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ANTI-IMPLANTATION EFFECT OF A BONE-MARROW CYTOKINE - BIM

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Abstract : Successful implantation of blastocyst is dependent upon a cytokine induced localized immunosuppression at uterus. BIM, a bone marrow secreted bio-immunomodulator (BIM) has been observed to have positive immunomodulatory activity in immunosuppressed cases. As pregnancy is associated with immunosuppression upregulation of the suppressed immune system by injection of BIM (conc. 0.08 µg/g b.wt once in rats b.wt. <150 gm or 0.2 µg/g b.wt thrice in rats weighing > 160 gm) is believed to prevent implantation. The anti-implantation action of BIM is probably mediated via mononuclear cells at site of uterus, the effect is reversible and a single dose did not affect the estrous cycle. Multiple dose of BIM however, produce prolonged diestrous and this is probably an autonomic phenomena.

Key words : anti-implantation bone-marrow cytokine

INTRODUCTION

Immunosuppression, a prerequisite for successful blastocyst implantation, is produced by a cascade of cytokines (1–14) in the uterus with the aid of progesterone (15, 16). One approach to inhibit implantation is directed towards the development of cytokine receptor antagonist (17). But this process has limitation, as *in vivo* introduction of this receptor antagonist might disrupt normal physiological function at sites other than uterus. Our logical approach to this probelm is to upregulate the suppressed immune system so as to prevent implantation.

In our laboratory we have isolated an immunomodulatory rodent bone marrow cytokine (BIM) that improved suppressed immune conditions (18–20). BIM showed both autocrine (on bone marrow) and paracrine (on thymus, brain, kidney) effect (21, 22). In this study attempts have been made to upregulate the immune system by BIM at time of implantation which could be successfully used to prevent implantation in rodents.

METHODS

Selection of animals

Adult experienced Charles Foster male rats (b.wt. 200 ± 5 gm) and non-pregnant

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females (b.wt. 160 ± 10 gm) having regular estrous cycles were allowed to mate and the number of pups per rat were noted. Those females with litter size less than eight were rejected. After the pups were weaned, the mothers were separated and given rest for two weeks.

The female pups, born in the laboratory, were reared separately from the male litter mate and the virgin females with regular estrous cycles were used in some of the experiments (e.g. noting the number of corpus luteum; pontamine sky blue reaction etc).

Preparation of BIM

The bone marrow cytokine BIM was prepared as per method described earlier (19, 20). In brief, the peptide is isolated from the supernatant of the serum free bone marrow cell culture by Sephadex G_{10} (Sigma) column chromatography. Three protein fractions were obtained of which the first fraction (mol wt. 12 KD) showed the desired immunomodulatory activity.

Dose applied

Previous studies from our laboratory have shown that the effective immunomodulatory dose of the bone marrow cytokine, BIM, in rodents and chicken was 0.08 µg/g b.wt. In the present study on implantation different doses of BIM ranging from 0.08-0.5 µg/g b.wt. was used (Table I).

Female rats were exposed to male (2:1) on proestrous day. Vaginal smear was checked for the presence of sperm in the next morning and the sperm +ve animals were divided into (1) Control and (2) Experimental groups.

(1) *Control group :* These animals (n=10) were injected with saline. The number of doses and the volume injected was similar to that of BIM treated group, keeping noninjected controls.

(2) *Experimental group :* The experimental group were treated with BIM either in

(a) *Single dose :* These animals were treated with a single dose (0.08 µg/g b.wt. for rats weighing <150 g or 0.2 µg/g b.wt. for >160 gm, i.p.) of BIM on the sperm positive day and separated from their male partner. Signs of pregnancy and number of pups/litter, if any, was noted.

(b) *Multiple dose :* In rats with an average b.wt. of 160 gm a single dose of either 0.08 µg/g or 0.2 µg/g b.wt. or multiple doses of 0.08 µg/g b.wt. failed to prevent implantation. It was observed that a minimum dose of 0.02 µg/g b.wt. for three days (1st, 2nd, 3rd, or 1st, 2nd and 4th) is essential for preventing implantation.

