AJRI 1996; 36:33–39
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American Journal of Reproductive Immunology
ISSN 8755-8920

Maternal Antibodies Protect Immunoglobulin Deficient Neonatal Mice From Mouse Hepatitis Virus (MHV)-Associated Wasting Syndrome

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Gustafsson E, Blomqvist G, Bellman A, Holmdahl R, Mattsson A, and Mattsson R. Maternal antibodies protect immunoglobulin deficient neonatal mice from mouse hepatitis virus (MHV)-associated wasting syndrome. AJRI 1996;36:33–39 © Munksgaard, Copenhagen

PROBLEM: Neonatal mice nursed by dams lacking immunoglobulins (Igs) may often suffer from lethal runting if raised under conventional conditions.The present study was performed in order to clarify a) the cause of the wasting syndrome and b) the protective role of antigen-specific milk antibodies.

METHOD: Ig-deficient mouse embryos in a conventional environment were embryo-transferred to specified pathogen free (SPF) dams. Neonatal growth, mortality, and health status of mice from both environments was recorded. Suspected presence of mouse hepatitis virus (MHV) was tested by RT-PCR. Protective effects on neonatal mortality of milk containing different titers of anti-MHV antibodies were investigated in cross-fostering experiments.

RESULTS: The SPF colony of Ig-deficient mice exhibited no breeding problems, whereas Ig-deficient neonates in the conventional environment suffered from lethal wasting syndrome. Serological screening of the mice kept in the two environments revealed that mice in the conventional room had high titers of antibodies against mouse hepatitis virus. Presence of MHV in runting neonates was confirmed by pathological examinations and RT-nested-PCR using MHV genome specific primers. Milk containing high titers of anti-MHV antibodies, when provided for 8 days or more, completely prevented Ig-deficient neonates from developing wasting syndrome in the conventional environment.

CONCLUSION: These findings show that the neonatal wasting syndrome is associated with the presence of MHV and that neonates are efficiently protected by MHV-specific antibodies in the milk.

Key words:
Immunoglobulin-deficient mice, immunoglobulins, lactation, milk antibodies, mouse hepatitis virus, wasting syndrome

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Submitted December 4, 1995; accepted March 1, 1996.

INTRODUCTION

It is well established that the immune system of fetal and new-born mammals is not fully developed and as a result fetuses and new-born animals are more susceptible than adults to most infections.[1] Despite this, most neonates survive the postpartum period even though they are exposed to a variety of environmental pathogens. It has been suggested that during the first weeks of life, neonates are protected against a wide spectrum of pathogens by maternally derived passive immunity.[1,2] During midgestation pregnant mice begin to prepare for transmission of passive immunity to their fetuses by increasing the number of immunoglobulin-secreting cells.[3–5] It is also clear that pregnancy proceeds without any complications in the absence

of maternal immunoglobulin secretion.[6,7] However, in a conventional animal house environment, it appears as if postnatal transmission of maternal immunoglobulins is almost essential for neonatal survival. This stems from the observation that normal and Ig-deficient neonatal mice nursed by Ig-deficient dams normally suffer from growth retardation and high mortality (wasting syndrome) if kept under conventional conditions.[8] However, if Ig-deficient neonates are nursed by immune dams, which provide them with passive immunity, neonatal development will be normal.[8] We also observed that highly purified monoclonal anti-collagen II IgG antibodies positively affected the resistance to runting.[9] These antibodies would be expected to have little relevance for protection against environmental pathogens.

One of the most common reasons for runting in neonatal mice is infection, caused by viruses such as epizootic diarrhea of infant mice (EDIM) virus, lymphocytic choriomeningitis (LCM) virus, reovirus type 3, and mouse hepatitis virus (MHV).[10–12] MHV is extremely contagious and is probably the most common of these viruses, and has been found in laboratory breeding populations throughout the world.[13,14] Age, genotype, immune status of the mouse and the MHV strain influence the course and severity of the disease, ranging from subclinical infection with minimal lesions to acute infection with severe lesions.[10,11] Several different viral strains have been described, but the high mutation rate of the coronaviruses leads to a diverse, ever changing population of MHV strains.[15]

The present study was performed to clarify a) the possible pathogenic reasons for the wasting syndrome of Ig-deficient neonatal mice, and b) the protective role of pathogen-specific antibodies in the maternal milk.

