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Mouse major histocompatibility class I gene expression begins at midsomite stage and is inducible in earlier-stage embryos by interferon

(mouse embryos/major histocompatibility complex class I antigen expression)

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ABSTRACT **To determine the timing of major histocompatibility complex class I gene expression during embryonic development, binding of anti-class I antibodies and appearance of class I gene transcripts were examined in mouse embryos from the egg-cylinder stage through day 16 of gestation. By using two series of monoclonal antibodies reactive with monomorphic and polymorphic determinants of class I antigens, it was found that cell-surface expression of the antigens becomes detectable at a low level only after midsomite stage on gestation day 10, at a time when embryos are developed beyond primordial organogenesis and have partial blood circulation. In agreement with the above finding, a low level of class I mRNA became detectable in day 9 and older embryos in blot hybridization. The level of class I transcripts in embryos at least to day 13 remained less than 1/50th that in adult spleen cells. Cells from head-fold stage embryos (gestation day 8), which otherwise do not have an appreciable amount of class I mRNA or surface antigens, begin to express a high level of antigens upon treatment with mouse α/β or γ interferon. This induction of class I antigen expression appears to be stage specific in that embryos in an earlier egg-cylinder stage (day 6) failed to express the antigens after interferon treatment. A possible role of interferons in activating class I genes during *in vivo* embryonic development is suggested.**

The major histocompatibility complex (MHC) class I antigens play an essential role in immune responses and are highly polymorphic; more than 26 class I (or related) genes are found in the classic MHC and the adjacent *Qa/Tla* region in mouse (1, 2). Class I genes are evidently activated during embryonic development because cleavage embryos do not have the antigen (3), whereas at birth most somatic cells display the antigens on their surface. Our knowledge on the timing and mechanisms of class I antigen expression during embryonic development is conflicting and incomplete (see reviews in refs. 4 and 5–11). This issue warrants further reexamination because the class I antigens have been implicated to have a role in morphogenesis and cell-to-cell interaction in embryonic development (12) and because this question extends to a long-acknowledged immunological enigma of pregnancy—namely, the fact that the fetal allograft is not rejected by the immunocompetent mother (13). The issue seems even more urgent in the recent immunological view that self-class I and -class II antigens influence the development of T-cell immune repertoire during ontogeny (14). Previous difficulties in studying this problem include the necessity to use polyclonal allosera, which often contain antibodies of unknown reactivities (4–11), and the lack of specific DNA probes to test transcripts. In the present work we examined two criteria of class I gene expression: appearance of class I mRNA and display of surface antigens in embryos from the egg-cylinder stage through day 16 of gestation. We found that the antigens appear comparatively late in embryogenesis. This result led us to test activation of class I genes in early embryos by interferons (IFNs), molecules known to augment the level of class I antigens (15–18). We report that IFNs induce a high level of antigen in embryonic cells, suggesting a possible role for these molecules in normal embryonic development.

MATERIALS AND METHODS

Mouse Embryos and Cells. C3H/HeJ females (8–12 wk old) were mated with BALB/c males (10–30 wk old). The presence of a vaginal plug was taken as day 0 of pregnancy. Embryos at egg cylinder stage were obtained after a 4-day *in vitro* culture of preimplantation embryos harvested from superovulated pregnant mice at gestation day 4 (19, 20). The embryos were incubated in CMRL medium (GIBCO) supplemented with sodium pyruvate, glutamine, gentamicin, and a selected fetal bovine serum (10%) at 37°C in humidified 5% CO_2/95% air. Embryos at later stages (from gestation day 8) were dissected from the uterus. Placental tissues and yolk-sac membranes were removed in Dulbecco's modified Eagle's medium (DME medium). Embryos were then treated with 0.02% trypsin and 1 mM EGTA in phosphate-buffered saline containing 1% glucose and 25 mM Hepes for 10 min at room temperature, followed by an additional 10-min incubation at 37°C. Trypsin treatment, under these conditions, did not alter the level of surface class I antigen in control mouse L cells cultured *in vitro* (data not shown). Single-cell suspensions in DME medium containing 15% fetal bovine serum, which showed >90% viability, were used for further experiments. Fetal liver cells were prepared by teasing the organ without trypsin treatment.

