Immunology 1994 **82** 287–293

Differences in non-MHC alloantigens promote tissue rejection but fail to mediate allogeneic co-operation and autoimmunity in mice neonatally injected with semi-allogeneic F_1 B cells

A. RAMOS, R. MERINO* & J. MERINO *Servicio de Inmunología, Hospital Universitario 'Marqués de Valdecilla', Santander, Spain*

SUMMARY

Mice injected at birth with semi-allogeneic lymphoid cells develop a lupus-like autoimmune syndrome in which donor B cells are polyclonally activated by host alloreactive $CD4^+$ T cells, producing autoantibodies and immune complex-mediated glomerulonephritis. It has been demonstrated that the recognition of major histocompatibility complex (MHC) class II alloantigens triggers the development of a complete disease. But differences in either MHC class I molecules or Mls-1 antigens are not sufficient to induce production of autoantibodies. Here we have investigated whether differences in other non-MHC alloantigens could induce a similar autoimmune disease and whether the maternal environment could modulate the T–B allogeneic co-operation in this model. For this purpose (BALB/c × BC20)F_1 hybrid females were backcrossed with BC20 males. R_2 mice obtained in this backcross were neonatally injected with 10^8 (C57BL/6 × BALB.Ig^b)F_1 spleen cells and the tolerance against maternal derived BALB/c alloantigens as well as the development of autoimmune manifestations were subsequently evaluated. In contrast to R_2 mice injected at birth with (C57BL/6 × BALB.Ig^b)F_1 cells, control R_2 mice rejected skin grafts from BALB/c mice and B cells from (C57BL/6 × BALB.Ig^b)F_1 mice, independently of their H-2 haplotype (H-$2^{b/d}$ or H-$2^{b/b}$). Nevertheless, after neonatal injection of (C57BL/6 × BALB.Ig^b)F_1 cells, none of 19 H-$2^{b/d}$ R_2 injected mice presented autoimmune manifestations, in contrast with the typical autoimmune disease observed in all neonatally injected H-$2^{b/b}$ R_2 mice (26 mice). These results support that the development of autoimmunity in this model depends exclusively upon differences in MHC class II alloantigens and that the relationship between mother and fetus, through the pregnancy or the breast suckling, is not sufficient to inhibit cytolytic and allo-helper responses against non-inherited maternal-derived alloantigens.

INTRODUCTION

The induction of tolerance to alloantigens by neonatal injection of semi-allogeneic lymphoid cells in parental mice is followed by the development of an autoimmune syndrome characterized by lymphadenopathy, splenomegaly, thrombocytopenia, high levels of autoantibodies (autoAb), and immune complex-mediated glomerulonephritis.[1–3] In these mice, it has been demonstrated that host $CD4^+$ T cells are responsible for the polyclonal activation of donor B cells.[4–7] A split tolerance mechanism has been claimed to explain this phenomenon. Indeed, a drop in the frequencies of cytolytic T-cell precursors and in the interleukin-2 (IL-2) producer T-cell precursors (Th1) correlates with an increase in the frequency of IL-4 producer T-helper type 2 (Th2) cell precursors.[8–9] Moreover, the treatment of F_1 cell-injected mice with an anti-IL-4 monoclonal antibody (mAb) prevented the triggering of the autoimmune syndrome in these mice.[10] By contrast, it has been reported that donor T cells seem to be irrelevant for autoimmunity.[11] Previously, we have demonstrated that differences at the level of I-A or I-E class II alloantigens, but not in class I or Mls-1 alloantigens, play a central role in the production of the autoimmune syndrome in neonatally injected mice.[12,13] However, at present, the possibility that the recognition of other non-MHC alloantigens could contribute to the abnormal interaction between host $CD4^+$ Th2 cells and donor B cells has not been evaluated.

The experiments reported in the present paper were designed to analyse the role of non-MHC alloantigens in the

Received 9 December 1993; revised 9 February 1994; accepted 12 February 1994.

*Present address: Department of Pathology, M5240 Med. Sci., University of Michigan, Ann Arbor, MI 48109–0602, U.S.A.

Abbreviations: AMGG, heat-aggregated mouse IgG; AutoAb, autoantibodies; CIC, circulating immune complexes; Igh, Ig heavy chain; NIMA, non-inherited maternal alloantigens; ssDNA, single-stranded DNA; TU, titration units; Th2, IL-4 producer T-helper type 2 cells.

Correspondence: Dr J. Merino, Servicio de Inmunología, Hospital Universitario 'Marqués de Valdecilla', 39008 Santander, Spain.

production of autoimmune manifestations in parental mice neonatally injected with F_1 spleen cells. Likewise, this model allowed us to evaluate the influence of the maternal environment in the induction of host $CD4^+$ T-cell tolerance to donor alloantigens. In this regard, it was suggested that the maternal environment (i.e. passage of cells or soluble antigens from the mother through the placenta or by breast feeding) could facilitate the induction of tolerance to MHC alloantigens in humans.[14–16] However, in rodents, experiments performed by Tyzzer[17] showed that the pregnancy does not modify the cytolytic response to non-inherited maternal alloantigens (NIMA) in the F_2 progeny.

