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Autoimmune syndrome after induction of neonatal tolerance to alloantigens: analysis of the role of donor T cells in the induction of autoimmunity

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SUMMARY

The injection of (C57BL/6 × BALB/c) F1 spleen cells into BALB/c newborn mice leads to activation of persisting F1 donor B cells and development of a lupus-like syndrome in tolerized BALB/c mice. This syndrome is characterized by hypergammaglobulinaemia, high levels of anti-DNA and anti-Sm antibodies, circulating immune complexes and deposits of immunoglobulin in renal glomeruli. The role of donor T cells in this model was investigated by injecting the newborn mice with F1 cells depleted in different T cell subsets by using specific monoclonal antibodies (MoAbs). Tolerance, as shown by an absence of H-2^b-specific CTL alloreactivity and persistence of immunoglobulin bearing the donor allotype were observed in mice injected with F1 cells previously depleted in the $CD4^+$ and/or $CD8^+$ T cell subsets as well as in those which received Thy-1^+-depleted F1 spleen cells. In these mice, a typical autoimmune syndrome was found, including splenomegaly and lymphadenopathy, anti-ssDNA and anti-aortic myosin IgG antibodies and renal deposition of immunoglobulin. However, some quantitative changes were seen: the levels of anti-aortic myosin antibodies were lower in mice tolerized with $CD4^+$-depleted F1 cells than in those receiving untreated F1 cells. Conversely, higher levels of these autoantibodies were observed in mice tolerized with $CD8^+$-depleted F1 cells. These results suggest that mature donor T cells are not necessary neither for the establishment of neonatal tolerance to alloantigens nor for the activation of F1 donor B cells in the production of the autoimmune syndrome in tolerant mice, but they may contribute in the regulation of the expression of autoreactive B cell clones.

Keywords neonatal tolerance autoimmunity T cells

INTRODUCTION

The injection of semiallogeneic (C57BL/6 × BALB/c) F1 spleen cells into parental newborn BALB/c mice induces a state of specific cytolytic T lymphocytes (CTL) unresponsiveness to H-2^b alloantigens (Feng *et al.*, 1983), but also the development of a lupus-like autoimmune syndrome (Hard & Kullgren, 1970; Goldman *et al.*, 1983). These mice show hypergammaglobulinaemia, high titres of anti-DNA antibodies, circulating immune complexes and renal deposits of immunoglobulins (Luzuy *et al.*, 1986; Merino *et al.*, 1987), associated with splenomegaly and lymphadenopathy (Simpson *et al.*, 1974). In previous experiments it was demonstrated that all the autoantibodies were produced by F1 donor B cells in these tolerized mice (Luzuy *et al.*, 1986).

A role of T cells in the activation of persisting autoreactive donor B cells was suggested by a predominance of T dependent antibody isotypes (Luzuy *et al.*, 1986) and by the prevention of the autoimmune syndrome following treatment of tolerized mice with a monoclonal anti-CD4 antibody (Merino *et al.*, 1987). Further experiments emphasized the role of host T cells in the activation of F1 B cells. Indeed, athymic BALB/c nude mice, neonatally injected with normal (C57BL/6 × BALB/c) F1 spleen cells, failed to develop the autoimmune manifestations seen in euthymic tolerized mice. In addition, a lupus-like syndrome was triggered after injection of these tolerized nude mice at 3 weeks with purified $CD4^+$ T cells from tolerant or control euthymic BALB/c mice (Merino *et al.*, 1989).

The purpose of the present studies was to investigate the possible role of donor T cells in this model. This problem was approached by depleting the F1 hybrid donor mice in different T cell subsets before transferring spleen cells.

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MATERIALS AND METHODS

Mice

Mice of the inbred strains BALB/c and C57BL/6 were purchased from Bomholtgard Laboratories (Ry, Denmark) (C57BL/6 × BALB/c) F1 hybrid mice were produced in our laboratory. Congenic BALB. Ig^b mice (Igh.1^b) were kindly provided by the Walter & Elisa Hall Institute, Melbourne, Australia.

