognize antigens in the context of self MHC class I and class II determinants.[7] Potentially autoreactive thymocytes expressing anti-self TcR V genes are deleted in the thymus upon engagement of their TcR with the self-antigens. Thymocytes expressing high levels of TcR have been shown to be susceptible to the deletion signal.[4] Not only self-reactive but MHC and Mls alloreactive T cells have also been shown to be eliminated in certain mouse strains.[8,9] However, tolerance to self or alloantigens can also arise from non-deletional mechanisms.[10,11]

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We have demonstrated that tolerance to alloantigens can be induced in neonates by immunization of the mothers prior to and during pregnancy.[12,13] The offspring from immunized mothers were able to resist graft-versus-host disease (GVHD) upon challenge with allogeneic cells 24–48 hr after birth. Their lymphocytes tested in adult life were unresponsive to the challenge and certain third-party lymphocytes in mixed lymphocyte cultures (MLC) and did not develop cytotoxic capacity. This was shown to be due to the combined effects of specific suppression and possibly clonal anergy.[14,15] Higher levels of Lyt-2$^+$ cells were shown to be present in the lymphoid organs of the mice which resisted GVHD than in normal controls; lymphocytes from the GVHD-resistant mice were able to suppress the *in vitro* responses of normal mice to the allogeneic lymphocytes.

In the present report we demonstrate that BALB/c (H-2$^d$, Mls$^b$) mothers preimmunized prior to and during syngeneic pregnancy with DBA/2 (H-2$^d$, Mls$^a$) lymphocytes give rise to offspring which are resistant to runting disease upon neonatal challenge with DBA/2 splenocytes and several weeks after birth their lymphocytes are unresponsive *in vitro* to Mls$^a$-induced stimulation. We also demonstrate that the offspring of the hyperimmunized mothers, whether challenged at birth with DBA/2 lymphocytes or not, have a significant increase in the

percentage of the Lyt-2$^+$ and a decrease in the L3T4$^+$ T-cell subsets when compared to age-matched controls. We traced this alteration of the lymphocyte profile to the cortisone-resistant thymic T-cell population in both the challenged and unchallenged groups, suggesting that the alterations occurred in the foetus under the influence of maternal alloimmunization.

## MATERIALS AND METHODS

*Mice*

BALB/c (H-2$^d$, Mls$^b$), BALB.D2MA (H-2$^d$, Mls$^a$), DBA/2 (H-2$^d$, Mls$^a$) and AKR (H-2$^k$, Mls$^a$) mice were used between 6 and 10 weeks of age.

*Immunization of female BALB/c mice prior to and during pregnancy*

BALB/c females were given two intraperitoneal (i.p.) injections of $4 \times 10^7$ DBA/2 splenocytes at weekly intervals. They were then mated with BALB/c males and the immunization protocol was continued until parturition.

*Treatment of offspring from immunized and normal BALB/c mice*

Neonatal BALB/c mice from the preimmunized mothers and normal controls were injected i.p. within 24–48 hr of birth with $1–2 \times 10^7$ DBA/2 splenocytes. They were observed daily for signs of runting disease. Neonatal mice from preimmunized mothers, some challenged and some not challenged, were maintained until adult life for examination of their immunological responses and lymphocyte profiles. The offspring from the preimmunized mothers which were not challenged will henceforth be called BALB/c-P-DBA/2, and those that were challenged BALB/c-Ch-DBA/2.

*Corticosteroid treatment of mice*

Weight-matched groups of 8–10-week-old normal BALB/c, BALB/c-P-DBA/2 and BALB/c-Ch-DBA/2 were injected i.p. with 250 mg/kg body weight of hydrocortisone acetate 48 hr prior to testing.

