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Synthesis of H-2 Antigens by Preimplantation Mouse Embryos[1]

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ABSTRACT

An enzyme-linked immunosorbent assay (ELISA), which utilized anti-H-2 monoclonal antibody, was used to detect H-2 antigens on preimplantation mouse embryos. All embryonic stages studied, including unfertilized eggs and 1-cell, 2-cell, 8-cell, and blastocyst-stage embryos, showed the presence of H-2 antigens. To prove that the H-2 antigens were not cytophilically adsorbed to the embryos, blastocysts were treated with papain to strip off the H-2 antigens, and then the embryos were further incubated to allow the H-2 antigens to regenerate. After a 3-h incubation time, 60% of the H-2 antigens on the embryos had reappeared, proving that the H-2 antigens were synthesized by the embryos themselves.

INTRODUCTION

The MHC (major histocompatibility complex, H-2 in the mouse) has been the subject of intense study during the last decade. All animals possess a MHC that codes for the predominant surface proteins (antigens) on cells, tissues, and organs of each member of the species. Except for inbred animals and identical twins, the MHC antigens are unique for each individual and serve as a biologic marker to distinguish self from nonself. The protein and gene structure of many of the products of the H-2 complex have recently been elucidated (Hood et al., 1983). The function of MHC products in the immune response is well documented (Klein et al., 1983), and recently attention has been focused on nonimmune as well as immune functions of the MHC (Edidin, 1983). For instance, in the mouse, genes in the H-2 complex influence various traits associated with reproduction and development (Yamasaki et al., 1976; Melnick et al., 1981; Goldbard et al., 1982; Bonner and Tyan, 1983).

One area that has not yet received adequate attention is the ontogeny of the MHC antigens. The literature has been highly controversial on the subject of whether or nor preimplantation mouse embryos express H-2 antigens (Heyner, 1983). Most of the controversy has been generated by the diversity of antibodies and immunoassays used in the various studies. However, in the past few years, more and more positive reports have appeared supporting the view that H-2 antigens are indeed expressed on early mouse embryos (Searle et al., 1976; Krco and Goldberg, 1977; Webb et al., 1977; Cozad and Warner, 1981, 1982; Goldbard et al., 1984; Warner and Spannaus, 1984). These recent studies have used congenic alloantisera and monoclonal antibodies, coupled with highly sensitive techniques, to demonstrate unequivocally the presence of H-2 antigens on the embryonic cell surface. It now seems clear that earlier negative results were due to the use of poor antisera or insensitive assays.

This paper reports the results of our research on the question of whether H-2 antigens, detectable on mouse blastocyst embryos, are synthesized by the embryos themselves, or are merely cytophilically bound to the embryonic cell surface. We found using monoclonal antibodies and an enzyme-linked immunosorbent assay (ELISA), that H-2 antigens are detectable on unfertilized ova, as well as on 1-cell, 2-cell, 8-cell, and blastocyst-stage embryos. Moreover, we found that H-2 antigens may be stripped off the surface of blastocyst-stage embryos with papain, and then detected again after a 3-h regeneration time. This proves that H-2 antigens are actively synthesized by preimplantation mouse embryos.

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MATERIALS AND METHODS

Embryos

Embryos for this study were collected from CF-1 mice (Charles River Breeding Laboratories, Wilmington, MA) and C57BL/10Sn mice (Jackson Laboratory, Bar Harbor, ME). Mice were housed in a photoperiod-controlled room (14L:10D). Mature female mice were superovulated with 5 IU PMS (pregnant mare's serum; Sigma, St. Louis, MO) at 1300 h followed 48 h later by 5 IU hCG (human chorionic gonadotropin; ICN Biochemicals, Cleveland, OH). Females were placed individually with a single male immediately after hCG injection and checked for the presence of a vaginal plug the following morning. Embryos were collected into Whitten and Biggers (1968) medium (WB) 89, 65, 41, and 17 h post-hCG injection, corresponding to the blastocyst, 8-cell, 2-cell, and 1-cell stages of development.