Induction of neonatal tolerance

Spleen cells (10^8) from 2- to 3-month-old (C57BL/6 × BALB/c) F1 hybrid mice, prepared as previously described (Merino *et al.*, 1987) were injected intraperitoneally into newborn BALB/c mice within 24 h after birth. Control mice were untreated littermates.

Serum samples

Mice were bled by retro-orbital sinus puncture under ether anaesthesia, and blood was left for 90 min at room temperature to clot. The serum was then separated by centrifugation at 1500 *g* for 10 min and stored at −70°C until use.

Generation of CTL in mixed leucocyte culture (bulk CTL assay)

The CTL unresponsiveness to F1 alloantigens in BALB/c mice neonatally injected with F1 spleen cells was assessed in a 5-day mixed leucocyte culture (MLC), followed by a cytolytic ^{51}Cr release assay as previously described (Feng *et al.*, 1983).

Estimation of cytolytic T lymphocyte precursor (CTL-p) frequency

The frequencies of alloreactive CTL-p in spleen cells were investigated in a micro-MLC under limiting dilution conditions as previously described (Ryser & MacDonald, 1979). CTL-p frequencies were calculated by the maximum likelihood method (Fazekas de St Groth, 1982).

Preparation of anti-IgCH allotype antisera

Anti-Igh^b and anti-Igh^a allotype antisera were obtained in BALB/c mice immunized with C57BL/6 IgG and in SJL mice immunized with BALB/c IgG respectively, following the method described by Lieberman (1978). These antisera were purified on a sepharose–protein-A column (Pharmacia Fine Chemicals, Uppsala, Sweden) and the resulting IgG fractions were then conjugated to alkaline-phosphatase (AP) (Sigma Chemicals, St Louis, MO) in the presence of glutaraldehyde (Serva Lab, New York, NY) (Avrameas, 1969).

Determination of immunoglobulin allotype

To evaluate the levels of Ig bearing the Igh^b allotype in the serum of tolerant mice, a previously described (Luzuy *et al.*, 1986) ELISA was employed. Results were expressed in mg-equivalent/ml of Igh^b-bearing immunoglobulin from normal C57BL/6 serum.

Solid-phase anti-DNA assays

To measure anti-ssDNA IgG antibodies, an ELISA assay was employed as previously described (Luzuy *et al.*, 1986). Calf thymus DNA (type V, Sigma) was heat-denatured and used as ssDNA. Results were expressed in titration units (tU) referring to a standard curve obtained by serial dilutions of a serum pool from 6- to 8-month-old MRL lpr/lpr mice or of a serum pool from 6- to 8-month-old male B × SB mice for anti-ssDNA IgG2a determination.

Anti-myosin antibodies

An ELISA method was employed, similar to that used for anti-DNA. Myosin, highly purified by affinity chromatography from ox aorta (Pollard, Thomas & Niederman, 1974), was generously provided by Dr G. Benzonana, University of Geneva. For the assays, polystyrene microtitre plates (Limbro, Flow), were coated with 50 μl of aortic myosin (5 μg/ml), diluted in BBS. The AP conjugate used was a goat anti-mouse gamma chain specific antibody (1 μg/ml). Results of anti-myosin antibodies were expressed in tU referring to a standard curve obtained from the same serum pool of MRL lpr/lpr mice used for anti-ssDNA antibodies. Units were chosen arbitrarily by referring to these sera.

In vivo treatment with anti-T cell subset antibodies

The rat GK 1.5 clone producing anti-CD4 IgG2b MoAb was a generous gift from Dr Frank W. Fitch, University of Chicago, IL (Dialinas *et al.*, 1983). The H.35-17.2 clone (rat anti-CD8 IgG2b MoAb) (Pierres, Goridis & Goldstein, 1982) was kindly donated by Dr H. R. McDonald, Ludwig Institute, Epalinges, Switzerland. The TIB-107 hybridoma line, producing a rat anti-Thy-1.2 IgG2b MoAb, obtained by Ledbetter & Herzenberg (1979), was purchased from the American Type Culture Collection (Rockville, MD). Hybridoma ascitic fluids were used for *in vivo* treatment. These fluids were diluted in DMEM containing 10 mM HEPES to an immunofluorescence titre of 1:1000. Eight- to 10-month-old (C57BL/6 × BALB/c) F1 mice were treated intraperitoneally with 1 ml of GK-1.5, H-35 or TIB-107 MoAbs daily for 5 days. The efficacy of these treatments was checked by cytofluorometry (Epics V; Coulter Electronics, Hialeah, FL) following the method previously discussed (Merino *et al.*, 1987).