Here, we present evidence that the relationship between mother and fetus does not prevent either the rejection of allogeneic tissues or the development of autoimmunity in F_1 cell-injected mice. Moreover, differences in histocompatibility antigens other than MHC class II antigens are unable to trigger the development of a lupus-like autoimmune syndrome in mice injected at birth with semi-allogeneic spleen cells.

MATERIALS AND METHODS

Mice

BALB/c and C57BL/6 were purchased from IFFA Credo España (Barcelona, Spain). Congenic BALB.Ig^b mice (IgCH-1^b) were kindly provided by the Walter & Elisa Hall Institute (Melbourne, Australia). Congenic BC20 mice (IgCH-1^a) were provided by The Jackson Laboratory (Bar Harbor, ME). The (C57BL/6 × BALB.Ig^b)F_1 and (BALB/c × BC20)F_1 hybrid mice, as well as the R_2 mice, obtained by backcrossing (BALB/c × BC20)F_1 females with BC20 males, were produced in our animal facilities.

Neonatal injection of F_1 cells

10^8 spleen cells from 2–3-month-old (C57BL/6 × BALB.Ig^b)F_1 (Igh^b) hybrid mice, were injected intraperitoneally (i.p.) into newborn R_2 (Igh^a) mice within 24 hr of birth, as previously described.[5] Control mice were uninjected littermates.

H-2 typing of R_2 mice

The characterization of the H-2 haplotype in R_2 mice was performed at 6 weeks of age. Blood samples (200 μl) were collected on tubes containing 50 μl of calcium heparin (25,000 U/ml) and incubated during 30 min in ice with an adequate concentration of FITC-conjugated MK.D6 (mouse IgG2a anti-mouse I-A^d) mAb.[18] Then, 2 ml of a lysis solution (FACS Brand lysing solution; Becton Dickinson, Mountain View, CA) was added to the pellet and incubated during 10 min at room temperature and washed twice in phosphate-buffered saline (PBS). Cell frequencies were analysed by flow cytometry using a FACScan (Becton Dickinson), with Lysys II computer equipment. R_2 mice having significant frequencies of I-A^d-positive cells were considered heterozygous (H-$2^{b/d}$) for the MHC complex, whereas those lacking these cells were evaluated as homozygous (H-$2^{b/b}$).

Serological analysis

Levels of IgG subclasses were determined by ELISA as described elsewhere.[4] Results are expressed in mg/ml by referring to normal mouse reference serum. To evaluate the levels of Ig bearing the donor Igh^b allotype in the serum of F_1 cell-injected R_2 mice, a previously described ELISA[4] was employed using an alkaline phosphatase-conjugated anti-Igh^b allotype antiserum, obtained as previously reported.[19,20] Results are expressed in mg-equivalent/ml of Igh^b bearing Ig from normal C57BL/6 serum. IgG and IgG subclass anti-single-stranded (ss) DNA antibody (Ab) and the Ig allotype of these Ab were determined by ELISA as described previously.[7] Results are expressed in titration units (TU), referring to a standard curve obtained by serial dilutions of a serum pool from 6–8-month-old MRL lpr/lpr mice or 6–8-month-old BXSB male mice. Levels of circulating immune complexes (CIC) were determined by conglutinin binding in solid phase, using the ELISA methodology previously described.[5] Bovine conglutinin was obtained from normal adult bovine serum, according to Maire *et al.*[21] Results are expressed in μg-equivalent by reference to a standard curve obtained by serial dilutions of heat-aggregated mouse IgG (AMGG), previously incubated for 45 min at 37° with fresh normal mouse serum as a source of complement.

Kidney studies

Kidney deposition of IgM, IgG subclasses and C3 was assessed by direct immunofluorescence on kidney cryocut sections (5–7 μm) using a fluoresceinated rabbit anti-mouse IgM (Sigma Chemical Co., St Louis, MO) or rabbit anti-mouse IgG subclass-specific antisera (Cappel Lab., Westchester, PA). The presence of C3 was detected with a fluoresceinated rabbit anti-rat C3 Ab cross-reactive with mouse C3 (Cappel Lab.).

Skin grafts

R_2 mice (6–8 weeks old) were implanted with tail skin grafts from three different donors: (1) syngeneic C57BL/6 (H-2^b) mice; (2) BALB/c (H-2^d) mice bearing the maternal alloantigens to which tolerance was analysed; and (3) third-party allogeneic B10.M (H-2^f) mice. In ketamine-anaesthetized mice (Ketalar, Parke Davis, Barcelona, Spain; 150 μg/g of body weight), three pieces of skin were removed from the back, replaced by the graft and stitched with fine surgical thread. Recipients were examined 14 and 21 days after grafting.

