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Anti-cardiolipin antibodies induce pregnancy failure by impairing embryonic implantation

(antiphospholipid syndrome/systemic lupus erythematosus/fetal loss)

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ABSTRACT **The antiphospholipid syndrome is characterized by thrombocytopenia, thrombosis, and recurrent fetal loss in association with anti-cardiolipin antibodies (ACAs) or lupus anti-coagulants. However, the causal role of these antibodies in the disease and the mechanisms by which the ACA may induce the syndrome are not clear. Recently, we have established an experimental mouse antiphospholipid syndrome induced by the mouse IgM monoclonal ACA designated 2C4C2. In the present study, we focused on the effects of immunization with the monoclonal ACA 2C4C2 on the outcome of pregnancies in BALB/c female mice. Four weeks after active immunization with the monoclonal ACA, a severe gestational failure with low pregnancy rates, low number of fetuses, and a high rate of resorptions was observed. Moreover, embryos obtained from the ACA-immunized females on day 3.5 of pregnancy were severely impaired, demonstrating developmental delay and abnormal morphology. These abnormal embryos failed also to develop in an *in vitro* implantation model. Furthermore, specific binding of the 2C4C2 ACA to the trophectoderm cell lineage of *in vitro* implanting normal embryos was observed. Thus, our studies demonstrate that the severe ACA-induced gestational failure results from an impairment of implantation and suggest that the ACA may react directly with the preimplantation embryos.**

Anti-cardiolipin antibodies (ACAs) and lupus anti-coagulants are a heterogeneous group of autoantibodies directed against phospholipids found in antiserum of patients with systemic lupus erythematosus (SLE) or in patients without any evidence of autoimmune disorders. These antibodies have been shown to be associated with thrombosis, thrombocytopenia, and recurrent fetal loss, which together comprise the antiphospholipid syndrome (APS) (1–4). The APS associated with SLE is defined as secondary APS, whereas primary APS occurs in patients without any autoimmune disturbances (3, 4). In spite of the close relationship between the presence of the lupus anti-coagulant or ACA and the clinical features of APS, the role of these autoantibodies in the pathogenesis of the disease is not clear (4, 5). Recently, we produced and characterized a monoclonal ACA designated 2C4C2 that was isolated from mice in which experimental SLE was induced by injection of a monoclonal antibody (mAb) against a common idiotype (Id)—namely, the 16/6-Id (6, 7). We have demonstrated that the latter ACA was capable of inducing a secondary APS characterized by the presence of APS-related clinical features in addition to SLE-associated symptoms (7). The establishment of this experimental model allowed dissection of the mechanisms involved in the pathogenesis of the various clinical manifestations of APS. In the present study, we demonstrate a severe failure of allogeneic pregnancies in mice with ACA-induced experimental APS. Moreover, we determine here that ACA immunization leads to abnormalities in preimplantation embryonic development, causing the pregnancy failure.

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MATERIALS AND METHODS

Mice. BALB/c female and (C57BL/6J × DBA/2)F_1 (B6D2F1) male mice were purchased from Olac (Bichester, U.K.). All mice were used at the age of 12–16 weeks.

mAbs. 2C4C2 is a murine IgM monoclonal ACA. This mAb also reacts with single-stranded DNA and has lupus anticoagulant activity (7). A6 is a control murine IgM mAb directed against the major Id on antibodies to the synthetic polypeptide (Tyr, Glu)-Ala—Lys without any cardiolipin reactivity (8). The human 16/6-Id mAb is an anti-DNA antibody that does not react with cardiolipin (6, 9).

Assay of ACAs. ACAs were detected by a solid-phase ELISA according to the method of Harris *et al.* (10) and modified by ourselves (7). Background values, representing nonspecific binding to "no-antigen" wells coated with ethanol without cardiolipin, were subtracted in all assays.

Active Immunization. BALB/c female mice (10–18 mice per group) were immunized with phosphate-buffered saline (PBS), 14 μg of mAb 2C4C2, or 14 μg of mAb A6 in complete Freund's adjuvant (CFA) into the hind footpads. Four weeks later, the mice were bled and the leukocytes and thrombocytes were quantified with a cell counter (Ortho Instruments).