MATERIALS AND METHODS

Mice

Three types of immunodeficient mice were used in this study; outbred Ig-deficient mice,[16] $\beta_2$microglobulin-deficient[17–19] and MHC class II-deficient[20] C57BL/6J mice. Normal C57BL/6J mice were used as controls. The Ig-deficient mice were kindly been provided by Klaus Rajewsky and Werner Müller (Cologne, Germany). These mice were kept as an outbred colony (originally from C57BL/6J, 129/Sv, and C3H.Q mice) and had been selected for brown coat colour only. The $\beta_2$microglobulin-deficient mice (lacking functional $CD4^-8^+$ T cells and neonatal Fc-receptors) and MHC class II-deficient mice (lacking functional $CD4^+8^-$ T helper cells and the ability to produce antibodies to T cell dependent antigens) were purchased from GenPharm International (Mountain View, CA) and were tested for pathogens before arrival by Anmed Biosafe, Inc. (Rockville, MD). Specified pathogen free (SPF) C57BL/6J mice were purchased from BomMice, Ry, Denmark.

Mice were housed in a conventional animal facility as well as in a barrier-protected isolation room (SPF room). Mice in both environments were kept in autoclaved cages with free access to rodent chow and water at 22°C and exposed to a 12 hr:12 hr light/dark cycle. Mice in the SPF room were kept in a sterile-filtered air unit and chow and water was autoclaved.

Experimental Design

At the beginning of the experiment it was known that neonates nursed by Ig-deficient dams suffered from wasting and death in conventional animal rooms. To try to eliminate the pathogen(s) causing this wasting syndrome, we embryo-transferred Ig-deficient embryos to pseudopregnant C57BL/6J foster dams (health-controlled mice of BomMice origin) in the SPF room (see below). Thereafter, homozygous Ig-deficient mice bred in the two environments were compared in terms of neonatal weight gain and mortality.

The period of milk Ig transmission needed for preventing lethal wasting syndrome in the conventional environment was determined by transferring day 2 postpartum Ig-deficient neonates to immune C57BL/6J foster dams and then back again to their biological Ig-deficient dams after different periods of time.

The effects on neonatal mortality of different titers of relevant antibodies provided by A) Ig-deficient, B) MHC class II-deficient C), $\beta_2$microglobulin-deficient, and D) C57BL/6J foster dams in the conventional environment were recorded. In these cross-foster experiments, the pups were first nursed by the Ig-deficient dam for 1 day, whereupon the pups were divided into two groups, with one group being transferred to a foster dam and the other kept with the Ig-deficient dam as controls. This procedure was done to ensure uniformity at the beginning of the experiment. The experiment was designed to evaluate the importance of specificity of the maternal milk antibodies in protecting pups from the wasting syndrome.

At the end of the experiment, the health status of C57BL/6J mice kept in the two separate rooms was evaluated (see below). In addition, the IgG and IgM levels and MHV titers of adult control C57BL/6J and the three types of immunodeficient mice kept in the two environments were determined (see below).

In all experiments, pregnant mice were placed in separate cages before delivery. The day of detection of a vaginal plug was defined as day 1 of pregnancy, and the day of delivery was defined as day 1 postpartum.

Mouse Embryo Transfer

Ig-deficient females kept in the conventional room were superovulated and allowed to mate with Ig-deficient males. Fertilized eggs were prepared from the oviducts, treated with hyaluronidase and thoroughly washed in medium M2. The embryos were then transferred to pseudopregnant C57BL/6J foster dams (health-controlled mice of

BomMice origin) in the SPF room. The superovulation and embryo transfer procedures were performed according to standard methods.[21]

Health Evaluation of Mice

Four C57BL/6J female mice of the same age 2 and 4 months old) from each environment were health evaluated by the National Veterinary Institute (Uppsala, Sweden) at the end of the experiment. Microscopic examination of the lung and liver from three neonatal Ig-deficient mice suffering from the lethal wasting syndrome were performed in order to determine the cause of death. Throughout the experiment the SPF colony was MHV antibody monitored using the immunocompetent C57BL/6J mice as sentinels. The serum analyses were performed by BomMice (Ry, Denmark).

In addition, Ig-deficient and control neonates nursed by an Ig-deficient dam for 3 days and control neonates nursed by an immune C57BL/6J dam were analyzed for the presence of MHV by a nested DNA amplification of cDNA after reverse transcription from total RNA prepared from lung tissue. The primer sequences, termed OMHV1B, OMHV2A, OMHV3 and OMHV4, were selected from the E1 gene of MHV strain A-59 (G. Blomqvist, manuscript in preparation).

