Clin. exp. Immunol. (1990) **79**, 130–134

Reduction of graft-*versus*-host disease in neonatal F1 hybrid mice

L. DEGIORGI, J. A. HABESHAW*, S. POVEY† & A. MATOSSIAN-ROGERS
*Department of Immunology, London Hospital Medical College, London; *MRC, Clinical Research Centre, Harrow; and †MRC Human Biochemical Genetics Unit, Galton Laboratory, University College, London, England*

(Accepted for publication 15 August 1989)

SUMMARY

Pre-immunization of BALB/c (H-2^d) mothers with C57BL/10 (H-2^b) or CBA/H (H-2^k) spleen cells partially protected the F1 hybrid offspring of (BALB/c × C57BL/10) or (BALB/c × CBA/H) matings from graft-*versus*-host-disease (GVHD) induced by neonatal intraperitoneal inoculation with spleen cells of the paternal strain. The effects achieved were manifest as a reduction in mortality. Experiments to establish whether the phenomenon was antibody mediated were performed by passive pre-immunization of BALB/c mothers with alloantisera obtained from BALB/c previously immunized with C57BL/10 spleen cells. Alloantisera produced an equivalent reduction in GVHD mortality. Some of the F1 mice that survived challenge with paternal strain spleen cells were proven to be haemopoietic chimaeras using immunofluorescence with anti-MHC monoclonal antibodies and polymorphism of the enzyme glucose–phosphate–isomerase present in the strains used. The possible mechanisms of protection from GVHD in our mouse model are discussed.

Keywords maternal isoimmunization abrogation of GVHD

INTRODUCTION

Graft-*versus*-host-disease (GVHD) was first described by Simonsen (1957), who also established the genetic basis of the reaction in chickens. Subsequent studies demonstrated the same phenomenon in mice (Billingham, 1968).

The injection of mature spleen or bone marrow cells into recipient animals incapable of their rejection initiates GVHD.

It is now well established that T lymphocytes are responsible for GVHD, and in clinical practice T cell depletion of donor bone marrow is used routinely to prevent GVHD in humans (Butturini & Gale, 1988).

GVHD can be prevented experimentally by inducing tolerance neonatal recipients to parental MHC antigens. This is classically obtained by injecting immunologically incompetent T cells of the parental strain. Neonatal tolerance can also be achieved by inducing tolerance of the mother during pregnancy, whereby immune unresponsiveness is transmitted to the neonate (Auerbach & Clark 1975), or by immunizing the mother during pregnancy, to alter the expression of allotypic (Herzenberg & Herzenberg, 1974; Herzenberg, Okomuza & Metzler, 1975) and idiotypic determinants in neeonatal F1 progeny (Wilker *et al.*, 1980). In addition, passive immunization with anti-idiotypic antibody in the neonate (Berek, 1983; Takemori & Rajewsky, 1984) or in young adult mice (Bernabe *et al.*, 1981) alters the immune repertoire of the recipient mice significantly by suppressing the strain-specific immunodominant idiotype.

The precise way by which immunological manipulations during pregnancy produce effects such as the transmission of tolerance is not known, although permanent alterations can be produced in the neonatal T cell immune repertoire by preventing exposure of T cells to strain-specific immunoglobulin idiotype. This has been achieved by suppression of B cell development with anti-μ heavy chain antiserum (Sy *et al.*, 1984; Martinez *et al.*, 1984, 1985).

The neonatal immune system, including the repertoire of B and T cell antigen receptors (idiotypes) is thus mutable either by exposure to passively administered antibody or by antibody acquired through transplacental or colostral passage. Adult F1 rats can be made resistant to lethal semi-allogeneic GVHD by prior exposure to small doses of parental T lymphocytes, or to bone marrow cells. This resistance is radioinsensitive and adoptively transferrable by serum (Bellgrau, Smilek & Wilson, 1982).

We have similarly attempted to induce resistance in mice to lethal semi-allogeneic GVHD by maternal immunization with paternal strain spleen cells. Anti-H-2 alloantiserum was also used for repeated maternal immunization.

The F1 hybrid offspring of mothers pre-immunized with allogeneic spleen cells or passively immunized with anti-H-2 alloantiserum proved partially resistant to the induction of GVHD following intraperitoneal inoculation with letal doses of paternal strain spleen cells.

