Anti-Mac-1 antibodies and early pregnancy loss in mice

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This study focused on the role of adhesion molecules in early pregnancy in mice. Injection of anti-Mac-1 antibodies during early pregnancy resulted in early pregnancy loss (only 30.7% of mice in the group injected with anti-Mac-1 antibody were pregnant compared with 87.5% of controls), while mice treated with anti-Mac-1 antibodies during late pregnancy did not show a significant abortive effect (68.8% mice in the treated group were pregnant compared with 92.9% of control mice). Anti-LFA-1 α , LFA-1 β or mouse Ag-Eb antibodies, when injected during early pregnancy, caused a nonsignificant decrease in pregnancy rate ranging between 15% and 25% ($P > 0.05$), while anti-Thy-1.2 antibodies demonstrated a marginal effect only. Staining of uterine tissue sections, collected on days 4–6 of pregnancy, with anti-Mac-1 antibodies, demonstrated antibody bound to cells in the deep endometrium and in the myometrium but not in the uterine area close to the lumen or on the surface of the blastocyst. These results indicate a possible role for the Mac-1 antigen in early pregnancy.

Introduction

Early pregnancy loss occurs quite frequently; however, the reasons for it are usually unclear. It has been estimated that more than 30% of all known human pregnancy losses may be due to failure of the blastocyst to implant (Toder *et al.*, 1991). The process of implantation in mice involves intensive contact between trophoblast cells and the epithelial surface layer of the endometrium (Denker *et al.*, 1989; Gossler, 1992; Denker, 1993). Although it is generally agreed that such interactions are governed by adhesion mechanisms located at and outside the plasma membrane of the interacting cells, very little is known about the identity of the molecules involved in the process (Hyafil *et al.*, 1981; Denker, 1993). Adhesion molecules are cell surface receptors that are involved in cell–cell, cell–substrate or cell–soluble ligand binding (Springer, 1990; Takeichi, 1990; Hynes, 1992). It has been shown that several adhesion molecules are expressed and appear to play a role in early embryogenesis. These include E and P cadherins (Nose and Takeichi, 1986; Vestweber *et al.*, 1987; Ozawa, 1992), integrins of the β_1 and β_4 subfamilies (Ozawa *et al.*, 1985; Denker, 1993) and embigin and neural cell adhesion molecule (N-CAM) of the immunoglobulin superfamily (Huang *et al.*, 1990; Kimber *et al.*, 1994).

Although these data have shed some light on the expression and characterization of various adhesion molecules involved in embryogenesis, no data are available about whether anti-adhesion molecule antibodies affect the early stages of embryogenesis *in vivo* and cause pregnancy loss. Thus, the effects of

antibodies against various adhesion molecules on the early stages of pregnancy *in vivo* were investigated, concentrating on adhesion molecules belonging to the leucocyte integrins and immunoglobulin superfamily particularly the Mac-1 antigen, which is a member of the β_2 family of leucocyte integrins. Mac-1 is expressed by macrophages and neutrophils, serving as a receptor for the iC3b component of complement (Springer, 1990; Hynes, 1992). Both macrophages and neutrophils are known to be present in the mouse uterus during pregnancy and are thought to fulfil multiple immunological and nonimmunological functions, affecting various stages of pregnancy (Finn and Pope, 1991; Stewart and Mitchell, 1991; Orlando-Mathur and Kennedy, 1993; Hunt, 1994). Therefore, any alteration in those functions might disturb the normal course of pregnancy, resulting in pregnancy loss or resorptions. Antibodies against LFA-1 and Thy-1, which are expressed by leucocytes and T cells, respectively, and against Ag-Eb, a new cell surface antigen, which is expressed mainly by nucleated erythrocytes and also by epithelial and endothelial cells in various fetal and adult organs, were also used.

Materials and Methods

Animals

Male and female ICR mice (8–12 weeks of age) were purchased from Tel Aviv University and kept under standard conditions. Females were checked for oestrus, caged overnight with males and examined for a vaginal plug on the next morning. The day on which the plug was present was considered as day 1 of pregnancy.

⁺Deceased.

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Table 1. Antibodies used in the study

Antibody	Clone/Isotope	Family	Expression	Ligand
Thy-1.2	30H12/IgG ₂ b	Immunoglobulin superfamily	Lymphocytes	Unknown
Thy-1.1	HO-22-1/IgM	Immunoglobulin superfamily	Lymphocytes	Unknown
Mac-1	M1/70.15.11.5.HL/IgG ₂ b	Leucocyte integrins	Macrophages, neutrophils	iC3b
LFA-1 α^a	M17/4.4.11.9/IgG ₂ a	Leucocyte integrins	Haemopoietic cells	ICAM-1
				ICAM-2
LFA-1 β^a	M18/2.a.12.7/IgG ₂ a	Leucocyte integrins	Haemopoietic cells	ICAM-1
				ICAM-2
Ag-Eb	MAE15 (anti-mouse) ^b /IgG	Unknown	Nucleated erythroid cells	Unknown
Ag-Eb	HAE9 (anti-human) ^c /IgM	Unknown	epithelial cells, endothelial cells	Unknown

^aKind gift from N. Hollander (Department of Human Microbiology, Tel Aviv University). ^bIevleva *et al.*, 1976. ^cMechetner *et al.*, 1987.