In vitro *depletion of Thy-1$^+$ lymphocytes*

Thy-1^- F1 spleen cells were prepared by incubating the cells (20×10^6/ml) for 15 min in an ice bath with 20 μl/ml of AT-83 ascitis (rat anti-Thy-1 IgM MoAb, immunofluorescence titre $1:10^5$). After two washes, cells were incubated at 37°C in 5% CO_2 for 60 min with 10% low toxic rabbit complement (Cederlane Laboratories, Ontario, Canada). The treated spleen cells were washed three times and then injected intraperitoneally into newborn BALB/c mice. The efficiency of the treatment was documented by cytofluorometry, following the method described by Merino *et al.* (1987).

Tissue studies

Tissue-bound IgM and IgG subclasses were studied by immunofluorescence on kidney sections using a fluoresceinated rabbit anti-mouse IgM or rabbit anti-mouse IgG subclasses. As second step, a FITC-conjugated goat anti-rabbit immunoglobulin was used. All these antibodies were purchased from Tago (Burlingame, CA).

Statistical analysis

Significance analyses were performed using the Wilcoxon rank test.

Table 1. Effects of anti-CD4, anti-CD8 or anti-Thy-1 monoclonal antibodies (MoAbs) on spleen T cell subsets in (C57BL/6 × BALB/c) F1 mice

Treatment	Thy-1$^+$ cells (%)	CD4$^+$ cells (%)	CD8$^+$ cells (%)	sIgM$^+$ cells (%)
None (control)	36	25	12	32
Anti-CD4 MoAb	43	< 1	42	NT
Anti-CD8 MoAb	38	40	< 1	NT
Anti-CD4 + anti-CD8 MoAbs	2	< 1	< 1	43
Anti-Thy-1 MoAb (AT-83)	< 1	< 1	< 1	68

(C57BL/6 × BALB/c) F1 mice were injected daily with anti-CD4 and/or anti-CD8 MoAbs for 5 days and then killed for transferring spleen cells to newborn BALB/c mice. For depletion of Thy-1$^+$ cells, F1 spleen cells were treated *in vitro* with an anti-Thy-1 IgM MoAb plus complement before transfer into newborn BALB/c mice. The efficacy of these treatments was followed by cytofluorometric analysis of the cellular pool injected into the different groups of BALB/c mice. The results are expressed in percentage of fluorescent cells after substraction of background values.

(NT) Not tested.

Table 2. Effects of depletion of different T cell subsets in the F1 donor spleen cells on the cytolytic T lymphocyte (CTL) alloreactivity in neonatally injected BALB/c mice

Groups	*n*	CTL activity in MLC* (lU/culture)	CTL-p frequency†
BALB/c control	10	141 (50–500)	1/6043 (1/2816–1/9020)
BALB/c + 10^8 F1 cells	27	< 5	1/62528 (1/17320– < 1/100000)
BALB/c + 10^8 CD4$^-$ F1 cells	12	< 5	1/16228 (1/2782–1/40809)
BALB/c + 10^8 CD8$^-$ F1 cells	11	< 5	1/70799 (1/30572–1/100000)
BALB/c + 10^8 CD4$^-$ CD8$^-$ F1 cells	19	< 5	1/35258 (1/6293– < 1/100000)
BALB/c + 10^8 Thy-1$^-$ F1 cells‡	8	< 5	1/64732 (1/46697–1/91537)

* BALB/c mice were neonatally injected with (C57BL/6 × BALB/c) F1 cells and, 4 weeks later, spleen cells from these animals were stimulated with irradiated (2000 rad) C57BL/6 spleen cells in mixed leucocyte culture (MLC). CTL activity was tested on MBL-2.9 (H-2^b) tumour target cells in a 3·5-h ^{51}Cr release assay. Results are expressed in lytic units (lu) per 5×10^6 responder spleen cells.