Correspondence: Dr L. DeGiorgi, Department of Immunology, London Hospital Medical College, Turner Street, London E1 2AD, England.

In some F1 mice a state of cellular chimaerism was detected, and most mice surviving initial challenge remained healthy for over 1 year.

MATERIALS AND METHODS

Mice

BALB/c (H-2[d]), GPI[a] (Glucose–phosphate–isomerase), C57BL/10 (H-2[b]), GPI[b] and CBA/H (H-2[k]), GPI[b] mice were obtained from either St Mary's Hospital Medical School Animal Department or the Animal Unit of The London Hospital Medical College. They were used at between 6 and 8 weeks of age (15 to 20 g weight). (BALB/c × C57BL/10) and (BALB/c × CBA/H)F1 hybrids were generated from the appropriate matings.

Cells

Spleens were removed using sterile technique, from C57BL/10 and CBA/H mice and the cells suspended in RPMI tissue culture medium (Gibco, Grand Island, NY).

Immunization of BALB/c females

Allogeneic spleen cells. In most experiments, immunization was performed by i.p. injections of 4×10^7 washed viable spleen cells/mouse, once a week on four occasions prior to pregnancy. Various other schedules of immunization, including supplementary injections during and after pregnancy were tried, but none seemed to offer significant advantages. Control animals were not immunized.

Alloantiserum. Female BALB/c mice received four weekly i.p. injections of 0·1 ml of pooled antiserum raised in BALB/c against C57BL/10 lymphocytes. They were then mated with C57BL/10 males and the pregnant females received 3–4 further injections of the pooled serum. As control a group of female BALB/c maintained under the same conditions as the experimental mice received repeated i.p. injections of 0·1 ml of autologous BALB/c serum, obtained from young animals.

Test challenge of F1 hybrid mice

A number of litters of animals born to treated or control females were injected i.p. with $2–4 \times 10^7$ cells of paternal genotype within 48 h of birth. These animals were inspected daily, and any mortality was recorded.

Isoenzyme and fluorescence studies

Surviving mice selected at random were studied for expression of H-2[d] alloantigen and GPI isoenzyme profile at intervals throughout the experiment. With (BALB/c × C57BL/10)F1 hybrids, spleen cells were analysed by immunofluorescence microscopy for expression of H-2[d] class I antigens of the BALB/c strain, and were also examined for the presence of excess GPI of type *b* electrophoretic mobility. (BALB/c × CBA/H)F1 hybrids were studied only with reference to the isoenzyme pattern.

Immunofluoresence. Fifty microlitres of spleen cell suspension containing 1×10^6 cells were stained with 50 μl of a 1:40 dilution of monoclonal anti-H-2D[d] antibody (kindly donated by Professor H. Festenstein, Department of Immunology, London Hospital Medical College, London). Staining was continued for 30 min at 4°C; the cells were then washed three times in a large excess of tissue culture medium, and reacted with a 1:50 dilution of FITC-coupled goat anti-mouse immunoglobulin (Coulter Clone, Hialeah, FL) for a further 30 min at 4°C. In each experimental determination, the same cell suspension was treated with the same dilution of goat anti-mouse immunoglobulin antibody, but with the omission of the primary anti-H-2D[d] monoclonal. After three further washes, the cells were resuspended in 50 μl of phosphate-buffered saline (PBS) and examined by fluorescence microscopy (Leitz Laborlux 12). Results were expressed as the means of multiple determinations ± s.d.

GPI. Blood lysates or aqueous extracts of spleen (approximately 1:1, w/v) were analysed by horizontal starch gel electrophoresis in a gel containing 11·5% hydrolysed starch (Connaught) in 0·01 M PBS, pH 7, run at 3·5 V/cm for 20 h with cooling to 6°C. The bridge buffer was 0·1 M, pH 7. GPI activity was detected using an overlay gel of 2% noble agar (Difco) containing the reaction mixture (fructose-6-phosphate, NADP glucose-6-phosphate dehydrogenase, PMS (Boehringer), and MTT (Sigma) according to the method described by Harris & Hopkinson (1976).

