

Analysis of mRNA Levels for the MHC Class I-Like Molecules CD1 and FcRn in Preimplantation Mouse Embryos

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PROBLEM: Major histocompatibility complex (MHC) antigens expressed on preimplantation embryos are important for the control of development, reproduction, and allo-recognition of the embryo by the mother. Four types of MHC class I and MHC class I-like antigens have recently been defined: class Ia, class Ib, class Ic, and class Id, based on their similar three-dimensional protein structures. Class Ia and class Ib antigens are encoded in the MHC, whereas class Ic and class Id antigens are encoded by genes on other chromosomes. Both class Ia and class Ib MHC antigens are expressed on preimplantation mouse embryos. The function of the class Ia antigens on embryos is unknown, but the function of one class Ib antigen, Qa-2, the product of the *Ped* gene, has been found to control the rate of early cleavage division and subsequent embryo survival. The expression of class Ic and class Id antigens on preimplantation embryos has not yet been evaluated. In the present study, we report the analysis of mRNA expression of two class Id genes, *CD1* and *FcRn*, in preimplantation mouse embryos.

METHOD OF STUDY: A reverse transcription-polymerase chain reaction (RT-PCR) assay was performed to analyze mRNA levels for *CD1* and *FcRn* in 1-cell, 2-cell, 8-cell, and blastocyst stage embryos from C57BL/6 mice.

RESULTS: No expression of *CD1* mRNA was found in any of the preimplantation embryos tested. As a by-product of this study, we found a mistake in the published sequence of the mouse *CD1* gene: nucleotide 746 in the cDNA is a G not a C. This base change is in a site recognized by the restriction enzyme *PstI*, thereby eliminating a *PstI* cleavage site. Expression of mRNA for *FcRn* was found in all preimplantation stages tested. Higher levels of mRNA for *FcRn* were detectable in 2-cell and 8-cell embryos compared to 1-cell and blastocyst stage embryos.

CONCLUSION: This study shows that mRNA for *FcRn* but not for *CD1* is found in preimplantation mouse embryos.

Key words:
Major histocompatibility complex, mammalian development, pregnancy

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INTRODUCTION

The mechanisms by which the fetal allograft escapes immunological rejection by the mother remain elusive. The classical paper on this subject by Medawar¹

suggested that the immunological interaction between the mother and the fetus is suppressed, either by lack of antigenicity of the fetus or by suppression of the maternal immune system. The idea that the maternal immune system is suppressed has recently been challenged.² Sacks et al.² have hypothesized that the innate immune system is actually activated during pregnancy, sometimes leading to pregnancy complications such as infection and preeclampsia, and only the adaptive immune system is relatively suppressed. The first Medawar suggestion, that the fetus is missing certain antigenic molecules, has also been challenged. Many years of research on mouse and human fetuses has shown that the developing fetus expresses paternally derived alloantigens. Thus, other mechanisms than the lack of expression of alloantigens must exist to protect the fetus from rejection by the maternal immune system.

The fetal expression of one set of alloantigens, those encoded by the major histocompatibility complex (MHC), has received particular attention. Recently, MHC and MHC-like class I antigens have been classified into four groups: class Ia, class Ib, class Ic, and class Id.³ The class Ia antigens are encoded in the *HLA-A,B,C* regions in the human and the *H-2K,D,L* regions in the mouse. The class Ib antigens are encoded in the *HLA-E,F,G* regions in the human and the *H-2Q,T,M* regions in the mouse. The genes encoding the class Ic proteins (*MICA*, *MICB*, *Hfe* in the human and *Hfe* in the mouse) and class Id proteins (*CD1*, *FcRn*, *Zinc α 2-glycoprotein*, *MRI* in the human and *CD1* and *FcRn* in the mouse) are not located in the MHC. However, the class Ic and class Id genes encode proteins with a similar three-dimensional structure to the MHC-encoded class Ia and class Ib proteins, including an association with β_2 -microglobulin.

