Persistence of Shigella Antigen in Subcellular Fractions of Spleens from Immunologically Unresponsive Mice.* (28566)

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Immunologic unresponsiveness has been observed in a variety of experimental animals following either embryonic, neonatal, or postnatal administration of relatively large quantities of non-living antigen, including serum proteins, erythrocytes, and microbial products(1). Many aspects of such unresponsiveness are similar to those observed in mice following injection of pneumococcal polysaccharide(2) which results in a specific suppression of immunity termed immunologic paralysis.

A number of investigators believe that such unresponsiveness or paralysis, as well as classical immunologic tolerance to tissues, may be directly related to persistence of "excess" antigen in critical sites of the antibody forming mechanism(1,3,4). If all of these phenomena are associated with excess persisting antigen, it would be expected that such antigen could be detected by sensitive serologic techniques. This has not been demonstrated experimentally, although a relationship between dosage of antigen administered and duration of resulting unresponsiveness has been established by several groups with non-living antigens(5,7). It is thought that failure to detect persisting excess antigen during a specific unresponsive state may often be due either to low sensitivity of methods available, or to the fact that "overloading" quantities of antigen maintaining unresponsiveness may exist in an intracellular site(7).

There have been various demonstrations of localization of antigenic material in subcellular fractions of tissues from immunized animals shortly after injection of antigen. Such antigenic materials usually have been detected by means of radioisotope tracer methods(8,9), although immunogenic determinants of antigen have been detected by serologic means in several experiments(9,11).

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The present investigation was undertaken to determine whether persisting antigen could be detected in tissues obtained from young mice exhibiting a state of specific immunologic unresponsiveness following a relatively large inoculum of soluble Shigella antigen at birth(7,12). A cell transfer technique was utilized in the hope that it would prove to be sufficiently sensitive to detect possible Shigella antigens persisting in association with subcellular fractions of unresponsive mice. Similar cell transfer experiments(13) with rabbit lymphoid cells have indicated that as little as 10^{-7} mg of similar Shigella soluble antigen can sensitize suspensions of normal lymph node cells *in vitro* to form anti-Shigella agglutinins upon transfer to non-immunized recipient rabbits. The use of this method for the apparent detection of Shigella agglutinogens in subcellular fractions of spleen cells obtained at various times from neonatally injected mice is reported here.

Materials and methods. Newborn NIH mice were injected intraperitoneally with 0.1 ml of soluble Shigella antigen containing 20 μg N(7). Several mice in each litter were injected with 0.1 ml saline as a control. Serum specimens were obtained from each mouse by retro-orbital puncture(12) at various intervals during the next 8 weeks or prior to sacrifice and anti-Shigella agglutinins determined by standard tube agglutination tests. Groups of such Shigella injected and control mice were sacrificed at one day, 1, 2, 4, 6, and 8 weeks of age and spleens removed. Cell suspensions were prepared by teasing (12) in Hanks' balanced salt solution. Cell-free homogenates were prepared by homogenization with Teflon tipped tissue homogenizers at 4°C. Nuclei and mitochondria-rich fractions were prepared from the homogenates by differential centrifugation, while ribonucleoprotein (RNP) and deoxyribonucleoprotein (DNP)-rich fractions were ex-

TABLE I. Anti-Shigella Agglutinin Titers of Non-Immunized Adult Mice Receiving Normal Adult Mouse Spleen Cells Incubated *In Vitro* with Splenic Fractions from Unresponsive Mice Injected at Birth with Shigella Antigen.

Unresponsive donor mice*		Recipient mean peak agglutinin titer†					
		Age of unresponsive mice at sacrifice					
Spleen cell fraction	No. of mice	1 day	1 wk	2 wk	4 wk	6 wk	8 wk
Homogenate	48	1:184	1:63	1:18	1:12	<8	<8
Nuclei	35	1:85	1:71	1:30	8	<8	<8
Mitochondria	28	1:81	1:34	1:16	<8	<8	<8
RNP	46	1:173	1:79	1:94	1:84	1:58	1:21
DNP	40	1:28	1:31	1:16	<8	<8	<8

* Received intraperitoneally 0.1 ml soluble Shigella antigen (20 μg N) within 12 hr of birth.
† Received 2-9 $\times$ 10^7 normal mouse spleen cells incubated *in vitro* with spleen cell fractions (0.4-0.9 mg N) from unresponsive donor mice.

tracted from supernatant and nuclear fractions by salt extraction procedures(14,15).