RESULTS

Effect of maternal pre-immunization with allogeneic paternal spleen cells on the development of GVHD in neonatal F1 mice

Two groups of experiments were carried out using C57BL/10 and CBA/H mice as paternal strains. Female BALB/c were pre-immunized with paternal spleen cells from either C57BL/10 (group 1) or CBA/H (group 2) before and during pregnancy (Table 1). Their F1 offspring received, 48 h after birth, high doses of paternal spleen cells ranging from 1×10^7 to 4×10^7 cells per mouse. The mice were observed daily for gross evidence of GVHD. In the first group of experiments (Table 1) 66 (BALB/c × C57BL/10)F1 mice from mothers pre-immunized with C57BL/10 were injected with paternal spleen cells; 48 (72%) of these mice survived without evidence of GVHD.

In the second group 87 (BALB/c × CBA/H)F1 mice were challenged with CBA/H spleen cells and 70 (85%) survived without evidence of GVHD.

In both groups after paternal spleen cell inoculation the F1 mice showed an initial failure to gain weight between days 5 and 11, but the majority thereafter fully recovered and survived GVHD for over 1 year. The mice that died showed gross and microscopic evidence of GVHD with loss of coat condition, diarrhoea, weight loss and finally death between 17 and 23 days after inoculation of paternal cells. For control purposes F1 mice from mothers pre-immunized with paternal spleen cells were challenged with maternal spleen cells and F1 mice from non-immunized mothers were challenged with paternal spleen cells. In both these groups all mice died of GVHD (Table 1). F1 mice from mothers pre-immunized with third-party H-2 antigens were challenged 48 h after birth with paternal spleen cells, and all (100%) survived GVHD. There was cross-protection, probably due to a cross-reactivity of maternal response to both strains of mice C57BL/10 and CBA/H. Maternal pre-immunization did not adversely affect the course of pregnancy in either (BALB/c × C57BL/10) or (BALB/c × CBA/H) matings. There was no significant effect on litter size or sex ratio at birth. No differences were observed in subsequent growth rate or post-natal development between F1 mice from non-immunized mothers and untreated F1 mice from pre-immunized mothers.

Table 1. Survival from graft-*versus*-host disease (GVHD) of (BALB/c × C57BL/10) and (BALB/c × CBA/H)F1 hybrid mice given i.p. injections of 10^7 to 4×10^7 paternal strain spleen cells 48 h after birth

Experimental groups	Maternal immunization	Paternal strain injection in F1 mice	Maternal strain injection in F1 mice	F1 offspring injected	F1 mice surviving GVHD	
				n	*n*	%
1	+ C57BL/10	C57BL/10	–	66	48	72
	–	C57BL/10	–	37	2	5
	+ C57BL/10	–	+ BALB/c	9	0	–
	+ CBA/H*	C57BL/10	–	11	11	100
2	+ CBA/H	CBA/H	–	87	70	80
	–	CBA/H	–	95	5	5
	+ CBA/H	–	+ BALB/c	7	0	–
	+ C57BL/10*	CBA/H	–	8	8	100

Some BALB/c mothers were immunized against paternal lymphocytes (see Materials and Methods) and some with third-party lymphocytes for specificity controls. F1 mice from pre-immunized mothers receiving i.p. maternal strain spleen cells did not survive GVHD.

* Immunization of female BALB/c with third-party lymphocytes.

Table 2. Survival from graft-*versus*-host disease (GVHD) of (BALB/c × C57BL/10)F1 hybrid mice given i.p. injections of 3×10^7 C57BL/10 spleen cells 48 h after birth

Experiment	Paternal strain	Maternal immunization by i.p. injection of serum	No. of F1 injected 48 h after birth	F1 mice surviving GVHD	
				n	%
1	C57BL/10	BALB/c anti-C57BL/10 serum	17	14	82
2	C57BL/10	Autologous serum	6	0	0

Some mothers were immunized with anti-paternal serum raised in BALB/c background against C57BL/10 antigens and some, as control, with autologous serum.

