The Production of Migration Inhibitory Factor and Reproductive Capacity in Allogeneic Pregnancies

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Migration inhibitory factor (MIF) is produced during allogeneic pregnancies but not during syngeneic pregnancies. Removal of the paraaortic or paraaortic and renal lymph nodes significantly decreased MIF production whereas splenectomy did not. Removal of these regional lymph nodes decreased the mean litter size and increased the variance in the weights of the offspring, with the greatest changes occurring when both the paraaortic and renal lymph nodes were removed; splenectomy did not alter either parameter. None of the surgical procedures affected the gestation period significantly, but removal of the paraaortic and renal lymph nodes greatly reduced the rate of conception and increased the incidence of stillbirths. These findings support the proposition that a vigorous immune response occurs during allogeneic pregnancies and that this response provides reproductive advantages to the offspring. (Am J Pathol 88:333–344, 1977)

THERE IS CONSIDERABLE EVIDENCE that an immunologic response is evoked in the pregnant female by the histocompatibility antigens of the father—hemagglutinating antibodies,[1,2] leukoagglutinating antibodies,[3-5] and lymphocytotoxic antibodies [6,7] have been found in pregnant animals and humans.[8] This immune response has also been defined morphologically by the enlargement of the lymph nodes draining the uterus, by the proliferation of blast cells (particularly among the thymus-derived populations),[8-19] and by the large numbers of plasma cells in the medullary cords.[20-22] Similar evidence was obtained from ova transfer experiments which showed that the weights of the fetoplacental units in DA and (DA × F344) F_1 hybrids developed in the uteri of (F344 × DA) F_1 hybrid females were quite similar.[12] Recent studies [23,24] showed that a cell-mediated immune response, as measured by the production of migration inhibitory factor (MIF), occurs in females during heterozygous pregnancies but not during homozygous ones.

The proposal has been advanced that the genetic disparity between the fetus and the mother affords the fetus a selective advantage over genetic-

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ally compatible fetuses in terms of the number of concepti that implant and survive to term and the weight of the offspring produced.[25] There is a contradictory school of thought,[26-30] however, which states that all of the advantages of the hybrid fetus are due to heterosis and not to an activation of the immune response.

The present experiments were designed to explore the role of immunologic activation during pregnancy by measuring the production of MIF *in vivo*. The ability to measure continuously the production of a lymphokine as a reflection of immunologic activity may provide a significant advantage in studying the immune response at various times during pregnancy, since multiple measurements can be made in 1 animal and the response can be quantitated. The site of production of MIF was explored by removing different groups of lymph nodes and the spleen. The effects of these various surgical procedures on the litter size and the weight of the individual offspring were also examined.

Materials and Methods

To confirm previous work on the lymphatic drainage of the uterus,[22,31-33] 30 female rats of different strains (LEW, DA, MNR, and KGH) were injected with a 1% solution of Pelikan ink (Gunther-Wagner No. C11/1431a). In the first set of experiments, the dye was introduced into the lumina of the uterine horns via the cervix with a spinal needle, and the cervix was then ligated. A second set of experiments was done by injecting the dye subserosally into the wall of one or both uterine horns. These experiments were also performed in animals in which the paraaortic lymph nodes, including the caudal nodes, had been removed. The localization of the dye in the lymph nodes was determined by gross inspection and by histologic examination.