The first dose of BIM was injected on the day the vaginal smear showed presence of sperm. In a set of experiments the females (b.wt. 160 ± 5 gm; n=10). were separated from the males and the BIM was repeated as per above schedule. Signs of pregancey and number of pups, if any, noted.

In another set of experiment, BIM (conc. 0.2 µg/g b.wt.) was injected on the sperm positive day but the males were not removed from the females (b.wt. 150 ± 2 gm; n=5). These animals were injected with BIM every alternate 4 days and signs of pregnancy, if any, noted. The animals were kept together for one month and then the BIM dose was either reduced (0.05 µg/g b.wt.) or totally

withdrawn and signs of pregnancy and number of pups born, if any, of each animal noted. The pups were weighed at birth, and abnormality, if present, noted.

Pontamine sky blue test

Mainly virgin females (n=15, b.wt. 160 ± 5) were used in this experiment. PSBT (1% w/v, 0.3 ml, i.v.) was performed on uterus of ether anesthetized rats on the 5th day positive presence of sperm. Before injection of the dye a drop of blood was collected from the tail vein for routine differential count of WBC (DC) and the number of corpus luteum on ovary was counted macroscopically. The dye was then injected i.v. and after a time lapse of 15 minutes the uterus was examined for the presence of any blue spots.

Histological studies of uterus

In a separate set of experiment, both horns of the uterus of anesthetized animals on the 6th day post coitus were removed. One horn, cut into small pieces was dipped in Bouin's fluid for routine histological examination. The other horn was flushed with distilled water either on glass slides or in Eppendorf tubes containing Alsevier's solution. The slides were stained with Nadi reagent for myeloperoxidase, counterstained with Giemsa and the cells in the Eppendorf tubes were allowed to rosette with sheep red blood cells in cold to find out if it is of T cell variety (23). The rosetted cells were viewed on hemocytometer.

RESULTS

The anti-implantation effect of BIM seems to be body-weight dependent. Female rats with a body wt < 150 gm and injected with BIM (conc. 0.08 µg/g b.wt.) before the 5th day of pregnancy failed to show implantation ($P < 0.001$). However, rats of b.wt. > 160 gm required a minimum of 3 days BIM treatment (conc. 0.2 µg/g b.wt.) to prevent implantation ($P < 0.001$; Table-IA & B).

When females (b.wt. 150 ± 2 gm) were kept with males and BIM (conc. 0.2 µg/g b.wt.) injected at an interval of 4 days starting from the first sperm positive day it was observed that the animals did not get pregnant ($P < 0.01$) till the BIM dose is reduced (0.05 µg/g b.wt.) or withdrawn. However, a minimum of 15-20 days after the last BIM injection is essential for the females to have normal litters (n=12±1, survival rate 98%). It was observed that

TABLE 1A : Contraceptive effect of BIM in female rats (b.wt. 145±5 gm).

Group	*BIM Conc :*	*No. of doses*	*Pontamine Sky Blue Test :*	*No. of Corpus luteum*	*No. of pups*	*Stat. Significance*
BIM injected (n = 10)	0.08 µg/gb.wt.	1	Nil	9 ± 1	Nil*	
Control saline (n = 5)	–	1	9 ± 2	9 ± 1	8 ± 2	
Conrol (n = 5)	–	–	9 ± 2	9 ± 1	8 ± 1	P<0.001

*One animal had 2 pups. Mother did not suckle. Dead after 72 hrs.
@One injection either on 1st or 2nd day post mating.
No significant reduction in body wt. observed.

TABLE 1B : Effect of BIM on implantation in rats (b.wt. 160 ±2 gm).

