Suppression of Developmental Anomalies by Maternal Macrophages in Mice

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Summary

We tested whether nonspecific tumoricidal immune cells can suppress congenital malformations by killing precursor cells destined to cause such defects. Pretreatment of pregnant ICR mice with synthetic (Pyran copolymer) and biological (Bacillus Calmette-Guérin) agents significantly suppressed radiation- and chemical-induced congenital malformations (cleft palate, digit anomalies, tail anomalies, etc.). Such suppressive effects were associated with the activation of maternal macrophages by these agents, but were lost either after the disruption of activated macrophages by supersonic waves or by inhibition of their lysosomal enzyme activity with trypan blue. These results indicate that a live activated macrophage with active lysosomal enzymes can be an effector cell to suppress maldevelopment. A similar reduction by activated macrophages was observed in strain CL/Fr, which has a high spontaneous frequency of cleft lips and palates. Furthermore, Pyran-activated maternal macrophages could pass through the placenta, and enhanced urethane-induced cell killing (but not somatic mutation) in the embryo. It is likely that a maternal immunosurveillance system eliminating preteratogenic cells allows for the replacement with normal totipotent blast cells during the pregnancy to protect abnormal development.

During embryonic development, the different cell types become determined in their proper places, and the pattern of the body is set up on a small scale and then grows (1). The appearance of outliers in such processes can result in congenital malformations, but such outliers are effectively reduced or eliminated by homeostatic surveillance mechanisms. In experimental animals, radiation- and chemical-induced and spontaneously developed malformations are cured or prevented by vitamins (2, 3), caffeine (2, 4–6), and other bioresponse modifiers (2, 7), probably due to normalization, or more likely to killing of the damaged cells responsible for maldevelopment. It is expected that such cellular alterations responsible for producing congenital malformations in the embryos are also under the control of the immunosurveillance system of the pregnant animals (7). In the present study, pregnant mice were pretreated with Pyran copolymer and Bacillus Calmette-Guérin (BCG)[1] vaccine which are known to activate macrophages (8, 9), or they were post-treated with macrophages activated by these agents, and then examined to determine whether such immunological treatments can suppress radiation- and chemical-induced, and also spontaneously developing, congenital malformations. Furthermore, placental transfer of maternal macrophages and their effects on embryonic mutation and cell killing were examined with specific tester strains of mice.

Materials and Methods

Mice. ICR (F_{30+27}) and CL/Fr (F_{20+24}) mice were used for detecting congenital malformations, and PT and HT mice for detecting in vivo somatic mutation and cell killing. Inbred ICR mice were established by T. Nomura. CL/Fr mice were provided by Dr. K. Esaki (Central Laboratory for Experimental Animals, Kanagawa, Japan) at F_{20}, and then maintained by brother × sister mating (Department of Radiation Biology, Osaka University). Approximately 30% of CL/Fr mice develop cleft lips and palates spontaneously (2). PT and HT mice were provided by Dr. M. F. Lyon and Dr. A. G. Searle (Radiobiology Unit, Medical Research Council, Harwell, UK) in 1978, and were maintained by brother × sister inbreeding (Department of Radiation Biology, Osaka University). Coat color genes of these mice were *a/a*, *b/b*, pc^{ch}/pc^{ch}, and *d/d* for PT, and *a/a*, *ln/ln*, *pa/pa*, and *pe/pe* for HT. Mice were maintained with mouse diet CRF-1 (Charles River Japan, Kanagawa, Japan) and chlorinated water in the complete barrier condition at 23 ± 1°C. An estrous female (2 mo old) was selected by the appearance of her vaginal orifice, and mated with males in the evening. The next morning, a vaginal plug was examined to determine the first day of pregnancy (day 0) (2–5, 10). The mouse room was illuminated from 4:00 to 18:00 so that fertilization occurred at about 2:00 on the day of a vaginal plug (2, 3, 10).

X-Ray Exposure. X-irradiation (KC-18-2A; Toshiba, Tokyo, Japan) was performed at a distance of 51.5 cm, operating at 20 mA and 180

[1] *Abbreviations used in this paper:* BCG, Bacillus Calmette-Guérin; MNU, *N*-methyl-*N*-nitrosourea.