Enzyme-Linked Immunosorbent Assay (ELISA)

A sandwich-ELISA, performed basically as previously described,[22] was used for determining serum IgG and IgM levels. In brief, goat anti-mouse IgG (U. S. Biochem. Corp.) was coupled to immunoplates overnight. After coating with albumin (Sigma), purified IgG, or IgM (Sigma), control sera and test sera of various concentrations were added to the plate. The presence of IgG and IgM was visualised using peroxidase-conjugated goat anti-mouse Ig (Sigma) and 1,2-phenylendiamine dihydrochloride (Fluka, AG Switzerland). The colour of the product was then measured in a spectrophotometer at 492 nm. The Ig preparations used as standards in ELISA were polyclonal mouse IgG (reagent grade, no. I5381; Sigma Chemical Co., St. Louis, MO) and polyclonal mouse IgM (Sigma no. M 1520).

Statistical Analyses

Statistical differences in neonatal survival were tested by a Generalized Wilcoxon Test (Survival Analysis module, True Epistat 4.01 for DOS). If statistical changes in the whole material were detected, pairwise comparisons between the different test groups were performed.

RESULTS

Health Status

Results of the health evaluation of C57BL/6J mice kept in the conventional and SPF rooms are summarized in Table I. Mice in the SPF room were devoid of all screened microorganisms except for *Proteus mirabilis*. Mice kept in the conventional room had high titers of MHV antibodies and also present were *Protaeus mirabilis, Escherichia coli* and, in small quantities, the endoparasite *Aspicularis tetraptera*.

Mice kept in the SPF room did not show any pathological changes in the organs analyzed, whilst some changes were observed in mice kept in the conventional room. In the 2-month-old mice, focal chronic interstitial pneumonia was seen. In the 4-month-old mice, signs of chronic pyelitis and a limited increase in the number of Kuppfer cells were seen.

The microcopic findings in all neonates suffering from the lethal wasting syndrome was subacute hepatitis and hyperplasia in the bile duct. The lungs showed a mild acute interstitial pneumonia and presence of *Pneumocystis carinii*. These findings strongly indicated infection with MHV.

The Ig-deficient and control neonates nursed by an Ig-deficient dam for 3 days were positive for viral RNA in the lungs, while the lungs of control neonates nursed by an immune C57BL/6J control dam in the same room were negative.

Ig Levels and MHV Titers of Mice Kept Under Conventional and SPF Conditions

Table II summarizes Ig levels and MHV titers in adult, control, and immunodeficient mice kept in the two environments. Serum IgG levels were generally lower in the SPF room, with the exception of the $\beta_2$microglobulin-deficient mice. C57BL/6J mice in the conventional room contained high serum titers of anti-MHV antibodies; $\beta_2$microglobulin-deficient mice contained low titers, and MHC class II-deficient mice did not contain detectable anti-MHV titers. The SPF colony of mice was continuously monitored and was negative for anti-MHV antibodies throughout the experiments.

Weight Gain and Mortality of Ig-Deficient Neonatal Mice

Ig-deficient pups kept in the SPF room showed normal growth, whereas growth of the pups kept in the conventional animal room was retarded (Fig. 1). In addition, the Ig-deficient pups in the conventional room had a very high neonatal mortality, and by day 20 postpartum almost all of the pups were dead. The Ig-deficient neonates in the SPF room did not show any neonatal mortality, and homozygous breeding was successful in this environment (Fig. 2).

Minimum Period of Lactation Needed for Protection From Lethal Wasting Syndrome

Ig-deficient new-born mice were taken from their biological mother on the day of birth and transferred to a con-

TABLE I. Results of the Health Evaluations Performed by the National Veterinary Institute (Uppsala, Sweden) at the End of the Experimental Period

Screened organism	Conventional room	SPF room
VIRUS (serum antibodies)		
Ectromelia virus (mouse-pox virus)	–	–
Lymphocytic choriomeningitis virus (LCM)	–	–
Mouse hepatitis virus (MHV)	+[a]	–
Minute virus of mice (MVM)	–	–
Pneumonia virus of mice (PVM)	–	–
Reovirus type 3	–	–
Sendai virus	–	–
BACTERIA		
Bacillus	–	–
Bordetella	–	–
Corynebacteria	–	–
Escherichia coli	+[b]	–
Klebsiella	–	–
Proteus	+[c]	+[c]
Yersinea	–	–
Other enteribacteria	–	–
Erysiopelothrix	–	–
Listeria	–	–
Pasteurella	–	–
Pseudomonas	–	–
Salmonella	–	–
Staphylococcus	–	–
Streptococcus	–	–
MYCOPLASMA		
Mycoplasma pulmonis	–	–
PARASITES (gut)		
Ectoparasites (arthropodes)	–	–
Endoparasites	+[d]	–