In the experiments on the production of MIF during pregnancy, four groups of 20 virgin females of the LEW strain, age 14 weeks, were test bled and then mated with DA males of similar age in a ratio of 4 : 1. The first group were controls and were used to demonstrate the normal MIF response to a heterozygous pregnancy and to provide data for the normal litter size and weight. The second group consisted of females who had been splenectomized 1 month prior to mating. The surgery in this and in all subsequent groups was performed under fluothane anesthesia. In the third group, the paraaortic lymph nodes, including both the lateral and caudal lymph nodes, were removed 1 month before mating. In the fourth group, the paraaortic lymph nodes, including both the lateral and caudal lymph nodes, and the renal lymph nodes were removed 1 month before mating. Since the rate of conception in this group was quite low, an additional 15 females of the same age were added later, and data from the two separate sets of matings were combined. The females were examined every morning for the presence of copulation plugs by taking smears of the vaginal contents and staining with Giemsa stain. The presence of copulation plugs [34] was taken as the indication that mating had occurred. The animals were test bled on Days 12 and 20 after mating, and the sera were stored frozen until used. Immediately after delivery, the litters were separated from the mothers, and the offspring were individually weighed 3 hours later.

The MIF assays were performed with sera from animals at different stages of pregnancy using a method that has been described in detail previously.[35] The sera, which were stored frozen until used, were incorporated into agar, and the mixture was poured into sterile Falcon culture flasks. Peritoneal exudate cells from WKA rats were placed on the surface of the agar in two spots. The distance of migration from the edge of each spot was measured under a dissecting microscope after incubation for 72 hours, and the results were averaged. The inhibition of migration was determined by comparing the distance that the cells migrated on agar that contained pregnancy serum with the distance that they migrated on agar that contained normal serum. The controls were done each time that the pregnancy sera were studied. The variation in the controls was minimal, and all inhibition of migration greater than 10% was significant.[35] When significant production of MIF was found, all of the sera studied contained MIF.

Hematocrits were measured prior to pregnancy and on Days 12 and 20 by the capillary method using a microcapillary centrifuge.

Various statistical procedures were used to establish the significance of the findings.[36,37] The differences in mean litter sizes were compared by the Mann-Whitney U test. The mean weights of the animals in each litter were compared by an analysis of variance. The variances among the weights of each animal within a litter and among animals from various litters were evaluated using Bartlett's test.

Results

In the animals in which dye was introduced into the uterine lumen, no dye was observed in any of the lymph nodes. When the dye was injected subserosally into the walls of the uterus, it was generally found in the lateral paraaortic nodes, occasionally in the caudal nodes, and always in the renal lymph nodes on the same side. In approximately 50% of these animals, the dye was also found in the anterior mediastinal lymph nodes. Histologic studies of all of the lymph nodes showed that the dye was predominantly in the macrophages lining the sinusoids. There were usually two to four lateral paraortic lymph nodes on either side of the aorta just above the bifurcation. The caudal lymph nodes were located within the bifurcation of the aorta, and they usually varied from one to three in number. The renal lymph nodes were found above both renal veins; on the left side there was usually only one rather large lymph node, while on the right side there were generally two to three smaller lymph nodes.

Removal of the paraaortic lymph nodes (Group 3) or the paraaortic and renal lymph nodes (Group 4) caused a significant decrease in mean litter size, and the largest decrease occurred when both the paraaortic and the renal lymph nodes were removed (Table 1). Although there were no differences among the mean weights of the offspring, the variances of the weight within each litter and among litters from different mothers were different for the various surgical procedures (Table 1). An analysis of the various components of the average variances by the Bartlett test (Table 2) showed that the variance of the weights of the splenectomized animals (Group 2) is not different from the controls (Group 1) but did show that there are significant differences in the two groups in which the lymph nodes were removed (Groups 3 and 4). There are no significant differences in the variance of the weights among the litters from different mothers.

Table 1—Influence of Splenectomy and Removal of the Paraaortic and Renal Lymph Nodes on Litter Size and Mean Weight of Offspring From LEW × DA Matings*

				Mean litter size		Mean weight (g)	
Group	Surgical procedure	No. of mothers	No. of offspring	Average ± SD	Difference from control†	Average ± SD	Difference from control‡
1	None (control)	12	133	11.08 ± 1.56		5.68 ± 0.46	
2	Splenectomy	17	156	9.17 ± 3.53	0	5.58 ± 0.58	0
3	Paraaortic lymph nodes removed	16	125	7.81 ± 3.35	±	5.38 ± 0.64	0
4	Paraaortic and renal lymph nodes removed	10	55	5.50 ± 2.83	+	5.51 ± 0.74	0

* The mean litter size and mean weights, respectively, of the offspring from LEW × LEW matings (18 mothers) are 9.67 ± 3.65 and 5.72 ± 0.46 g, and from DA × DA matings (18 mothers) they are 5.17 ± 2.41 and 4.97 ± 0.46 g. The levels of significance are: +, $P < 0.01$; ±, $0.01 < P \leq 0.05$, and 0, $P > 0.5$.