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kVp, with a filter of 0.5 mm copper and 0.5 mm aluminum. The average dose rate was 0.54 Gy/min. The dose was measured by an r-meter (570; Victoreen, Inc., Cleveland, OH) adjusted by Fricke dosimetry.

Chemicals. Urethane and *N*-methyl-*N*-nitrosourea (MNU) were used for inducing congenital malformation. Urethane (ethyl carbamate; Wako Pure Chemical Ind. Ltd., Osaka, Japan) was dissolved in distilled water in a 10% concentration, and MNU (Sigma Chemical Co., St. Louis) in 0.1 M sodium phosphate buffer solution (pH 5.8) in a 0.1% concentration. These solutions were prepared just before injection.

To activate macrophages, Pyran copolymer (XA 146-85-2, NSC 46015; Hercules Research Center, Wilmington, DE) and freeze-dried glutamate BCG (Japan BCG Lab., Tokyo, Japan) were used. Pyran copolymer was dissolved in a 0.9% NaCl solution, and the pH of the solution was adjusted to 7.2 with a 10% NaOH solution. The final concentration of the Pyran copolymer in a 0.9% NaCl solution was 0.01, 0.1, and 0.5%. BCG was suspended in a 0.9% NaCl solution at a 0.1% concentration.

Pretreatment with Pyran and BCG before X-Ray or Chemical Treatment. Pyran copolymer was injected intraperitoneally into pregnant ICR mice on day 3 of gestation at a dose of 0, 1, or 10 μg/g, and then 1.23, 1.43, or 1.85 Gy of X-rays, or a single subcutaneous injection of urethane (1.0 mg/g) or MNU (10 μg/g) was given at 14:00 on day 9 of pregnancy. Macrophage activation measured by tumoricidal effects becomes maximum 6 d after intraperitoneal injection of Pyran copolymer (9). BCG (10 μg/g) was injected intraperitoneally into female ICR mice (2 mo old), and then they were mated with ICR males at various intervals from 6 to 46 d after BCG treatment. Pregnant mice were treated with urethane (1.0 mg/g) or X rays (1.43 Gy) at 14:00 on day 9.

Collection of Peritoneal Macrophages. Pyran copolymer (50 μg/g) was injected intraperitoneally into adult (2-mo-old) ICR mice, and peritoneal exudates were collected 6 d after Pyran treatment, since macrophage activation measured by tumoricidal effects becomes maximum on that day (9). Cells were washed once with an ice-cold 0.9% NaCl solution and resuspended in Whitten's embryo culture medium (3 ml/mouse) (11). Peritoneal macrophages were purified by adherence on tissue culture dishes. After 1 h of incubation at 37°C in 5% CO_2 and air, adherent cells were gently scraped with a soft rubber policeman into Whitten's medium, adjusted to 10^7 viable cells per ml, and kept in an ice bath before use. More than 95% of the cells prepared in this manner ingest dextran sulfate, which was stained metachromatically by toluidine blue 0 (Chroma, Stuttgart, FRG) (8). For the collection of normal macrophages, glycogen (0.5 mg/g, Wako Pure Chemical Ind. Ltd.), instead of Pyran, was injected intraperitoneally into 2- or 12-mo-old ICR mice. For the disruption of activated macrophages by supersonic waves, cells were treated with a sonifier disruptor (B-12; Branson Sonic Power Co., Danbury, CT) at 20 kHz for 30 s in an ice bath. Microscopically, no cells were detected. To inhibit the activity of lysosomal enzymes of activated macrophages, trypan blue (100 μg/g, Wako Pure Chemical Ind. Ltd.) was injected intraperitoneally into 2-mo-old ICR mice 48 h before collection of peritoneal exudates (8). Activated peritoneal macrophages were also collected from PT and CL/Fr mice treated intraperitoneally with 50 μg/g of Pyran and purified by adherence on culture dishes as described above.

Peritoneal macrophages were also collected 35 or 45 d after intraperitoneal injection of BCG (10 μg/g), purified, and adjusted to 10^7 viable cells/ml. These activated or normal (nonactivated) macrophages (5×10^6 cells/mouse) were injected intravenously into pregnant ICR mice 0.5 or 48 h after urethane or X-ray treatment at 14:00 on day 9 of pregnancy. Pyran-activated macrophages (5×10^6 cells/mouse) were also injected intravenously into pregnant CL/Fr mice on day 9, a critical stage for inducing congenital malformations by radiation and chemicals (2–5).