For the collection of unfertilized ova, mice were superovulated as described above but were not mated. The eggs were obtained from the ampullary portion of the oviducts and treated with a solution of 300 U/ml hyaluronidase (Sigma) in WB medium to remove cumulus cells.

Determination of Cell Number Per Embryo

Embryos of 4 cells or less were scored visually. In order to assess the number of cells per embryo at more advanced stages, embryos were subjected to the Tarkowski (1966) method. Briefly, the embryos were fixed and stained, after which the number of nuclei were counted using a Zeiss phase-contrast microscope at 400X magnification.

Tissue Culture Cells

Cells known to express H-2 antigens were used as a positive control to compare with the data collected on the embryos. The cells used were P815 (H-2^d mastocytoma cells obtained from the American Type Culture Collection (Rockville, MD), and maintained in D-MEM (Dulbecco's Modified Eagle's Medium/GIBCO, Grand Island, NY), supplemented with 10% fetal calf serum (FCS; GIBCO), 1% antibiotic/antimycotic (GIBCO), and 0.5% gentamicin (Schering, Kenilworth, NJ).

Rabbit antimouse serum. Rabbit antimouse serum was prepared by injection of 1×10^8 C57BL/10Sn spleen cells into the marginal ear vein of an 8-pound female New Zealand rabbit. Three injections were given (on Days 1, 10, and 20) with a test bleeding performed on Day 30. Three more booster injections of 1×10^8 spleen cells were given about 6 wk apart, with a cardiac puncture performed after the final booster injection. The serum was collected and heat-inactivated at 56°C for 30 min.

Monoclonal antibodies. Monoclonal antibody M1/42.3.9.8 (anti-H-2) and 53-7.313 (anti-Ly1.2) (hereafter abbreviated M1/42 and 53-7) cell lines were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in suspension culture in D-MEM, 10% FCS, 1% antibiotic/antimycotic, and 0.5% gentamicin at 37°C in 5% CO_2 in air. Both antibodies are rat monoclonal antibodies and both were purified from culture supernatant by a 45% ammonium sulfate precipitation, followed by Sephadex G-200 chromatography (Goldbard et al., 1984). The statement that M1/42 recognizes H-2 antigens is used for convenience throughout this paper. In fact, M1/42 may detect all class I antigens including Qa and T1a antigens (Good et al., 1983).

Rabbit antirat serum. Rabbit antirat serum (affinity purified) was purchased from Zymed (Burlingame, CA).

Enzyme-linked Immunosorbent Assay Procedure

The ELISA procedure used in this study was developed in our laboratory and has been described in detail (Goldbard et al., 1984). Monoclonal antibody M1/42 was used to detect H-2 antigens because it recognizes a public specificity common to all H-2 antigens (Stallcup et al., 1981). Monoclonal antibody 53-7 has no reactivity with H-2 antigens and was therefore used as the negative control (Goldbard et al., 1984). A brief description of the ELISA method for cells and embryos follows.

Cell ELISA. The P815 cells were fixed with glutaraldehyde on microtiter plates at 5×10^6 cells/ml. The plates were stored in 200 μg/ml gelatin/1% sodium azide, in phosphate-buffered saline, at 4°C until they were used. The ELISA was started by adding 100 μl of appropriately diluted antibody (conventional or monoclonal) to the wells and incubating for 1.5–2 h at 37°C. After extensive washing, the second antibody, rabbit antirat serum at a 1:200 dilution, was added and the plates were incubated for 1 h at 37°C. After washing, a 1:400 dilution of protein A-β-galactosidase (Zymed) was added, the plates were incubated for 1 h at 37°C and washed, and the substrate *O*-nitrophenyl-β-D galactopyranoside (Sigma) was added. After 1 h incubation at 37°C the reaction was stopped by the addition of 0.1 M Na_2CO_3 and color intensities were measured spectrophotometrically at 410 nm.

Embryo ELISA. The procedure on the embryos was performed in depression slides. The embryos were manipulated with micropipets, using a dissecting microscope, through the various reagents and washing procedures. The assay conditions on embryos were the same as those on cells except that the rabbit antirat serum was used at a 1:100 dilution, and the protein A-β-galactosidase was used at a 1:50 dilution. In the last step of the assay, the embryos were transferred to microtiter plate wells, the substrate solution was added, and the results were read spectrophotometrically at 410 nm.