Reagents

Rosewell Park Memorial Institute-1640 (RPMI-1640), penicillin, streptomycin, fungizone, fetal calf serum (FCS), L-glutamine, sodium pyruvate and nonessential amino acids (NEAA) were purchased from Biological Industries (Beth Haemek). 2-Mercaptoethanol (2-ME) and sodium azide (NaN₃) were purchased from Fluka (Buch) and BSA, glycerol-gelatin solution, casein, 3-aminopropyltriethoxysilane and diaminobenzidine (DAB) were purchased from Sigma (St Louis, MO). Paraformaldehyde and hydrogen peroxide (H₂O₂) were purchased from Merck (Darmstadt) and streptavidin-conjugated horseradish peroxidase (HRP) was from Zymed Laboratories (San Francisco, CA). Tissue culture supplies were from Greiner (Murtlingen), except for tubes for fluorescence activated cell sorter (FACS) analysis which were from Sarstedt (Numbrecht).

Antibodies

The antibodies against adhesion molecules used throughout the study are listed in Table 1. All antibodies were used as rat or mouse hybridoma cell line supernatants. The amount of the antibodies in the supernatants was detected by the ELISA technique, using enzyme-conjugated antibodies to the various isotypes of immunoglobulins used. In some experiments commercial anti-Mac-1 antibodies (rat anti-mouse Mac-1; clone – M1/70, IgG₂b; Pharmingen, San Diego, CA) were used for comparison. Both the hybridoma supernatants and the commercial antibody were used at a dose of 20 μ g per mouse and the results obtained were similar in both cases. Biotin-conjugated mouse anti-rat IgG (Fab fragment) was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Treatment of mice with anti-adhesion molecule antibodies

Mice were injected i.p. (1 ml per mouse) with the antibodies listed in Table 1 or with complete medium used for the growth of the hybridoma cell lines (PRMI-1640 supplemented with 100 iu penicillin ml⁻¹, 100 μ g streptomycin ml⁻¹, 0.25 μ g fungizone ml⁻¹, 1 mmol L-glutamine l⁻¹, 1 mmol sodium pyruvate l⁻¹, 1 mmol NEAA l⁻¹, 5 $\times$ 10⁻⁵ mol 2-ME l⁻¹

and 10% (v/v) FCS). Three injections of the antibodies were given either early in pregnancy (before implantation, days 2–5) or during late pregnancy (days 7–11). The experiments were terminated on day 13–14 of pregnancy and the number of implantation sites and the percentage of postimplantation loss were calculated; the masses of embryos and placentae were determined and embryos were examined for malformations.

Immunohistochemistry

Uteri collected from animals treated with anti-Mac-1 antibodies or complete medium were snap-frozen in liquid nitrogen. The samples were cut into 5–7 μ m thick sections, layered on pretreated 3-aminopropyltriethoxysilane slides and fixed in 2% (w/v) paraformaldehyde in PBS for 15 min at 4°C. After prolonged washing in tap water, the sections were incubated with 0.3% (v/v) H₂O₂ in PBS for 15 min to block endogenous peroxidase activity. The sections were then washed in 0.5% (w/v) casein and 0.1% (w/v) NaN₃ in PBS twice for 5 min and treated for 20 min with FCS diluted 1:5. The excess of serum was shaken off without washing and the slides were incubated with the anti-Mac-1 antibody for 50 min at room temperature in a humid chamber. After four washes of 5 min duration in the casein/NaN₃/PBS solution, the sections were incubated with biotin-conjugated mouse anti-rat IgG (2.5 μ g ml⁻¹) for 50 min at room temperature in a humid chamber. After another wash as described above, the sections were incubated with streptavidin-conjugated HRP (10 μ g ml⁻¹) for 20 min at room temperature, washed again and developed with 0.2 mg DAB ml⁻¹ and 0.05% (v/v) H₂O₂ in PBS (20 min, in the dark). The sections were washed well in tap water and the signal enhanced by incubation in 8% (w/v) CuSO₄ (for 20 min at room temperature). After another wash in tap water, the sections were counterstained with haematoxylin and covered with coverslips using a glycerol-gelatin solution.

Statistical analysis

Statistical analysis of the data was performed by Student's *t* test. Significance was considered at the *P* < 0.05 level.