Examination of Congenital Malformations. Pregnant ICR mice were killed on day 18 of gestation by cervical dislocation. After hysterectomy, implants, early deaths, late (fetal) deaths, and living fetuses were recorded. Living fetuses were weighed and examined for external malformations and for skeletal malformations using a soft X-ray apparatus (SOFRON Type SRO-M50; Sohken, Tokyo, Japan). Details of these procedures were reported previously (2–5). The predominant types of induced congenital malformations were cleft palate, tail anomalies (kinky and short), and digit anomalies (poly-, olygo-, or syndactyly). Pregnant CL/Fr mice were killed on day 17 to detect both cleft lip and cleft palate.

Detection of In Vivo Somatic Mutation and Cell Killing. An estrous PT female was mated with HT males. Pregnant PT females were treated subcutaneously with urethane (1.0 mg/g) at 14:00 on day 10, and 0.5 h later, 5×10^6 Pyran-activated macrophages were injected intravenously into half of them. Concurrent controls were not treated with urethane or activated macrophages. Activated macrophages were collected from PT mice treated intraperitoneally with 50 μg/g of Pyran and purified by adherence on culture dishes. Somatic mutation and cell killing were detected as coat color spots 6 wk after birth. When a mutation occurred in a melanoblast of the [PT × HT]F_1 (hereafter designated PTHTF_1) embryo heterozygous at seven coat color genes (*a/a*, *In/+*, *pa/+*, *b/+*, $pc^{ch}/+$, *d/+*, *pe/+*), a spot with different coat color, which derived from the mutated melanoblast, appears on the nonagouti black background after birth. Killing of a melanoblast results in a pigment vacancy in the hair, and a white spot appears on the ventral midline of the coat (white midventral spots) (5, 12). Details of the experimental procedures were reported previously (5).

Results

Suppression of X-Ray-, Urethane-, and MNU-induced Malformations by Pyran Pretreatment. Pyran copolymer (1 and 10 μg/g) was injected intraperitoneally into pregnant ICR mice on day 3 of gestation, before X-ray or chemical treatment on day 9, when macrophage activation by Pyran becomes maximum. As shown in Fig. 1, X-ray-induced congenital malformations were suppressed slightly but significantly by the Pyran pretreatment at a high dose. 98.6% of the X-ray-induced malformations were tail anomalies, some of which were accompanied with meningoceles (1.9%). The remainders were cleft palates (0.7%), digit anomalies (0.4%), gastroschises (0.12%), and exencephalus (0.06%). More dramatic reductions were observed with urethane- and MNU-induced cleft palates, tail anomalies, and digit anomalies. Pyran pretreatment reduced 40–100% of urethane- and MNU-induced congenital malformations. However, Pyran pretreatment reduced neither the average number of living fetuses (see legends to Figs. 1, 2, and 4, and Table 1) nor average body weight of the fetuses (data not shown).

Suppression of Urethane- and X-Ray-induced Congenital Malformations by Pyran-activated Macrophages. To examine the association of macrophage activation with antiteratogenic activity by Pyran, peritoneal macrophages that had been activated by Pyran and purified by adherence on tissue culture dishes

