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

S. Savion1, J. Irlin2, J. Shepshelovich1, M. Brengauz1 and V. Toder1

1Department of Embryology and Teratology, Sackler School of Medicine, Tel Aviv University, Ramat Aviv, Tel Aviv 69978, Israel; and 2Laboratory of Immunology, The Research Institute, Ariel 44820, Israel

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-1α, LFA-1β 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 β1 and β2 subfamilies (Ozawa et al., 1985; Denker, 1993) and embryonic 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 β2 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 fulfill multiple immunological and nonimmunological functions, affecting various stages of pregnancy (Finn and Pope, 1991; Stewart and Mitchell, 1994; 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.

1Deceased.
Revised manuscript received 7 December 1995.
Antibodies benzidine were penicillin, sorter (San horseradish chased solution, Haemek).

conjugated comparison.

The commercial isotypes of the hybridoma 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 dianinobenzidine (DAB) were purchased from Sigma (St Louis, MO). Parafomaldehyde 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 (Mutingen), except for tubes for fluorescence activated cell sorter (FACS) analysis which were from Sarstedt (Numbrecht).

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 dianinobenzidine (DAB) were purchased from Sigma (St Louis, MO). Parafomaldehyde 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 (Mutingen), 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 µg 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).

Table 1. Antibodies used in the study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone/Isotope</th>
<th>Family</th>
<th>Expression</th>
<th>Ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thy-1.2</td>
<td>30H12/IgG,b</td>
<td>Immunoglobulin superfamily</td>
<td>Lymphocytes</td>
<td>Unknown</td>
</tr>
<tr>
<td>Thy-1.1</td>
<td>HO-22-1/IgM</td>
<td>Immunoglobulin superfamily</td>
<td>Lymphocytes</td>
<td>Unknown</td>
</tr>
<tr>
<td>Mac-1</td>
<td>M1/70.15.11.5.HL/IgG2,b</td>
<td>Leucocyte integrins</td>
<td>Macrophages, neutrophils</td>
<td>iGb</td>
</tr>
<tr>
<td>LFA-1a</td>
<td>M17/4.1.11.9/IgG2a</td>
<td>Leucocyte integrins</td>
<td>Haemopoietic cells</td>
<td>ICAM-1</td>
</tr>
<tr>
<td>LFA-1b</td>
<td>M18/2.a.12.7/IgG2a</td>
<td>Leucocyte integrins</td>
<td>Haemopoietic cells</td>
<td>ICAM-2</td>
</tr>
<tr>
<td>Ag-Eb</td>
<td>MAE15 (anti-mouse)/IgG</td>
<td>Unknown</td>
<td>Nucleated erythroid cells</td>
<td>Unknown</td>
</tr>
<tr>
<td>Ag-Eb</td>
<td>HAE9 (anti-human)/IgM</td>
<td>Unknown</td>
<td>epithelial cells, endothelial cells</td>
<td>Unknown</td>
</tr>
</tbody>
</table>


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 placenta 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 µm 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/NaNO3/PBS solution, the sections were incubated with biotin-conjugated mouse anti-rat IgG (2.5 µg 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 µg 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.
Role of Mac-1 in early pregnancy

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

<table>
<thead>
<tr>
<th>Stage of pregnancy</th>
<th>Control complete medium</th>
<th>Anti-Mac-1</th>
<th>Anti-Thy-1.2</th>
<th>Anti-LFA-1α</th>
<th>Anti-LFA-1β</th>
<th>Anti-mouse Ag-Eb</th>
<th>Anti-Human Ag-Eb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early</td>
<td>77/88 (87.5)</td>
<td>18/59 (30.7*)</td>
<td>39/48 (81.3)</td>
<td>14/23 (60.9)</td>
<td>14/20 (70.0)</td>
<td>11/15 (73.3)</td>
<td>7/7 (100)</td>
</tr>
<tr>
<td>Late</td>
<td>14/15 (92.9)</td>
<td>11/16</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*P < 0.001.

Table 3. Pregnancy characterization after treatment with anti-Mac-1 antibody

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control complete medium</th>
<th>Anti-Mac-1 antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of implantation sites per mouse (mean ± SD)</td>
<td>11.7 ± 2.1</td>
<td>10.2 ± 2.8</td>
</tr>
<tr>
<td>Post-implantation loss (%)</td>
<td>5.4</td>
<td>4.6</td>
</tr>
<tr>
<td>Embryo mass (g) (mean ± SD)</td>
<td>0.82 ± 0.08</td>
<td>0.78 ± 0.12</td>
</tr>
<tr>
<td>Placenta mass (g) (mean ± SD)</td>
<td>0.11 ± 0.01</td>
<td>0.10 ± 0.01</td>
</tr>
</tbody>
</table>

*The 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-1α, LFA-1β 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).

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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References
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