Production of Congenital Malformations Using Tissue Antibodies. I. Kidney Antisera.* (26390)

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(Introduced by J. M. Coon)
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Embryologists have attempted to use tissue antibodies *in vitro* as growth inhibitors and disorganizers, and *in vivo* as teratogenic or abortogenic agents. There is evidence that specific and non-specific antigen-antibody reactions can cause fetal death or early resorption(1,2,3). Others have attempted to utilize organ or tissue antibodies as teratogenic agents specifically directed against that developing organ or tissue in order to produce specific congenital malformations(3, 4,5). The results of the latter experiment are somewhat controversial because of the low percentage of specific malformations produced by this technic.

Attempts to produce malformations in mice, using injected brain, have been reported to be successful(2,4), although no evidence was presented that anti-brain antibodies were developed or that they reached the fetus(2). Furthermore, the "malformations" of the central nervous system described were the same as those seen in fetuses dying or retarded from many noxious agents(2).

Lens malformations(3,5) were produced in mammals utilizing anti-lens antisera, but only in an extremely small percentage of cases (3 malformed eyes out of 611 fetuses in Miller's experiments). In any event, the reported instances of antibody teratogenesis would certainly classify antibodies as very ineffective teratogenic agents.

Methods. In our laboratory, we have been utilizing tissue antibodies for 3 years as an experimental embryological tool. Antigens were made from adult and fetal rat tissues: 21-day placenta, fetal liver, skin and brain; adult rat muscle, brain, kidney, liver, testes, plasma and red blood cells. The tissues were prepared by saline perfusion, homogenization and lyophilization, and stored in the deep freeze as a dry powder. Two injections of 200 mg of resuspended tissue powder were injected intraperitoneally into albino rabbits twice weekly, without any adjuvant, over a period of 3 months, followed by periodic bleeding. The presence of antibody was evaluated by agar diffusion plates and a serial dilution precipitin test.

Anti-kidney sera was obtained by multiple bleedings from 4 rabbits. Ten quantities of anti-kidney sera were obtained which gave reactions varying from very weak to extremely potent, as measured by number and density of bands in the agar diffusion plates, and the titers of the sera in the precipitin test.

Thirty-one pregnant rats were injected intravenously with .2 to 1.0 ml of rat kidney antisera on the 8th day of gestation. Number and location of the embryos were determined by laparotomy on the 8th day. At the 21st day of gestation, the mothers were sacrificed and fetuses were removed, fixed in buffered formalin and dissected.

The injection of teratogenic anti-kidney antibody was combined with the technic of vascular clamping of one horn of the pregnant uterus (Fig. 1g). The clamping technic was previously described and demonstrated that clamping the vascular supply to the pregnant uterus for one-half hour on the 9th gestational day produced insignificant gross changes in the fetus(6). The antiserum was injected intravenously just after isolating one horn of the uterus from the circulation, allowing the unclamped uterine horn to be in contact with the circulating kidney antiserum for the first one-half hour, while the clamped horn was isolated from the antiserum during this period. This technic enables one to evaluate the rapidity of action of embryonic growth inhibiting or malform-

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ing agents, since a greater effect on the unclamped side indicates that a significant effect has occurred during the period of clamping.

Gamma globulin was prepared from the anti-kidney plasma by the salting-out procedure using ammonium sulfate and reconstitution after dialysis with pH 7.4 borate buffer. The purified gamma globulin was labelled with I^{131} by the technic of Helmkamp *et al.*(7). Iodination was carried out utilizing 2 ml of gamma globulin and 1 mc of Na I^{131}. The labelled globulin was injected intravenously into pregnant and non-pregnant rats. From 12 to 172 hours later, the animals were anesthetized and perfused with 500 ml of saline. Samples of tissue (100-300 mg) were weighed and placed in 2 ml of 2 N NaOH and counted in a scintillation well counter.

Results. Fourteen of the 31 litters injected with antisera had quite severe malformations, consisting of anencephaly, anophthalmia, encephalocele, hydrocephalus, aplastic cerebral hemispheres, evisceration, chest wall defects, hypoplastic lungs, hypoplastic auricle, tear drop heart, right-sided aortic arch, fused kidneys, absent kidneys, absent ears, situs inversus, omphalocele, cleft lip, club feet and aplastic tail (Fig. 1a, b, c, d). Four samples of antisera produced 100% malformations if the dose was between .4 and .7 ml. Doses of .7-1.0 ml of potent antisera caused complete resorption. Growth retardation was frequently associated with malformation, although some malformations occurred in normal-sized fetuses at term. When maternal rat plasma was evaluated for presence of injected antibody following injection, none could be detected. This meant that either it reacted rapidly with the tissues, or the tests used in our laboratory were not sensitive enough to measure the antibody when it was diluted into the maternal rat circulation. In either event, teratogenic antibodies can be present in an organism and not be easily detected.