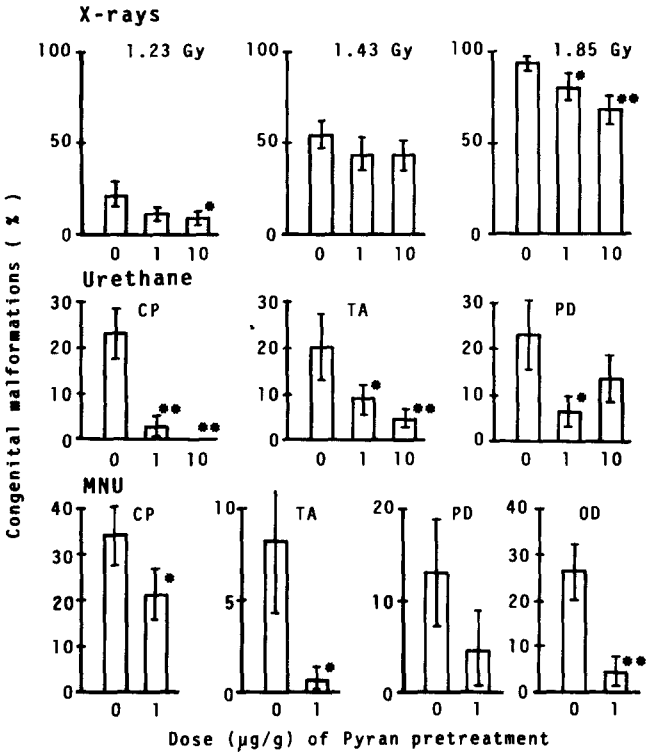

Figure 1. Suppression of X-ray-, urethane-, and MNU-induced congenital malformations by the pretreatment with Pyran copolymer. (*X-rays*) Histograms for the total incidence of congenital malformations (mostly tail anomalies, see text) at X-ray doses of 1.23, 1.43, and 1.85 Gy. (*Urethane*) Histograms for cleft palates (*CP*), tail anomalies (*TA*), and polydactylies (*PD*). (*MNU*) Histograms for cleft palates, tail anomalies, polydactylies, and oligo-syn-dactylies (OD). The number of fetuses examined (pregnant mice in parentheses) is 129 (11), 150 (14), and 111 (10) for 1.23 Gy of X-rays; 198 (18), 99 (11), and 130 (11) for 1.43 Gy; and 89 (10), 107 (11), and 91 (11) for 1.85 Gy at 0, 1, and 10 μg/g of Pyran, respectively. For urethane, the number is 155 (14), 114 (10), and 141 (13) at 0, 1, and 10 μg/g of Pyran; and for MNU, 118 (11) and 125 (11) at 0 and 1 μg/g of Pyran, respectively. Vertical bars indicate 90% confidence intervals of the mean computed from the *t* distribution of malformed fetuses in each litter. (*)$p < 0.05$; (**)$p < 0.01$ against experimental groups without Pyran treatment. A student's *t* test was applied after testing variance ratio.

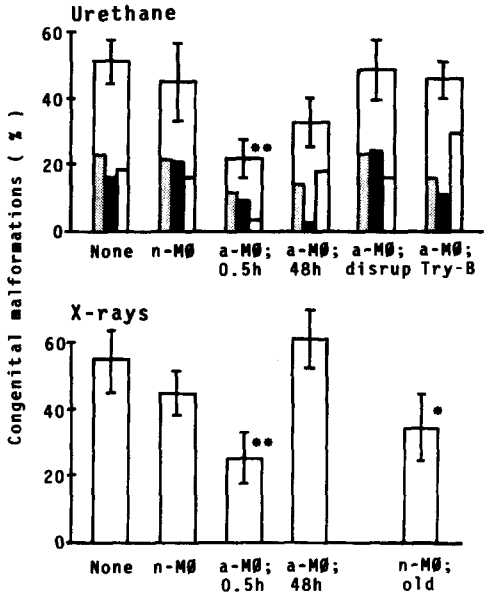

Figure 2. Association of macrophage activation by Pyran with the suppression of urethane- and X-ray-induced congenital malformations. Pregnant ICR mice were treated with 1.0 mg/g of urethane or 1.43 Gy of X-rays at 14:00 on day 9, and then received intravenous injection of 5×10^6 activated macrophages (*a-MØ*) (10^7 cells/ml) 0.5 or 48 h after urethane or X-ray treatment. Equal numbers of normal (glycogen-elicited) macrophages (*n-MØ*) and disrupted (*a-MØ;disrup*) or trypan blue–treated (*a-MØ;Try-B*) activated macrophages were also injected intravenously into pregnant mice 0.5 h after urethane or X-ray treatment. Macrophages elicited by glycogen from 12-mo-old non-SPF ICR mice were also injected 0.5 h after X-ray exposure (*n-MØ;old*). The number of fetuses (pregnant mice) examined was 247 (23), 111 (11), 151 (12), 155 (14), 129 (11), and 195 (17) for urethane-treated groups with no macrophages (*None*), n-MØ, a-MØ;0.5 h, a-MØ;48 h, a-MØ;disrup, and a-MØ;Try-B, respectively, and 198 (18), 153 (14), 131 (11), 161 (14), and 140 (12) for X-ray-treated groups with no macrophages (*None*), n-MØ, a-MØ;0.5 h, a-MØ;48 h, and n-MØ;old, respectively. Vertical bars indicate 90% confidence intervals of the mean. Urethane: (▨) cleft palate; (■) tail anomaly; (□) polydactyly. X-rays: mostly tail anomalies (see text). (*)$p < 0.05$; (**)$p < 0.01$ against urethane- or X-ray-treated groups without macrophages (*None*) and also with normal macrophages (*n-MØ*).