Effect of maternal pre-immunization with alloantiserum against H-2 paternal antigens on GVHD development in F1 hybrid mice

To demonstrate the involvement of antibody in the protection of F1 hybrid neonatal mice from GVHD unprimed BALB/c females were immunized weekly for 7–8 weeks with antiserum obtained from BALB/c mice previously immunized with C57BL/10 spleen cells. The immunized BALB/c females were then mated with C57BL/10 males; the F1 offspring received 3×10^7 paternal C57BL/10 spleen cells 48 h after birth.

Table 2 shows that of 17 F1 progeny of these matings injected with paternal strain spleen cells, 14 (82%) survived GVHD and remained healthy for over 1 year. As control, (BALB/c × C57BL/10)F1 mice from BALB/c mothers pre-immunized with autologous serum, did not survive GVHD.

Immunofluorescence

The number of spleen cells expressing the H-2^d antigen and the number of cells positive by expression of surface immunoglobulin are given in Table 3. Spleen cells from C57BL/10 (H-2^b) were negative for H-2^d expression within the limits of this technique, whereas BALB/c spleen cells were positive, as expected. The normal F1 hybrid (BALB/c × C57BL/10) (H-$2^{d,b}$) showed the majority of spleen cells positive with this antibody, whether derived from normal matings or from pre-immunized mothers. Between 36 and 105 days after birth, seven experimental (BALB/c × C57BL/10)F1 hybrids (from mothers immunized with C57BL/10 spleen cells) which had subsequently received C57BL/10 spleen cells 48 h after birth showed significantly depressed expression of H-2^d compared with normal controls. Differences in H-2^d antigen expression in these animals amounted to a 37% decrease in H-2^d-expressing cells and indicated either the presence of a substantial H-2^b-positive C57BL/10 derived spleen cell population, or, alternatively, an active suppression of expression of the H-2^d antigen in some F1 hybrid recipients of C57BL/10 spleen cells.

The immunofluorescent profile of the spleen cell population in the F1 mice considered as chimaeric resembled that of the control normal C57BL/10 mice.

GPI polymorphism

The results obtained from this study are illustrated in Fig. 1. A total of 14 mice, (BALB/c × C57BL/10)F1 which received paternal spleen cells C57BL/10 48 h after birth, were studied between 36 and 150 days and showed unequivocal evidence of chimaerism. A further group, of 48 mice analysed between 150 days and beyond 200 days after birth failed to show any evidence of chimaerism. An example of the results obtained by this technique is given in Fig. 1. In this example, a clear dominance of the fast (b) form of GPI from a 'chimaeric animal' (track 5, Fig. 1) is evident when compared with the normal F1 (track 4, Fig. 1). The relative intensity of the different isoenzymes suggested a high degree of cellular chimaerism (>40%). F1 hybrid recipients of paternal strain spleen cells

Table 3. Immunofluorescence analysis of MHC class I antigens detected by monoclonal anti-H-2D^d on spleen cells of control and experimental F1 hybrid mice surviving graft-*versus*-host disease after injection with paternal spleen cells

Mice	Expressed H-2	No. of mice	Mean percentage* positive cells with Anti-2D^d	Mean percentage* positive cells with Goat anti-mouse immunoglobulin	Mean percentage* negative cells with anti-H-2D^d
C57BL/10	b	4	10±5	9±4	89±5
BALB/c	d	4	87±2	11±4	12±2
(BALB/c × C57BL/10)F1	db	5	66±5	7±4	34±5
(BALB/c × C57BL/10)F1 injected with C57/BL/10 spleen cells	db+b	7	29±4	12±5	71±4

* ±s.d.