In vivo *evaluation of B-cell tolerance*

Cell transfer and peritoneal washes were done according to the methodology described elsewere.[22] Briefly, 2×10^7 spleen cells from 6–8-week-old (C57BL/6 × BALB.Ig^b)F_1 (Igh^b) mice depleted of T cells by *in vivo* anti-Thy-1.2 treatment (30H12 mAb),[23] were injected in the following groups of mice bearing the Igh^a allotype: BALB/c, (BALB/c × BC20)F_1, and R_2 mice. Peritoneal cells were recovered 6 days later by peritoneal washing of live mice with 10 ml of PBS. Cell suspensions were stained with FITC–MB.86 mAb (mouse IgG1 anti-mouse IgM^b)[24] and analysed by flow cytometry as described above. The number of donor cells present in the volume recovered was reported for 10 ml.

Statistical analysis

Comparison of serological parameters between groups was performed with the Wilcoxon two-sample test. For the

comparison of cytometry test, the Student's *t*-test was used. These analyses were done using the STATA computer software. Probability values greater than 5% were considered insignificant.

RESULTS

Experimental design

To study the influence of the maternal environment and the role of non-MHC alloantigens in the autoimmune syndrome developed in parental mice injected at birth with semi-allogeneic cells, (BALB/c × BC20)F_1 females were backcrossed with BC20 males. In this backcross two populations of H-$2^{b/b}$ homozygous and H-$2^{b/d}$ heterozygous R_2 mice were obtained. Both populations of R_2 mice, however, were genetically heterogeneous for maternal BALB/c non-MHC alloantigens. Interestingly, these R_2 mice had been developed in a common (BALB/c × BC20)F_1 maternal environment which had afforded them the opportunity to incorporate alloantigens from the BALB/c during the prenatal period, delivery or breast feeding. This fact could have mediated the tolerization of R_2 mice to NIMA (BALB/c) . It should be noted that all R_2 mice bear the BC20 genetic background.

One group of R_2 mice received at birth 10^8 spleen cells from (C57BL/6 × BALB.Ig^b)F_1 mice. Uninjected R_2 mice served as controls. At 6 weeks of age all animals were analysed for H-2 haplotype. Tolerance to NIMA was tested *in vivo* in R_2 (Igh^a) mice, injected at birth or not with (C57BL/6 × BALB.Ig^b)F_1 cells, using two procedures: (1) skin-graft from BALB/c (H-2^d), C57BL/6 (H-2^b) and third-party B10.M (H-2^f) mice, and (2) i.p. injection of B cells from (C57BL/6 × BALB.Ig^b)F_1 (Igh^b) mice, followed 6 days later by peritoneal washing and a donor IgM^b-positive B-cell count by flow cytometry.

Finally, the development of autoimmune manifestations in R_2 mice injected at birth with (C57BL/6 × BALB.Ig^b)F_1 spleen cells was evaluated by serological and histological studies.

The response against NIMA was not prevented by the maternal environment

Since R_2 mice had been developed in a (BALB/c × BC20)F_1 maternal environment, tolerance to NIMA (BALB/c) was evaluated firstly by studying the survival of skin grafts. R_2 mice injected at birth (eight mice) with 10^8 spleen cells from (C57BL/6 × BALB.Ig^b)F_1 mice, and R_2 control mice (17 mice), received skin grafts from C57BL/6, BALB/c and third-party B10.M mice. The persistence of these grafts was evaluated 3 weeks later (Table 1). All non-injected R_2 mice rejected BALB/c skin grafts independently of their H-2 haplotype. By contrast, homozygous R_2 mice injected at birth with (C57BL/6 × BALB.Ig^b)F_1 cells accepted BALB/c implants (Table 1). The third-party B10.M skin graft was rejected in all instances (data not shown). Because all R_2 mice shared BC20 alloantigens, skin grafts from syngeneic C57BL/6 mice were always accepted.

The induction of tolerance to NIMA was analysed secondly by the injection of R_2 (Igh^a) mice with B cells from (C57BL/6 × BALB.Ig^b)F_1 (Igh^b) donors. Six days later the persistence of donor B cells was measured in peritoneal exudates by flow cytometry with an anti-IgM^b mAb (Fig. 1). The recovery of donor B cells in R_2 mice injected at birth with (C57BL/6 × BALB.Ig^b)F_1 cells was similar to that found in syngeneic F_1 (Igh^a) mice ($P = 0{\cdot}7$). By contrast, the number of donor B cells obtained in peritoneal exudates from non-injected R_2 mice was significantly lower ($P < 0{\cdot}05$). This reduction was more marked in the group of H-$2^{b/b}$ homozygous R_2 mice.

Table 1. Evaluation of skin grafts in R_2 mice

	Source of skin grafts	
Groups of mice	C57BL/6	BALB/c
R_2 (H-$2^{d/b}$)	9/9	0/9
R_2 (H-$2^{b/b}$)	8/8	0/8
R_2 + F_1 cells at birth	8/8	8/8

R_2 mice, homozygous (H-$2^{b/b}$) and heterozygous (H-$2^{b/d}$) for MHC, were injected or not with spleen cells from (C57BL/6 × BALB.Ig^b)F_1 mice in the neonatal period. These mice were grafted with tail skin from syngeneic C57BL/6 mice and from BALB/c mice bearing NIMA. The persistence of skin grafts was evaluated 3 weeks later. Results show the number of mice in which the skin graft persisted, in relation to the number of grafted mice.