were injected intravenously into pregnant mice after urethane or X-ray treatment on day 9 (Fig. 2). Urethane- and X-ray-induced congenital malformations were significantly suppressed by the activated macrophages injected 0.5 h after treatment. Normal macrophages that were elicited by glycogen and purified similarly had no effect on malformation frequency. Such suppressive effects of activated macrophages on congenital malformations diminished or disappeared when macrophages were injected 48 h after urethane or X-ray treatment, suggesting that the teratogenic process was determined during these periods. It is noted that such suppressive effects of activated macrophages disappeared after the disruption of cells by supersonic waves. When Pyran-activated macrophages were treated in vivo with trypan blue, an inhibitor of lysosomal enzymes, 48 h before collection, they did not suppress congenital malformations (Fig. 2). These results indicate that a live activated macrophage with functioning lysosomal enzyme activities can be the critical effector cell for the suppression of congenital malformations.

Suppression of Spontaneously Developing Malformations by Activated Macrophages. A reduction of congenital malformations was observed similarly in strain CL/Fr, which has a high spontaneous frequency of cleft lips and palates. When Pyran-activated macrophages were injected intravenously into pregnant CL/Fr mice on day 9 (the critical stage for induction of cleft palates), the incidence of congenital malformations (35 cleft lips [18.2%] and 34 cleft palates [17.7%] among 192 fetuses) was significantly suppressed in comparison with that of untreated controls (58 cleft lips [28.2%] and 61 cleft palates [29.6%] among 206 fetuses; $p < 0.02$).

Suppression of Congenital Malformations by BCG and BCG-activated Macrophages. Similar effects on congenital malfor-

mations were observed with a biological agent, BCG vaccine (Fig. 3). Pretreatment with BCG significantly suppressed urethane- and radiation-induced malformations even when it was injected 6–46 d before pregnancy, although such suppressive effects of BCG were prominent on cleft palates and tail anomalies, but not on polydactylies. BCG-activated peritoneal macrophages collected ~40 d after BCG injection also suppressed urethane- and X-ray-induced congenital malformations. It is likely that chronic BCG infection could continuously activate macrophages to suppress maldevelopment.

Moreover, normal macrophages collected from older (12 mo of age) mice nursed in the conventional mouse rooms did suppress X-ray-induced malformations, although those collected from 2-mo-old mice in the SPF condition did not show significant suppressive effects (Fig. 2).

Effects of Activated Macrophages on Urethane-induced Somatic Mutation and Cell Killing. To ascertain the mechanism, a specific tester strain ($PTHTF_1$) of mice was used for detecting somatic mutation and cell death of the melanoblast in the embryo. As shown in Table 1, a large and significant increase of urethane-initiated cell deaths was observed by the intravenous injection of Pyran-activated macrophages, while activated macrophages showed no effects on the untreated embryo. However, activated macrophages did not affect the incidence of somatic mutation in both urethane-treated and untreated embryos. Consequently, it is strongly suggested that activated macrophages killed susceptible target cells with urethane-induced cellular (but not DNA) alterations for maldevelopment.

Detection of Activated Macrophages in the Embryo. To confirm the placental transfer of immune cells, activated and normal macrophages were first labeled with dextran sulfate, injected intravenously into pregnant ICR mice on day 9, which were killed 0.5–24 h later. About 75,000 serial sections were stained by toluidine blue. As shown in Fig. 4, metachromatically stained macrophages were first found in the maternal blood flow, and 6 h later, detected in the chorionic villi of the fetal placenta, indicating that macrophages can pass through the placental barrier. A few cells with metachromatic granules of dextran sulfate were found in the blood flow and interstitial tissue of the embryo.