Monoclonal Anti-Class I Antibodies. *Alloantibodies.* Mouse monoclonal antibodies reacting with polymorphic determinants of classic H-2 antigens used in this study have been described (21). For detection of maternal H-2K^k and D^k antigens, a combination of 16.1.2 and 3.83 (both anti-K^k, D^k) was used. For measuring the paternal antigens, a mixture of 34.1.2 (anti-K^d, D^d), 34.5.8 (anti-D^d), and 30.5.7 (anti-L^d) or a mixture of 20.8.4 (anti-K^d), 34.2.12 (anti-D^d), and 28.14.8 (anti-L^d) was used. For detection of class II (Ia) antigens, antibodies reactive with I-A^k, I-A^d, and I-$E^{k,d}$—i.e., 10.2.16 (obtained from the American Type Culture Collection), 25.9.17, and 14.4.4 (21)—were used. Embryos at later stages were tested with individual antibodies. All of the antibodies are IgG2a. A monoclonal IgG2a antibody reacting with rat neurophysin, negative for mouse tissues, was used as a control.

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Abbreviations: IFN, interferon; MHC, major histocompatibility complex.

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Table 1. Onset of class I antigen expression in mouse embryogenesis

Monoclonal antibody	^{125}I-labeled antibody binding, cpm per tube				
	Day 6 (egg-cylinder stage; 1×10^5 cells)		Day 8 (head-fold stage; 1×10^5 cells)	Day 9 (somite stage; 2.5×10^5 cells)	Day 10 (midsomite stage; 5×10^5 cells)
	Dissociated	Undissociated			
Rat, xeno					
K44	135 ± 15	160 ± 11	165 ± 15	237 ± 15	590 ± 51
K204	123 ± 10	155 ± 15	207 ± 23	210 ± 10	581 ± 43
42.3.9.8	140 ± 13	ND	230 ± 21	232 ± 13	593 ± 30
Negative control	175 ± 15	153 ± 12	241 ± 20	207 ± 18	280 ± 21
Mouse, allo					
Mixture (anti-K^k,D^k,K^d,D^d)	165 ± 20	215 ± 15	125 ± 14	181 ± 20	365 ± 18
Anti-K^k,D^k (maternal)	150 ± 13	ND	139 ± 13	198 ± 18	383 ± 20
Anti-K^d,D^d (paternal)	182 ± 21	220 ± 20	143 ± 10	153 ± 15	379 ± 31
Anti-L^d	ND	ND	ND	ND	321 ± 15
Negative control	180 ± 20	240 ± 18	131 ± 15	181 ± 19	330 ± 23

Xeno antibodies. Rat monoclonal antibodies reacting with common determinants of the antigens were used to insure detection of less polymorphic class I antigens encoded in the *Qa/Tla* region, which may be expressed only in embryos (22). K44, K204, and K260 were raised in Fisher rats immunized against mouse 3T3 cells by T. Hasegawa (National Cancer Institute, National Institutes of Health). The IgG antibodies showed reactivities characteristic of those against class I antigens as assessed by immunoprecipitation, reactivity to various established cells, and tissue/strain distribution, details of which will be presented elsewhere. 42.3.9.8, another rat anti-mouse class I antigen described by Springer (23), was obtained from the American Type Culture Collection. Both mouse and rat antibodies were in culture supernatants and were used at saturating concentrations determined by assays on adult spleen cells. Control IgG antibody K260 reacts with a cytoplasmic component of mouse cells and is negative for cell surface (unpublished data).