Pronase Treatment of Embryos—Time Course

Blastocyst-stage embryos were treated with pronase [Sigma; 0.5% solution in WB medium without bovine serum albumin (BSA)] for 2, 5, 7, and 10 min. After each time point, embryos were removed from the enzyme and washed twice in WB medium with BSA and twice in PBS before using them in the ELISA procedure.

H-2 Removal and Recovery Experiments

For all the H-2 removal and recovery experiments, embryos were first treated with Tyrode's solution according to Thadani (1982) to remove the zonae pellucidae.

Pronase. Embryos were treated for 6 min with a 0.5% solution of pronase in WB medium without BSA, after which they were washed as described earlier, and

used in the ELISA procedure with 5 embryos per well. Part of the embryos were treated only with WB medium (no-treatment controls) and others were washed and left to recover for 3 h at 37°C in 5% CO_2 before the ELISA procedure was performed (recovery controls). The same protocol was followed with the P815 cells.

Papain. Removal of H-2 antigens with papain was performed based on the method of Gromkowski et al. (1983). Zonae-free embryos were treated with enzyme treatment medium [ETM (pH 7.4): Earl's balanced salt solution/15 mM HEPES/20 mM L-cysteine/1% fetal calf serum/3 mg/ml papain (Sigma)] for 30 min, after which they were washed and used in the ELISA procedure. No-treatment controls were embryos treated with enzyme-free ETM. After enzyme treatment, some embryos were washed and placed in the incubator (37°C, 5% CO_2) for 3 h for recovery. The same protocol was used for P815 cells except that they were washed and incubated in D-MEM instead of WB medium.

RESULTS

Expression of H-2 Antigens during Development

H-2 expression during development was detected using the embryo ELISA, and plotted as absorbance per embryo (Fig. 1).

H-2 Removal and Recovery Experiments

The effect of the zona removal process on H-2 antigen expression was assessed by comparing zonae-free embryos (previously treated with Tyrode's solution; see *Materials and Methods*) with intact embryos. It can be observed from Fig. 2 that the removal of the zonae enhanced absorbance in the ELISA test, probably indicating increased access of the antibody to the embryonic cells.

For the pronase recovery experiments, the time of incubation with enzyme was determined from the experiment shown in Fig. 3. It was

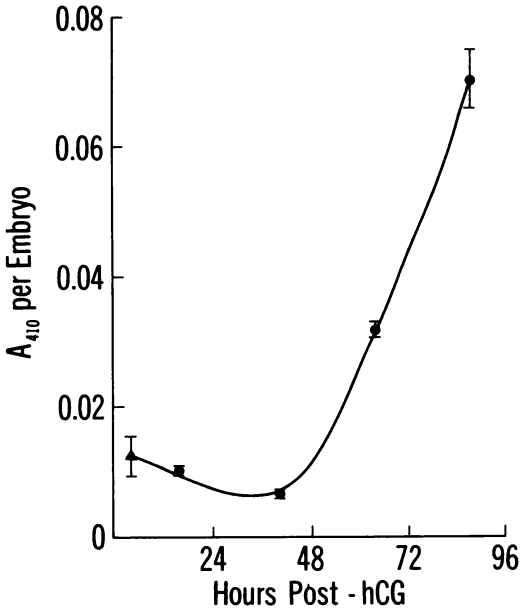

FIG. 1. Detection of H-2 antigens on unfertilized eggs (▲) and preimplantation mouse embryos (●) by the ELISA method, as described in the text.