Differences in non-MHC alloantigens failed to induce an autoimmune syndrome in R_2 mice injected at birth with F_1 spleen cells

The role of non-MHC alloantigens in the development of autoimmunity after induction of neonatal tolerance with semi-allogeneic lymphoid cells was studied. For this purpose, 26 H-$2^{b/b}$ homozygous R_2 mice and 19 H-$2^{b/d}$ heterozygous R_2 mice were injected at birth with spleen cells from (C57BL/6 ×

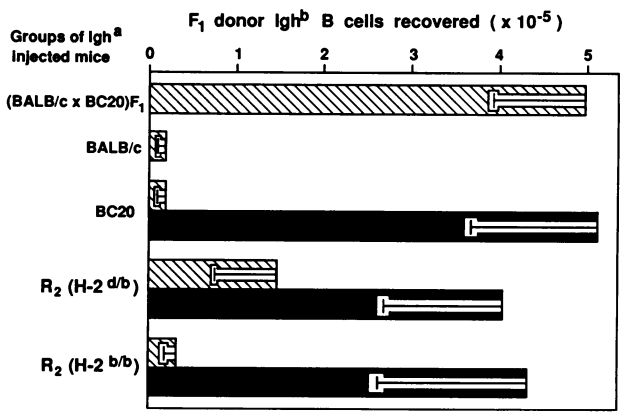

Figure 1. *In vivo* tolerance to F_1 donor B cells was analysed in several groups of adult mice (▨) bearing the Igh^a allotype. Some of these mice (■) had been injected at birth with spleen cells from (C57BL/6 × BALB.Ig^b)F_1 mice. For this purpose, all these mice were injected i.p. with 2×10^7 Thy-1-depleted spleen cells from (C57BL/6 × BALB.Ig^b)F_1 mice, bearing the Igh^b allotype. Peritoneal washes were performed in these mice and the number of recovered donor B cells was evaluated by flow cytometry with a FITC–anti-IgM^b mAb. Bars represent mean values (4–13 mice/group) and SEM.

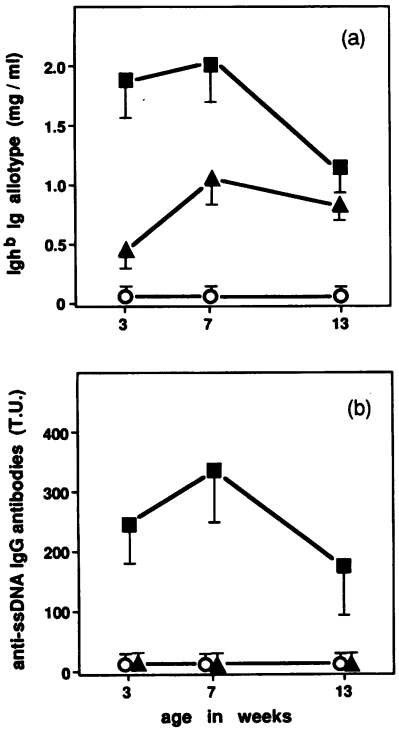

Figure 2. Serum levels of Ig bearing the F_1 donor Igh^b allotype (a) and of anti-ssDNA IgG Ab (b) in R_2 (Igh^a) mice. Groups of these mice, either homozygous (■) or heterozygous (▲) for the H-2 haplotype, were injected at birth with spleen cells from C57BL/6 × BALB.Ig^b)F_1 (Igh^b) mice. Unmanipulated R_2 mice were used as controls (○). For Igh^b allotype, results are expressed in mg-equivalent/ml Igh^b-bearing Ig from normal C57BL/6 serum. For anti-ssDNA IgG Ab, results are expressed in titration units. Means of 10–26 mice/group are represented (± SEM).

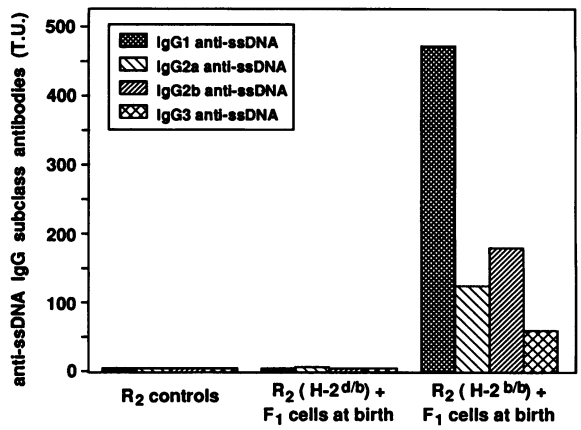

Figure 3. Serum titres of anti-ssDNA IgG subclasses in R_2 mice, either homozygous or heterozygous for the H-2 haplotype, injected at birth with spleen cells from (C57BL/6 × BALB.Ig^b)F_1 mice. Unmanipulated R_2 mice served as control. Results are expressed in titration units. Means of 10–26 mice/group are represented.