Preparation and Electrophoresis of RNA. Total RNA from embryos was prepared by the guanidinium thiocyanate procedure of Chirgwin *et al.* (24). More than 75 embryos were used to prepare RNA from day 8, 9, and 10 embryos. About 30–40 μg of RNA from each sample was heated at 60°C in 50% formamide/20% formaldehyde in 4-morpholinepropanesulfonic acid buffer and then electrophoresed in 1.1% agarose gel containing 15% formaldehyde in the running buffer.

Class I Gene Probe and Hybridization of RNA Blot. A single-stranded DNA probe, corresponding to a 242-base-pair sequence of the second external domain of the *H-2L^d* gene, was labeled with ^{32}P by a primer extension reaction. A synthetic oligomer G-C-C-C-G-C-G-G-C-C-C-C-T-G-C-A-C-C was annealed to 1 μg of the single-stranded *H-2L^d* gene cloned in phage M13 mp9 vector (25). Extension reaction was carried out with [α-^{32}P]dATP (800 Ci/mmol; 1 Ci = 37 GBq) at 25°C for 30 min as described (25). The labeled probe was isolated in polyacrylamide gel electrophoresis after digestion with *Nar* I. The filters were hybridized with the probe (3×10^6 cpm) at 42°C in buffer containing 20% formamide, 1 mM EDTA, 0.85 M NaCl, 0.1 M NaH_2PO_4, 0.1% $NaDodSO_4$, 7.5% dextran sulfate, 20 μg of salmon sperm DNA per ml, and 10× Denhardt's solution (0.2% polyvinylpyrrolidone/0.2% Ficoll/0.2% bovine serum albumin). The blot was then exposed to Kodak XAR-5 film for 1–7 days.

IFNs. Mouse IFN-γ produced in *Escherichia coli* by a cloned IFN-γ gene (26) was generously provided by Genentech. The material (lot no. 1551/67, ≈1.75 mg/ml) was stored at 4°C and was diluted immediately before use to 10, 100, and 1000 units/ml in the medium described below. A mixture of mouse IFN-α and IFN-β (IFN-α/β) was purchased from Lee Biomolecular Laboratories (San Diego, CA). Effects of IFNs on class I antigen expression were examined with single-cell suspension of embryos incubated in 96 flat-bottom microtiter plates (Falcon) at the concentration of 1–2 × 10^5 cells per 100–150 μl in DME medium supplemented with 15% fetal bovine serum, glutamine, and other ingredients (21). Cells were incubated at 37°C in 10% CO_2/90% air for up to 60 hr.

RIA. One to 5×10^5 dissociated embryonic cells were incubated with 50 μl of monoclonal antibodies for 60 min at 4°C. After being rinsed, cells were further incubated with ^{125}I-labeled sheep anti-mouse Ig or ^{125}I-labeled sheep anti-rat Ig (both from Amersham) for 60 min at 4°C. The sensitivity of the assay was at least a magnitude higher than that of complement-mediated cytotoxicity assay combined with a developing anti-mouse Ig reagent or immunocytochemistry tested with frozen sections of adult thymus. In the RIAs a significant signal was detected with 50–100 cultured mouse L cells (data not shown). Adult thymocytes rather than adult spleen cells were used as a positive control, because of their low background.

Cytofluorography. Cytofluorography was performed as described (25) with biotin-labeled anti-mouse Ig or anti-rat Ig (diluted 1:50; Vector Laboratories, Burlingame, CA) in combination with fluoresceinated avidin (diluted 1:100; Vector Laboratories).

RESULTS

Onset of Surface Class I Antigen Expression in Midsomite-Stage Embryos. To determine the time at which class I gene expression begins in mouse embryogenesis, the appearance of surface class I antigens was examined in C3H × BALB/c F_1 embryos from day 6 (egg-cylinder stage) to day 16 of gestation. Embryos at an earlier blastocyst stage have been reported to be negative for the antigen, although data are conflicting (4–6, 9, 11). Two types of monoclonal antibodies were used: (*i*) rat xeno antibodies reacting with common determinants of class I antigens necessary to detect less polymorphic Qa/Tla antigens, and (*ii*) mouse alloantibodies reacting with polymorphic H-2 antigens in classic MHC, capable of distinguishing paternal and maternal antigens (see *Materials and Methods*).