† CTL-precursor (CTL-p) frequency was tested in MLC under limiting dilution conditions in the presence of additional interleukin-2 (IL-2). Results represent means and ranges of five to 12 individual mice.

‡ CTL activity in MLC and CTL-p frequency in spleen cells of 9-week-old BALB/c mice neonatally injected with F1 cells depleted of Thy-1$^+$ cells by *in vitro* treatment with AT-83 MoAb.

RESULTS

Depletion of F1 donor spleen cells in CD4$^+$ and/or CD8$^+$ T cells before neonatal injection in parental mice does not significantly affect the development of the autoimmune syndrome

Spleen cells (10^8) from (C57BL/6 × BALB/c) F1 mice which were previously depleted of CD4$^+$ and/or CD8$^+$ T cell subsets by *in vivo* treatment with specific MoAbs, were injected intraperitoneally into newborn BALB/c mice. The efficacy of these treatments with MoAbs was confirmed by cytofluorometric analysis at the time of spleen cell collection (Table 1).

The effects of the depletion of F1 donor cells in different T cell subsets on their capacity to induce tolerance to H-2^b alloantigens and autoimmune manifestations were investigated at 4 weeks of age. An absence of alloreactivity to H-2^b target cells was observed in MLC under bulk conditions in all groups of BALB/c mice injected with F1 cells (Table 2). The frequency of alloreactive CTL-p measured in limiting dilution assay was

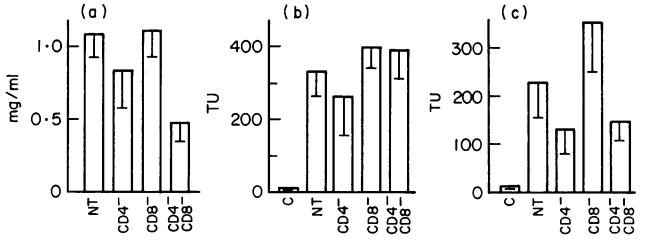

Fig. 1. BALB/c mice were injected at birth with 10^8 spleen cells from (C57BL/6 × BALB/c) F1 hybrids previously treated by an i.p. injection of specific anti-T cell subset MoAbs. Mice were bled at 4 weeks and tested in ELISA for the presence of (a) immunoglobulin bearing the donor Igh^b allotype; (b) anti-ssDNA IgG antibodies; and (c) anti-aortic myosin IgG antibodies. There was no detectable Igh^b in control BALB/c mice. Results of Igh^b allotype are expressed in milligram-equivalent/ml of Igh^b-bearing immunoglobulin from normal C57BL/6 serum. Results of anti-ssDNA and anti-myosin IgG antibodies are expressed in titration units (TU). Mean values from groups of nine to 19 mice and standard error of the mean are represented. C, BALB/c control; NT, BALB/c + untreated F1 spleen cells; $CD4^-$, BALB/c + $CD4^-$ F1 spleen cells; $CD8^-$, BALB/c + $CD8^-$ F1 spleen cells; and $CD4^-$ $CD8^-$, BALB/c + $CD4^-$ $CD8^-$ F1 spleen cells.

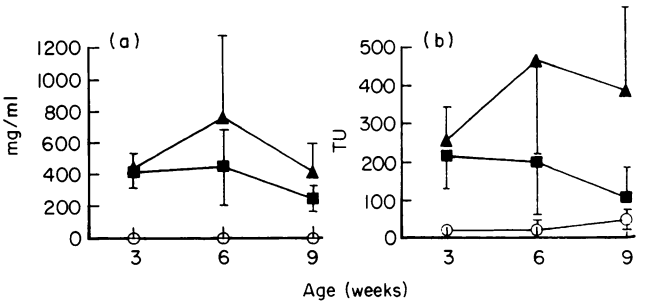

Fig. 2. BALB/c mice were injected at birth with 10^8 spleen cells from (C57BL/6 × BALB/c) F1 after *in vitro* treatment with rat anti-Thy-1 IgM MoAb plus complement. BALB/c injected with untreated F1 cells served as control. Mice were bled at 3, 6 and 9 weeks and tested in ELISA for (a) immunoglobulin bearing donor Igh^b allotype and (b) anti-ssDNA IgG antibodies. Results for anti-ssDNA antibodies are expressed in titration units (TU). Bars are ± s.e.m. of groups of seven to eight mice. ○, BALB/c control; ▲, BALB/c + untreated F1 spleen cells; and ■, BALB/c + Thy-1⁻ F1 spleen cells.