BALB.Ig^b)F_1 mice. In H-$2^{b/b}$ R_2 mice donor cells always differed in H-2 antigens and randomly in non-MHC antigens. In H-$2^{b/d}$ R_2 mice, donor cells shared MHC molecules but differed randomly in non-MHC antigens. The persistence of donor B cells was confirmed by the presence of significant serum levels ($P < 0.005$) of the donor Igh^b allotype in both H-2 homozygous and heterozygous R_2 mice (Fig. 2).

Serum levels of IgG subclasses remained in the normal range in the 19 H-$2^{b/d}$ heterozygous R_2 mice, injected with (C57BL/6 × BALB.Ig^b)F_1 cells at birth. However, all the 26 H-$2^{b/b}$ homozygous R_2 mice neonatally injected with (C57BL/6 × BALB.Ig^b)F_1 cells developed a marked hypergammaglobulinaemia compared with R_2 control mice. At 3 weeks of age, there was a 12-fold increase of IgG1 levels in (C57BL/6 × BALB.Ig^b)F_1 cell-injected H-$2^{b/b}$ R_2 mice compared with age-matched R_2 control mice and (C57BL/6 × BALB.Ig^b)F_1 cell-injected H-$2^{b/d}$ R_2 mice (Table 2).

The production of anti-ssDNA IgG Ab was also analysed in these mice. All R_2 H-$2^{b/b}$ mice showed abnormal levels ($P < 0.001$) of anti-ssDNA IgG Ab after neonatal injection of (C57BL/6 × BALB.Ig^b)F_1 cells (Fig. 2). These anti-ssDNA IgG Ab were exclusively of the Igh^b F_1 donor allotype (data not shown), and were predominantly of the IgG1 isotype (Fig. 3). By contrast, anti-ssDNA IgG Ab were not detected in heterozygous H-$2^{b/d}$ R_2 mice injected at birth with (C57BL/6 × BALB.Ig^b)F_1 cells (Fig. 2). Similar results were obtained when the levels of CIC were analysed in R_2 mice neonatally injected with (C57BL/6 × BALB.Ig^b)F_1 cells. Abnormal levels of CIC were found in all H-$2^{b/b}$ homozygous R_2 injected mice (33·4 ± 28·8 μg-equivalent AMGG/ml), in comparison to H-$2^{b/d}$ heterozygous R_2 mice injected with (C57BL/6 × BALB.Ig^b)F_1 cells ($P < 0.001$) which showed normal values

Table 2. Serum levels of IgG subclasses in R_2 mice injected at birth with F_1 cells

Groups of mice	Number of mice	IgG1*	IgG2a*	IgG2b*	IgG3*
R_2 controls	10	1·03 ± 0·20	0·04 ± 0·01	0·15 ± 0·03	0.09 ± 0·03
R_2 (H-$2^{d/b}$) + F_1 cells at birth	19	1·30 ± 0·63	0·21 ± 0·22	0·24 ± 0·05	0·15 ± 0·06
R_2 (H-$2^{b/b}$) + F_1 cells at birth	26	12·80 ± 6.80†	5·86 ± 1·81†	0·63 ± 0·32†	0·88 ± 0·51†

3-week-old R_2 mice, homozygous (H-$2^{b/b}$) or heterozygous (H-$2^{b/d}$) for the MHC locus, were injected at birth with spleen cells from (C57BL/6 × BALB.Ig^b) F_1 mice. Serum levels of IgG subclasses were analysed in these mice by ELISA.

*Results of 10–26 mice/group expressed in mg/ml as mean ± 1 SD.

†$P < 0.001$ compared with R_2 control mice or (C57BL/6 × BALB.Ig^b)F_1 cell-injected H-$2^{b/d}$ R_2 mice.

(1·9 ± 0·2 μg-equivalent AMGG/ml, versus 2·1 ± 0·6 μg-equivalent AMGG/ml in the R_2 controls; $P > 0.05$). Immunofluorescence studies on kidney sections, performed at 5 and 20 weeks of age (three to seven mice in each group), showed prominent deposits of C3, IgM and all IgG subclasses in the renal glomeruli of H-$2^{b/b}$ R_2-injected mice, whereas in heterozygous H-$2^{b/d}$ R_2 mice neonatally injected with (C57BL/6 × BALB.Ig^b)F_1 cells only scarce deposits of IgM were found (data not shown).

All these results indicate that allogeneic MHC antigens are responsible for the production of the autoimmune syndrome in this model, and exclude the possibility that non-MHC alloantigens could contribute to the abnormal interaction between host alloreactive $CD4^+$ T cells and donor B cells, responsible for the autoimmune manifestations in mice neonatally injected with semi-allogeneic lymphoid cells.