† Mann-Whitney U test.

‡ Analysis of variance.

This is to be expected in Groups 2, since the splenectomy did not have any effect on the weights of the offspring themselves. In Groups 3 and 4, the removal of the lymph nodes caused a large and significant variation in the weights of the individual offspring, and the effect of this surgical procedure was the same in all of the mothers so treated (Table 2).

The gestational period in this particular mating combination was 21 to 23 days (Table 3), and the various surgical procedures did not alter it significantly. In Group 4, in which both the paraaortic and renal lymph nodes were removed, the rate of the conception was greatly reduced. Only 20 to 30% of the females became pregnant, whereas 60 to 80% of the females in the other three groups became pregnant. In addition, Group 4

Table 2—Estimates of the Various Components of the Average Variance in Weights of Offspring From LEW × DA Matings*

		Within each litter		Among different litters	
Group	Surgical procedure	Variance	Difference from control†	Variance	Difference from control†
1	None (controls)	0.0962		0.1149	
2	Splenectomy	0.1244	0	0.2211	0
3	Paraaortic lymph nodes removed	0.3141	+	0.3434	0
4	Paraaortic and renal lymph nodes removed	0.2794	+	0.2622	0

* Data derived from the matings shown in Table 1.

† Bartlett test.

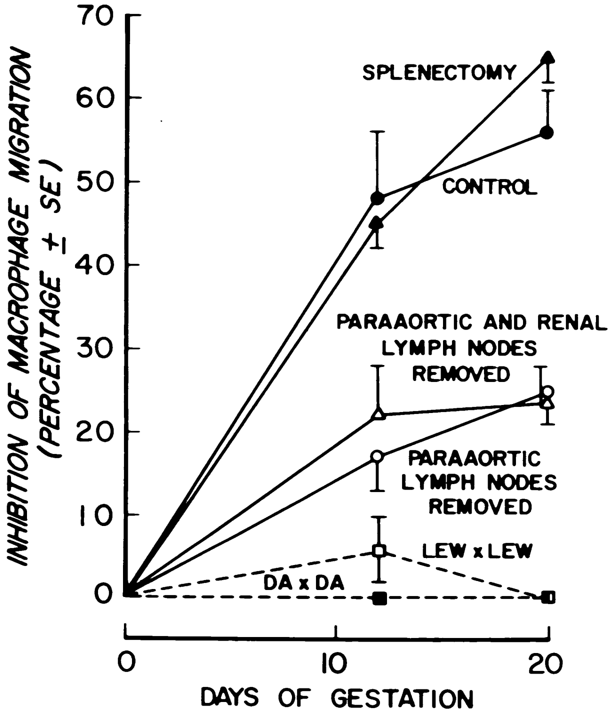
Table 3—The Gestation Period for the Various Groups of Animals From LEW × DA Matings

Group	Surgical procedure	Total No. of litters	Gestational period in days (No. of litters)						
			19	20	21	22	23	24	25
1	None (controls)	13	1		6	2	4		
2	Splenectomy	14	1		4	6	2	1	
3	Paraaortic lymph nodes removed	15			1	8	3	2	1
4	Paraaortic and renal lymph nodes removed	4			2		1	1	
Total No.		46	2		13	16	10	4	1

was the only one in which stillbirths occurred, and 7% of the offspring were stillborn.