The fractions were tested serologically for detectable Shigella antigens by precipitin, complement fixation, and agglutination-inhibition tests using high titered rabbit anti-Shigella antisera as a reagent(12). Fractions were also tested for Shigella agglutinins by standard tube agglutination and tanned cell agglutination tests(12). Aliquots of each fraction (0.4 to 0.9 mg N per ml) were injected intravenously into groups of non-immunized adult recipient mice to determine whether the fractions could directly stimulate anti-Shigella agglutinin formation. Similar aliquots were incubated as 1.0 ml quantities with 1.0 ml suspensions of normal adult mouse spleen cells (2-9 $\times$ 10^7 cells) in Hanks' solution containing 30% normal rabbit serum for one hour at 37°C. Following incubation, the mixtures were washed 3 times with Hanks' solution by serial centrifugation and injected intravenously as 20% suspensions into other groups of non-immunized recipient NIH mice, 6 to 10 weeks of age. One recipient was used for each subcellular fraction-spleen cell suspension preparation. Serum samples were obtained from each recipient at close intervals for 2 to 3 weeks following transfer and formation of anti-Shigella agglutinins determined by tube agglutination tests. Titers were expressed as reciprocals of the highest dilution giving complete agglutination.

Results. No serologically detectable Shigella antigens could be found in sera or spleen cell fractions from neonatally injected mice after one week of age. No anti-Shigella agglutinins could be detected in any serum or fraction sample. Injection of the subcellular fractions into fresh adult recipients rarely resulted in Shigella agglutinin formation, except with preparations obtained at one day or occasionally one week following Shigella injection of the neonatal donors. However, incubation of normal whole spleen cell suspensions with fractions obtained from unresponsive donor spleen cells, followed by washing and transfer to fresh recipient mice, usually resulted in readily detectable formation of anti-Shigella agglutinins in recipients (Table I). Most mice receiving spleen cell suspensions incubated with subcellular fractions obtained from unresponsive mice up to 4 weeks of age following neonatal injection formed detectable anti-Shigella agglutinins. Agglutinins appeared within 3 to 4 days following transfer and reached maximum titers at 5 to 7 days.

Incubation of spleen cells with donor RNP fractions resulted in the most frequent appearance of agglutinins in recipients. Such RNP fractions, obtained from mice as long as 6 weeks after neonatal injection, still sensitized fresh spleen cells to form anti-Shigella agglutinins in other recipients. However, at 8 weeks following neonatal injection, when unresponsive mice were first becoming capable of positive immune responses to challenge injections with Shigella antigen(7,12), most RNP fractions were usually negative for agglutinogenic activity.

Control experiments with similar splenic subcellular fractions obtained from mice of various ages following neonatal injection and incubated with normal spleen cells heated at

56°C for 30 minutes prior to incubation resulted in no detectable formation of agglutinins following transfer to recipients. A comparison of these results with similar experiments using adult mice injected with Shigella antigen (20 μg N per gram mouse) indicated that there was a more prolonged persistence of detectable Shigella agglutinogenic activity in fractions obtained from neonatal and early post-natal injected mice as compared to spleen fractions from adult injected mice.

Discussion. The results obtained indicate that Shigella agglutinogens apparently may be detectable in spleen cell fractions up to 40 or 50 days following injection of neonatal mice with a concentration of soluble Shigella antigen sufficient to induce a state of immunologic unresponsiveness. Although the Shigella antigen was known to be quite toxic (12), it was necessary to administer a dosage which usually resulted in 20 to 30% mortality in order to establish unresponsiveness (12). Utilization of such a toxic antigen may interfere with comparison of these results with other studies using less toxic antigens, such as serum proteins, since an immunologic selection may have occurred in the surviving mice used in this study.

One advantage of using this soluble antigenic material is that the typical immune response it induces in mice can be readily detected by a standard agglutination test whereby a dilute suspension of alcohol-killed whole Shigella is specfically agglutinated by positive serum. The same agglutination procedure was used in this study to detect agglutinin responses in recipients receiving subcellular fractions suspected of containing Shigella agglutinogens. The use of this procedure has indicated that immunogenic quantities of Shigella antigen exist for some time in spleens of neonatally injected mice and can stimulate fresh spleen cells to form anti-Shigella agglutinins upon transfer to new, non-immunized mice.