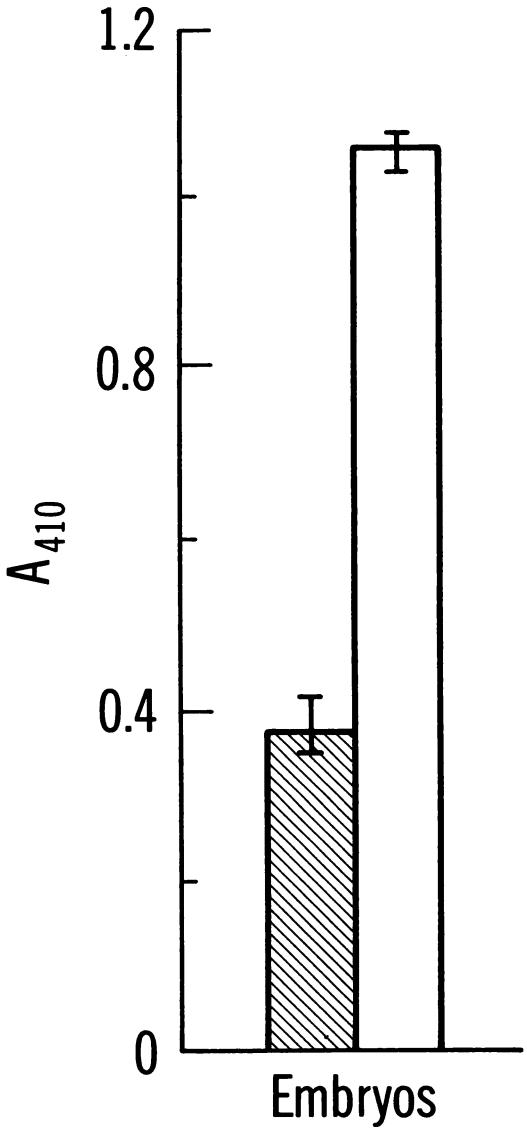

FIG. 2. Effect of zona pellucida removal on the ELISA method. A comparison of H-2 antigen level detected on mouse blastocysts with (*batched bar*) and without (*open bar*) the zona pellucida is shown.

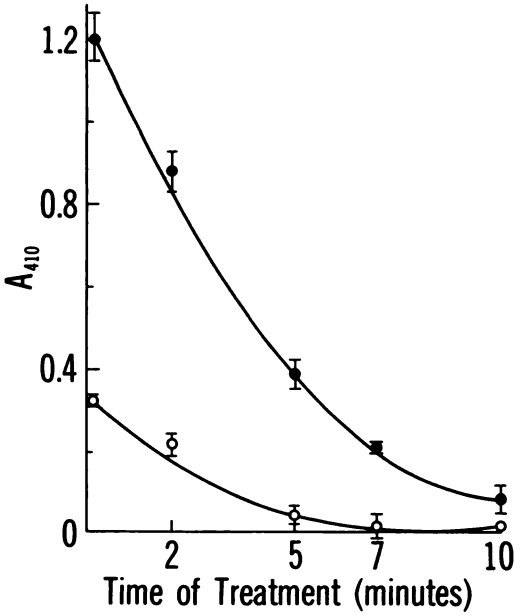

FIG. 3. Time course of treatment of mouse blastocysts with pronase. After pronase treatment the embryos were assayed by the ELISA procedure with either monoclonal antibody M1/42 (•) or PBS (○), as described in the text.

found that the complete removal of surface antigens reacting in the ELISA method is obtained between 5 and 7 min after enzyme treatment. Therefore 6 min was used as the time of pronase treatment for the recovery experiments. In the case of papain, 30 min incubation was used since this was the optimum time reported for the removal of H-2 antigens from P815 cells (Gromkowski et al., 1983).

Pronase

P815 cells, treated 6 min with pronase, showed a 50% decrease of reactivity with anti-H-2 M1/42 monoclonal antibody (Fig. 4A). The decrease was marked when polyclonal rabbit antimouse antibody was used in the assay (Fig. 4A). This shows that the enzyme removed most surface antigens in a nonspecific manner. However, a 3-h recovery period brought the absorbance back to its original level. Fig. 4B shows that the embryos were much more susceptible to the enzyme than were P815 cells. The enzyme removed all surface antigens detectable by both types of antibodies (M1/42 and rabbit antimouse polyclonal). Furthermore, no recovery was shown by the embryos after a 3-h incubation period. A longer incubation period (6 h) did not change the results. (Embryos treated with pronase for 6 min are not dead. They develop to term when transferred to pseudopregnant foster females.)