The removal of the lymph nodes (Groups 3 and 4) caused a significant ($P < 0.01$) decrease in MIF production, but splenectomy did not alter MIF production. The data are shown in Text-figure 1.

The hematocrit in all groups decreased by 10% on Day 12 and by 20% on Day 20 during pregnancy, and there was no difference in the amount of the decrease among the four groups.



Text-figure 1—The production of migration inhibitory factor during pregnancy and the role of various lymphoid organs in its production. Splenectomy (*solid triangles*) did not alter MIF production, but removal of the paraaortic (*open circles*) or paraaortic and renal (*open triangles*) lymph nodes significantly ($P < 0.01$) decreased MIF production. The homozygous matings LEW × LEW (*open squares*) and DA × DA (*solid squares*) did not elicit any MIF production (significant response is ≥10% inhibition).[35]

Discussion

The endometrium has no lymphatics;[38,39] hence, there was no drainage of dye introduced into the lumen of the uterine horn. After subserosal injection of the dye, it was found in the paraaortic and renal lymph nodes, and when the paraaortic lymph nodes were removed (Group 3), it was always found in the renal lymph nodes. This finding suggests that the renal lymph nodes are not just the efferent drainage of the paraaortic lymph nodes but also drain the uterus directly. The appearance of dye in the anterior mediastinal lymph nodes following subserosal injection into the uterus presents a question in interpretation. In the cases in which some leakage into the peritoneal cavity could be demonstrated, the finding of dye in these lymph nodes would be expected, since the lymphatics which drain the peritoneum drain into the anterior mediastinal lymph nodes.[31,40,41] In the cases in which we could not find any evidence for peritoneal leakage, there are two possible interpretations. First, we could not detect a small amount of peritoneal leakage. Second, there may be some lymphatic connections draining the uterus directly into the anterior mediastinal lymph nodes. There is some evidence for the latter interpretation, because the lymphatic drainage from a particular site may bypass several groups of regional lymph nodes and go directly to distant lymph nodes. For example, Tilney [31] has shown that the lymphatic drainage of the testes occasionally bypassed one or more groups of lymph nodes and drained into distant lymph nodes. This interpretation would explain the low, but significant, level of MIF in the animals from which the paraaortic or paraaortic and renal lymph nodes had been removed (Figure 1).

An increase in the size of the paraaortic lymph nodes during allogeneic pregnancies has been observed in rats,[8,14,42-44] rabbits,[44] mice,[26] hamsters,[8,19] and humans,[8] whereas, it was not seen in the presence of syngeneic fetuses. Since allogeneic pregnancies result in larger litters and a larger mean weight of the offspring,[19,25,45] the suggestion has been made that activation of the immune system of the mother brings about beneficial conditions for implantation and intrauterine development of the fetuses. The corollary of this proposition is that abrogation of the mother's capacity to mount an immune response by the removal of the lymph nodes involved should lead to loss of the advantage that allogeneic fetuses have over syngeneic fetuses. The present results support the proposition that the lymph nodes draining the uterus participate in providing a selective advantage to histoincompatible fetuses in terms of the number of concepti implanted and sustained to term: removal of the regional lymph nodes is followed by evidence for a decreased cell-mediated immune response (decreased MIF production) (Figure 1), by a significant decrease in litter size (Table 1) and by uneven fetal development as shown by an increase in the variance of the weights of individual offspring (Table 2). The mean weights of the offspring were not affected, and they are different from those previously reported because they were measured after birth rather than at 18 days of gestation.[25]

The role of the paraaortic lymph nodes in extending selective advantage to genetically alien fetuses has been shown by Beer and Billingham,[12,25] but the role of the renal lymph nodes in this process has not been established. As shown in Table 1, removal of the paraaortic and renal lymph nodes decreased the mean litter size more than did the removal of the paraaortic lymph nodes alone. This is consistent with histologic studies in mice [32] which showed that during heterozygous pregnancies the highest level of immune reactivity occurred in the renal lymph nodes.