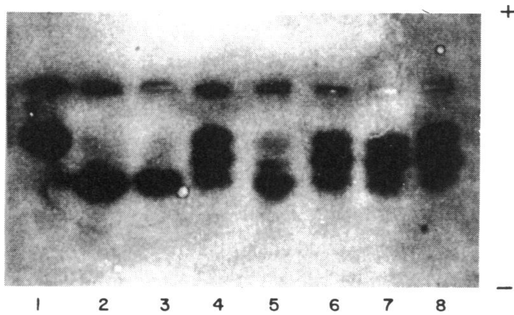

Fig. 1. Starch–gel electrophoresis of glucose–phosphate–isomerase (GPI) in mouse spleen of (1) BALB/c (GPI phenotype a); (2) C57BL/10 (phenotype b); (3) CBA/H (phenotype b); (4) (BALB/c × C57BL/10)F1 (phenotype ab); (5) Experimental group 1 (Table 1): (BALB/c × C57BL/10)F1 receiving at 48 h after birth C57BL/10 spleen cells (phenotype ab + b); (6) (BALB/c × CBA/H)F1 (phenotype ab); (7) Experimental group 2 (Table 1): (BALB/c × CBA/H)F1 receiving at 48 h after birth CBA/H spleen cells (phenotype ab); and (8) (BALB/c × CBA/H)F1 from pre-immunized mothers but not challenged with CBA/H paternal spleen cells (phenotype ab).

which were not chimaeric (track 7, Fig. 1) had a GPI profile indistinguishable from the F1 hybrid controls (tracks 6, 8, Fig. 1).

DISCUSSION

Hyperimmunization of female mice with spleen cells of an H-2 allogeneic paternal strain, before and during pregnancy, confers to the newborn F1 mice the capacity to resist GVHD upon injection of the H-2 allogeneic paternal strain lymphocytes.

The mechanism at present is not clear, but preliminary experiments using alloantiserum against paternal antigens (Table 2) as an immunogenic agent for unprimed female BALB/c, led us to the conclusion that the phenomenon described in this report could be antibody mediated. At this stage we do not know the nature of the specific antibodies involved, nor the mechanism which blocks GVH reactions. The network hypothesis of Jerne (1974) and the concept of antibody cascade resulting in the development of anti-idiotypic antibodies specific for the antigen binding site (Jerne, Roland & Cazenave, 1982) could explain the blocking of GVHD in F1 mice.

The observed effects are due to the passage of some humoral component, probably maternal immunoglobulins (particularly of IgG class) to the F1 offspring through the placenta, yolk sac and milk (Adeniyi-Jones & Ozato, 1987).

The level of protection of the F1 hybrids recorded by this technique, ranging between 72 to 82% (Tables 1 and 2) is similar to that described by Batchelor & Howard (1965) in which 71% of (A × C57)F1 hybrids survived when challenged with 5×10^7 spleen cells, together with anti-A serum prepared in the C57 parental strain.

The precise experimental procedure described here has not to our knowledge been previously reported. The two new features of our mouse model are immunization prior to and during pregnancy with paternal antigens, either by injection of paternal strain spleen cells or serum from BALB/c mice immunized against paternal lymphocytes; and protection from GVHD of neonate F1 hybrids following inoculation of paternal spleen cells and not maternal strain spleen cells (Table 1). For specificity studies BALB/c mothers of (BALB/c × C57BL10)F1 mice were pre-immunized with CBA/H and those of (BALB/c × CBA/H)F1 mice were pre-immunized with C57BL/10. The F1 mice were challenged with the respective paternal strain lymphocytes. Our data show that 100% of these F1 mice survived GVHD (Table 1), which indicates that antibodies against private H-2 specificities may not be directly involved in the abrogation of GVHD.

Several mechanisms may be responsible for the protective effect of prenatal immunisation: the development of anti-H-2 cytotoxic antibodies; T cell receptor blockade by anti-idiotypic antibodies; development of suppressor cells; and elimination of activated cells *in vivo* by lymphokine-activated killer (LAK) cells formed in the offspring of hyperimmunized mothers.

At present we cannot distinguish between the latter three possibilities. We can, however, assume that the development of cytotoxic antibodies against paternal cells is unlikely to be the cause. The specificity control data together with the presence of paternal cellular chimaerism detected in a small number of F1 mice surviving GVHD would exclude this possibility. We

cannot explain why only few mice which survived GVHD express paternal grafted cells. This may be due to the limitations of our techniques which can only detect cellular chimaerism of above 10%.

Although we need more information to identify the mechanisms responsible for the protection against GVHD of newborn mice after pre-immunization of the mother, we believe that this mouse model can offer a new approach to the study of the induction of transplantation tolerance and the elucidation of the roles of the major and minor histocompatibility loci in GVHD. The protocol is simple to perform and obviates the need to employ T cell depletion of donor inocula or immunosuppression of the recipient mice.