Papain

Fig. 5A shows that papain specifically removed H-2 antigens from the surface of cells since more than an 80% decrease in absorbance was observed with the M1/42 antibody. Moreover the decrease in absorbance with the polyclonal antimouse serum was lower, showing the presence of non-H-2 murine antigens after papain treatment. After a 3-h recovery period more than 95% of the original level of absorbance was detected as a result of the resynthesis of H-2 antigens by the cells.

Treatment of the embryos with papain showed very similar results (Fig. 5B). A very sharp decrease in absorbance was observed when M1/42 antibody was used, but less decrease was observed when the polyclonal serum was used. This shows that the blastocysts are expressing both H-2 and non-H-2 antigens. The embryos, as well as the cells, recovered after 3 h. Again, further incubation (6 h) did not change the results.

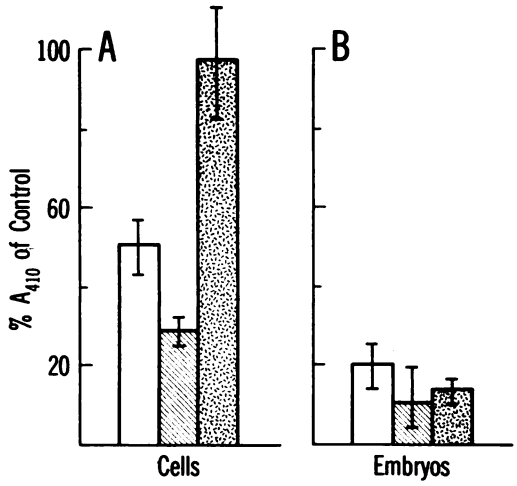

FIG. 4. Treatment of cells (*A*) or embryos (*B*) with pronase. The cells or embryos were either subjected to the ELISA procedure immediately after pronase treatment, using monoclonal antibody M1/42 (*open bars*) or polyclonal rabbit antimouse antibody (*hatched bars*), or after a 3-h recovery period (*speckled bars*), after which they were assayed with M1/42 monoclonal antibody.

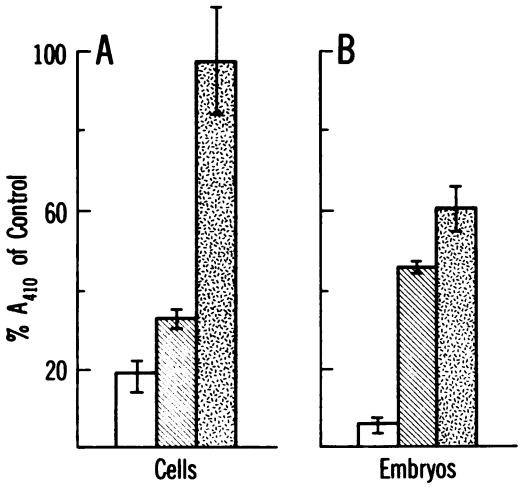

FIG. 5. Treatment of cells (*A*) or embryos (*B*) with papain. The cells or embryos were either subjected to the ELISA procedure immediately after papain treatment, using monoclonal antibody M1/42 (*open bars*) or polyclonal rabbit antimouse antibody (*batched bars*), or after a 3-h recovery period (*speckled bars*), after which they were assayed with M1/42 monoclonal antibody.

DISCUSSION

In this study H-2 antigens are shown to be present on unfertilized eggs, and on embryos as early as the 1-cell stage of development (see Fig. 1). It was possible to obtain these results because we used a highly sensitive ELISA technique developed in our laboratory (Goldbard et al., 1984). The results in Fig. 1 are plotted on a per-embryo basis. We determined the number of cells per embryo, and calculated the results on a per-cell basis (see Table 1). It can be seen that the increase in H-2 per embryo reflects an increase in the number of cells per embryo up to the 65-h time point, at which time the amount of H-2 antigen per cell seems to decrease. However, from electron microscopic studies, using immunoperoxidase labeling (Searle et al., 1976; Warner and Spannaus, 1984), we know that H-2 antigen concentration on the trophectoderm of blastocysts is at least as high as that on 8-cell embryos. This paradox is resolved by assuming that the ELISA reagents cannot penetrate the blastocoele and are therefore detecting H-2 antigens only on the outside of the blastocyst. Recalculation on the basis of number of "outside" cells clearly indicates that blastocysts express at least equivalent amounts of H-2 on their outside cells as do 8-cell embryos.

