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International Immunopharmacology 2 (2002) 325–332

International Immunopharmacology

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Review

Maternal immune stimulation in mice decreases fetal malformations caused by teratogens

S.D. Holladay [a,*], L.V. Sharova [a], K. Punareewattana [a], T.C. Hrubec [a], R.M. Gogal Jr. [a], M.R. Prater [a], A.A. Sharov [b]

[a] *Department of Biomedical Sciences and Pathobiology, Virginia–Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061-0442, USA*

[b] *Department of Entomology, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061-0442, USA*

Abstract

For unknown reasons, non-specific stimulation of the maternal immune system in pregnant mice has what appears to be a broad-spectrum efficacy for reducing birth defects. Immune stimulation by diverse procedures has proven effective, including footpad injection with Freund's complete adjuvant (FCA), intraperitoneal (IP) injection with inert particles to activate resident macrophages, IP injection with attenuated Bacillus Calmette-Guerin (BCG), and intrauterine injection with allogeneic or zenogeneic lymphocytes. Morphologic lesions that were significantly reduced included cleft palate and associated craniofacial defects, digit and limb defects, tail malformations, and neural tube defect (NTD). Teratogenic stimuli to induce these lesions included chemical agents (2,3,7,8-tetrachlorodibenzo-*p*-dioxin [TCDD], ethyl carbamate [urethane], methylnitrosourea [MNU], cyclophosphamide [CP], and valproic acid [VA]), physical agents (X-rays, hyperthermia), and streptozocin (STZ)-induced diabetes mellitus. Limited information is available regarding mechanisms by which such immune stimulation reduced fetal dysmorphogenesis. The collective literature suggests the possibility that immunoregulatory cytokines of maternal origin may be the effector molecules in this phenomenon.

Keywords: Teratology; Neural tube defect; Cleft palate; Immunostimulation; Immune protection

1. Introduction

Historically, the mammalian fetus has been thought of as a genetically preprogrammed entity that derives nutrition from the maternal organism, but otherwise, largely directs its own development as codified by its inherited unique DNA ''blueprint''. This process requires highly regulated cell proliferation, differentiation, and migration. Limited specific morphologic defects occur with disruption of these events during early phases of embryogenesis, prior to organogenesis. Instead, death of the embryo caused by damage to undifferentiated cells is the major effect. The time of greatest susceptibility to induction of gross anatomic defects occurs later, during germ layer formation and organogenesis. The type of morphologic defect is then determined by multiple factors including nature of the teratogen and the precise developmental stage of the

* Corresponding author. Tel.: +1-540-231-3372; fax: +1-540-231-6033.

E-mail address: holladay@vt.edu (S.D. Holladay).

1567-5769/02/$ - see front matter
PII: S1567-5769(01)00183-7

fetus at the time of teratogen exposure, with early events in organ formation being most sensitive [1].

Reports in the literature have suggested that developmental outcome may be positively affected by relatively minor manipulation of maternal dietary conditions. For instance, supplementation with vitamins [2], caffeine or xanthines [2–4], retinoic acid [2], or nicotinamide [5] has reported efficacy for reducing spontaneous or induced malformations in experimental animals. More recently, studies in humans and rodents have established the importance of folic acid in the prevention of neural tube defect (NTD). Up to 70% of human NTDs can be prevented by supplementation with folic acid in the periconceptual period; folic acid supplementation in pregnant mice likewise reduced NTDs in the fetuses [6].

A growing literature database provides compelling evidence that maternal immune manipulation is an additional factor capable of dramatically improving developmental outcome in teratogen-challenged fetuses. Several recent reports suggest the possibility that this effect may at least in part be mediated by immunoregulatory cytokines secreted by activated maternal immune cells.