DISCUSSION

The results presented here clearly emphasize the central role of MHC class II antigens in the allogeneic collaboration between alloreactive $CD4^+$ T cells and semi-allogeneic B cells. In previous studies, we have demonstrated that the injection of semi-allogeneic spleen cells from (C57BL/6 × BALB/c)F_1 (H-$2^{b/d}$) mice into parental newborn BALB/c (H-2^d) mice results in both the development of a self-limited autoimmune syndrome and the induction of cytolytic unresponsivenees to allogeneic cells.[3] In this model, host $CD4^+$ T cells are responsible for the activation of autoreactive donor B cells.[7,11,25] These donor B cells are polyclonally activated through an allogeneic interaction, as demonstrated in experiments using hapten–carrier immunizations, and produce the totality of autoAb detected in these animals.[4] Interestingly, the predominance of IgG1 and IgE isotypes in the Ig response,[4,26] together with the therapeutic effect of anti-IL-4 mAb,[10] and the increased frequency in IL-4 producing $CD4^+$ T cells,[8,9] indicate that host Th2 cells are essential in the induction of autoimmune manifestations in this model. Moreover, using recombinant mouse strains it has been demonstrated that the injection of newborn mice with semi-allogeneic B cells, differing only at the level of MHC class II molecules, promotes a typical lupus-like syndrome.[12,13] In contrast, the injection of cells differing only at the level of MHC class I or Mls-1^a antigens was accompanied by the induction of specific cytolytic T-cell tolerance, but not by the presence of an autoimmune disease.[12] However, the possibility that other alloantigens not related with the MHC gene locus could be involved in the allogeneic interactions between host alloreactive Th2 cells and donor B cells has not been evaluated previously.

According to the experimental design described above, a population of R_2 mice was obtained in which about half of the animals were homozygous or heterozygous for the H-2 haplotype. However, these two groups were statistically heterogeneous for the rest of non-MHC alleles. This model allowed us to evaluate whether non-MHC NIMA are able to trigger allogeneic reactions, either cytolytic responses on tissue grafts or helper responses on semi-allogeneic B cells. Thus, it was shown that unmanipulated R_2 mice were able to reject skin grafts or B cells bearing maternal antigens, independently of their MHC compatibility, providing evidence of the capacity of non-MHC antigens to mediate cytolytic allogeneic responses. This ability was efficiently inhibited in all R_2 mice after neonatal injection of maternal-derived (C57BL/6 × BALB.Ig^b)F_1 cells, as documented by the persistence of donor allografts and the evidence of chimerism.

The capacity of the non-MHC NIMA to trigger an allohelper co-operation was evaluated further in R_2 mice by studying the development of autoimmune manifestations after neonatal injection with (C57BL/6 × BALB.Ig^b)F_1 (H-$2^{b/d}$) spleen cells. In this respect, a clear dichotomy was observed between MHC compatible or incompatible R_2 mice. Thus, neonatally injected homozygous H-$2^{b/b}$ R_2 mice presented a typical autoimmune syndrome,[4,26] in which donor B cells selectively produced autoAb, preferentially of the IgG1 isotype. By contrast, heterozygous H-$2^{b/d}$ R_2 mice injected at birth with (C57BL/6 × BALB.Ig^b)F_1 (H-$2^{b/d}$) spleen cells were unable to develop autoimmune manifestations despite the presence of functional donor B cells. These results were confirmed using a different strain combination [BALB/c and C3H (H-2^k) mice] and the same experimental protocol. In this experiment only H-$2^{k/k}$ R_2 mice, but not H-$2^{k/d}$ R_2 mice, injected at birth with (C3H × BALB.Ig^b)F_1 (H-$2^{k/d}$) spleen cells, developed autoimmune manifestations (unpublished results).

The present results are in agreement with our previous observations showing that parental mice, injected at birth with semi-allogeneic cells differing exclusively in either class I or Mls-1^a antigens, failed to develop an autoimmune syndrome.[12] All these observations indicate that the allogeneic interaction between alloreactive host Th2 cells and semi-allogeneic donor B cells observed in this model occurs uniquely through the recognition of allogeneic MHC class II molecules. In addition, the recognition of other alloantigens presented by MHC class II molecules does not trigger the polyclonal activation of donor B cells. Although one cannot totally exclude the possibility that other alleles, closely linked to the H-2 complex, could be important in the allogeneic co-operation in this model, the fact that none of the 19 heterozygous H-$2^{b/d}$ R_2 mice developed an autoimmune syndrome argues against such a possibility.