As opposed to the fetus, there are many fewer studies on possible mechanisms of protection of the preimplantation embryo from rejection by the maternal immune system. Although it was originally reported that class Ia MHC antigens were not expressed on preimplantation embryos, later work, using monoclonal antibodies and highly sensitive techniques such as ELISA and immuno-polymerase chain reaction (PCR), showed that this was clearly not the case.⁴⁻⁶ Moreover, not only are MHC class Ia antigens expressed on early embryos, but they can be recognized by cytotoxic T cells after removal of the zona pellucida causing embryo lysis.⁷

Although the function of class Ia antigens in preimplantation development is unknown, the function of one class Ib antigen that is expressed by preimplantation mouse embryos, Qa-2, has been well defined. Qa-2 antigen is the product of the *Ped* gene, a gene

that controls the rate of cleavage division of preimplantation mouse embryos and their subsequent survival (reviewed in^{8,9}). Thus, at least one MHC class I protein plays an important role in development and reproduction.

The possibility that class Ic and class Id MHC-like proteins may be expressed in preimplantation embryos has not yet been examined. The purpose of the present study was to analyze preimplantation mouse embryos for the presence or absence of mRNA for two MHC class Id molecules, CD1 and FcRn.

MATERIALS AND METHODS

Mouse Embryos

C57BL/6 mice were housed in a day/night cycled room (lights on 4:00 AM to 6:00 PM standard time) with food and water *ad libitum*. The mice were maintained according to the NIH guidelines in an American Association for the Accreditation of Laboratory Animal Care approved facility at Northeastern University. Mice that were 2–6 months old were superovulated with 5 IU eCG (Sigma, St. Louis, MO) at the 11th hour (3:00 PM) of the light cycle followed 48 hr later with 10 IU hCG. One-cell, 2-cell, 8-cell, and blastocyst C57BL/6 embryos were collected in Whitten–Biggers medium, under 5% CO₂, 5% O₂, 90% N₂, then washed three times in 1 mL of sterile PBSA (10% bovine serum albumin in phosphate-buffered saline). Next, embryos were transferred in as little PBSA as possible into microfuge tubes. Each tube contained 160 1-cell, 80 2-cell, 20 8-cell, or 8 blastocyst stage embryos.

RNA Isolation

To each tube containing the embryos, 100 μ L of denaturing solution containing freshly added β -mercaptoethanol (Micro RNA Isolation Kit, Stratagene, La Jolla, CA) was added and the tube was immediately vortexed, frozen in liquid nitrogen, and stored at -70°C . For RNA preparation, frozen embryos were thawed and 1×10^6 copies of a synthetic RNA (1 μ L of pAW109, Perkin Elmer, Branchburg, NJ) were added to each tube to serve as an internal control. Next, the following reagents (Micro RNA Isolation Kit, Stratagene) were added to each tube: 10 μ L 2M sodium acetate (pH 4.0), 100 μ L water-saturated acid phenol, and 30 μ L chloroform:isoamyl alcohol. The samples were then vortexed at high speed for 30 s. A 5 min centrifugation at 13,000g was carried out. At this point, 2 μ g glycogen (Stratagene) and 100 μ L isopropanol (Stratagene) were added to each tube. The sample was then centrifuged for 45 min at 9,400g.

As much of the supernatant as possible was removed and the pellet washed with 200 μ L 75% ethanol (150 μ L absolute ethanol plus 50 μ L DEPC-dH₂O). The wash was removed and discarded before drying the pellets for 5 min in a speed-vac. Each pellet was resuspended in 4.25 μ L DEPC-dH₂O and in some cases stored overnight at 4°C.

cDNA Synthesis (RT Step)

cDNAs were synthesized from the isolated RNAs. A cDNA assay master mix was produced from which an aliquot was taken for each re-hydrated RNA sample. cDNA was made in a final volume of 20 μ L of 5 mM MgCl₂, 1 \times PCR buffer without MgCl₂ (GIBCO, Grand Island, NY), 4 mM total dNTPs (GIBCO), 20 units RNase inhibitor (Perkin Elmer), 50 units MuMLV-reverse transcriptase (GIBCO), and 0.05 μ g random hexamers (Perkin Elmer). Samples were layered with 35 μ L of autoclaved mineral oil, and then placed in a DNA Thermal Cycler 480 (Perkin Elmer) for the following cycles: 10 min at 25°C, 30 min at 42°C, 5 min at 99°C, and 5 min at 5°C. Negative controls of reagents without RNA and RNA without RNA polymerase were run. One PCR amplification reaction of pAW109 cDNA was carried out per embryo pool as a check that the RNA pellet was not lost or degraded during the isolation process. The conditions for this amplification were the same as for the CD1 or FcRn PCR being carried out that day. Positive control RNAs were isolated from C57BL/6 mouse liver for CD1, and the proximal end of the intestine from 8-day-old C57BL/6 mice for FcRn. The isolations were carried out using a Micro RNA Isolation Kit (Stratagene) and the manufacturer's protocol was followed. cDNAs were made as described above.