2. Immunostimulation and reduced fetal malformations

Nomura et al. [7] reported that stimulation of peritoneal macrophages in pregnant, inbred ICR mice reduced fetal malformations caused by chemical agents or X-rays. Specifically, intraperitoneal (IP) injection of a synthetic copolymer (pyran) or a biological agent (attenuated Bacillus Calmette-Guerin (BCG)) was used to activate maternal peritoneal macrophages. Significant reductions in cleft palate and digital and tail anomalies were observed in fetuses from dams receiving a teratogen exposure (urethane, methylnitrosourea [MNU], or radiation) together with macrophage activation, compared to pregnant mice exposed to teratogens without macrophage activation. Since this early report, many investigators have confirmed the efficacy of diverse methods and timing of maternal immune stimulation to reduce chemical-induced morphologic lesions in mice (summarized in Table 1).

Table 1 data demonstrate the sometimes remarkable level of protection against teratogenesis reported in mice as a result of maternal immune stimulation. In two cases (urethane-induced digit defects and VA-

Table 1
Immunoprotection against teratogenesis

Teratogen	Immune stimulant (IS)	Mouse model	Defect studied	Defect incidence (%)		Ref.
				Without IS	With IS	
TCDD	Pyran	B6C3F1	Cleft palate	86	67	[12]
CP	Rat splenocytes	ICR-O	Craniofacial and limb defects	81	49	[20]
	GM-CSF			78	50	[13]
Urethane	Pyran	ICR-I	Cleft palate and digit defects	25	6	[7]
	BCG	ICR-O	Digit defects	19	0	[12]
	IFNγ	ICR-O	Cleft palate	70	48	[12]
	FCA	ICR-O	Cleft palate	70	26	[12]
Hyperthermia	Rat splenocytes	ICR-O	Exencephaly	28	13	[11]
			Resorptions	21	8	
			Open eyes	21	9	
Diabetes	Rat splenocytes	ICR-O	NTD + other	9	2	[10]
	GM-CSF			50	22	[a]
MNU	Pyran	ICR-I	Cleft palate and digit defects	35	20	[7]
		B6C3F1	Digit defects	56	38	[12]
		ICR-O	Digit defects	22	7	[12]
Valproic acid	FCA	ICR-O	Exencephaly	53	0	[12]
			Open eyes	78	15	
X-rays	Pyran	ICR-O	Tail defects	55	28	[7]

ICR-O and ICR-I refer to outbred and inbred ICR mouse models, respectively.

[a] Punareewattana, unpublished date.

induced exencephaly), the chemical-induced morphologic defects were totally blocked (reduced to 0%) by the immune intervention. These reports also demonstrate protection against morphologic lesions from causes other than chemical teratogens, for instance, diabetes mellitus.

The incidence of malformed newborns in women with insulin-dependent diabetes mellitus (IDDM) is 6–10%, approximately three to five times higher than among non-diabetic women [8]. Streptozocin (STZ)-induced IDDM in ICR mice prior to breeding is accompanied by similar increased percentages of fetuses displaying morphologic anomalies, thus, this model has been used to study mechanisms by which diabetes increases birth defects [9]. Defects observed in the offspring of IDDM ICR mice include craniofacial anomalies such as exencephaly, anophthalmia, open eyelids, agnathia, micronathia, and cleft palate. In a study with considerable methodological parallels to previous reports using chemical teratogens, Torchinsky et al. [10] demonstrated that maternal immune stimulation reduced these defects associated with diabetes in ICR mice. Female mice in these experiments were immunostimulated by intrauterine injection of splenocytes from male rats 3 weeks prior to mating. Malformed fetuses decreased over four-fold, from 8.9% in non-immunized diabetic mice to 2.1% in immunized diabetic mice. A similar level of protection against IDDM-induced malformations was recently observed in fetuses from ICR mice that were immunostimulated by footpad injection with Freund's complete adjuvant (FCA) prior to breeding or by IP injections with the cytokine, granulocyte-macrophage colony stimulating factor (GM-CSF) before breeding and during gestation (Punareewattana, personal communication).