Results of highly sensitive RIAs carried out on dissociated embryonic cells (1–5 × 10^5 cells per tube) are presented in Table 1. Neither rat nor mouse antibodies bound embryonic cells at gestation day 6 and 8, which represent embryos at egg-cylinder and head-fold stage, respectively. The egg-cylinder embryos, obtained after a 4-day culture of preimplantation embryos, underwent differentiation into endoderm and ectoderm and showed trophoblast outgrowth (19, 20). Day 8 embryos dissected from the uterus have head fold, heart,

Table 1. *Continued*

^{125}I-labeled antibody binding, cpm per tube									
Day 12 (2.5 × 10^5 cells)			Day 13 (5 × 10^5 cells)			Day 16 (5 × 10^5 cells)			Control adult thymocytes
Fetal liver	Body trunk	Brain	Liver	Body trunk	Brain	Liver	Body trunk	Brain	(1 × 10^5 cells)
583 ± 30	396 ± 21	280 ± 19	719 ± 51	564 ± 18	303 ± 31	811 ± 40	651 ± 60	450 ± 21	2455 ± 140
563 ± 38	423 ± 23	338 ± 35	699 ± 44	559 ± 27	310 ± 20	821 ± 30	ND	ND	2382 ± 133
ND	ND	ND	685 ± 30	582 ± 23	ND	688 ± 40	590 ± 30	ND	2258 ± 105
190 ± 18	221 ± 19	251 ± 28	230 ± 20	250 ± 15	285 ± 33	255 ± 23	280 ± 25	383 ± 15	110 ± 5
321 ± 11	215 ± 15	ND	ND	ND	ND	ND	ND	ND	1290 ± 75
338 ± 15	ND	ND	698 ± 61	555 ± 23	321 ± 15	630 ± 36	581 ± 34	ND	923 ± 50
398 ± 18	208 ± 13	ND	819 ± 63	605 ± 11	358 ± 23	770 ± 52	599 ± 23	ND	895 ± 29
ND	ND	ND	570 ± 22	410 ± 25	ND	592 ± 33	ND	ND	313 ± 21
159 ± 13	115 ± 12	ND	360 ± 11	301 ± 10	285 ± 30	340 ± 30	313 ± 19	ND	190 ± 13

C3H × BALB/c embryos were dissociated by a mild trypsin treatment, and 1–5 × 10^5 cells per tube (as indicated) were tested for antibody binding. The values represent the binding of ^{125}I-labeled antibody (cpm per tube), given as the mean of triplicate determinations ± SDs. Results in this table are a summary of 51 independent experiments. ND, not determined.

and 1–6 somites and are in the process of forming forgut and blood islets (27). The average number of cells recovered from an embryo at this stage was about 3 × 10^4, in approximate agreement with a previous report (28). Day 9 embryos in which a functional heart with circulating blood became visible were also negative for class I antigens (Table 1). Embryos at this stage, having an average of 2–5 × 10^5 cells per embryo, are in the process of rapid organogenesis to form neural crest, intestinal tract, nephritic vesicles, and primordial germ cells (27). The size of embryos at this stage varied, and cell recovery ranged between 2 × 10^5 and 5 × 10^5. Slightly positive binding was found with some rat antibodies at this stage in two of six experiments, which is probably attributable to occasional asynchrony of embryonic development (27).