*Mixed lymphocyte responses*

Lymph node lymphocytes were used as responders and were cultured at a concentration of $2 \times 10^6$ cells/ml with 2000 rads irradiated splenocytes at the same concentration in RPMI-1640 containing 10% foetal calf serum and antibiotics. Responders and stimulators were mixed in equal proportion in a final volume of 200 $\mu$l in round-bottomed microtitre plates. They were incubated for 24 or 72 hr at 37° in a 5% $CO_2$ atmosphere; 1 $\mu$Ci [$^3$H]thymidine (Amersham, Amersham, Bucks, U.K.) was added to each well and the cultures were maintained for a further 16 hr. They were then harvested on filter paper strips and uptake of radioisotope was measured in a scintillation counter. Stimulation index was calculated as described previously.[16]

*Suppression of mixed lymphocyte responses*

Lymph node responder and 2000 rads irradiated stimulator lymphocytes were suspended at $2 \times 10^6$/ml in RPMI-1640 containing 10% FCS and antibiotics. The suspensions were mixed in equal proportions and 100 $\mu$l volumes of the mixed cultures were aliquoted into 96-well round-bottom microtitre plates. A total volume of 100 $\mu$l of irradiated cells were added to the mixed cultures. These additional cells, at $2 \times 10^6$/ml, comprised either entirely of 2000 rads irradiated responder lymphocytes (a) or mixtures of responder and BALB/c-Ch-DBA/2 or BALB/c-P-DBA/2 lymphocytes in various proportions (b). The cultures were incubated for 72 hr at 37° in a 5% $CO_2$ atmosphere; 1 $\mu$Ci [$^3$H]thymidine was then added to each well and the cultures were incubated for a further 16 hr. They were then harvested on filter paper strips and uptake of radioisotope was measured in a scintillation counter. Percentage inhibition of MLR was calculated as follows:

$$\% \text{ inhibition} = 100 - \frac{\text{SI in presence of additional cells B}}{\text{SI in presence of additional cells A}} \times 100.$$

*Immunofluorescent staining and FACS analysis*

Lymphocyte, total thymocyte and corticosteroid-resistant thymocyte populations were used in various experiments. The cells were suspended in RPMI-1640 medium containing 1% foetal calf serum and 0·1% $NaN_3$. They were incubated for 45 min on ice at a concentration of $10^6$ cells per well in microtitre plates with saturating levels of fluorescein-conjugated monoclonal anti-Lyt-1, anti-Lyt-2, anti-L3T4 and anti-Thy-1.2.

The monoclonal reagent 44-22-1 (anti-V-beta 6) kindly donated by Dr H. Hengartner, University Hospital, Zurich, Switzerland was used as undiluted hybridoma culture supernatant. Cells labelled directly with fluorescein-conjugated antibodies were washed once and analysed by flow cytometry. Thymocytes treated with 44-22-1 were washed three times after the 45 min incubation period and then allowed to react for a further 30 min with fluorescein-conjugated goat anti-rat immunoglobulin. Control samples were treated with the second reagent alone. The cells were then washed once and analysed by flow cytometry.

## RESULTS

### Prevention of Mls$^a$ induced runting disease in BALB/c mice by maternal preimmunization with DBA/2 splenocytes

Female BALB/c mice were hyperimmunized prior to and during syngeneic pregnancy with DBA/2 splenocytes. Litters from the hyperimmunized mothers were challenged 24–48 hr after birth with DBA/2 splenocytes. Control litters from normal BALB/c mothers were challenged in the same manner. The former group of mice thrived and reached a normal mean weight of $21·2 \pm 1$ g 6 weeks after birth, while the latter group developed splenomegaly, runting disease and a mean weight of $14·9 \pm 1·3$ g. Fourteen neonates from normal BALB/c mice were challenged and all developed runting disease, while 22 neonates from preimmunized mothers were challenged and all thrived normally (Table 1).