ACKNOWLEDGMENTS

We are indebted to Professor A. J. S. Davies for his support and critical evaluation of the work in progress. We should also like to thank Professor H. Festenstein for the gift of the monoclonal antibody and Denny Williams for preparing the manuscript. We gratefully acknowledge the Medical Research Council and the Leukaemia Research Fund for financial support.

REFERENCES

Adeniyi-Jones, S.C.A. & Ozato, K. (1987) Transfer of antibodies directed to paternal major histocompatibility class I antigens from pregnant mice to developing foetus. *J. Immunol.* **138,** 1408.

Auerbach, R. & Clark, S. (1975) Immunological tolerance: transmission from mother to offspring. *Science,* **189,** 811.

Batchelor, J.R. & Howard, J.G. (1965) Synergic and antagonistic effects of isoantibody upon graft-versus-host disease. *Transplantation,* **3,** 161.

Bellgrau, D., Smilek, D. & Wilson, D.B. (1982) Specific systemic graft-versus-host resistance: failure to induce in adult F_1 rats tolerised at birth to anti-MHC receptors on parental T cells. *Adv. exp. Med. Biol.* **149,** 563.

Berek, C. (1983) Antibodies specific for different T15 idiotopes induce neonatal suppression of the T15 idiotype. *Eur. J. Immunol.* **13,** 766.

Bernabe, R.A., Coutinho, A., Cazenave, P.A. & Forni, L. (1981) Suppression of a "recurrent" idiotype results in profound alterations of the whole B cell compartment. *Proc. natl Acad. Sci. USA,* **78,** 6416.

Billingham, R.E. (1968) The biology of Graft-versus-Host reactions. *Harvey Lectures,* **21,** 62.

Butturini, A. & Gale, R.P. (1988) T cell depletion in bone marrow transplantation for leukaemia: current results and future directions. *Bone Marrow Transpl.* **3,** 185.

Harris, H. & Hopkinson, D.A. (1976) The enzymes. In *Handbook of Enzyme Electrophoresis Human Genetics.* North Holland, Amsterdam.

Herzenberg, L.A. & Herzenberg, L.A. (1974) Short-term and chronic allotype suppression in mice. *Cont. Top. Immunobiol.* **3,** 41.

Herzenberg, K.A., Okumura, K. & Metzler, C.M. (1975) Regulation of immunoglobulin and antibody production by allotype suppressor T cells in mice. *Transplant. Rev.* **27,** 57.

Jerne, N.K. (1974) Towards a network theory of the immune system. *Ann. Immunol. (Paris)* **125,** 373.

Jerne, N.K., Roland, J. & Cazenave, P.A. (1982) Recurrent idiotypes and internal images. *EMBO J.* **1,** 243.

Martinez, A.C., Bernabe, R.R., DeLaHera, A., Pereira, P., Cazenave, P.A. & Coutinho, A. (1985) Establishment of idiotypic T helper cell repertoire in early life. *Nature,* **317,** 721.

Martinez, A.C., Pereira, P., Bernabe, R.R., Bandeira, A., Larsson, E.L., Cazenave, P.A. & Coutinho, A. (1984) Internal complimentaries in the immune system: regulation of expression of T helper cell idiotypes. *Proc. natl Acad. Sci. USA,* **81,** 4520.

Simonsen, M. (1957) The impact on the developing embryo and newborn animal of adult homologous cells. *Acta Pathol. Microbiol. Scand.* **49,** 480.

Sy, M.S., Lowy, A., Hayglass, K., Janeway, C.A., Gurish, M., Greene, M.I. & Benacerraf, B. (1984) Chronic treatment with rabbit anti-mouse μ chain antibody alters the characteristic immunoglobulin heavy chain restriction of murine suppressor T cell factors. *Proc. natl Acad. Sci. USA,* **81,** 3846.

Takemori, T. & Rajewsky, K. (1984) Mechanism of neonatally induced idiotype suppression and its relevance for the acquisition of self tolerance. *Immunol. Rev.* **79,** 103.

Wikler, M., Demur, C., Dewasme, G. & Urbain, J. (1980) Immunoregulatory role of maternal idiotypes. Ontogeny of immune networks. *J. exp. Med.* **152,** 1024.