The cell transfer procedure used in this study appears capable of detecting antigen at a concentration many-fold lower than that which can be detected with less sensitive immunologic methods. Experiments by Harris and Harris have shown that very little Shigella antigen is required for *in vitro* stimulation of rabbit lymphoid cells. They have estimated(13) that approximately 100 molecules of a relatively similar Shigella antigen per lymph node cell are sufficient for stimulation of *de novo* agglutinin formation. The observation in this study that Shigella agglutinogenic activity may persist in association with nucleoprotein-rich fractions is in general agreement with suggestions by others that immunogenic determinants may exist as nucleoprotein-antigen complexes during various phases of antibody formation or suppression.

Summary. Spleen cell nuclei, mitochondria, ribunucleoprotein and deoxyribonucleoprotein-rich fractions have been prepared from immunologically unresponsive NIH mice which had received a large inoculum of Shigella soluble antigen at birth. Presence of Shigella agglutinogens in these subcellular fractions has been inferred by use of an indirect method in which the fractions were incubated with fresh spleen cell suspensions, followed by transfer to new recipient mice. A rapid rise in specific anti-Shigella agglutinins in these recipients suggested the presence of Shigella agglutinogens in the original fractions. Such agglutinogenic activity was detected in most fractions for 2 to 4 weeks following neonatal injection, whereas ribonucleoprotein fractions exhibited detectable agglutinogenic activity for 6 to 8 weeks.

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Maintenance of Pregnancy with Subcutaneous Pellets of Progesterone in Ovariectomized Mice.* (28567)

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Bilateral ovariectomy of the mouse at any stage of pregnancy is promptly followed by death or abortion of the fetuses(1,2). Functional corpora lutea are essential for maintenance of pregnancy in the mouse(3). However, if bilateral ovariectomy of this animal is preceded and followed by adequate administration of progesterone, pregnancy can continue(4,5,6,7,8,9). Some question remains as to the minimal amount of exogenous progesterone necessary at various stages of pregnancy to forestall loss of some or all of the fetuses. The experiments to be described were addressed primarily to this problem. To simulate the uninterrupted release of progestin supposedly occurring in the intact pregnant animal, pellets of progesterone were implanted subcutaneously for continuous absorption.

Materials and methods. Adult virgin female mice of the CHI and Brown Belt (BB) stocks were mated with males of corresponding stocks. The females were inspected each morning for vaginal plugs; the day of finding the plug was counted as day 1 of pregnancy. Mice later found not to be pregnant were excluded from the experiment. All animals received Purina Lab Chow and water *ad libitum.*

Sixty pregnant CHI and 89 pregnant BB mice served as controls. Of these, 33 CHI and 52 BB mice, intact and untreated, were allowed to go to term and deliver their young. Eight CHI and 9 BB mice, also intact and untreated, were killed on day 19 and were inspected for live and dead fetuses. Sham ovariectomies (exposure and manipulation, but not removal, of ovaries) were performed *via* a dorsal skin incision on days 10, 14, or 16 of pregnancy in 9 CHI and 13 BB mice; no pellets were given. Body weights were recorded daily, and these animals were killed on day 19. Finally, 10 CHI and 15 BB mice were ovariectomized on days 10, 14, or 16 and received no pellets. Body weights were also determined daily for these mice, which were killed on day 19.

Cylindrical pellets were made by the compression of crystalline progesterone or of 17*a*-hydroxyprogesterone in a device which ensures that each pellet is subjected to the same amount of compression(10). One or more pellets, usually of 9-11 mg each, were weighed and then were discharged from a large (#13) hypodermic needle under the dorsal thoracic or cervical skin of each experimental mouse during light ether anesthesia. Two days later the recipient was bilaterally ovariectomized, care being taken not to disturb the pellet(s). Implantation of hormone and removal of ovaries were carried out on days 8-10, 9-11, 10-12, 11-13, 12-14, and 14-16 of pregnancy. Seventy-five CHI mice and 26 BB mice received ½, 1, 2, 4, or 6 progesterone pellets. Two or 4 pellets of 17*a*-hydroxyprogesterone were given to 22 BB mice.

At autopsy of control and experimental mice the live and dead fetuses were removed, counted, freed from membranes and placentae, lightly blotted, and weighed individually. The uteri were inspected for implantation sites. The percent of living fetuses in a total representing live fetuses plus dead fetuses plus

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