### *In vitro* unresponsiveness to the tolerogenic lymphocytes of BALB/c mice born from hyperimmunized mothers

Newborn mice from BALB/c mothers hyperimmunized with DBA/2 lymphocytes were either challenged at birth with DBA/2 cells or left unchallenged. The former were operationally termed BALB/c-challenged DBA/2 (BALB/c-Ch-DBA/2), and the latter BALB/c preimmunized DBA/2 (BALB/c-P-DBA/2). At 6 weeks of age, normal BALB/c mice, BALB/c-Ch-DBA/2 and BALB/c-P-DBA/2 were tested in mixed culture for their proliferative responses to each other and to DBA/2 stimulators.

Table 2 demonstrates that both BALB/c-P-DBA/2 and BALB/c-Ch-DBA/2 stimulated normal BALB/c responder lym-

**Table 1.** Protection from runting disease of offspring from BALB/c (H-$2^d$ $Mls^b$) mothers preimmunised with DBA/2 (H-$2^d$, $Mls^a$) spleen cells

| Immunization of BALB/c mothers | Challenge of offspring | Litters tested | No. mice tested | No. mice runting | Mean weight (g ± SD) |
|---|---|---|---|---|---|
| — | DBA/2 | 2 | 14 | 14 | 14·9 ± 1·3 |
| DBA/2 | DBA/2 | 4 | 22 | 0 | 21·2 ± 1 |

The offspring were challenged at birth with high doses of DBA/2 spleen cells.

**Table 2.** Mixed lymphocyte responses of BALB/c mice born of preimmunized mother, unchallenged (BALB/c-P-DBA/2) and challenged (BALB/c-Ch-DBA/2) at birth with DBA/2 spleen cells

| Responding lymphocytes | Irradiated stimulator lymphocytes c.p.m. ± SD (SI)* | | | |
|---|---|---|---|---|
| | BALB/c | DBA2 | BALB/c-P-DBA/2 | BALB/c-Ch-DBA/2 |
| BALB/c | 1015 ± 60 | 11868 ± 96 (11·6) | 4654 ± 821 (4·5) | 5708 ± 440 (5·6) |
| BALB/c-P-DBA/2 | 3674 ± 904 (4·1) | 5734 ± 323 (6·4) | 885 ± 4 | 2998 ± 416 (3·3) |
| BALB/c-Ch-DBA/2 | 7320 ± 9 (2·0) | 10780 ± 1344 (3·2) | 3763 ± 255 (1·1) | 3347 ± 222 |

* SI = stimulation index.

phocytes with stimulation indices of 4·5 and 5·6, respectively, indicating alteration of their antigenic profile due to maternal preimmunization. It may be more surprising, however, that BALB/c-P-DBA/2 and BALB/c-Ch-DBA/2 lymphocytes respond with stimulation indices of 4·1 and 2·0 to normal BALB/c stimulators. There is thus cross-responsiveness between control BALB/c and the offspring of the preimmunized mothers. Both BALB/c-P-DBA/2 and BALB/c-Ch-DBA/2 lymphocytes showed reduced responsiveness to DBA/2 irradiated stimulators, with stimulation indices of 6·4 and 3·2, respectively, compared to responses of normal BALB/c lymphocytes (SI 11·6). The greater unresponsiveness of BALB/c-Ch-DBA/2 compared to BALB/c-P-DBA/2 was a common feature of all mice tested. Furthermore, the lymphoid organs of BALB/c-Ch-DBA/2 mice contained three to fourfold higher numbers of blast-transformed lymphocytes compared to BALB/c-P-DBA/2 and normal BALB/c. This was reflected in the higher levels of [$^3$H]thymidine uptake of lymphocytes from BALB/c-Ch-DBA/2 cultured *in vitro* with autologous irradiated lymphocytes in all experiments.