Mating and Evaluation of Pregnancy. Four weeks after active immunization or the night before i.v. injections, BALB/c female mice were paired overnight with B6D2F1 males. Mating was evidenced by the appearance of a vaginal plug on the following morning (day 0.5 of pregnancy). All plugged females were caged separately and sacrificed on day 14 (or, when indicated, on day 16) of pregnancy and the uteri were examined for the presence of viable or resorbed fetuses as described (11, 12). In several experiments, pregnancy evaluation was done on day 3.5 for assessment of early embryonic development. Embryos (3.5 days old) were flushed from uteri and immediately examined microscopically, and their developmental stage was defined. When indicated, embryos were transferred to microtiter plates and cultured as described (11). Under these conditions, blastocysts hatch from the zona pellucida, adhere, and spread their trophectoderm that differentiates into giant trophoblast tissue. These processes are considered to be an *in vitro* model of implantation (13, 14).

Passive Immunization. Plugged BALB/c female mice (8–12 mice per group) were injected i.v. (50 μg per mouse into the tail) with mAb 2C4C2 or A6 on days 1 and 4 after mating. In some experiments, the mAbs were i.v. injected (150 μg per mouse) on day 10 of pregnancy. Pregnancies were evaluated

Abbreviations: ACA, anti-cardiolipin antibody; APS, antiphospholipid syndrome; CFA, complete Freund's adjuvant; Id, idiotype; mAb, monoclonal antibody; SLE, systemic lupus erythematosus.

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on day 14 (for the early injections; days 1 and 4) or on day 16 (for the late injections; day 10) as described above.

Immunoperoxidase Staining. Uteri (day 3.5) and placenta (day 16) obtained from normal BALB/c pregnant mice were frozen in liquid nitrogen. Frozen cryostat sections (5 μm) were air dried, fixed in 2% paraformaldehyde, and permeabilized in −20°C acetone before staining. Embryos 3.5 days old obtained from normal BALB/c pregnancies were cultured on glass coverslips (13). Seventy-two hours later, embryo outgrowths were fixed as described above. The uteri, placenta, or embryos were incubated with the 2C4C2, A6, or the 16/6-Id mAbs at 10 μg/ml for 60 min and processed with the avidin–biotin peroxidase complex (Vector Laboratories) as described (15).

RESULTS

To assess the possible effect of active immunization with ACA on the outcome of pregnancy, BALB/c female mice were injected with the mAb 2C4C2, the control mAb A6, or with PBS, all in CFA. An additional group was treated with PBS only. Four weeks after immunization, mice were paired and mated females were separated. At that time, the mAb 2C4C2-immunized mice revealed normal counts of thrombocytes ($681 \times 10^3 \pm 47 \times 10^3/mm^3$; mean ± SD) and leukocytes ($7200 \pm 1300/mm^3$; mean ± SD) similar to the control groups. On the other hand, the serological evaluation of these mice revealed high titers of ACA in the mAb 2C4C2-immunized mice but not in the control groups: 0.238 ± 0.129 as compared to 0.019 ± 0.017, 0.039 ± 0.036, and 0.030 ± 0.027 (mean ± SD) in the mAb A6-, CFA/PBS-, or PBS-injected groups ($P < 0.003$, 0.001, and 0.005, respectively). ACAs of both IgM and IgG isotypes were detected in the sera of the mAb 2C4C2-injected mice as was determined by goat anti-mouse isotype-specific antibodies. In addition, significant but low anti-single-stranded DNA antibody titers could be detected in the antisera of the mice at that time. Evaluation of the pregnancies of the above mice on day 14 demonstrated a remarkable gestational failure in the mAb 2C4C2-immunized groups of mice (Table 1). These mice exhibited low pregnancy rates (infertility) as well as highly significant percentages of resorbed fetuses in the pregnant animals. The number of fetuses per pregnant female was also lower in the mAb 2C4C2-immunized mice. Consequently, as shown in Fig. 1, the fertility index of the mice injected with the monoclonal ACA was found to be extremely low in comparison to that of the control groups. Moreover, 80% of the control mated mice had successful pregnancies in contrast to only 6.2% in the mAb 2C4C2-immunized mice. The pregnancy impairment appeared to correlate with the levels of ACA in the antisera of the mated mice. Thus, active immunization with the 2C4C2 monoclonal ACA causes severe gestational (day 14) impairment.