Embryos obtained from gestation day 10 had a larger brain, prominent branchial arches, and limb buds (27). It is in embryos at this stage when low but significantly positive antibody binding was first observed. Binding was detected only with the rat xeno antibodies, and the level was about 1/30th to 1/50th that of adult control thymocytes (Table 1). Immediately thereafter, in embryos at gestation day 11, binding of both mouse and rat antibodies became positive. Cytofluorography analysis (Fig. 1) showed that day 11 embryos contain a small population of distinctly antigen-positive cells, although the antigen level was again quite low. In contrast, most of adult F_1 thymocytes showed strong fluorescein signals (data not shown). On gestation day 12 and onward embryos develop a liver (the main fetal hematolymphopoietic organ), which can be separated by dissection, and have rapidly forming spleen, pancreas, primordial thymus, and extremities. Antigen expression was tested in three different parts of embryos in these older embryos. Significant binding was noted for fetal liver and body trunk in day 12 and day 13 embryos with both rat and mouse antibodies, binding being always higher in fetal liver than in body trunk, although fetal liver was still much lower than adult thymocytes. The brain region was, in general, negative for antigen expression (Table 1). Up to gestation day 16, the level of antigen expression did not change much and remained distinctly lower than that of adult thymocytes.

Detection of Class I mRNA in Day 9 and Older Embryos. Because surface class I antigens became detectable in embryos only on day 10 of pregnancy, we examined the level of class I mRNA starting with embryos at gestation day 8 through day 13 by blot hybridization with a single-stranded DNA probe corresponding to the *C1* domain of the *H-2L^d* gene. Results in Fig. 2 show the presence of the expected mRNA of about 1.8 kilobases in adult spleen RNA [both total and poly(A)$^+$ RNA]. In contrast, RNA preparations from all embryonic stages tested contained a strikingly low level of class I mRNA. However, a faint band at 1.8 kilobases was detectable in RNA from day 9 and older embryos. These RNA preparations from embryos and adult spleen used in the above experiments contained a similar level of mRNA for the β-actin (data not shown). These results are in agreement with those in the preceding section on antibody binding and suggest that class I genes are not transcribed significantly up to day 9 of embryonic development, and the mRNA levels remain low up to day 13 of gestation. It is possible, however, that the mRNA is present in earlier embryos at the level below the detection limit.

Induction of Class I Antigen Expression by IFN in Day 8 but Not in Day 6 Embryos. IFNs augment the level of class I antigen expression in a variety of tissue culture cells, and IFN-γ is also known to induce class II antigens in certain cells (15–18). In this work the effect of IFN-γ was examined on embryonic cells at day 6, 8, and 9, at which time no class I antigens were detectable (see Table 1). Dissociated embryonic cells were plated in a microtiter plate and incubated with IFN-γ (10–1000 units/ml) up to 38 hr. Upon plating, more than 90% of the embryonic cells, heterogeneous in morphology, adhered to the plastic within 6 hr and remained adherent throughout the experiments. A high level of class I antigens was demonstrated in cells obtained from embryos at gestation day 8 and day 9, 38 hr after the initiation of IFN-γ treatment (Fig. 3). The antigen induction was observed by alloantibodies to K, D, and L of classic MHC (Fig. 3) and by xenoantibodies (data not shown). The induction was detectable even 24 hr after the treatment but became more pronounced after a longer period of incubation. Thirty-eight hours after the incubation, the level of the antigens was comparable to or higher than that of adult cells.

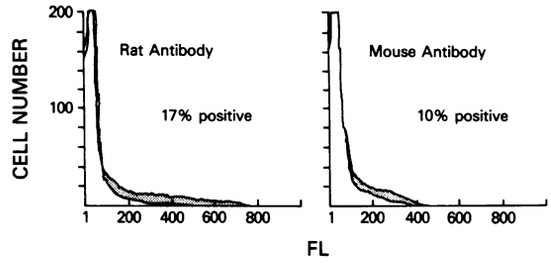

FIG. 1. Cytofluorography profile of class I antigen expression in day 11 embryos. Dissociated day 11 embryos were tested for binding of rat xeno (K44) and mouse allo (a mixture, anti-K^k,D^k,K^d,D^d) monoclonal antibodies. FL, fluorescence. Shaded areas represent antigen-positive cells.