**Effect of maternal preimmunization of BALB/c mice with DBA/2 splenocytes on the thymic expression of V-beta 6 in the BALB/c offspring**

To test whether reduced responsiveness to DBA/2 alloantigens of the offspring of BALB/c mothers preimmunized with DBA/2 splenocytes is a result of a reduction of $Mls^a$-responsive T cells derived from the thymus, thymocytes from normal adult BALB/c, BALB/c-P-DBA/2 and BALB/c-Ch-DBA/2 were examined by immunofluorescent staining and FACS analysis for the expression of V-beta 6. This T-cell receptor beta chain fragment has been shown previously to characterize the majority of T cells which respond to $Mls^a$-encoded antigens.[4] Table 3 demonstrates that approximately 6·5% of normal BALB/c thymocytes and slightly higher percentages of BALB/c-P-DBA/2 and BALB/c-Ch-DBA/2 thymocytes were stained with anti-V-beta 6. These data indicate that the suppressed responses of mice born of preimmunized mothers are not due to the deletion of $Mls^a$-reactive T-cell clones during thymic ontogeny.

**Specific suppression of BALB/c responses by the addition of BALB/c-Ch-DBA/2 and BALB/c-P-DBA/2 in mixed lymphocyte cultures**

Since clonal deletion does not appear to account for the reduced responses of BALB/c-Ch-DBA/2 lymphocytes to irradiated DBA/2 stimulators, we tested whether clonal anergy or active suppression may explain the suppressed responses of these mice. To mixed lymphocyte cultures of normal BALB/c lymphocytes responding to DBA/2 stimulators, BALB/c-Ch-DBA/2 irradiated cells were added to ascertain their putative suppressor properties. To 100 $\mu$l of a mixed culture of BALB/c and irradiated DBA/2 lymphocytes a total of 100 $\mu$l of various proportions of irradiated BALB/c and BALB/c-Ch-DBA/2 lymphocytes were added. Table 4 demonstrates that in the presence of an additional 100 $\mu$l of irradiated BALB/c lymphocytes the stimulation index of the mixed lymphocyte response was 10·0. When 20 $\mu$l, 50 $\mu$l and 80 $\mu$l of the additional cells were replaced by irradiated BALB/c-Ch-DBA/2 lymphocytes the stimulation index of normal BALB/c responding to DBA/2 was reduced to 6·5, 4·6 and 3·6, respectively, the latter figure representing a 64% inhibition of the mixed lymphocyte response. The same numbers and proportions of BALB/c and BALB/c-Ch-DBA/2 irradiated cells added to a culture of BALB/c lymphocytes responding to AKR stimulators did not affect the anti-AKR response, even though AKR shares $Mls^a$ with DBA/2. These results indicate that specific suppressor cells play a role in the reduced responsiveness of BALB/c-Ch-DBA/2 to DBA/2 stimulator cells.

**Table 3.** Expression of V-beta 6 on thymocytes of BALB/c mice born of mothers preimmunized with DBA/2 splenocytes, unchallenged (BALB/c-P-DBA/2) or neonatally challenged (BALB/c-Ch-DBA/2) with DBA/2 splenocytes

| | Percentage V-beta 6 positive thymocytes (±SD) | |
|---|---|---|
| Mice | Mouse 1 | Mouse 2 |
| BALB/c | 6·7±0·4 | 6·5±0·2 |
| BALB/c-P-DBA/2 | 8·0±0·2 | 7·9±0·2 |
| BALB/c-Ch-DBA/2 | 7·3±0·1 | 6·7±0·7 |

Data are presented as % positive cells after substraction of background staining with the fluorescent anti-Ig conjugate alone.

Similar mixing experiments were also carried out using BALB/c-P-DBA/2 irradiated cells to determine the role of the neonatal challenge with DBA/2 splenocytes in the development of the suppressor cells. Table 5 demonstrates that the addition of increasing amounts of irradiated BALB/c-P-DBA/2 to stimulation cultures of BALB/c responding to DBA/2 or BALB.D2MA resulted in correspondingly higher levels of suppression of the mixed lymphocyte response. As with BALB/c-Ch-DBA/2, the addition of BALB/c-P-DBA/2 did not affect the response of BALB/c to AKR stimulators.