Injection of normal rabbit plasma, potent rabbit anti-rat plasma antisera and rabbit anti-rat erythrocyte antisera on the 8th gestational day served as control experiments. In spite of anaphylaxis, hemolytic anemia and apneic periods produced in the mother by the antisera, no congenital malformations were produced in the fetuses, and fetal weights were the same as non-injected animals that had laparotomies on the 8th day (Fig. 1e).

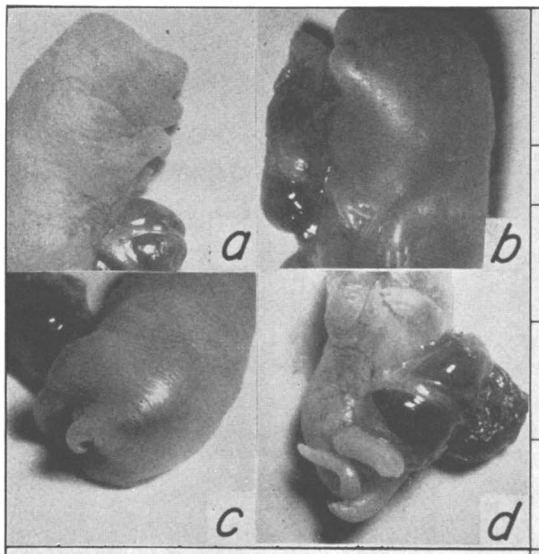
The animals that were injected with anti-kidney antiserum after clamping one horn of the uterus showed a higher incidence of malformations and fetal deaths on the unclamped side (Fig. 1g). Weights of the fetuses that survived on the unclamped side were significantly smaller than on the clamped side.

The localization of the I^{131} labelled anti-kidney serum confirmed previous reports of localization in the kidney, adrenal and spleen(8). In the pregnant animal, there was also localization within the placenta (Fig. 1f). The fetal placenta consistently localized a larger amount of the anti-kidney antibody than did the maternal placenta. The measured specificity of the anti-kidney serum for the placenta was diluted by the rapid growth of the placenta during the uptake studies. There were differential localizations in fetal tissues, but the quantitative amounts were insignificant compared to the labelled antibody in maternal kidney and fetal placenta (Fig. 1f).

Discussion. There is no doubt that some fraction of the anti-kidney antisera is teratogenic. This is not due to antibodies against red blood cells and/or sera trapped in the post-perfused kidney. There are several mechanisms by which the kidney antiserum may be teratogenic:

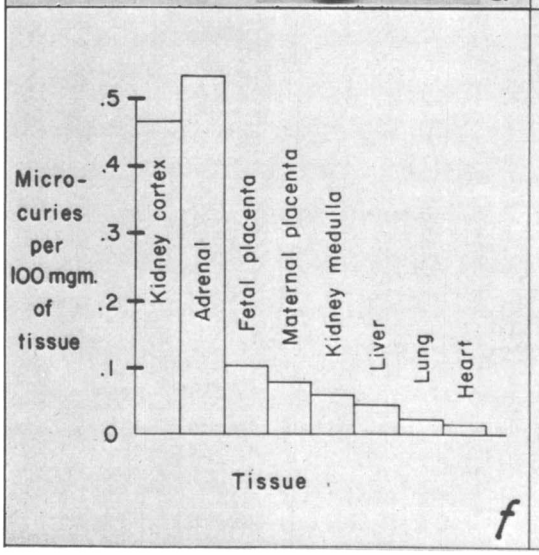
1) by producing severe disease in the mother which then indirectly affects the fetus (maternal rats exhibited marked proteinuria and weight loss following kidney antisera injections). 2) by directly interfering with embryonic growth and differentiation. 3) by interfering with placental function and permeability.