In addition to birth defects caused by chemical teratogens, X-rays, or IDDM, hyperthermia-induced fetal malformations in ICR mice were reduced following maternal immune stimulation [11]. Mice in these studies received two consecutive 10-min exposures to 43.6 °C on day 9 of gestation. Fetuses from non-immunostimulated mothers displayed 28% and 21% encephalocele and open eyes, respectively, on day 19 of gestation. Pregnant females treated identically but immunostimulated prior to mating by intrauterine rat splenocyte injection displayed 13% and 9% encephalocele and open eyes, respectively. These authors also detected a significant increase in the heat shock protein HSP60 in fetuses from non-immunized females as compared to immunized females, and a maximum level of heat shock-induced apoptosis in embryos of non-immunized females of 30%, compared to 7% in embryos of immunized mice.

The diversity of techniques used to stimulate the immune system of mice in the above studies is noteworthy, as is the time of stimulation relative to pregnancy. Immune stimulators and time of administration have included: inert polymer particles administered by IP injection on day 3 of gestation [7,12]; intrauterine injection with GM-CSF on day 1 of gestation followed by a second IV GM-CSF injection on day 10 of gestation [13]; IP injection with GM-CSF prior to mating and in mid-gestation (Punareewattana, personal communication); INFγ by IP injection immediately prior to teratogen treatment on day 10 of gestation [12]; FCA by footpad injection on days 5 and 3 before mating [12]; intrauterine injection with allogeneic or xenogeneic splenocytes 3 weeks prior to mating [10,11,13]; and IP injection with BCG 6 to 46 days prior to mating [7,12].

3. Hypothesized mechanisms of immune protection

In their early report, Nomura et al. [7] hypothesized that immune protection against chemical-induced teratologenesis may be the result of a maternal immunosurveillance system, whereby activated peritoneal macrophages cross the placenta, find and eliminate pre-teratogenic cells. Questions were raised, however, regarding surveillance of the fetus by maternal immune cells as a mechanism leading to a reduced teratogenesis [12]. The latter authors suggested that operating mechanisms (signals) were unclear by which fetal pre-teratogenic cells may selectively recruit maternal immune cells across the placenta. Flow cytometry and a cell-tracking probe were used in an attempt to demonstrate activated maternal immune cells in the circulation of teratogen-exposed fetal mice, with negative results (no maternal immune cells identified in the fetal mice). These authors also reported a significant reduction of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-induced cleft palate in fetal mice from immune stimulated mothers [12]. The cleft palate lesion caused by TCDD has been

associated with failure of apoptosis of epithelial cells lining the palatal shelves (an event required prior to proliferation and fusion of underlying mesodermal cells) [14,15]. Thus, no pre-teratogenic cells as such would appear to be associated with this particular morphologic lesion, but instead, phenotypically normal cells that did not receive signal to apoptose. This observation raised questions regarding how these cells might be immunologically distinguished from other fetal cells by maternal leukocytes that entered fetal circulation.

The possibility that maternal immune stimulation may reduce chemical-induced morphologic lesions by an indirect effect, e.g., via regulatory cytokine products of activated immune cells, has been suggested by several investigators. In mice, cyclophosphamide (CP) exposure during development causes limb malformations ranging from oligodactyly to amelia. Savion et al. [13] observed a significant decrease in such malformations if the mice were dosed with GM-CSF prior to breeding. This cytokine treatment resulted in enhanced maternal splenocyte proliferation and increased interleukin (IL)-2 and IL-3 production, leading the authors to suggest a possible role of regulatory molecules produced by activated immune cells in protection against CP-induced fetal malformations. In this regard, CP treatment in pregnant rodents has previously been associated with inappropriate apoptotic cell death in fetal rodent limb buds, leading to teratogenesis [16]. However, intrauterine immunization of pregnant CP-treated mice with either semiallogeneic (paternal) or xenogeneic (rat) splenic lymphocytes reduced such apoptotic nuclei in developing fetal limbs, and increased fetal survival [17]. Increased levels of maternal cytokines including GM-CSF were again implicated in the protective effect. Based on these observations, the authors speculated that regulation of apoptosis during limb development might, in part, be dependent on fetomaternal immune interactions.