Product Amplification (PCR Step)

CD1 PCR. Nested PCR was performed on 14 μ L of embryo cDNA or 2 μ L of positive control cDNA. The primers used are shown in Table I. They were either designed by using Oligo 5.0 software for the PC (National Bioscience, Plymouth, MN) or were taken from the literature. Final concentrations for first-round PCR reactions were 1 \times PCR buffer without MgCl₂ (GIBCO), 0.7 mM MgCl₂, 1 mM total dNTPs (GIBCO), 1.25 units Taq polymerase (Perkin Elmer), and 0.4 μ M of each primer. PCR samples were each layered with 45 μ L of autoclaved mineral oil before starting the PCR cycling. Step-down PCR was employed in the first amplification reaction. First-round PCR machine settings were as follows: one cycle at 96°C for 30 s; three cycles at 94°C for 60 s, at 69°C for 60 s, and at 72°C for 90 s; three cycles each for annealing temperatures of 66, 63, 60, and 57°C with

other settings remaining the same: 20 cycles at 94°C for 60 s, at 56°C for 60 s, and at 72°C for 90 s; one final extension at 72°C for 5 min; and 4°C soak. The second-round PCR amplification involved the inner primers (Table I). Final concentrations for the second-round PCR reactions were 1 \times PCR buffer without MgCl₂ (GIBCO), 0.7 mM MgCl₂, 1 mM total dNTPs (GIBCO), 1.25 units Taq polymerase (Perkin Elmer), and 0.68 μ M of each primer. PCR samples were each layered with 45 μ L of autoclaved mineral oil before starting the PCR cycling. One cycle at 96°C for 30 s was followed by the machine settings defined by Balk et al.¹⁰

FcRn PCR. PCR was performed on 14 μ L of embryo cDNA or 2 μ L of positive control cDNA. Final concentrations for PCR reactions were 1 \times PCR buffer without MgCl₂ (GIBCO), 0.7 mM MgCl₂, 1 mM total dNTPs (GIBCO), 1.25 units Taq polymerase (Perkin Elmer), 0.68 μ M of each primer. PCR samples were each layered with 45 μ L of autoclaved mineral oil before starting the PCR cycling. The primer pairs used are shown in Table I. Hot start PCR was used and the machine settings were as follows: one cycle at 96°C for 30 s; five cycles at 94°C for 60 s, at 58°C for 30 s, and at 72°C for 90 s; 35 cycles at 94°C for 30 s, at 58°C for 30 s, and at 72°C for 90 s; one final extension at 72°C for 5 min; and 4°C soak.

Product Analysis

The identity of the CD1 and FcRn RT-PCR amplification products of liver and neonatal C57BL/6 intes-

TABLE I. Primers for RT-PCR Analysis of mRNA for CD1 and FcRn

Primer	Upper (U)		Source
	Lower (L)	Sequence (5'-3')	
CD1 outer	U	TCTGAAGCCCAG CAAAG	Oligo 5.0
	L	TATCAAGACATC CGGTGACTC	Oligo 5.0
CD1 inner	U	AATTACACCTTC CGCTGCCT	Ref. ¹⁰
	L	CAGGATATCATC CTCTACTG	Ref. ¹⁰
FcRn	U	TGGTTGGGTCCT CAGCAGTA	Oligo 5.0
	L	GCTCCGGACAAG AGTTTAGCA	Oligo 5.0

TABLE II. Expected and Actual Sizes (bp) of *PstI* Digestion Products from RT-PCR of *CD1* and *FcRn*