Moreover, our data could shed some light on the discussion about the transmission of tolerance to NIMA. In previous reports it was suggested that, in humans, the maternal environment influences the induction of tolerance to NIMA.[14] More recently the passage of immunocompetent live cells from mother to newborn through breast feeding has been documented, and it has been suggested that a breast feeding history could have beneficial effects in patients that receive a maternal renal allograft.[27] In this respect, a recent study showing that in patients waiting for a renal allograft there was a preferential unresponsiveness to NIMA is relevant, indicating that the mother can induce partial but lifelong tolerance in some of her children.[15] However, other authors have shown that kidney grafts coming from the mother have a worse outcome than those from paternal origin.[16]

In mice, several authors have also described the placental passage, from the mother to the fetus, of a significant number of cells which could play a role in the establishment of an adequate tolerance to NIMA.[28,29] Conversely, recent experiments indicated that such a passage is a rare event and generally related to placental abnormalities.[30,31] Nevertheless, it has been reported that mothers can transfer to newborns signals able to neutralize allogeneic reactions. Thus, offspring from mothers immunized with alloantigens failed to develop graft-versus-host disease[32,33] and runting disease[34] upon

neonatal challenge with allogeneic cells. The nature of such a negative signal remains elusive. Whereas certain authors claim that the protection is mediated by Ab,[33,34] others propose that it is related to the transmission of suppressive T-cell factors through the placenta.[35] Although this situation is not comparable to our model, in which mothers are not manipulated before or during pregnancy, the present studies indicate that the hypothetical transmission of both maternal antigens and possible suppressive factors is not sufficient either to induce an effective tolerance to NIMA or to prevent allohelper reactions.

In summary, we present evidence that the neonatal tolerization to non-MHC alloantigens, which can mediate the rejection of tissue grafts, is not followed by the triggering of a polyclonal activation of donor B cells, as has been demonstrated after induction of neonatal tolerance to class II MHC alloantigens. Furthermore, the maternal environment (i.e. placental passage or breast suckling), which could represent for the fetus a potential source of alloantigens or suppressive factors, does not prevent the neonatal Th2 alloreactivity to non-inherited maternal-derived class II MHC alloantigens.

ACKNOWLEDGMENTS

We thank Mr G. Brighouse (C.M.U., University of Geneva) for providing bovine conglutinin; Mrs Blanca Renedo (Hospital Universitario Marqués de Valdecilla, Santander) for technical assistence; Dr Veronique Thomas-Vaslin (Institute d'Embriologie Cellulaire et Moleculaire, Nogent sur Marne, France) and Drs José Luis Fernández Luna and Francisco Leyva-Cobián (Hospital Universitario Marqués de Valdecilla, Santander) for helpful and stimulating discussions.

This work has been supported by the 'Comisión Interministerial de Ciencia y Tecnología', Spain (grant no. PM89–0153) and the 'Fundación Ramón Areces', Madrid, Spain. A. Ramos was partially supported by the 'Fundación Marcelino Botín', Santander, Spain.