### FACS analysis of lymphocyte profiles of the offspring of BALB/c mothers preimmunized with DBA/2

To analyse the cellular basis of the suppressed anti-DBA/2 responses in the offspring of the preimmunized mothers, FACS analysis of the T-cell subset composition was performed of the offspring which were left untreated at birth and those that were neonatally challenged with DBA/2 splenocytes. At 8–10 weeks of age lymphocyte populations were stained with monoclonal anti-Lyt-1, anti-Lyt-2, anti-L3T4 and anti-Thy-1.2. Twelve mice that were left unchallenged and 12 that were challenged at birth were examined. The results are shown in Table 6. In both the challenged and unchallenged groups two patterns of altered T-cell profiles were discernible. The dominant pattern represented in the larger number (9/12) of BALB/c-P-DBA/2 mice involved more than a 100% increase in the percentage of Lyt-2$^+$ T cells and no considerable alteration in the other subsets. The remaining three mice showed a 50% increase in the Lyt-2$^+$ subset but also a 20% decrease in the L3T4$^+$ subset. A similar pattern of T-cell subset alterations was detected in the BALB/c-Ch-DBA/2 mice. In this group a similar proportion of mice showed an increase in the percentage of Lyt-2$^+$ cells which, however, was considerably greater than in the BALB/c-P-DBA/2 group (171%). Four of the 12 mice showed a 64% increase in the Lyt-2$^+$ subset and a 22% decrease in the L3T4$^+$ subset. These data suggest that the prevention of runting disease in the neonatally challenged mice and the suppressed mixed lymphocyte responses in both the challenged group of mice and those that had not been challenged are due to immunoregulatory mechanisms characterized by the presence of active suppression or a combination of suppression and decreased helper function.

### FACS analysis of mature thymocyte profiles of BALB/c-P-DBA/2 and BALB/c-Ch-DBA/2 mice

Since the analysis of lymphocyte profiles of neonatally challenged and unchallenged offspring of the hyperimmunized mothers revealed that suppressor cells were present in the unchallenged group, we investigated the origin of the T-cell subset alterations in both these groups of mice. For this purpose the mice were injected with hydrocortisone acetate 48 hr prior to examination of the mature thymocyte profiles. Results from individual mice are shown in Table 6 and demonstrate that both increased levels of Lyt-2$^+$ T cells and decreased levels of L3T4$^+$ T cells originate from the mature medullary thymocyte population. A BALB/c-P-DBA/2 mouse which showed a 12·5% increase of the Lyt-2$^+$ medullary thymocyte subset had a 25·8% decrease in the L3T4$^+$ subset. Data from two other unchallenged mice demonstrate increases of 56% and 75% of the Lyt-2$^+$ mature thymocytes (Table 6). These results demonstrate that alterations in the lymphocyte profiles are a developmental phenomenon in the offspring of the hyperimmunized mothers.

## DISCUSSION

In this report we have demonstrated that tolerance to Mls$^a$ alloantigens can be achieved in the absence of clonal deletion of V-beta 6$^+$ thymocytes. BALB/c mothers hyperimmunized with DBA/2 splenocytes prior to and during syngeneic pregnancy gave rise to offspring which resisted runting disease on neonatal challenge with high doses of DBA/2 splenocytes (Table 1). Comparison at 6 weeks of age of V-beta 6 expression on the thymocytes of neonatally challenged and unchallenged offspring of the hyperimmunized BALB/c mothers revealed no differences between these two groups and age-matched control BALB/c (Table 3). Thus the neonatal tolerance of these mice to Mls$^a$ and their unresponsiveness in adult life to *in vitro* stimulation by DBA/2 lymphocytes cannot be explained by deletion of Mls$^a$-reactive T-cell clones. Recent reports have associated neonatal or cyclophosphamide-induced tolerance to Mls$^a$ determinants in Mls$^b$ mice to intrathymic clonal deletion of V-beta 6$^+$ T cells.[17,18] In the case of self[19] or neonatally induced tolerance by injection of DBA/2 spleen cells into BALB/c neonates, deletion or programmed death of Mls$^a$-reactive clones proceeded not only prenatally in the thymus but a few days after birth or after the neonatal injection of the allogeneic cells.[17]