Previous studies of H-2 antigen expression on embryos have concentrated on the detection of antigens on the cell surface. An extremely important question is whether the H-2 antigens are cytophilically adsorbed to the surface of the embryos, or if they are synthesized by the embryos themselves. The results given in Fig. 5 clearly show that embryos can be stripped of their H-2 antigens by papain, and can then regenerate the antigens after further incubation. Thus, there is no question that the H-2 antigens are synthesized by the embryos themselves. The embryos show results similar to cells in tissue culture, except that the maximum recovery is only about 60%, compared to 95% for the tissue culture cells. This could be due to differences in the rate of H-2 synthesis between both cell types, one being a transformed cell line (P815) and the other being normal embryonic cells. Other workers have found very low levels of mRNA specific for H-2 in trophoblast cell clones (Tanaka et al., 1983). This is in agreement with the slow regeneration time ob-

TABLE 1. H-2 on mouse embryos detected by ELISA method.

Time post-hCG (h)	Mean cell no./embryo (SEM)[a]	Theoretical no. of "outside" blastomeres[b]	ELISA results: A_{410} per		
			Embryo	Cell	"Outside" cell
17 (1-cell)	1.0 (0.0)	1	0.01	0.01	0.01
41 (2-cell)	2.0 (0.0)	2	0.007	0.0035	0.0035
65 ("8-cell")	6.8 (0.3)	7	0.032	0.004	0.004
89 (blastocyst)	39.3 (1.8)	19	0.070	0.002	0.004

[a]Determined by Tarkowski (1966) method, as described in the text. A minimum of 30 embryos were scored for each point.

[b]Extrapolated from Handyside (1981).

served in our studies. It would be interesting to measure mRNA levels directly for H-2 antigens in embryos. This may be possible using existing cDNA probes, if enough embryos could be collected.

An interesting contrast to the results with papain, which specifically acts on proteins with globular domains, such as H-2 or immunoglobulins, are the results with pronase. Pronase removes all cell surface proteins, regardless of conformation. The generalized removal of all proteins by pronase versus the specific removal of H-2 antigens by papain is highlighted by comparing the results in Figs. 4 and 5. Also of interest is the inability of embryos to regenerate H-2 antigens after removal with pronase, as shown in Fig. 4B. This may be due to the higher proteolytic activity of pronase, compared to papain.

Expression of MHC antigens on early embryos suggests a possible role for these molecules in development. Because it is now clear that early embryos actively express H-2 antigens, nonrejection of early embryos by the mother cannot be simply due to absence of these highly immunogenic molecules, as has been proposed by some workers (reviewed by Beer and Sio, 1982). Other, as yet unknown, mechanisms must be invoked.

It is not yet known whether the H-2 antigens detected on embryos are the same as those found on adult cells. Some unexpected cross-reactions have been found among embryonic H-2 antigens (Cozad and Warner, 1981). It is possible that a generalized or embryonic form of the H-2 antigens exists on embryos. However, there is no evidence for different MHC gene arrangement in germ line (sperm) DNA compared to MHC gene arrangement in adult somatic cells (unpublished results quoted in Hood et al., 1983). This is an interesting area that certainly merits further study.

In conclusion, expression of H-2 antigens by unfertilized ova, as well as by all preimplantation stages of development, has been demonstrated. Most important is the demonstration that the H-2 antigens are actively synthesized by the embryos themselves. The molecular structure of these antigens, as well as their function in development, remains to be elucidated.

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REFERENCES

Beer, A. E. and Sio, J. O. (1982). Placenta as an immunological barrier. Biol. Reprod. 26:15–27.

Bonner, J. J. and Tyan, M. L. (1983). Map of major histocompatibility complex subregions influencing vaginal septa frequency. J. Immunogenet. 10: 413–416.