decreased in all mice injected with treated or untreated F1 cells ($P < 0.05$). However, it was significantly less decreased ($P < 0.025$) in BALB/c mice injected with $CD4^+$-depleted F1 spleen cells. The persistence of B cell chimaerism was demonstrated in all groups of tolerant mice as shown by the existence of significant levels of immunoglobulins bearing the donor Igh^b allotype (Fig. 1a). BALB/c mice injected with $CD4^-$ $CD8^-$ F1 cells had levels of Igh^b allotype lower than the other groups of tolerant mice ($P < 0.05$).

Enlargement of spleen and lymph nodes was observed in all groups of mice injected with F1 cells as compared with uninjected controls (spleen weight 50 ± 19 mg in BALB/c controls; 185 ± 95 mg in BALB/c + F1 cells; 138 ± 49 mg in BALB/c + $CD4^-$ F1 cells and 145 ± 84 mg in BALB/c + $CD4^-$ $CD8^-$ F1 cells). This splenomegaly was more marked in BALB/c mice injected with $CD8^+$-depleted F1 spleen cells (251 ± 67 mg, P 0·05).

Anti-ssDNA IgG antibodies were observed in all groups of tolerized BALB/c mice without any significant effect of the T subset depletion of F1 donor cells (Fig. 1b). Anti-aortic myosin IgG antibodies were also observed in all groups of injected BALB/c mice (Fig. 1c) but, unlike anti-ssDNA antibodies, BALB/c mice injected with $CD8^-$ F1 cells had the highest levels of anti-aortic myosin antibodies ($P < 0.05$), whereas those injected with $CD4^-$ F1 cells had the lowest levels ($P < 0.05$).

By immunofluorescence studies, dense IgM and IgG deposits were observed in the renal glomeruli of all groups of BALB/c mice injected with treated or untreated F1 spleen cells.

Injection of Thy-1-depleted F1 spleen cells can induce neonatal tolerance and autoimmunity

BALB/c mice were injected intraperitoneally at birth with 10^8 (C57BL/6 × BALB.Ig^b) F1 spleen cells depleted of Thy-1^+ cells by *in vitro* treatment with an anti-Thy-1 IgM MoAb plus complement. The efficiency of the treatment was documented by cytofluorometry, as shown in Table 1.

The effects of the injection of Thy-1^+-depleted F1 spleen cells were studied at 3, 6 and 9 weeks of age. At 9 weeks, spleen cells from these injected mice showed no cytolytic reactivity against H-2^b target cells in MLC, and the frequency of alloreactive CTL-p was very low, similar to that exhibited by BALB/c mice tolerized with cells from untreated F1 mice (Table 2). Significant levels of immunoglobulins bearing the Igh^b donor allotype were found in all BALB/c mice injected with Thy-1^- F1 spleen cells (Fig. 2a), indicating the persistence of donor B cells.

Lymphoid hypertrophy was observed in all mice injected with F1 spleen cells depleted in Thy-1^+ cells, as compared with uninjected controls (spleen weight at 9 weeks 122 ± 23 mg in BALB/c controls, *versus* 328 ± 166 mg in BALB/c + Thy-1^- F1 cells). Anti-ssDNA IgG antibodies were not significantly different at 3 weeks in sera of BALB/c tolerized with Thy-1-depleted F1 cells compared with BALB/c tolerized with untreated F1 cells (Fig. 2b). However, they were significantly lower at 6 and 9 weeks ($P < 0.05$ and $P < 0.005$, respectively).

Immunofluorescence studies showed dense deposits of IgM and all IgG isotypes in renal glomeruli of BALB/c mice injected with Thy-1^- F1 spleen cells.