In our experimental system of tolerance induction, unresponsiveness to Mls$^a$ correlated with alterations in the proportional representation of CD4$^+$ and CD8$^+$ T-cell subsets both in the thymus and the peripheral lymphoid organs. These changes in lymphocyte and thymocyte profiles were not a result of the neonatal challenge, since unchallenged offspring of the hyperimmunized mothers showed similar alterations of lymphocyte and thymocyte profiles. The percentage of Lyt-2$^+$ cells was, however, higher in the neonatally challenged group (BALB/c-Ch-DBA/2) than in the unchallenged (Table 6). This may represent an anamnestic recall type of response indicating memory to the tolerogenic antigen and enhancing the immunological pathways leading to the maintenance of tolerance. Bandeira *et al.*[20] reported anamnestic lymphocyte hyperactivity in tolerized mice accepting a second specific skin allograft challenge. Both activated CD4$^+$ and CD8$^+$ T cells were involved in the tolerant immune response. In our experiments, the majority of the unchallenged offspring of the hyperimmu-

**Table 4.** Suppression of the mixed lymphocyte response (MLR) of BALB/c lymphocytes responding to DBA/2 stimulators by the addition of BALB/c-Ch-DBA/2 irradiated cells

| Responder cells (volume μl) | Stimulator cells (volume μl) | Additional irradiated cells (volume μl) | | MLR c.p.m. ± SD | SI | Inhibition of MLR (%) |
|---|---|---|---|---|---|---|
| BALB/c | DBA/2 | BALB/c | BALB/c-Ch-DBA/2 | | | |
| 50 | 50 | 100 | — | 14742 ± 670 | 10·0 | — |
| 50 | 50 | 80 | 20 | 9539 ± 870 | 6·5 | 35 |
| 50 | 50 | 50 | 50 | 6679 ± 450 | 4·6 | 54 |
| 50 | 50 | 20 | 80 | 5286 ± 118 | 3·6 | 64 |
| BALB/c | AKR | BALB/c | BALB/c-Ch-DBA/2 | | | |
| 50 | 50 | 100 | — | 18380 ± 645 | 12·6 | — |
| 50 | 50 | 80 | 20 | 17983 ± 1578 | 12·3 | 2·3 |
| 50 | 50 | 50 | 50 | 18011 ± 919 | 12·4 | 1·6 |
| 50 | 50 | 20 | 80 | 17783 ± 1481 | 12·2 | 3·2 |

Stimulation indices were calculated on the basis of [$^3$H]thymidine uptake of 1451 ± 341 (c.p.m. ± SD) by BALB/c responders cultured with irradiated autologous stimulators.

**Table 5.** Suppression of mixed lymphocyte responses of BALB/c lymphocytes by the addition of BALB/c-P-DBA/2 irradiated cells

| Responder cells (volume μl) | Stimulator cells (volume μl) | Additional irradiated cells (volume μl) | | MLR c.p.m. ± SD | SI | Inhibition of MLR (%) |
|---|---|---|---|---|---|---|
| BALB/c | DBA/2 | BALB/c | BALB/c-P-DBA/2 | | | |
| 50 | 50 | 100 | — | 7793 ± 671 | 16·6 | — |
| 50 | 50 | 80 | 20 | 4630 ± 314 | 9·8 | 40·9 |
| 50 | 50 | 50 | 50 | 2865 ± 377 | 6·1 | 63·3 |
| BALB/c | BALB.D2MA | BALB/c | BALB/c-P-DBA/2 | | | |
| 50 | 50 | 100 | — | 2735 ± 215 | 5·8 | — |
| 50 | 50 | 80 | 20 | 1746 ± 124 | 3·7 | 36·2 |
| 50 | 50 | 50 | 50 | 1187 ± 135 | 2·5 | 56·8 |
| BALB/c | AKR | BALB/c | BALB/c-P-DBA/2 | | | |
| 50 | 50 | 100 | — | 6475 ± 559 | 13·8 | — |
| 50 | 50 | 80 | 20 | 6563 ± 485 | 14·0 | 0 |
| 50 | 50 | 50 | 50 | 5789 ± 610 | 12·3 | 10·8 |