–, *not detectable.*
[a]*Serum titer of MHV antibodies 1:1250.*
[b]*Limited growth of* Escherichia coli *in the gut.*
[c]*Limited numbers of* Proteus mirabilis *in the gut.*
[d]*Limited numbers of* Aspicularis tetraptera *in the gut.*

trol, serological MHV positive C57BL/6J foster dam. The neonates were returned to an Ig-deficient dam after different time periods. As summarized in Table III, a minimum period of 8 days of suckling from an immune foster dam was sufficient to confer complete protection from lethal wasting syndrome.

Effects of MHV Antibodies of Different Titers and Specificity on Neonatal Mortality

Pups of Ig-deficient dams born in the conventional room were split 1 day after birth and transferred to either A) Ig-deficient, B) MHC class II-deficient, C) $\beta_2$microglobulin-deficient, or D) immunocompetent C57BL/6J foster dams. The pups nursed by the Ig-deficient dam developed severe wasting syndrome, as expected, and by day 20 postpartum almost all of the pups were dead. Although the MHC class II-deficient dams completely failed to produce any detectable quantities of anti-MHV antibodies, their milk antibodies still reduced susceptibility to the neonatal wasting syndrome. The pups nursed by $\beta_2$microglobulin-deficient dams, which produce low anti-MHV titers, were not completely protected from infection, and by day 20 postpartum, mortality was 40%. Pups nursed by an immunocompetent C57BL/6J dam were completely free of any signs of acute infection. The results of this experiment, which was performed in the conventional room only, are summarized in Figure 3.

TABLE II. Serum IgM and IgG Levels, and MHV Antibody Titers in Adult Mice of Different Strains Housed in the Two Environments

	Conventional room			SPF room		
Mice	IgM[a]	IgG[a]	MHV titer[b]	IgM[a]	IgG[a]	MHV titer[b]
Ig-deficient	0	0	–	0	0	–
MHC class II-deficient	0.8 ± 0.2	4.6 ± 0.2	–	0.5 ± 0.2	1.7 ± 0.5	–
β_2m-deficient	0.5 ± 0.2	2.6 ± 0.2	+	0.8 ± 0.2	3.0 ± 0.7	–
C57BL/6J	0.4 ± 0.2	3.7 ± 1.2	++	0.3 ± 0.1	1.9 ± 0.3	–

[a]*Serum Ig in mg/ml are presented as mean ± SD, n=3 adult mice of the same age*
[b]*MHV antibodies detected by ELISA (BomMice, Ry, Denmark), n=6.*
–, Serum titer less than 1:20; + , serum titer 1:20–1:80; ++ , serum titer exceeding 1:80.

DISCUSSION

In this study we attempted to identify the underlying cause of runting in neonatal mice fostered by Ig-deficient dams in conventional animal rooms. By using embryo transfer methods, we studied Ig-deficient neonatal mouse development in the two environments. The Ig-deficient neonates nursed by Ig-deficient dams showed growth retardation and had a very high mortality in the conventional room, whereas pups nursed in the SPF environment were successfully raised during homozygous breeding. These results suggests that the wasting syndrome is caused by pathogens in the conventional room. Results from the health monitoring revealed pathogenic differences between mice kept in the two environments. *Proteus mirabilis* is an opportunistic gram-negative pathogen that can remain latent in the intestinal tracts of mice,[23] and was present in mice from both environments. Mice in the conventional room had high titers of anti-MHV antibodies and were positive for *Escherichia coli* and *Aspicularis tetraptera*. *Escherichia coli* is not a major pathogen of mice[23] and *Aspicularis tetraptera* is a common mouse parasite and infection is usually asymptomatic.[23] These results indicate that particularly MHV could be involved in the lethal wasting syndrome of neonates nursed by Ig-deficient dams in the conventional room. An influence of other unidentified pathogens could not be excluded. It can also be assumed that Ig-deficient mice might have problems to completely eliminate this virus, since the mortality patterns of Ig-deficient mice kept under conventional conditions differ somewhat from time to time, but accumulated data from