The length of gestation has been reported to vary inversely with litter size.[46,47] McLaren and Michie [47] concluded that the effect of litter size on gestational length reflected the total mass of fetal tissue, and ova transfer experiments in rats [48] led to the same conclusion. We did not see this effect in the experiments described here (Table 3). It appears from our data that the immune response to the fetus plays the crucial role in the number of fetuses implanted and in their individual weights (Tables 1 and 2).

The production of MIF parallels the course of gestation, reaching a maximum at parturition and then rapidly decreasing.[23,24] Removal of the spleen did not affect the ability of the mother to respond to her genetically alien fetuses, but removal of the paraaortic and renal lymph nodes markedly did (Figure 1). These data provide functional evidence for a continued cell-mediated immune response during pregnancy. Whatever role this response plays in pregnancy, it is not necessary for the induction or maintenance of pregnancy, because no significant amount of MIF is produced during homozygous pregnancies.[23,24] Since MIF production following the removal of the regional lymph nodes did not completely disappear, the alien fetus must be stimulating lymph nodes other than those draining the uterus; one possibility is the anterior mediastinal lymph nodes, as discussed above.

Although pregnancy can occur successfully in the absence of an immune response, i.e., in the derivation of inbred stains, this situation is a biologically special case and probably represents a maximal stress on the reproductive mechanism. There are several reasons for this. First, in the initial selection of rats or mice for inbreeding from wild populations, a large number of mating pairs are unable to adapt to breeding in the laboratory, and these animals are selected out immediately.[48] Thereafter, it is common to select animals on the basis of their reproductive performance,[48,49] and this procedure introduces a continuing selective process. Second, the development and maintenance of inbred strains is frequently difficult or impossible because of reduction in litter size and in viability of the offspring due to adverse genetic or environmental effects on the various components of the reproductive cycle. In some strains of inbred rats [50] and mice,[51-54] the reduction in litter size is due to postimplantation losses. Another potent reducer of litter size and source of inherited partial sterility is chromosomal aberration.[55,56] Third, in the course of inbreeding, many of the females develop polycystic ovaries or a uterine environment inconducive to implantation either by normal fertilization or by ova transplantation.[57] Fourth, the mother can have difficulty with lactation and not be able to nurse beyond the first few days after delivery.[57,58] Finally, there are also defects in the male, such as loss of libido, which contribute to the difficulties of inbreeding.[57,58] Similar findings have been reported in attempts to inbreed rabbits.[57,59-62] In the experiments described here, removal of the lymphoid tissue draining the uterus decreases the reproductive capacity in allogeneic matings so that it resembles that of syngeneic matings.

The process of implantation follows the interaction of the fertilized ovum and the endometrium; it includes ovular maturation, endometrial receptivity, and tubal transport. Experiments with the transfer of ova have shown that asynchrony in the process of implantation affords the concepti initial developmental advantages that can be maintained throughout pregnancy. For example, in mice at Day 18 of gestation, concepti which arose from ova that were 1 day overdeveloped weighed approximately 23% more than those of normally aged ova.[63] However, the advantage obtained from the overdeveloped ovum may be offset by a compensatory shortening of the length of pregnancy.[64] Gates [65] reported that by the time ova in the same litter of inbred strains of mice reached the uterus, there was a spread in development of approximately 24 hours among them. Hybrid ova have a much narrower developmental spread than do the ova of inbred strains; therefore, the variances in the weights of individual offspring are smaller.[65] The experiments reported here show that removal of the regional lymph nodes decreased the cell-mediated immune response to pregnancy, as measured by MIF production, and concomitantly abrogated the reproductive advantages of the hybrids without significantly altering the time of gestation. The immune response to the fetus, particularly in the regional lymph nodes, may affect the time at which the ova implant or the state of the endometrium when they implant. Thus, this aspect of the selective advantage ascribed to heterosis may have an important immunologic component.

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