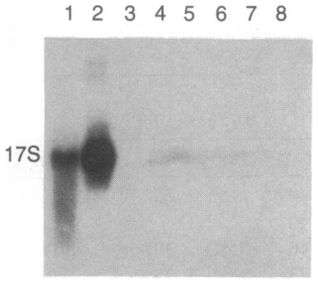

FIG. 2. Detection of class I mRNA in various stages of embryos. Total RNA (35 μg) (lane 1) and poly(A)$^+$ RNA (1.5 μg) (lane 2) from C3H × BALB/c F_1 adult spleen were compared with total RNA (30–40 μg each) from C3H × BALB/c F_1 embryos at gestation days 8 (lane 3), 9 (lane 4), 10 (lane 5), 11 (lane 6), 12 (lane 7), and 13 (lane 8) in blot hybridization with a class I gene-specific probe. 17S size marker is indicated.

In contrast, antigen expression remained at the background level in cells not treated with IFN. Class II antigens (I-A and I-E) were not induced in these cells under these conditions. Cells treated with IFN-γ were indistinguishable from untreated cells in their morphological appearance, and cell recovery was not significantly different between the two groups during the incubation period. Class I antigen induction was also noted by IFN-α/β in day 8 and day 9 embryos (data not shown). Results of cytofluorography analysis indicated that a large fraction of embryonic cells became positive for the antigens: up to 50–60% of cells were positive (data not shown). This indicates that IFN treatment induced class I antigen expression in a large fraction of embryonic cells in a short period of time.

In contrast, no antigen induction was found in earlier embryos at the egg-cylinder stage (day 6) as tested by either antibodies even when cells were assayed after 60 hr of incubation (Fig. 3). Most cells from this stage adhered to plastic and grew in the plate; cell viability was >85% regardless of IFN treatment, indicating that the failure of antigen induction was not due to gross deleterious effects of the culture conditions.

DISCUSSION

We report here that class I mRNA begins to be detectable in midsomite stage embryos at day 9 of gestation and that, in accordance, surface class I antigens start to appear at gestation day 10. This places the initiation of class I gene expression at or following major events in primordial organogenesis; embryos at these stages already possess a functional heart with a partial blood circulation and are in the process of forming brain, intestinal tract, nephritic vesicles, and germ cells (27). Further, we found that the level of class I mRNA and that of surface antigens remains much lower than those in adult tissues at least through gestation days 13–16.

The relatively late appearance of surface class I antigen found in this study is in contrast to previous reports in which antigen expression was detected in earlier embryos at preimplantation or blastocyst stage (4, 5, 7, 11). This discrepancy may be due in part to the use of undefined polyclonal sera in the previous studies. It is also possible that class I antigens are expressed transiently in earlier embryos or in day 6 embryos grown *in vivo*, which we did not assay. On the other hand, the present results support those reported by Buc-Caron *et al.* (6), Kirkwood and Billington (8), and Edidin (10), who found the first antigen appearance during a midgestational period, even though all of these studies used polyclonal sera.

The expression of polymorphic H-2 antigens was detectable by alloantibodies 1 day after class I antigens became measurable by xenoantibodies. The basis for this delay is not clear at present. One possibility is that only *Qa/Tla* region antigens are expressed during a brief period of embryogenesis (22). Alternatively, the apparent delay may be due to a differential affinity of the antibodies. Even if the former is the case, Qa/Tla antigens do not constitute the sole, predominant embryo-specific class I antigens as suggested (22), since a detectable level of class I mRNA was found in day 9 and older embryos, and classic H-2 antigens become detectable immediately thereafter.

The simplest interpretation for the late appearance and low levels of mRNA found in embryos (Fig. 2) would be that the class I genes are not transcribed significantly up to day 9 of embryogenesis and the rate of the transcription remains quite low for the following days. However, we cannot exclude at present the alternative possibility that class I mRNA are degraded much more rapidly in embryos than in adult cells or that mRNA levels below the detection limit are present in earlier embryo.