Table 1. Effects of active immunization with ACA on the outcome of pregnancy

Treatment	Pregnancy rate, %	No. of fetuses	Resorption, %
PBS	90	11.2 ± 1.71	14.5 ± 12.9*
CFA/PBS	80	11.6 ± 0.51	17.0 ± 13.5*
CFA/A6	91	11.0 ± 4.54	21.1 ± 29.2†
CFA/2C4C2	37.5	7.66 ± 6.0	75.1 ± 35.0*†

Groups of BALB/c females were immunized with mAb 2C4C2 or mAb A6 in CFA, with CFA/PBS, or treated with only PBS. Four weeks later, all mice were mated. Pregnancies were evaluated on day 14. Pregnancy rate denotes the ratio of pregnant females to the total number of mated (plugged) females in the group. Number of fetuses or percentage of resorptions per pregnant female (mean ± SD) is shown.
*$P < 0.001$.
†$P < 0.005$.

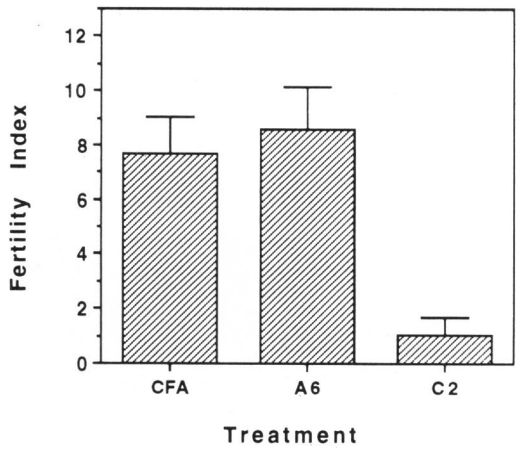

FIG. 1. Fertility index following active immunization with CFA, mAb 2C4C2 (C2), or mAb A6. Fertility index is defined as the mean (±SD) number of normal fetuses per mated female. $P < 0.0001$ between the C2-immunized mice and control groups.

To assess the earliest phase of pregnancy in which ACA injury can be detected, 3.5-day gestations were analyzed. These studies demonstrated a severe impairment of the preimplantation embryos. As shown in Table 2, a striking delay in the developmental stage of the embryos obtained from the mAb 2C4C2-immunized mice as opposed to the control group was demonstrated. A majority of the flushed embryos obtained from the mAb 2C4C2-immunized mice were at the compacted morula stage, which precedes the more mature developmental stage of the blastocyst. An opposite situation was observed in the control group, where the majority of the embryos were blastocysts (Table 2). In addition, most of the blastocysts obtained from the control group appeared to be mature expanded blastocysts, whereas the blastocysts obtained from the mAb 2C4C2-injected group were still at the early blastocyst stage. Moreover, ≈25% of the latter blastocysts appeared to be morphologically abnormal (Fig. 2*A*). Half of these abnormal blastocysts became necrotic upon culture *in vitro* and the remainder, although viable, demonstrated a deficient functional capacity to "implant" *in vitro* and to develop to the full outgrowth. The main abnormality observed was a defective trophectoderm cell lineage development (Fig. 2*B*). Thus, ACA-induced infertility and fetal wastage result from an early gestational impairment with a preimplantation embryonic failure.

To evaluate the possibility that the trophectoderm cell lineage may be the target tissue of the ACA, we studied direct binding of the mAb 2C4C2 to early embryos. Normal 3.5-day-old embryos obtained from untreated BALB/c female

Table 2. Developmental stage of 3.5-day embryos following active immunization with ACA

Treatment	Pregnancy rate, %	No. of embryos	Morulae, %	Blastocyst, %
CFA/A6	77.7	4.8 ± 2.0	30.4 ± 31.8*	54.3 ± 35.6†
CFA/2C4C2	66.8	4.4 ± 2.6	70.1 ± 38.2*	20.8 ± 27.6†

Groups of BALB/c females were immunized with the 2C4C2 or A6 mAbs, both in CFA, 4 weeks before mating. Embryos were flushed from uteri on day 3.5 of pregnancy. Embryos were examined microscopically, their developmental stage was defined, and the mean (± SD) ratios of morulae or blastocyst to the total number of embryos flushed per female were calculated. Pregnancy rate was determined as described in Table 1. Number of embryos is shown as mean ± SD.
*$P < 0.001$.
†$P < 0.003$.