REFERENCES

1. Goldman M., Feng H.M., Engers H., Hochman A., Louis J. & Lambert P.H. (1983) Autoimmunity and immune-complex disease after neonatal induction of transplantation tolerance in mice. *J. Immunol.* **131,** 25.
2. Stott D.I., Merino J., Schurmans S. & Lambert P.H. (1988) Expression of anti-DNA clonotypes and the role of helper T-lymphocytes in the autoimmune response in mice tolerant to alloantigens. *Autoimmunity*, **1,** 253.
3. De la Hera M., De la Hera A., Ramos A., Buelta L., Alonso J.L., Rodriguez-Valverde V. & Merino J. (1992) Self-limited autoimmune disease related to transient B cell chimerism in mice neonatally injected with semiallogeneic F1 cells. *Int. Immunol.* **4,** 67.
4. Luzuy S., Merino J., Engers H., Izui S. & Lambert P.H. (1986) Autoimmunity after induction of neonatal tolerance to alloantigens: role of B cell chimerism and F1 donor B cell activation. *J. Immunol.* **136,** 4420.
5. Merino J., Schurmans S., Luzuy S., Izui S., Vassalli P.A. & Lambert P.H. (1987) Autoimmune syndrome after induction of neonatal tolerance to alloantigens. Effects of *in vivo* treatment with anti-T cell subsets monoclonal antibodies. *J. Immunol.* **139,** 1426.
6. Merino J., Alonso J.L., Ramos A., Gonzalez A., Rodriguez-Valverde V. & De la Hera M. (1990) Depletion of $CD8^+$ T cells does not modify the evolution of the autoimmune syndrome observed in mice tolerized to alloantigens. *Allergy Immunol.* **9,** 227.
7. Merino J., Schurmans S., Duchosal M., Izui S. & Lambert P.H. (1989) Autoimmune syndrome after induction of neonatal tolerance to alloantigens: $CD4^+$ T cells from the tolerant host activate autoreactive F_1 B cells. *J. Immunol.* **143,** 2202.
8. Mosmann T.R. & Coffman R.L. (1989) TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.* **7,** 145.
9. Powell T.J. & Streilein J.W. (1990) Neonatal tolerance induction by class II alloantigens activates IL-4-secreting, tolerogen-responsive T cells. *J. Immunol.* **144,** 854.
10. Schurmans S., Heuser C., Qin H.-Y., Merino J., Brighouse G. & Lambert P.H. (1990) *In vivo* effects of anti-IL-4 monoclonal antibody on neonatal induction of tolerance and on a associated autoimmune syndrome. *J. Immunol.* **145,** 2465.
11. Merino J., Schurmans S., Wen L., Brighouse G., Luzuy S. & Lambert P.H. (1990) Autoimmune syndrome after induction of neonatal tolerance to alloantigens: analysis of the role of donor T cells in the induction of autoimmunity. *Clin. exp. Immunol.* **79,** 273.
12. Schurmans S., Brighouse G., Kramar G., Wen L., Izui S., Merino J. & Lambert P.H. (1991) Transient T and B cell activation after induction of neonatal tolerance to MHC class II or Mls alloantigens. *J. Immunol.* **146,** 2152.
13. Gonzalez A.L., Conde C., Revilla C., Ramos A., Renedo B. & Merino J. (1993) Autoimmune syndrome after induction of neonatal tolerance to I-E antigens. *Eur. J. Immunol.* **23,** 2353.
14. Desai R.G. & Creger W.P. (1963) Maternofetal passage of leukocytes and platelets in man. *Blood*, **21,** 665.
15. Claas F.H.J., Gijbels Y., van del Velden-de Munch J. & van Rood J.J. (1988) Induction of B cell unresponsiveness to noninherited maternal HLA antigens during fetal life. *Science*, **241,** 1815.
16. Panajotopoulos N., Ianhez L.E., Newmann J., Sabbaga E. & Kalil J. (1990) Immunological tolerance in human transplantation. The possible existence of a maternal effect. *Transplantation*, **50,** 443.
17. Tyzzer E.E. (1909) A study of inheritance in mice with reference to their susceptibility to transplantable tumours. *J. Med. Res.* **21,** 519.
18. Kappler J.W., Skidmore B., White J. & Marrack P. (1981) Antigen inducible, H-2 restricted, interleukin-2-producing hybridomas. *J. exp. Med.* **153,** 1198.
19. Lieberman R. (1978) Genetics of IgCH (allotype) locus in the mouse. *Springer Semin. Immunopathol.* **1,** 7.
20. Avrameas S. (1969) Coupling of enzymes to proteins with glutaraldehyde. Use of the conjugates for the detection of the antibodies. *Immunochemistry* **6,** 43.
21. Maire M.A., Barnet M. & Lambert P.H. (1981) Purification of bovine conglutinin using pepsin digestion. *Molec. Immunol.* **18,** 85.
22. Thomas-Vaslin V. & Freitas A. (1990) Population kinetics of peritoneal LPS-reactive B lymphocytes. *Int. Immunol.* **2,** 74.
23. Ledbetter J.A. & Herzenberg L.A. (1979) Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. *Immunol. Rev.* **47,** 63.
24. Nishikawa S., Sasaki Y., Kina T., Amagai T. & Katsura T. (1986) A monoclonal antibody against Igh 6.4 determinant. *Immunogenetics*, **23,** 137.
25. Martínez A.C. & Coutinho A. (1981) B cell activation by helper cells is a two step process. *Nature*, **290,** 60.
26. Goldman M., Abramowicz D., Lambert P., Vandervorst P., Bruyns C. & Toussaint C. (1988) Hyperactivity of donor B cells after neonatal induction of lymphoid chimerism in mice. *Clin. exp. Immunol.* **72,** 79.
27. Campbell D.A., Lorber M.I., Sweeton J.C., Turcotte J.G., Niederhuber J.E. & Beer AE. (1984) Breast feeding and maternal-donor renal allografts. Possibly the original donor-specific transfusion. *Transplantation*, **37,** 340.

28. Barnes R.D. & Holliday J. (1970) The morphological identity of maternal cells in newborn mice. *Blood*, **36,** 480.
29. Tuffrey M., Bishun N.P. & Barnes R.D. (1969) Porosity of the mouse placenta to maternal cells. *Nature*, **221,** 1029.
30. Jacoby D.R., Olding L.B. & Oldstone M.B.A. (1984) Inmunologic regulation of fetal-maternal balance. *Adv. Immunol.* **35,** 157.
31. Hunziker R.D., Gambel P. & Wegmann T.G. (1984) Placenta as a selective barrier to cellular traffic. *J. Immunol.* **133,** 667.
32. DeGiorgi L., Povey S., Habeshaw J.A. & Davies A.J.S. (1989) Prevention of graft-versus-host disease in neonatal F1 hybrid mice by pre-immunization of their mother with paternal spleen cells. *Transpl. Proc.* **21,** 3045.
33. DeGiorgi L., Habeshaw J.A., Povey S. & Matossian-Rogers A. (1990) Reduction of graft-versus- host disease in neonatal F1 hybrid mice. *Clin. exp. Immunol.* **79,** 130.
34. Matossian-Rogers A. & DeGiorgi L. (1991) Unresponsiveness to Mls^a induced in newborn Mls^b mice by maternal preinmunization. *Immunology*, **72,** 219.
35. Fujii Y. & Yamaguchi N. (1992) Maternal T cells of immunized pregnant mice induce immune suppression in their offspring. *Immunology*, **77,** 171.