BIM dose	*No. of doses / days post mating*	*No. of Corpus[a] Luteum*	*Pontamine Sky[b] Blue Test*		*Birt of Litter[c] (n=5) females / group*		*Changes in Body[d] wt. after inj.*	
0.08 µg/gb.wt. (n=10)	3/1,2,4	7 ± 2	8 ± 1		Number			
					Total	Dead	Alive	B.wt. in gm.
				7 ± 2	Nil	7 ± 2	4.8 ± 0.2	N.S.
0.1 (n=10)	"	"	3 ± 1	4 ± 2	3 ± 1	2 ± 1	<4.5 gm	N.S.
0.2 (n=15)	"	"	Nil		Nil			N.S.
0.4 (n=8)	"	"	Nil		Nil			145 gm*
0.5 (n=8)	"	"	Nil		Nil			< 130 gm*
Saline Control (n=10)	"	8 ± 3	9 ± 2	10 ± 1	Nil	10±1	>5.1 gm	N.S.
Control noninjected (n=10)	–	9 ± 2	9 ± 2	10 ± 1	Nil	10 ± 1	>5.1 gm	N.S.

*Prolonged diestrous, Mean 96 hrs., wasting of muscle mass.
P <0.001 't' test
[a]No statistical significant changes
[b-d]Statistical significant changes

those rats that became pregnant within two cycles of last BIM injection in the multidose treatment had 60% still births and the litter size is usually 4 ± 1, pups weighing < 4.5 gm. In some rats (n=5) prolonged diestrous was observed (mean 96 hours - Table IB). This study is, however, incomplete and requires detailed investigation. We have observed that after 5 or 6 BIM injections most of the female rats were reluctant to mate due to prolonged diestrous. Is it due to a change in hormonal profile or autonomic change, as discussed later?

Changes observed in blood : Differential count of WBC

The control pregnant rats showed a suppression in lymphocyte ($P < 0.01$) as early as on the 5th day of pregnancy compared to control non pregnant females. The BIM treated rats, however, had an

TABLE II : Effect of BIM administration on rat blood differential count on 5th day post mating.

Group	*Leukocyte Percentage*					*Statistical significance*
	N	L	E	M	B	't-test'
Control non-injected	42 ± 3	52 ± 2	2 ± 1	1 ± 1	0	N.S.
Control saline treated	43 ± 3	52 ± 2	2 ± 1	1 ± 1	0	N.S.
BIM treated (conc. 0.2 µg/g.b.wt.)	27 ± 5*	68 ± 7*	2 ± 1	1 ± 1	0	P < 0.001
Control non-pregnant	32 ± 4	65 ± 3	2 ± 1	1 ± 1	0	

increase in lymphocyte population ($P < 0.01$; Table II). No change in monocyte or eosinophil was observed.

Studies on uterine fluid

The uterine fluid of control pregnant rats contain lymphocytes that seem to be predominantly of T cell variety (23), while the BIM treated rats, showed an increased presence of myeloperoxidase +ve macrophages compared to lymphocytes in the flushed fluid as observed from stained slides.

Pontamine sky blue reaction

BIM treated sperm +ve rats demonstrated negative pontamine sky blue reaction ($P < 0.001$) whereas control pregnant rats showed dark implantation sites (Table I).

Fig. 1 : Implantation process in progress in control rat.

Histological observation

Histological observations of control pregnant uterus showed progestogenic character (Fig.1). It is well glandular and vasculated. The epithelial lining the lumen contain few mononuclear cells. Uterus of BIM treated animals is also progestogenic but the epithelial cell lining the lumen is heavily infiltrated with macrophages and mononuclear cells (Fig. 2).