<i>CD1</i>				<i>FcRn</i>			
Uncut (bp)		Cut (bp)		Uncut (bp)		Cut (bp)	
Expected	Actual	Expected	Actual	Expected	Actual	Expected	Actual
818	818	493	683	406	406	212	212
		190	–			194	194
		66	66				
		46	46				
		23	23				

tine, respectively, were first confirmed by digestion with *PstI* enzyme and analysis of the size of the products. The digestions were carried out at a $MgCl_2$ concentration of 4 mM and addition of 10 units of *PstI* in a 37°C water bath for 4 hr. At the end of the incubation, EDTA was added to a final concentration of 8 mM and the samples were stored at 4°C. Fifteen microliters of each RT-PCR product or digested RT-PCR product were mixed with 5 μ L of loading dye and run on a 6% polyacrylamide gel (5.28 mL dH_2O , 2mL 5 \times TBE, 2.5 mL 24% acrylamide, 210 μ L 3% ammonium persulfate, and 10 μ L TEMED). Gels were run at 25 mA, stained with EtBr solution for approximately 2 min, and destained in dH_2O for several hours to overnight. Photographs of gels were taken using a UV light box and Polaroid black and white film.

In addition, the CD1 RT-PCR product was analyzed by DNA sequence analysis. Two sequencing reactions were carried out, one using the upstream inner primer and the other using the downstream inner primer. Approximately 4 μ g of CD1 RT-PCR product, from C57BL/6 liver, was diluted in 1 \times reaction buffer (USB Sequencing Kit, Cleveland, OH) containing 1 μ M of primer for each reaction. The USB Sequencing Kit protocol was followed and the samples were stored at –70°C overnight. The following day, the samples were thawed and run on a 6% sequencing gel. The gel was dried and placed with film.

RESULTS

CD1 and FcRn RT-PCR Products in Control Samples

The first set of experiments was designed to validate the RT-PCR protocol by analyzing the cDNA products for *CD1* and *FcRn* in the control samples. The

results of a diagnostic *PstI* restriction enzyme digestion are shown in Table II. It is seen that the uncut product was of the correct size for both *CD1* and *FcRn*. However, whereas *FcRn* cDNA gave the correct size products after *PstI* digestion, the *CD1* DNA did not. Therefore, we undertook DNA sequence analysis of approximately 200 bp of the *CD1* cDNA product. The results (Table III) showed that there was a single nucleotide difference from the published sequence and this difference was at nucleotide position 746 of the cDNA sequence (in exon 4 encoding the $\alpha 3$ domain), directly inside one of the *PstI* sites.

CD1 and FcRn RT-PCR Products in Preimplantation Embryos

The results of the analysis of the cDNA products for *CD1* and *FcRn* in 1-cell, 2-cell, 8-cell, and blastocyst stage embryos are shown in Figs. 1 and 2. Also included in the figures are the amplification results of

TABLE III. DNA sequence of the *CD1* gene

Sequence-Ref. 10	5' CTGCAG 3' 3' GACGTC 5' ↑ Position 746
Sequence-This Paper	5' CTGCAC 3' 3' GACGTG 5' ↑ Position 746
<i>PstI</i> Cut Site	↓ 5' CTGCAG 3' 3' GACGTC 5' ↑

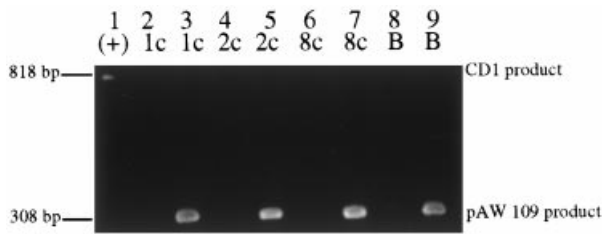


Fig. 1. *CD1* mRNA expression in preimplantation mouse embryos. Lane 1: positive control (liver); lane 2: 1-cell stage; lane 3: pAW109 internal control for 1-cell stage; lane 4: 2-cell stage; lane 5: pAW109 internal control for 2-cell stage; lane 6: 8-cell stage; lane 7: pAW109 internal control for 8-cell stage; lane 8: blastocyst stage; lane 9: pAW109 internal control for blastocyst stage. All RT-PCR reactions were performed as described in the text.

the internal control, pAW109 (product size: 308 bp), and the positive controls (liver cDNA for *CD1* [product size: 818 bp] and 8-day-old intestinal cDNA for *FcRn* [product size: 406 bp]). No *CD1* mRNA was detectable in any of the preimplantation embryos, even after using a nested RT-PCR reaction protocol. However, all stages of the preimplantation embryos had detectable mRNA for *FcRn* after just one round of PCR amplification.