Table 1. *Effects of Pyran-activated Macrophages on Urethane-induced Somatic Mutation and Cell Killing in the $PTHTF_1$ Mouse Embryo*

Urethane	Activated macrophage	Frequency per mouse: Somatic mutation	Frequency per mouse: Cell killing
mg/g			
1.0	0	25/195 (0.13)	12/195 (0.06)
1.0	5×10^6	29/190 (0.15)	33/190 (0.17)*
0	0	6/294 (0.02)	1/294 (0.003)
0	5×10^6	1/84 (0.01)	0/84 (0.0)

An estrous PT female was mated with HT males. Pregnant PT (56 females) were treated subcutaneously with urethane (1.0 mg/g) on day 10, and 0.5 h later, 5×10^6 Pyran-activated macrophages were injected intravenously into 27 of them. The remainders were not treated with activated macrophages. Effects of Pyran-activated macrophages on spontaneous frequency of somatic mutation and cell killing were similarly examined. Details for the experimental procedures are given in Materials and Methods.

* $p < 0.01$ by χ^2 test against urethane-treated group without activated macrophages.

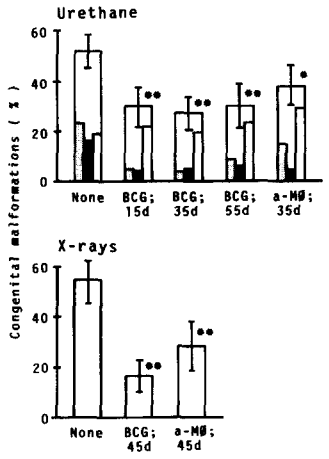

Figure 3. Suppression of urethane- and X-ray-induced malformations by BCG pretreatment and its association with macrophage activation. Pregnant mice were treated with urethane (1.0 mg/g) or X-rays (1.43 Gy) on day 9. The number of fetuses (pregnant mice) examined was 247 (23), 91 (8), 258 (26), 227 (22), and 141 (11) for urethane-treated groups without BCG (*None*), with BCG given 15, 35, or 55 d before urethane injection (*BCG;15 d, BCG;35 d, BCG; 55 d*), and with activated macrophages (*a-MØ;35 d*), respectively, and 337 (30), 184 (18), and 143 (13), for X-ray-treated groups without BCG (*None*), with BCG given 45 d before X-ray exposure (*BCG;45 d*), and with activated macrophages collected 45 d after BCG injection (*a-MØ; 45 d*), respectively. The experimental procedures are given in Materials and Methods. See legends to Figs. 1 and 2 for symbols and statistical analysis.

Discussion

Synthetic and biological agents that activate macrophages of host (pregnant) animals, and also resultant activated macrophages, cured both physically and chemically induced congenital malformations. Such suppressive effects of activated macrophages were not limited to radiation- and chemical-induced malformations, but extend to spontaneously developing congenital malformations. It is likely that activated macrophages killed precursor cells destined to cause congenital malformations. Such preteratogenic cells may be subsequently eliminated by macrophages and replaced by a normal totipotent blast cell, which plays its proper part in the multicellular society. Although the average number of living fetuses and their average body weight (data not shown) were not reduced by treatment with Pyran, BCG, or activated macrophages in the present study, cell destruction and replacement may cause postnatal functional damage in cured newborns, e.g., reduced life span, loss of immune competence, dementia, and so on. Such studies will be done in the future.

The problem is how activated macrophages can make contact with outliers. Chemicals of high molecular weight (>600) are known not to penetrate the placental barrier (13), but a possible placental transfer of some maternal immune cells has been suspected in man and mice (14, 15). In the present study, maternal macrophages could pass through the