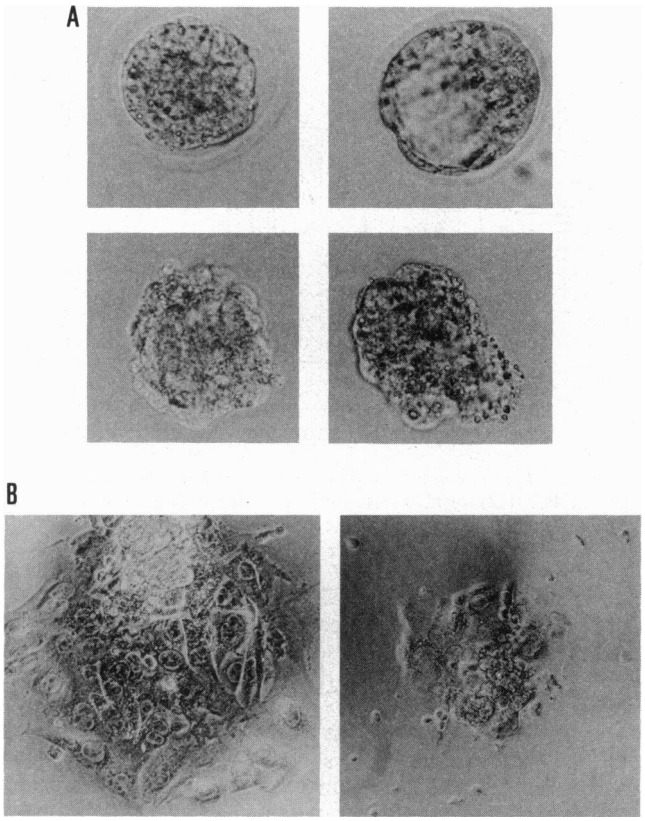

FIG. 2. Abnormal preimplantation embryos from mAb 2C4C2-immunized females. Preimplantation embryos were flushed from uteri, on day 3.5 of gestation, and examined microscopically. (*A*) Normal compacted morula (*Upper Left*) and normal blastocyst (*Upper Right*) encased in zona pellucida were obtained from A6-injected females. Abnormal embryos with no zona pellucida and irregular cellularity were obtained from mAb 2C4C2-immunized females (*Lower Right* and *Lower Left*). (×200.) (*B*) Blastocyst outgrowth (*in vitro* implantation). (*Left*) Normal outgrowth from a normal embryo. Note the large spread area of the trophoblast and the dense area of the proliferating inner cell mass. (*Right*) Underdeveloped outgrowth of an abnormal embryo flushed from a mAb 2C4C2-immunized female. Note the very limited trophoblast outgrowth. Phase-contrast microscopy. (×200.)

mice were cultured for 72 hr, fixed, and stained with 2C4C2, A6, or human 16/6-Id anti-DNA mAbs. As shown in Fig. 3, the trophectoderm part of the embryo outgrowth was strongly stained by the 2C4C2 mAb but not by the A6 or the 16/6-Id anti-DNA mAb. In contrast to the remarkable *in vitro* binding of our ACA to the embryos, staining experiments of the uteri (day 3.5) and mature placenta (day 16) did not show any binding of the mAb 2C4C2 (data not shown).