DISCUSSION

In the present study, we have shown that mRNA for the MHC class Id molecule *CD1* is not detectable in preimplantation mouse embryos, whereas mRNA for a second MHC class Id molecule, *FcRn*, is detectable throughout preimplantation development. *CD1* is a novel MHC-like molecule because it binds and presents lipids and glycolipids to T cells rather than binding and presenting short peptides, like class Ia and class Ib MHC molecules.¹¹ It is possible that

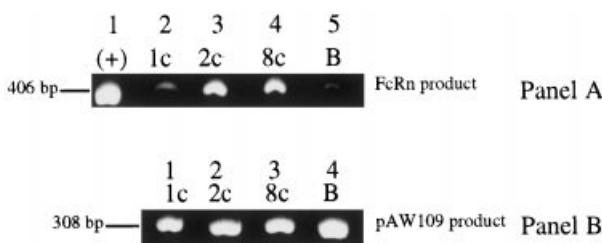


Fig. 2. *FcRn* mRNA expression in preimplantation mouse embryos. Panel A: Lane 1: positive control (8-day-old intestine); lane 2: 1-cell stage; lane 3: 2-cell stage; lane 4: 8-cell stage; lane 5: blastocyst stage. Panel B: Lane 1: pAW109 internal control for 1-cell stage; lane 2: pAW109 internal control for 2-cell stage; lane 3: pAW109 internal control for 8-cell stage; lane 4: pAW109 internal control for blastocyst stage. All RT-PCR reactions were performed as described in the text.

mRNA for *CD1* may be present below the sensitivity of our assay. However, we have never found any protein expressed for molecules that have undetectable mRNA levels by nested RT-PCR. Therefore, the lack of detectable mRNA for *CD1* in preimplantation embryos implies that the embryos do not express *CD1* protein. In the mouse, *CD1* has been found to be expressed mainly on hemopoietic-derived cells.¹² Other MHC molecules mainly expressed on hemopoietic cells, such as the class Ib antigen Qa-2, are, however, also expressed on preimplantation mouse embryos. Thus, one cannot generalize about tissue distribution of MHC antigens without testing each particular tissue. The absence of *CD1* mRNA in early embryos suggests that this molecule is not actively involved in the regulation of early mouse embryo development.

On the other hand, mRNA for the MHC class I molecule, *FcRn*, is found in 1-cell, 2-cell, 8-cell, and blastocyst stage embryos. Our experiments were set up so that each sample had approximately the same number of genome equivalents (e.g., 80 2-cell embryos or 20 8-cell embryos). Therefore, the gel shown in Fig. 2 shows that there is more *FcRn* mRNA per cell (genome equivalent) in 2-cell and 8-cell embryos than in 1-cell or blastocyst stage embryos. It will be interesting to analyze *FcRn* protein expression on the embryos to determine whether different mRNA levels correspond to different protein levels.

The role of *FcRn* on preimplantation embryos is a mystery. Because of the MHC-like character of *FcRn*, it is tempting to speculate that this molecule may play some role in the protection of the early embryo from rejection by the maternal immune system. The original described role of *FcRn* was transport of immunoglobulin G (IgG) across the neonatal rodent gut (hence the "n" in *FcRn*) (reviewed in^{13,14}). It came as a complete surprise when the gene sequence¹⁵ and then the X-ray crystallographic structure¹⁶ showed that *FcRn* is homologous to MHC class I molecules. This is not a feature shared by other Fc receptors, such as those of the *FcγR* family.¹⁷ It is now believed that *FcRn* has a much broader function than originally proposed, namely the maintenance of IgG homeostasis in serum.¹⁷ One speculation for a possible role of *FcRn* in development is that perhaps *FcRn* on embryos binds IgG by the Fc portion of the molecule, thereby preventing activation of complement components that may reside in the oviductal or uterine fluid. Since IgG passes freely through the zona pellucida,¹⁸ lysis by maternal antibody would be avoided. However, the true biological role of *FcRn* in preimplantation development remains to be determined.

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