Similar results were observed in BALB/c mice tolerized at birth with 10^8 spleen cells from (C57BL/6 × BALB/c) F1 mice treated *in vivo* during 5 days with an anti-Thy-1.2 (TIB-107) IgG2b MoAb (data not shown).

DISCUSSION

The present results indicate that the autoimmune syndrome, which appears after induction of tolerance to H-2^b alloantigens by neonatal injection of (C57 BL/6 × BALB/c) F1 spleen cells into parental BALB/c mice is not highly dependent on the presence of mature T cells from the F1 donor. The possible role of F1 T cells should be considered at two different levels: their capacity to induce a state of unresponsiveness to H-2^b alloantigens; and their involvement in the autoimmune response.

Tolerance, as measured by the generation of H-2^b-specific CTL activity in bulk MLC, by analysis of CTL-p frequencies or also by detection of persistent immunoglobulins bearing the donor allotype, has been observed after injection of F1 spleen cell populations deprived of $CD4^+$ and/or $CD8^+$ subsets. Moreover, Thy-1^+ depleted F1 spleen cells were also effective as

tolerogen. Therefore, these experiments do not support a major role of mature T cell subsets from the F1 donor in the induction of tolerance to alloantigens. These results somewhat differ from those observed by Weissmann, Jerabec & Greenspan (1984) which indicated the need for donor T cells in the induction of neonatal tolerance to male H-Y antigens in congenic females. However, our results are consistent with those of Ishizaka, Carnaud & Stutman (1986) who found that T donor cells were not essential to induce tolerance to alloantigens in neonatal CBA/HT6T6 mice.

It has also been suggested that CTL may be inactivated by the recognition of Thy-1$^-$ cells, possibly of the T cell lineage (Murakoa & Miller, 1980). These so called veto cells (Miller, 1980), might persist in our CD4$^-$ CD8$^-$ or Thy-1$^-$ F1 spleen cell inoculum.

The triggering of the autoimmune syndrome in tolerant mice does not appear to require the presence of CD4$^+$, CD8$^+$ or Thy-1$^+$ F1 donor cell populations. These results would not support the hypothesis of a major helper effect of the F1 donor T cells in the production of autoantibodies. However, the levels of anti-aortic myosin antibodies were lower in mice tolerized with CD4$^+$-depleted F1 cells. Conversely, it is possible that CD8$^+$ F1 cells exert a negative regulatory effect in this system since higher levels of anti-aortic myosin IgG antibodies were observed in mice tolerized with CD8$^-$ F1 spleen cells. Such regulatory effects were absent when Thy-1$^-$ F1 cells were used as tolerogen.

Our data differ from those of Abramowicz *et al.* (1987) who found an absence of hypergammaglobulinaemia and immunoglobulin glomerular deposits in BALB/c mice injected with Thy-1$^+$-depleted (BALB/c × A/J) F1 cells, although they observed a persistence of donor immunoglobulins and pathologic levels of anti-dsDNA antibodies and rheumatoid factor activity. The number of F1 cells injected at birth (three time less than in our study) may be the origin of this discordance. Tateno *et al.* (1985) also observed an absence of autoimmune manifestations in BALB/c mice after injection of T cell-depleted (C57BL/6 × BALB/c) F1 cells, but there was no evidence in these experiments of an effective tolerization.

Therefore, our present interpretation of the pathogenesis in this autoimmune syndrome is as follows. The neonatal injection of F1 spleen cells leads to an alloreactive CTL unresponsiveness for the corresponding alloantigens. This state of unresponsiveness probably occurs through a selective deletion of CD8$^+$ H-2^b-specific T cells in the thymus of these tolerized mice, as was recently documented for the establishment of perinatal tolerance to self-antigens (MacDonald *et al.*, 1988; Shimonkevitz & Bevan, 1988). In parallel, a B cell chimaerism takes place, including a persistence of autoreactive F1 B cells. As previously shown, CD4$^+$ T cells from tolerant mice are still capable *in vivo* (Merino *et al.*, 1989) and *in vitro* (Abramowicz *et al.*, 1987), of stimulating these autoreactive F1 B cells for the production of autoantibodies. However, T cells from the F1 donor would not be essential for this response, but may contribute in regulating the expression of autoreactive B cell clones.

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