Table 2. Abortifacient effect of anti-Mac-1 antibodies in mice

Stage of pregnancy	Proportion mice pregnant after treatment (%)							
	Control complete medium	Anti-Mac-1	Anti-Thy-1.2	Anti-LFA-1 α	Anti-LFA-1 β	Anti-mouse Ag-Eb	Anti-Thy-1.1	Anti-human Ag-Eb
Early	77/88 (87.5)	18/59 (30.7*)	39/48 (81.3)	14/23 (60.9)	14/20 (70.0)	11/15 (73.3)	7/7 (100)	13/15 (86.6)
Late	14/15 (92.9)	11/16 (68.8)	—	—	—	—	—	—

* $P < 0.001$.**Table 3.** Pregnancy characterization after treatment with anti-Mac-1 antibody

Parameter	Control complete medium	Anti-Mac-1 antibody
Number of implantation sites per mouse (mean $\pm$ SD)	11.7 $\pm$ 2.1	10.2 $\pm$ 2.8
Post-implantation loss (%) ^a	5.4	4.6
Embryo mass (g) (mean $\pm$ SD)	0.82 $\pm$ 0.08	0.78 $\pm$ 0.12
Placenta mass (g) (mean $\pm$ SD)	0.11 $\pm$ 0.01	0.10 $\pm$ 0.01

^aThe percentage of postimplantation loss was calculated from the total number of implantation sites in each experimental group.

Results

Only the anti-Mac-1 antibody, when injected during early pregnancy, caused a significant reduction (up to 60%) in the percentage of pregnant mice (Table 2). When injected during late pregnancy, anti-Mac-1 antibody reduced the pregnancy rate by about 20% only. Anti-LFA-1 α , LFA-1 β and mouse Ag-Eb antibodies, when injected during early pregnancy, caused a nonsignificant 15–25% reduction in pregnancy rate, while anti-Thy-1.2 antibodies, which are isotype-matched with anti-Mac-1 antibodies, demonstrated a marginal effect only. The effects of anti-Thy-1.1 and human Ag-Eb antibodies on pregnancy rate were similar to that of complete medium. Various pregnancy parameters, including the number of implantation sites per mouse, the percentage of postimplantation loss and the masses of embryos and placentae were not affected by the anti-Mac-1 antibody (Table 3), emphasizing its significant effect on pregnancy rate only. The antibody-binding cells were localized *in vivo* by collecting uterine tissue sections on days 4–6 of pregnancy and staining them with the anti-Mac-1 antibody. Figure 1a demonstrates a blastocyst in the uterine lumen, with no stained cells on its surface or in the uterine area close to the lumen. Some Mac-1-positive cells were found scattered around the endometrium, while most stained cells were located in the deep endometrium adjacent to the myometrium and in the myometrium itself (Fig. 1b).

Fig. 1. Uterine tissue sections collected on day 4 of pregnancy, stained with anti-Mac-1 antibodies, followed by streptavidin-conjugated horseradish peroxidase. (a) Blastocyst in the uterine lumen, with no anti-Mac-1 antibody-bound cells in its proximity. (b) Antibody-bound cells located in the myometrium. Scale bars represent 100 μ m.

Discussion

The present study examined the effect of antibodies against various adhesion molecules on the early stages of pregnancy, as reflected by changes in pregnancy outcome. To the best of our knowledge, this is the first demonstration of a significant reduction in the percentage of pregnant mice after anti-Mac-1

antibody injection during early but not late pregnancy. Since the antibody did not cause postimplantation loss, and had no effect on other pregnancy parameters, it is probable that it interferes with the preimplantation stages of embryo development or with the implantation process itself. The ability of antibodies administered to animals *in vivo* to reach their target organ and exert their effect has been demonstrated by Athanassakis *et al.* (1987), who showed that anti-T-cell antibodies can affect various placental cell functions, when administered *in vivo* to pregnant animals. Examination of uterine tissue sections collected on days 4–6 of pregnancy demonstrated the presence of numerous anti-Mac-1 antibody-stained cells in the deep endometrium adjacent to the myometrium and in the myometrium itself but not anywhere near the uterine lumen, where the blastocyst starts to implant. The results presented here are in agreement with the observations of Hunt (1994) and Rogers *et al.* (1992) concerning the redistribution of uterine macrophages and neutrophils, respectively, during implantation. Thus, on the basis of the immunohistochemistry data, the anti-Mac-1 antibody can be assumed to affect the implantation process indirectly by interfering with the normal activities of uterine Mac-1-positive cells, during early pregnancy. In addition, the possibility of some systemic effect triggered by the antibody cannot be excluded. Since Mac-1-positive cells (mostly macrophages) are known to be a major source of cytokines that affect various stages of pregnancy, it is possible that not only uterine macrophages but also para-aortic lymph node or spleen-derived macrophages may participate in this process.

In conclusion, the results reported here demonstrating the ability of anti-Mac-1 antibodies to cause pregnancy loss *in vivo* re-emphasize the role of adhesion molecules in regulating complex morphological events in the early stages of pregnancy.

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