Stimulation indices were calculated on the basis of [$^3$H]thymidine uptake of 468 ± 34 (c.p.m. ± SD) by BALB/c responders cultured with irradiated autologous stimulators.

**Table 6.** FACS analysis of lymphocyte profiles of normal BALB/c and the unchallenged and challenged offspring of BALB/c mothers hyperimmunized with DBA/2

| | | Immunofluorescent staining using monoclonal reagents against | | | |
|---|---|---|---|---|---|
| | | Lyt-1 | Lyt-2 | L3T4 | Thy-1·2 |
| Mice | No. tested | % positive cells ± SD (% increase ↑ or decrease ↓) | | | |
| BALB/c | 14 | 88 ± 0·4 | 14 ± 0·7 | 64 ± 1·4 | 90 ± 0·9 |
| BALB/c-P-DBA/2 | 12 < 9 | 88 ± 0·8 | 30 ± 2·0 (↑114) | 60 ± 1·0 | 94 ± 0·4 |
| | 12 < 3 | 86 ± 2·0 | 21 ± 0·4 (↑50) | 51 ± 0·1 (↓20·3) | 88 ± 0·4 |
| BALB/c-Ch-DBA/2 | 12 < 8 | 90 ± 0·2 | 38 ± 1·2 (↑171) | 66 ± 3·2 | 95 ± 0·5 |
| | 12 < 4 | 85 ± 0·4 | 23 ± 0·4 (↑64) | 50 ± 0·5 (↓22) | 94 ± 1·0 |

**Table 7.** FACS analysis of thymocyte profiles of BALB/c-P-DBA/2 and BALB/c-Ch-DBA/2 injected i.p. with 250 mg/kg body weight of hydrocortisone acetate 48 hr prior to testing

| Mice | Treatment with hydrocortisone | Immunofluorescent staining using monoclonal reagents against: Lyt-1 | Lyt-2 | L3T4 | Thy-1·2 |
|---|---|---|---|---|---|
| | | % positive cells ± SD (% increase ↑ or decrease ↓) | | | |
| BALB/c | – | 98 ± 0·7 | 81 ± 2·7 | 99 ± 0·1 | 92 ± 0·8 |
| BALB/c | + | 96 ± 0·4 | 16 ± 0·2 | 58 ± 1·6 | 92 ± 1·5 |
| BALB/c-P-DBA/2 | + | 86 ± 2·5 | 18 ± 0·8 (↑12) | 43 ± 1·1 (↓26) | 87 ± 0·7 |
| BALB/c-P-DBA/2 | + | 95 ± 1·2 | 28 ± 0·6 (↑75) | 55 ± 0·6 | 91 ± 2·4 |
| BALB/c-P-DBA/2 | + | 96 ± 0·5 | 25 ± 1·4 (↑56) | 56 ± 1·2 | 92 ± 1·4 |
| BALB/c-Ch-DBA/2 | + | 94 ± 2·1 | 24 ± 1·0 (↑50) | 62 ± 1·2 | 93 ± 0·1 |

nized BALB/c mothers had more than double the percentage of Lyt-2$^+$ lymphocytes compared to age-matched BALB/c controls. Some mice in the unchallenged group had only a 50% higher level of Lyt-2$^+$ T cells but also a 20% decreased level of L3T4$^+$ cells (Table 6). The maintenance of such altered lymphocyte profiles in adult mice corresponding to similar changes in the mature thymocyte profiles (Table 7) indicate that these alterations from the normal profiles are a result of thymic selection procedures during foetal development in the hyperimmunized mother.