Our results indicate that class I genes are developmentally regulated and are expressed in a stage-specific manner. In this sense, class I genes apparently do not belong to the broad category of "housekeeping" genes, which are expressed ubiquitously from very early stages of embryogene-

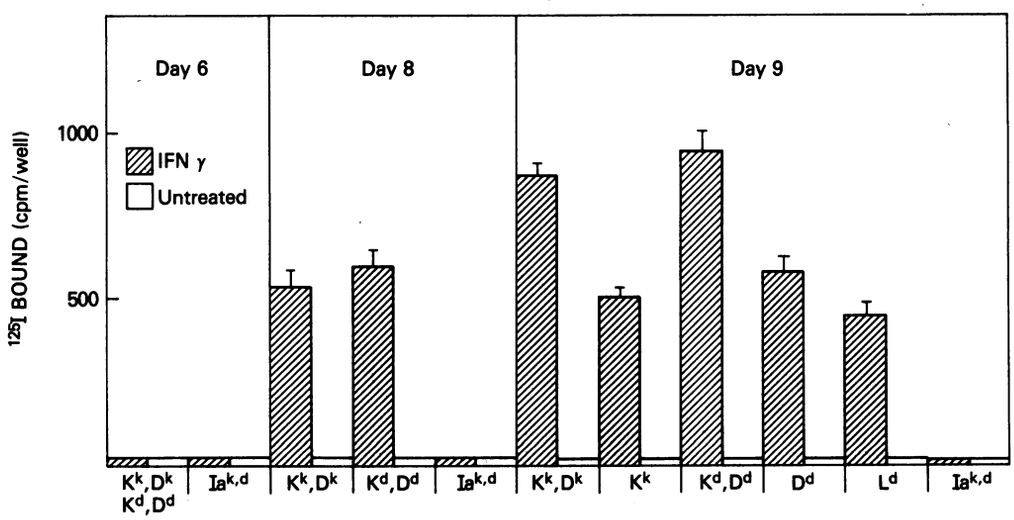

FIG. 3. Induction of class I antigens in early embryonic cells by IFN-γ. Dissociated embryonic cells from days 6, 8, and 9 were plated in a 96-well microtiter plate (1–2 × 10^5 cells per well). IFN-γ at 100 units/ml was added at the initiation of culture, and cells were incubated for 38 hr at 37°C in 10% CO_2/90% air. The values represent means of triplicates ±SD.

sis (29). The late appearance of the MHC class I antigens is even more striking when compared to minor histocompatibility antigens and β_2-microglobulin reported to be expressed in blastocyst or even earlier embryos (3, 4, 30). Our results cast doubt on the previously suggested major role of the MHC antigens in early embryonic morphogenesis (12) but rather may imply that the MHC genes are expressed in association with immunological maturation of embryos. The absence of the fetal allograft rejection in pregnancy may be partly due to low-level expression of class I alloantigens in the developing fetus that is insufficient to evoke actual graft rejection by the mother.

We show that IFNs are capable of inducing class I antigens in early embryonic cells at gestation day 8. The ability of IFNs to induce the antigens seems to be stage specific in that earlier embryos failed to express the antigens upon IFN treatment. These results support the previous finding that the antiproliferative effect of IFN-α is first demonstrable in cells cultured from day 8 embryos (31) and indicate that the ability to respond to IFN is also developmentally regulated. However, alternative explanations are possible—e.g., those egg-cylinder embryos developed *in vitro* may have lacked an unidentified component(s) necessary to respond to IFN. Our study indicates that the onset of the responsiveness to IFNs precedes that of class I gene expression. Since IFNs exert their effects through binding to specific cell-surface receptors (32, 33), the receptors for IFNs must be expressed earlier than MHC class I antigens. Thus, it is possible that class I antigen expression is regulated by the endogenous production of IFNs and by the expression of the IFN receptors. In humans the level of IFN-γ produced spontaneously in newborns is much higher than that in adults (34) and IFN-γ promotes class II antigen expression in fetal monocytes (35), suggesting a developmental role of IFN. It would be of interest to study the source of IFNs and expression of IFN receptors in embryos.

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