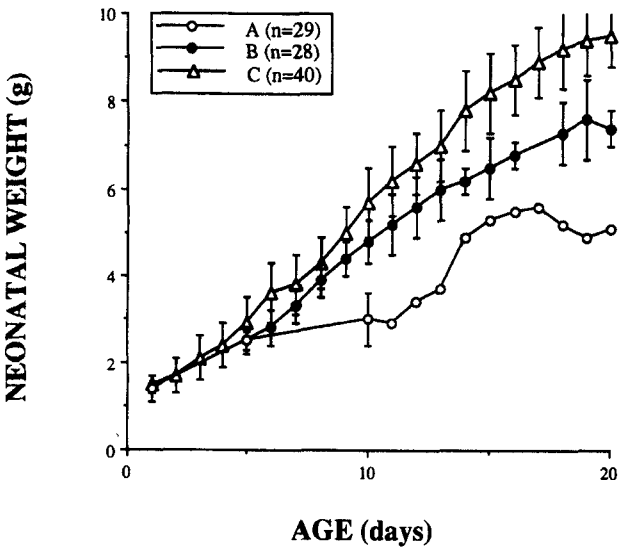

Fig. 1. Neonatal weight for homozygous Ig-deficient offspring nursed by their biological dams in the **A**) conventional and **B**) SPF room and **C**) Ig-deficient offspring nursed by immune foster dams in the conventional room. Data are presented as mean ± SD; n represents the number of litters in each experiment. For the Ig-deficient pups in the conventional room, no SD is given for days 11–20 owing to high mortality of the neonates.

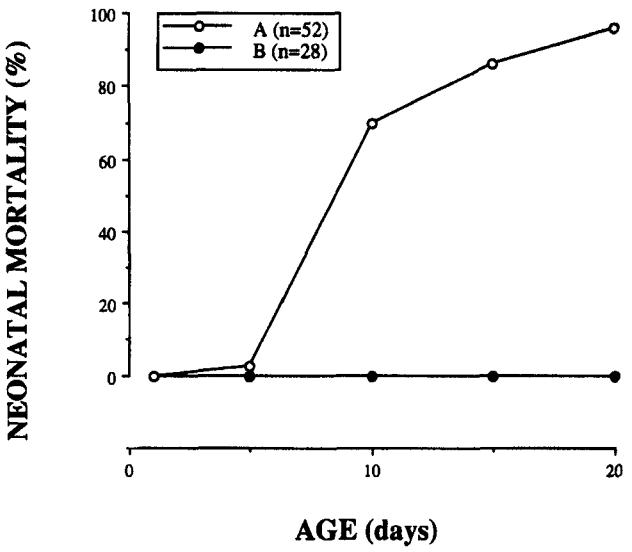

Fig. 2. Neonatal mortality for homozygous Ig-deficient offspring nursed by their biological mothers in **A**) the conventional and **B**) the SPF room. n is the number of litters in each experiment.

TABLE III. Nursing Period Needed for Complete Protection From Lethal Wasting Syndrome in Ig-deficient Offspring in a Pathogenic Environment*

Cross-fostered (days)	Neonatal mortality (%) Day 10	Day 15	Day 20	Day 35	n[a]
0	69	86	93	97	29
2	0	50	50	75	4
4	0	0	30	60	10
8	0	0	0	0	5
10	0	0	0	0	6

**Statistical differences in survival, tested by Generalized Wilcoxon Test, showed a P<0.000001 for the whole material; P=0.0067 for 2 days cross-fostering; P=0.0000067 for 4 days cross-fostering; P=0.00021 for 8 days cross-fostering; P=0.000073 for 10 days cross-fostering, all compared to 0 days cross-fostering.*

[a]Number of litters in each experiment.

longer time periods often are amazingly similar. However, no persistent MHV infections have yet been described. It is also known that MHV causes a wasting syndrome in other immunodeficient mice, such as athymic (nu/nu) and SCID (severe combined immunodeficient) strains.[24,25]

Intrauterine transmission of MHV may occur in infections of susceptible pregnant mice with polytropic MHV strains that are sufficiently virulent to induce disseminated infection in adult mice.[26] However, infection at this stage results in abortion and the likelihood of fetuses born with active MHV infection is remote.[26] In this study, the embryo transfer performed did eliminate the MHV infection, and if other pathogens were involved in the induction of the wasting syndrome it could be concluded that these hypothetical pathogens also were eliminated by the embryo transfer procedure.