Results which helped in evaluating the possible mechanisms were that teratogenic rat kidney antiserum, when labelled with



EXPERIMENTAL TECHNIQUE : 8th day	FETUS : 21st day	
	Mortality (percent)	Weight (grams)
Operative control	8.0	4.90 ± .37
Injected rabbit plasma	15.1	4.85 ± .26
Injected rat plasma antiserum	9.2	4.74 ± .37
Injected rat kidney antiserum	36.6	4.39 ± .51

e



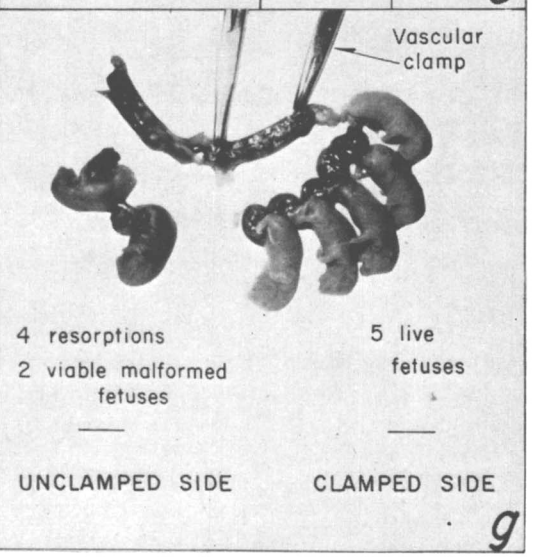


FIG. 1 (*a-d*). Newborn rats following 8th day inj. of rat kidney antiserum. *a*) Hydrocephalus, anophthalmia and absent external auditory canal. *b*) Anophthalmia, edema and evisceration. *c*) Hypoplasia of tail and evisceration. *d*) Evisceration and club feet. *e*) Control groups consisted of one-half hr laparotomy on 8th day; inj. of rabbit anti-rat plasma antisera and normal rabbit plasma on 8th day. Avg percent mortality and fetal weights in the group inj. with kidney antiserum was significantly different from control groups. *f*) Localization of I^{131} labelled kidney antiserum in rat tissues following intrav. inj. Corrected levels of I^{131} in placenta are actually much higher because of rapid placental growth in the 48 hr following inj. *g*) Clamping of one horn of uterus just before inj. of potent kidney antiserum results in a marked protective effect even when clamps are removed in one-half hr.

I^{131}, localized in maternal kidney cortex and in fetal placenta in very high concentrations and that the results of combining vascular clamping with injection of teratogenic kidney antibody showed a higher incidence of malformation and death on the unclamped side.

The fact that antibody 1) localized in fetal placental tissue and 2) that it had a significant effect in the first half hour after injection, tends to incriminate a disturbance in placental function as a major factor in production of these malformations, although neither of the other 2 postulated mechanisms can be presently eliminated. However, it is

significant that an antibody which was undetectable in maternal rat blood following intravenous administration, has proven to be so potent a teratogenic agent. From both theoretical and practical standpoints, it would be most important to understand the qualitative and quantitative mechanisms by which this biologically prepared and altered protein induces malformations.

Summary. Rabbit anti-rat kidney sera, in a prescribed dosage, injected intravenously into pregnant rats on the 8th day of gestation, resulted in severe congenital malformations in 100% of the fetuses. Larger doses caused complete fetal resorption. These antisera may produce teratogenesis by interfering with placental function.

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Biosynthesis of a Sulfobromophthalein Mercaptide with Glutathione.* (26391)

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Although glutathione mercaptides have long been suggested as intermediates in mercapturic acid synthesis(1-4), isolation and identification of a glutathione mercaptide from biological fluids has proved difficult.

The dye sulfobromophthalein (BSP) is rapidly taken up by the liver and secreted into the bile as pigmented metabolites(5-8). The main metabolite appearing in human bile has been identified as an unacetylated mercaptide formed through the –SH group of cysteine(9). The consistent demonstration of both glycine and glutamic acid in acid hydrolysates of this metabolite(5,7-9) has suggested that it may be a BSP-glutathione mercaptide(9,10). Positive identification of the glutathione mercaptide was difficult since both glycine and glutamic acid were found in acid hydrolysates of identically prepared BSP-free fractions from normal bile(5,9).

The present report describes the successful incorporation of isotopic glycine, glutamic acid and cysteine into the major BSP metabolite synthesized by the isolated perfused liver of the rat. The results indicate that BSP is excreted into bile primarily as a mercaptide with glutathione.

Methods. Intact livers were taken from Long-Evans male rats, weighing 400-450 g, and perfused(11). At 1 hr 50-125 μc of either glycine-U-C^{14}, glutamic acid-U-C^{14}, glutamine-U-C^{14}, cystine-S^{35} or glutathione-S^{35} (Schwartz Laboratories) was added to the perfusate. After 30 min equilibration, bile to be used in the controls was collected for 30 min and immediately frozen. BSP, 20 mg, was then introduced into the perfusate and equilibrated for 30 min. Experimental bile was collected during the subsequent 30 min interval. BSP levels were determined by the technic of Gaebler(12). Samples of bile or bile fractions, 10-50 μl, were plated and counted with an end-window Geiger-

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