The T-cell repertoire is a result of both positive and negative influences. Self-MHC class I and class II, TcR, CD4 and CD8 molecules are present in the thymus and affect T-cell development and both negative and positive selection. The presence of antibodies against these molecules in foetal and neonatal life has been demonstrated to affect repertoire selection and T-cell subset development.[21-26]

In our experiments we demonstrate for the first time the positive selection of CD8$^+$ T cells with antigen-specific suppressor properties and an analogous negative selection of CD4$^+$ helper cells in the offspring of BALB/c mothers hyperimmunized with DBA/2 splenocytes prior to and during pregnancy. The mature thymocyte and lymphocyte profiles of these offspring showed similar proportional alterations of the CD8$^+$ and CD4$^+$ subsets, indicating that these alterations in the lymphocyte profiles originate from thymocyte subsets which have preferentially migrated from the thymus to seed the peripheral lymphoid organs.

Tolerance to the H-Y antigen in male transgenic mice carrying the H-Y T-cell receptor transgene was shown to be associated with alteration in the CD4/CD8 phenotype of T cells in the lymph nodes. However, drastic depletion of the thymocyte population was also observed resulting in lower numbers of thymocytes in male but not female transgenic mice.[27]

We found no differences in total thymocyte numbers of BALB/c-P-DBA/2 compared to age-matched control BALB/c mice and also no differences in the percentage of thymocytes expressing V-beta 6, but the *in vitro* proliferative responses of BALB/c-P-DBA/2 to DBA/2 stimulator cells were severely suppressed (Table 2). Suppression of anti-Mls$^a$ responses in the absence of clonal deletion has been demonstrated in V-beta 8.1 transgenic mice[28] and also in adult Mls$^b$ mice immunized with Mls$^a$.[29] In both these experimental models T cells were rendered anergic rather than specifically suppressed. In contrast to these reports BALB/c-P-DBA/2 and BALB/c-Ch-DBA/2 lymphocytes responded well to stimulation by concanavalin A (data not shown), responded poorly to DBA/2 and specifically suppressed the responses of normal BALB/c lymphocytes to DBA/2 stimulator cells (Tables 4 and 5). The suppression appears to be mediated either by CD8$^+$ T suppressor cells or a combination of suppression and reduced levels of CD4$^+$ T-helper function. Webb & Sprent[30] have unequivocally demonstrated the capacity of CD8$^+$ T cells to respond to Mls$^a$ contrary to current opinion that only CD4$^+$ cells respond to Mls determinants.[31,32] Since lymphocytes from the unresponsive mice have high levels of CD8$^+$ cells and specifically suppress the response of normal BALB/c to DBA/2, BALB.D2MA but not to AKR, it appears that Mls-reactive CD8$^+$ T cells are MHC class I restricted.

The mechanism by which the offspring of hyperimmunized mothers undergo alterations in thymocyte profiles is not known. The cellular expression of the tolerogenic antigen is necessary in the thymic environment either naturally or by transgene manipulation for the induction of natural or experimental tolerance. The expression of these antigens on different cellular elements distinguished by their radiation sensitivities has been shown to influence the mechanism of tolerance induction, i.e. clonal deletion or clonal anergy.[10] In this report we have shown that tolerance can be generated in the thymus by a third mechanism involving positive selection of suppressor cells sometimes combined with negative selection of helper cells. Since hyperimmunization of the mother with the tolerogenic antigen is necessary and sufficient for the development of the altered T-cell repertoire and mature thymocyte profiles described in this report, transfer of maternal antibodies to the foetus must be the factor responsible for these alterations. Anti-Mls$^a$ antibodies are unlikely to have been produced even by hyperimmunization, thus the idiotype or IgV region of the preimmunized mother's antibodies representing the 'antigen image' of the receptors on the challenge strain T cells may be responsible for idiotypic imprinting of the neonatal T-cell repertoire. Characterization of the maternal antibodies is in progress.

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