Cozad, K. M. and Warner, C. M. (1981). Specificity of H-2 antigens expressed on mouse blastocysts. J. Exp. Zool. 218:313–320.

Cozad, K. M. and Warner, C. M. (1982). Detection of H-2 antigens on 8-cell mouse embryos. J. Exp. Zool. 221:213–217.

Edidin, M. (1983). MHC antigens and non-immune functions. Immunol. Today 4:269–270.

Goldbard, S. B., Verbanac, K. M. and Warner, C. M. (1982). Role of the H-2 complex in preimplantation mouse embryo development. Biol. Reprod. 26:591–596.

Goldbard, S. B., Gollnick, S. O. and Warner, C. M. (1984). A highly sensitive method for the detection of cell surface antigens on preimplantation mouse embryos. J. Immunol. Methods 68:137–146.

Gromkowski, S. H., Heagy, W., Sanchez-Madrid, F., Springer, T. A. and Hartz, E. (1983). Blocking of CTL-mediated killing by monoclonal antibodies to LFA-1 and Lyt-2,3. I. Increased susceptibility to blocking after papain treatment of target cells. J. Immunol. 130:2546–2550.

Handyside, A. H. (1981). Immunofluorescence techniques for determining the numbers of inner and outer blastomeres in mouse morulae. J. Reprod. Immunol. 2:339–350.

Heyner, S. (1983). Alloantigen expression on mouse oocytes and early mouse embryos. In: Immunology of Reproduction (T. G. Wegmann and T. Gill, Jr., eds.). Oxford University Press, New York, pp. 79–97.

Hood, L., Steinmetz, M. and Malissen, B. (1983). Genes of the major histocompatibility complex of the mouse. Annu. Rev. Immunol. 1:529–568.

Klein, J., Figueroa, F. and Nagy, Z. A. (1983). Genetics of the major histocompatibility complex: the final act. Annu. Rev. Immunol. 1:119–142.

Krco, C. J. and Goldberg, E. H. (1977). Major histocompatibility antigens on preimplantation mouse embryos. Transplant. Proc. 9:1367–1370.

Melnick, M., Jaskol, T. and Slavkin, H. C. (1981). The association of H-2 haplotype with implantation, survival, and growth of murine embryos. Immunogenetics 14:303–308.

Searle, F. M., Sellens, H., Elson, J., Jenkinson, E. J. and Billington, W. D. (1976). Detection of alloantigens during preimplantation development and early trophoblast differentiation. J. Exp. Med. 143:348–359.

Stallcup, K. C., Springer, T. A. and Mescher, M. F. (1981). Characterization of an anti-H-2 monoclonal antibody and its use in large scale antigen purification. J. Immunol. 127:924–930.

Tanaka, K., Ozato, K., Gilbert, J., Parnes, J. R., Ramanathan, L., Seidman, J. G., Chang, K.S.S. and Apella, E. (1983). Control of H-2 antigen and β_2-microglobulin gene expression in mouse

trophoblast cell clones. Proc. Natl. Acad. Sci. USA 80:5597–5601.

Tarkowski, A. K. (1966). An air-drying method for chromosome preparations from mouse eggs. Cytogenetics 5:394–400.

Thadani, V. M. (1982). Mice produced from eggs fertilized *in vitro* at a very low sperm:egg ratio. J. Exp. Zool. 219:277–283.

Warner, C. M. and Spannaus, D. J. (1984). Demonstration of H-2 antigens on preimplantation mouse embryos using conventional antisera and monoclonal antibody. J. Exp. Zool. 230:37–52.

Webb, C. G., Gall, W. E. and Edelman, G. (1977). Synthesis and distribution of H-2 antigens in preimplantation mouse embryos. J. Exp. Med. 146: 923–932.

Whitten, W. and Biggers, J. (1968). Complete development *in vivo* of the preimplantation stages of the mouse in a simple chemically defined medium. J. Reprod. Fertil. 19:399–401.

Yamasaki, K., Zayas, E. A. and Thomas, L. (1976). Control of mating preferences in mice by genes in the major histocompatibility complex. J. Exp. Med. 144:1324–1335.