In an outcome possibly related to the above, Gorivodsky et al. [18] noted that decreased fetal resorptions occurred in immune stimulated CP-treated pregnant mice compared to CP-treated pregnant mice receiving no immune stimulation. Further, the uteroplacental units of CP-treated mice displayed decreased colony stimulating factor-1 (CSF-1) mRNA as well as reduced expression of CSF-1 receptor (c-fms), an effect that was largely reversed in immunostimulated mothers. These authors extended this observation by reporting decreased uteroplacental transforming growth factor $\beta 2$ (TGF$\beta 2$) in mice with CP-induced pregnancy loss compared to control mice [19]. Immunostimulation increased TGF$\beta 2$ mRNA expression 2.0–3.2 fold in CP-treated mice, leading the authors to conclude that the beneficial effects of maternal immune stimulation may in part be due to increased synthesis of this growth factor.

Beyond altered growth factor levels in uteroplacental units, maternal immune stimulation has been shown to affect expression of such mediators in the fetus itself. Ivnitsky et al. [20] demonstrated increased tumor necrosis factor-α (TNF-α) expression in mouse fetal head tissues following developmental exposure to CP. Maternal immunostimulation diminished CP-induced brain and craniofacial anomalies in the fetal mice and decreased TNF-α mRNA transcripts in the head and brain of fetuses.

Collectively, the above observations suggest the possibility of unknown homeostatic and/or regulatory activity of the maternal immune system in fetal development. A hypothesis regarding indirect mechanisms by which immune stimulation may reduce birth defects could, therefore, involve increased synthesis and release of immune proteins with regulatory activity (e.g., TGFβ) which interact with placenta and/or fetus to alleviate or partially alleviate toxicant-mediated effects. It has been shown that TCDD alters regulatory proteins (e.g., EGF; TGFβ) in fetal palate that are required for timed expression of cell cycle genes [15]. Expression of such genes is critical for controlling waves of cellular proliferation, differentiation, or apoptosis necessary for closure of the palate. To test the hypothesis that maternal immune stimulation may affect expression of critical genes in target tissues, Sharova et al. [21] used urethane-induced cleft palate in a mouse model and evaluated a limited panel of genes that regulate cell cycle and apoptosis during palate closure. Fetuses from urethane-exposed mothers were characterized by decreased expression of genes bcl2α, bcl2β, pkCα, and p53. Maternal injection with the macrophage-stimulating protein IFNγ normalized expression of bcl2α, bcl2β, and pkCα in fetal heads to control levels. Urethane also decreased the bclα/p53, bclβ/p53, and pkCα/p53 gene expression ratios, an effect that was again reversed in fetuses

from mothers that received immune stimulation with either IFNγ or FCA.

Sharova et al. [21] used principal component analysis (PCA) [22,23] to further define interactions between urethane exposure, immune stimulation, and changes in expression level of the above genes. Three principal components (PC1–3) explained 92% of the variation in gene expression under the various treatments. The first principal component (PC1) represented average level of expression of bcl2α, bcl2β, and pkCα genes; PC2 represented RXRα and its ratio to p53; and PC3 represented p53 gene and its ratio to bcl2α and bcl2β. A scatter plot of PC1 and PC3 (Fig. 1) indicated that urethane caused an *x*-axis (PC1) shift of coordinate gene expression (large arrow in graph) reflecting reduced fetal expression of genes bcl2α, bcl2β, and pkCα. A *y*-axis shift reflecting increased p53/bcl2 gene expression ratio (i.e., a shift toward p53) was also associated with urethane exposure. Maternal immunization with FCA decreased the PC3 coordinate to the control level, while immunization with IFNγ decreased this component to lower than control and also partially prevented the PC1 change caused by urethane (small arrows in graph). The authors suggested that these results might indicate that FCA and IFNγ immunization affect different gene targets in the fetus, which may explain the differences in level of protection against urethane-induced cleft palate observed with these two methods of maternal immune stimulation.