Based on these observations, we investigated the possibility that passive injection of our ACA may also affect pregnancy outcome. Mated females were injected i.v. with mAb 2C4C2 or A6 on days 1 and 4 of gestation. Uteri were examined on day 14 for normal and resorbed fetuses. As shown in Table 3, repeated i.v. injections of mAb 2C4C2 resulted in a partial deleterious effect on the outcome of these pregnancies: the mean resorption rate in the mAb 2C4C2-injected group was higher and reached statistical significance ($P < 0.0377$). Additional experiments generated similar results. We also studied the effect of ACA injection at a later stage of pregnancy. Pregnant BALB/c females were i.v. injected with ACA 2C4C2 or A6 control mAbs on day 10 of pregnancy. The evaluation of these pregnancies, on day 16, demonstrated normal gestational outcome with no difference between the mAb 2C4C2- and the mAb A6-injected groups

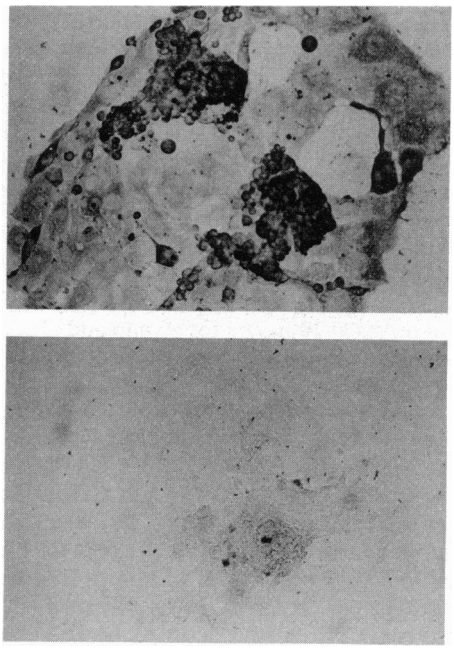

FIG. 3. Binding of the monoclonal ACA 2C4C2 to embryo outgrowth. Normal embryos, 3.5 days old, were flushed from uteri and cultured *in vitro* for 3 days, as detailed in text. Outgrowths were fixed and stained by immunoperoxidase following binding of the 2C4C2, A6, or 16/6-Id mAbs. (*Upper*) Strong binding of monoclonal ACA 2C4C2 to the trophoblast. (*Lower*) Absence of any binding of mAb A6 to trophoblast and background staining of the proliferating inner cell mass. Similar negative staining was obtained with the 16/6-Id mAb. (×200.)

(data not shown). Thus, passive immunization with monoclonal ACA 2C4C2 has a modest effect on the outcome of pregnancies. This effect is obtained only when the mice are exposed to the ACA during the early stages (preimplantation period) of pregnancy.

DISCUSSION

This study demonstrates that the immunization of BALB/c female mice with ACA, prior to mating with allogeneic males, deteriorates their capacity to impregnate and sustain a normal gestation. Moreover, we determine that these impaired pregnancies originate from an implantation failure due to abnormal preimplantation embryonic development. We further propose that the trophectoderm cell lineage of preimplantation embryos may be the target for the ACA.

Branch *et al.* (16) have recently shown that high doses of human IgG ACA induce premature delivery in pregnant mice. This effect, however, differs from the pregnancy loss observed in the human APS (2, 17) and in the present study. Blank *et al.* (18) and Bakimer *et al.* (19) reported induction of a primary APS with gestational disturbances after ACA administration. The effects described in the latter reports do

Table 3. Effect of passive immunization with ACA on the outcome of pregnancy

Treatment	Pregnancy rate, %	No. of fetuses	Resorption, %
A6	87.5*	12.1 ± 1.95*	7.67 ± 6.79†
2C4C2	77.7*	11.4 ± 1.98*	19.9 ± 0.12†

Plugged BALB/c females were i.v. injected with the 2C4C2 or A6 mAbs on days 1 and 4 after mating. Pregnancy was evaluated on day 14. Pregnancy rate, number of fetuses, and resorption are defined as per the legend to Table 1.
*Not significant.
†$P < 0.0377$.

not fully agree with our results. In those studies, the pregnancy impairment observed following active immunization with ACA was much weaker than that demonstrated after passive transfer of the antibodies. Since the authors used different mouse strains as well as ACA from different origins in the two reports, it is impossible to compare their two studies. In addition, the low number of mated and pregnant mice in those studies interferes with the interpretation of their results. Our study was performed with a well characterized monoclonal ACA derived from a mouse with experimental SLE (6, 7). Moreover, both passive and active administration of the ACA were done in the same mouse strain with a large number of animals. Therefore, our results provide an appropriate comparison between active and passive ACA immunization and offer a possible mechanism for ACA-induced pregnancy failure.