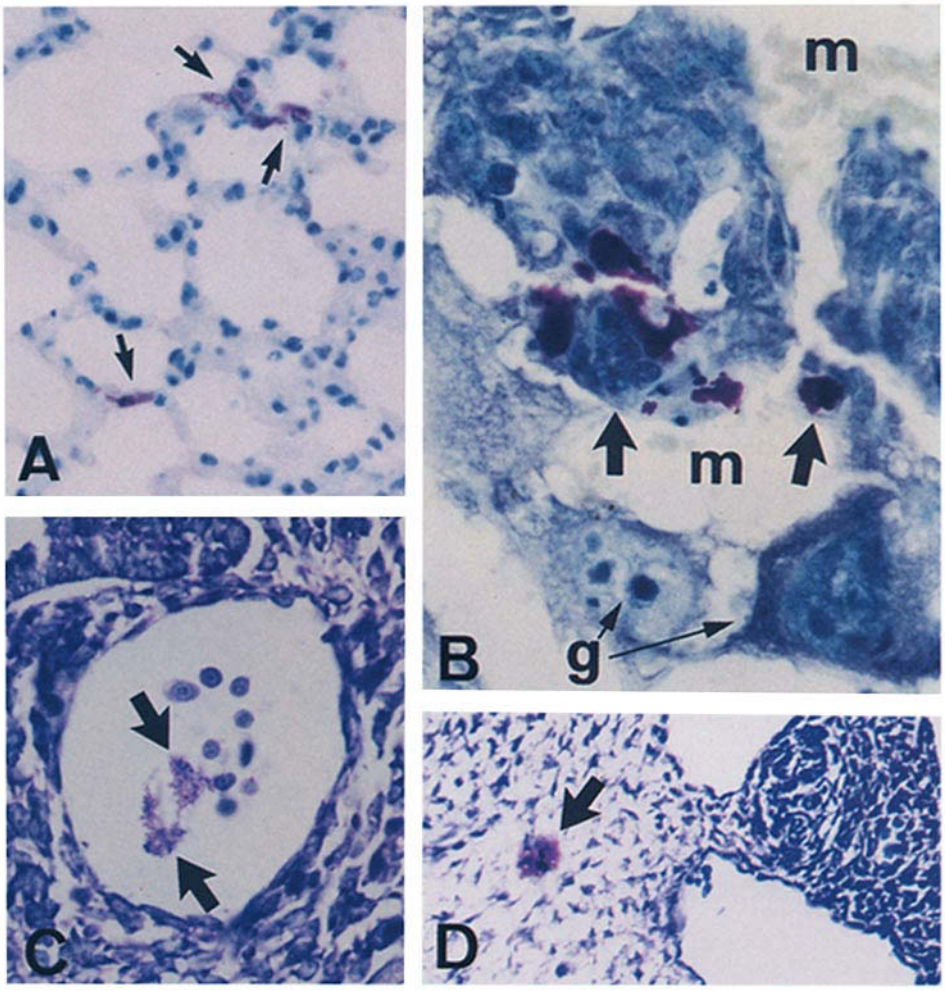

Figure 4. Placental transfer of activated macrophages. Dextran sulfate (25 μg/g; Pharmacia Fine Chemicals, Uppsala, Sweden) was injected intraperitoneally 24 h before the collection of Pyran (50 μg/g)-activated or normal macrophages (8). 10^7 cells were injected intravenously into pregnant ICR mice on day 9, and mice were killed 0.5, 2.5, 5, 6, and 24 h after injection. Serial sections of the pregnant uteri were made and stained by toluidine blue 0. (*A*) Macrophages stained metachromatically by toluidine blue (*arrows*) were seen in the capillary of the alveolar wall at 0.5, 2.5, 5, and 6 h after intravenous injection, but not after 24 h (×450). (*B*) Macrophages in the chorionic villi of the fetal placenta. Metachromatic cells are seen in the fetal chorionic villi (*arrows*) bathed in the maternal blood (*m*) 6 h after injection. *g*, giant cells (×450). (*C*) Macrophages in the sinus venosus of the day 9 embryo (*arrows*) (×450). (*D*) Metachromatic cell in the interstitial tissue of the day 9 embryo (*arrow*) (×225).

placenta, and a few were found in the embryo (Fig. 4). In contrast to chemical substances, living cells with amoeboid movements could pass through the trophoblast layer of the placental villus. However, it is still questionable whether such a small number of macrophages can kill preteratogenic cells, if direct contact with target cells is required for cell killing (8). Some unknown mechanisms might be involved in the suppression of congenital malformations by maternal macrophages.

It is noted that macrophages elicited by glycogen from aged mice nursed in the conventional mouse rooms did suppress X-ray-induced malformations, but those collected from young mice nursed in the SPF condition had little or no effect (Fig. 2). As in the case of BCG, potential infection may have activated macrophages slightly in non-SPF-aged mice. Consequently, it is not unreasonable to conclude that this homeostatic immunosurveillance system eliminating outliers (preteratogenic cells) can be naturally programmed for the protection of abnormal development in man and animals that are continuously exposed to biological modifying agents.

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