The precise relationship between the above normalized gene expression and protection against a morphologic defect remains speculative. However, it is recognized that the bcl2, pkCα, and p53 genes affected by maternal immune manipulation are important in fetal development. Protein products of bcl2 and p53 operate at the G1 cell cycle phase and are involved in assessment and regulation of DNA replication and repair [24]. The bcl2 gene is normally highly expressed in fetal tissues including CNS and rapidly proliferating epithelial cells [25]. The protein product of this gene plays a role in mediating the growth-inhibiting and apoptotic effects of the p53 gene [26]. A membrane-bound isoform of protein kinase C (pkCα) is involved in phosphorylation of proteins regulating bcl2 and p53 gene products, thus, the pkCα gene may be important in control of interactions between bcl2 and p53 [27,28]. The p53 gene is also highly expressed in fetal tissues including rapidly proliferating epithelia, where its expression is induced by TGFβ. Tightly regulated proliferation/apoptosis ratios that are critical for normal fetal development are further believed to depend more on bcl2/p53 gene expression ratios than on levels of expression of the individual genes. For

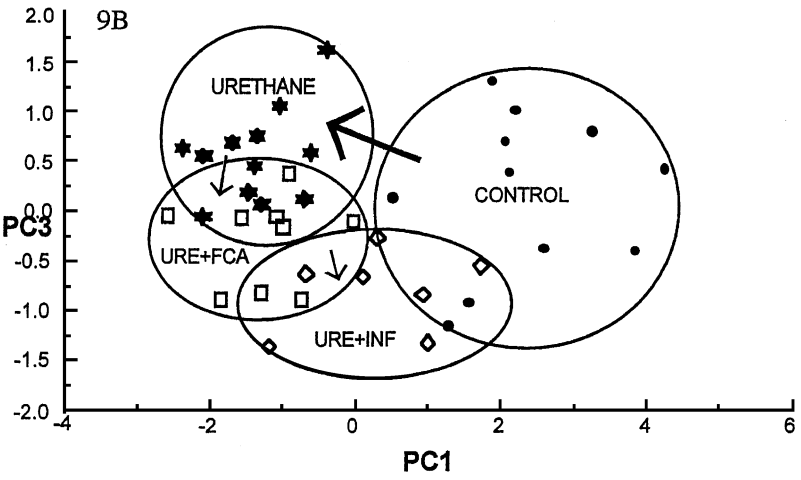

Fig. 1. Principal component analysis of the effects of urethane and maternal immune stimulation on fetal gene expression. Each dot in the Control circle represents gene expression data from one litter. In like manner, stars represent urethane-exposed animals; boxes represent urethane-exposed animals that received immunostimulation with FCA; triangles represent urethane-exposed animals that received immunostimulation with IFNγ. Modified from Sharova et al. [21].

instance, apoptosis induced by p53 protein can be blocked by an elevation in the level of bcl2 gene product [29]. Conversely, the apoptotic function of p53 is activated when the equilibrium between p53 and bcl2 favors p53 [30]. Thus, it may be noteworthy that maternal urethane treatment caused decreased bcl2α/p53, bcl2β/p53, and bcl2α + bcl2β/p53 expression ratios (i.e., a relative shift toward p53) that were in each case reversed by maternal injection with IFNγ or FCA [21].