Evaluation of day 14 pregnancies of BALB/c females, 4 weeks after immunization with the monoclonal ACA 2C4C2, demonstrated a severe gestational failure. The mAb 2C4C2-injected females, but not mice injected with control antibodies, revealed low pregnancy rates and a low number of fetuses per pregnant female with very high resorption rates (Table 1). As a result, the fertility index was extremely low in the mAb 2C4C2-immunized groups (Fig. 1). This pregnancy failure may result from an impairment at the stage of ovulation, implantation, or postimplantation. We therefore studied the fate of pregnancies of 2C4C2 or A6 mAb-treated females at the preimplantation stage (day 3.5). These experiments strongly suggest that a preimplantation failure is the cause for the ACA-induced pregnancy loss. In contrast to the low pregnancy rates and the low number of fetuses observed on day 14 (Table 1), the evaluation on day 3.5 revealed similar pregnancy rates and numbers of embryos in the mAb 2C4C2-immunized mice and in the mAb A6-injected control groups (Table 2), thus indicating that the mAb 2C4C2-induced pregnancy loss stems from a postfertilization disturbance. On the other hand, the early embryos, flushed from uteri of mAb 2C4C2-immunized females, were severely impaired, demonstrating a striking developmental delay (Table 2), abnormal morphology (Fig. 2*A*), necrosis, and a deficient capacity to implant, as shown by the limited trophectoderm outgrowth (Fig. 2*B*). It should be stressed that the successfully synchronized sequence of events during preimplantation development culminates at the point at which a hatched blastocyst is able to implant properly into a carefully prepared receptive endometrium. The very first phase of adherence to the maternal endometrium is mediated by the trophectoderm. Thus, any impairment of this cell lineage can cause an implantation failure, which may be reflected later on in gestation. Such a failure can range from a complete lack of implantation (manifested on day 14 as a low pregnancy rate or infertility) to a malimplantation that may lead to a resorption observed on day 14. Indeed, implantation impairments, as a cause for pregnancy failure and fetal loss, were previously reported by us in other experimental models (11, 12). The lack of any effect in the late (day 10) passive transfer experiments further supports an early gestational impairment. Embryo transfer studies may eventually reinforce our present results.

There are several possible mechanisms for the ACA-induced implantation failure. Some of them may represent maternal disturbances, while others may involve ACA effects on the embryos themselves either directly or indirectly via a hostile maternal environment. Specific binding of the 2C4C2 monoclonal ACA to the *in vitro* implanting embryos, particularly to the trophectoderm cell lineage (Fig. 3) and not to the mature placenta, may suggest a direct effect of ACA on the embryos as one of the mechanisms responsible for implantation impairment: such binding may interfere with the process of implantation. As demonstrated, the 16/6-Id (an anti-DNA mAb without cardiolipin reactivity) does not bind to the same embryos (Fig. 3). Interestingly, the latter mAb, which was shown to cause an experimental SLE (9), failed to induce gestational impairment in BALB/c females (unpublished observations), thus implying a correlation between ACA binding to embryos *in vitro* and a harmful biological significance.

The moderate effect of the passive immunization, as opposed to the strong consequences of the active one, points to the fact that the continuous exposure to ACA may be more harmful. The production of ACA, following active immunization, is advantageous over passive administration of the IgM mAbs. First, other isotypes of ACA, which are probably produced via the idiotypic network (20, 21), may be able to penetrate more readily the uterine lumen. Second, the active immunization with mAb 2C4C2 may lead to production of a variety of ACAs with different fine specificities, thus ensuring that the various epitopes of the embryonic antigen in question could be targeted in a more efficient manner. Alternatively, passive and active immunization may also differ in the degree of maternal pathological consequences, yet undefined, like a hostile uterine environment or an endocrinological deficiency. The normal platelet and leukocyte counts at mating rule out a significant role for these cells in pregnancy failure.

To conclude, establishment and analysis of *in vivo* systems for the ACA-induced pregnancy impairment, like the one described here, may help in formulation of diagnostic approaches and therapeutic modalities.

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