The results of the diagnostic microscopic examination of neonatal mice suffering from wasting syndrome showed that the neonates suffered from an acute MHV infection that was the major cause of death. In addition, a PCR assay indicated presence of MHV in the lung of neonates nursed by an Ig-deficient dam for three days during their first week of life in the conventional room. Lung tissue from control neonates fostered by an immune dam in the same environment was negative.

Some further observations strengthen our hypothesis that MHV is involved in the pathogenesis of the wasting syndrome. For instance, there appears to be a correlation between maternal titer of anti-MHV antibodies and protective effect on suckling neonates. C57BL/6J immune dams with high titers of anti-MHV antibodies provided complete protection if neonates were provided with milk for 8 days or longer. $\beta_2$microglobulin-deficient mice, which lack surface expression of MHC class I expression as well as CD8 positive T cells, produced MHV antibodies of low titer, and their milk provided marginal protection. The MHC class II-deficient mice which showed normal levels of immunoglobulins, but are unable to respond to T cell dependent antigens, lacked anti-MHV antibodies, and could not be expected to provide relevant passive immunity to any T cell dependent antigen. Despite this, their milk provided a low, but significant protection of Ig-deficient neonates. These results strengthen our previous observation that irrelevant immunoglobulins exert *a weak* low, but significant, health promoting effect on neonates raised under pathogenic pressure. These experiments also showed that the susceptibility of neonatal mice to MHV infection is unrelated to the lack of endogenous immunoglobulins and that only the immune status of the dam nursing the pups was relevant for the pups' survival.

As demonstrated in the present study, a minimum of 8 days of suckling from an immune foster dam was sufficient to completely protect Ig-deficient neonates from the lethal MHV-associated wasting syndrome. It seems clear that the supply of specific milk antibodies is a prerequisite for normal development and survival of mice raised in an MHV-infected environment. Previous studies have also shown that maternally derived passive immunity protects neonatal mice from disease and possibly also from infection.[11,27-32]

Interestingly, a comparison of growth patterns of mice bred as Ig-deficient homozygotes in the SPF room and Ig-deficient homozygotes nursed by an immune foster dam in the conventional room revealed that mice in the con-

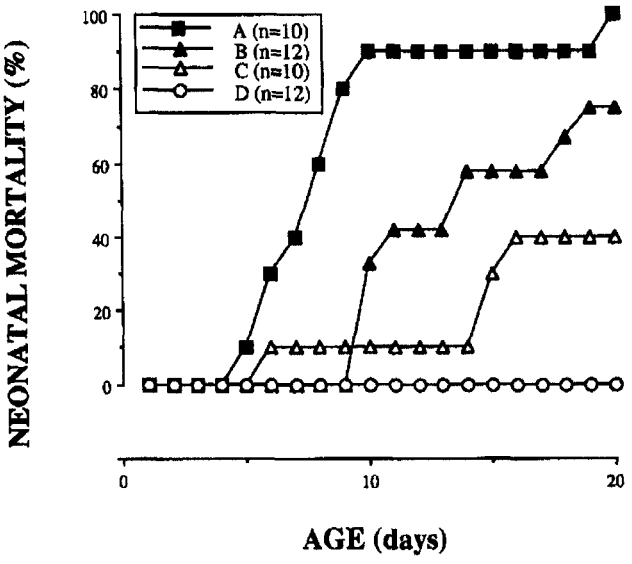

Fig. 3. Neonatal mortality for Ig-deficient offspring cross-fostered by **A)** Ig-deficient, **B)** MHC class II-deficient, C) $b_2$microglobulin-deficient, and **D)** C57BL/6J dams in the conventional room. n is the number of litters in each experiment. Statistical differences in survival, tested by Generalized Wilcoxon Test, showed a P=0.000011 for the whole material; P=0.000015 for A; P=0.00032 for B; P=0.019 for C, all compared to D.

ventional room grew larger. Ig-deficient mice raised in this way under pressure of environmental pathogens did not show apparent health problems as adults.

Although mechanisms of Ig-transfer differ considerably between mice and humans, basic concepts might be very similar. It is therefore tempting to speculate that immunoglobulin-supplements could be used to reduce the mortality of premature or immunologically defective babies.

Acknowledgments

We are grateful to Dr Lars Rydén and Uppsala University for financially supporting some of the health evaluations of our mice, and Stephen J Miller MSc, for linguistic corrections. This work was financially supported by the Swedish Natural Science Research Council, and the Helge Ax:son Johnson, Hierta-Retzius, Magnus Bergvall, Lennander and Von Hoffsten Foundations.

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