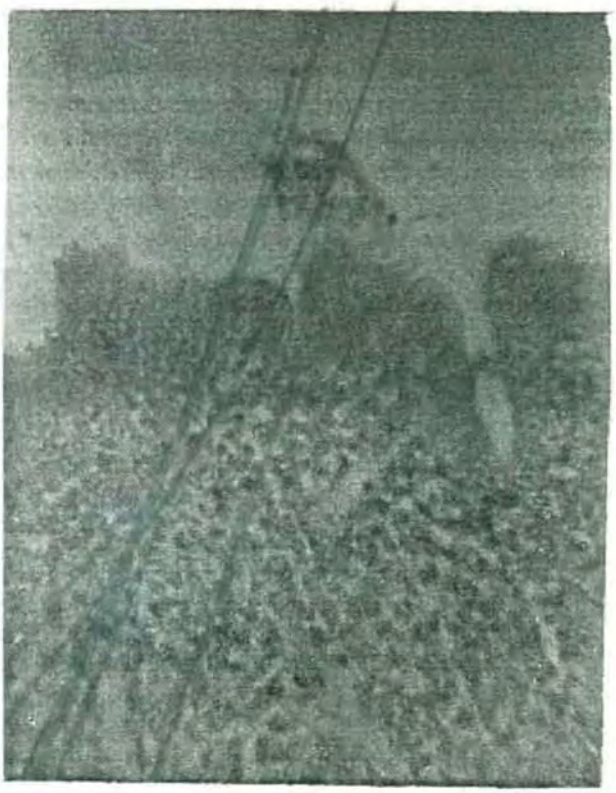

Fig. 2 : Implantation failure in BIM treated rat.

Studies on pups of BIM treated rats

Pups born of females previously treated with BIM showed no physical abnormality. The surviving pups grew normally and have normal reproductive capability. Their offsprings were also normal.

DISCUSSION

Implantation requires effective progesterone induced cytokine controlled immunosuppressive mechanisms to dampen fetal rejection (7, 15, 16).

BIM, induced both cell mediated and humoral immune responses (18-20). It also enhanced microbiocidal and phagocytic activities of macrophages (20) and neutrophils. This upregulation of the immune system seems to prevent blastocyst acceptance by the uterus (Table IA & B). The lymphopoiesis observed previously (18-20) in immunosuppressed BIM treated rodents is also seen here (Table II). BIM treated rats showed large number of mononuclear cells and neutrophils by the 6th day of pregnancy in the uterine endometrium (Fig. 2) and in the lumen with no concomitant increase in monocyte in the peripheral blood indicating that during pregnancy the activation of mononuclear cells (Mϕ and/or NK cells) is a local phenomena not reflected in blood (Table II). Similar site dependent enhanced mononuclear cell response was observed previously in mouse infected with tumor cell lines and treated with BIM (20) or other cytokines that elicited pathology by Th_1 response (7) or that initiated abortion or resorption of fetus (7, 24-27). These observations taken together seem to indicate that the anti-implantation effect of BIM is a mononuclear cell dependent immunological phenomena (acting probably via Th_1 pathway.) Uterus of control animals, however, do not show such heavy influx of mononuclear cells (27) and the process of implantation is evident (Fig. 1).

The anti-implantation effect of BIM is reversible. A single dose did not alter the estrous cycle and its anti-implantation action was found to be limited to one cycle.

Multiple doses in large and older animals, however, prolonged the diestrous phase for more than 3 days (Table I) which eventually shifted back to normal when BIM was withdrawn. The estrous cycle and uterine microenvironment is believed to be controlled by brain and administration of cytokine (viz IL-1) in the cerebroventricular region has been shown to initiate abortion (28). The secretion of BIM (*in vivo*) was observed to be under the control of vestibular nuclei situated at the base of the 4th ventricle (29) in the brain. BIM also modulated the activity pattern of brain ATPases (21, 22) when injected i.p. and, as observed in this paper induces pregnancy failure. This supports the previous observation (28) that cytokines can regulate implantation either by a local phenomena or via a central mechanism.

CONCLUSION

In conclusion, it seems that upregulation of the immune system by an immunomodulatory bonemarrow cytokine (BIM) at time of implantation evoked a cellular response at uterus that prevented implantation. This anti-implantation effect of the BIM is reversible.

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