Recent experiments with CP may support the hypothesis that maternal immune stimulation partially restores normal fetal gene expression in teratogen-challenged animals. Excessive apoptotic cell death in the heads of CP-treated fetal mice was associated with craniofacial dysmorphogenesis caused by this chemical [31]. These authors also noted increased levels of p53 protein and G1 arrest in the heads of fetuses exposed to CP. The growth factor TGFβ induces p53 gene expression in proliferating fetal tissues [28], thus, in subsequent experiments, the authors examined expression of TGFβ mRNA and TGFβ protein in CP-treated fetuses [32]. Both TGFβ2 mRNA and protein were elevated in embryos 72 h after the gestation day 12 injection with CP. Maternal immune stimulation by intrauterine injection with rat splenocytes 21 h before mating blocked the elevated TGFβ2 mRNA and protein levels in fetal tissues, leading the authors to conclude that immune protection against CP-induced morphologic defects may in part be realized by normalizing TGFβ2 gene expression in teratogen-targeted embryonic structures.

4. Valproic acid and neural tube defect

The antiepileptic drug valproic acid (VA) causes NTD in 1–2% of human fetuses exposed to this agent during early pregnancy [33]. Mice exposed to VA during development display increased exencephaly (an upper-end NTD) [23]. Exencephaly caused by VA in ICR mice was reduced from 53% in fetuses of non-stimulated mothers to 0% in fetuses of mothers receiving footpad injections with FCA shortly before mating [12]. Open eyes were present at day 17 of gestation in 15% of fetuses from mothers injected with FCA, compared to 78% open eyes in the VA-treated fetuses without maternal immunostimulation. Interestingly, 41% of fetuses from the immunostimulated mothers displayed anury (absence of a tail), a defect not typically seen in ICR mice exposed to VA [34] and not observed in non-immunostimulated fetuses. This is the first report of a defect apparently caused by maternal immune stimulation.

Specific mechanisms by which VA causes NTD have not been identified. However, disregulation of embryonic folic acid metabolism by VA, causing reduced proliferation of cells lining the neural tube, may contribute to NTD [6,35,36]. It has been clearly demonstrated that alterations in the normal cellular proliferative rate of tissues involved with neural tube closure can result in embryos with NTD [23,33,37]. In this regard, VA caused a 50% reduction in the proliferation of c6 glioma cells impeding the cell cycle during the G1 phase [38]. Wlodarczyk et al. [37] found that that exposure to VA altered the normal temporal pattern of gene expression in the teratogen-treated embryos such that mRNA levels were comparable to what would normally be observed 12 h later under control conditions. This accelerated expression was marked by elevated mRNA levels for transcription factors Emx-1, Emx-2, c-*fos*, c-*jun* and creb, and cell cycle genes p53 and bcl-2 consistent with a pattern of drug-induced inhibition of cell proliferation. In this regard, it was recently reported that urethane-induced cleft palate was associated with decreased bcl2/p53 gene expression ratio in fetal mouse heads, suggesting a relative shift toward p53 and, thus, toward decreased proliferation rates [21]. Maternal immunostimulation restored the expression ratio of these genes to the control level and reduced cleft palates from 70% in fetuses from urethane-treated mothers without immunostimulation to 26% in fetuses from urethane-treated mothers with immunostimulation. It is not known if maternal immune stimulation may similarly alter the above effects of VA on gene expression in cells lining the neural tube, or if such an effect may be related to the reported decrease in NTD in VA-treated mice receiving immunostimulation.

5. Conclusions

(1) Repeated studies from independent laboratories have verified the efficacy of maternal immune stimulation in reducing teratogen-induced morphologic

defects in mice. Operating mechanisms for this effect remain unclear, but may involve an effect of maternal immune proteins on fetal gene expression.

(2) The observation that maternal immune stimulation caused altered expression of critical genes in the fetus is in itself novel, and may suggest, heretofore, unrecognized regulatory activity of maternal immune proteins on normal fetal development.

(3) An early implication of these reports is that optimal maternal immune health may be important for protection against events leading to certain birth defects. In this regard, it may be important to note that many teratogens are also immunotoxic agents, raising questions about a possible contributing role of maternal immunosuppression to fetal morphologic lesions.

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