illustrates the diminution of enzymic activities following castration for 2 and 6 weeks. The effects of castration are seen to be quite similar to those of hypophysectomy. It is interesting to note that, unlike the marked decrease in activity of gulonolactone hydrolase found in hypophysectomized rats, the decrease in castrated males was of a similar magnitude to that of the other 3 enzymes.

Concomitant with the reduction of male enzymic activities following castration was a decrease in the tissue levels of ascorbic acid. As shown in Fig. 3, male rats castrated for 6 weeks had significantly diminished concentrations of tissue ascorbate which approach, or reach, those typical of females; in one case, *viz.*, bone, the level was even below that in females. Diminished concentrations of ascorbic acid are statistically significant for all tissues with the exception of the adrenals ($p<.05$). These decreased concentrations of tissue ascorbate which were found along with decreased hepatic enzymic activities in castrated males give further support to the hypothesis that tissue levels of ascorbic acid are dependent upon the rate of biosynthesis by the liver. The effects of hypophysectomy and castration clearly indicate a requirement of normal testicular secretion for the maintenance of the typically higher enzymic activities and tissue levels of ascorbic acid in intact males.

Summary. Hypophysectomy or castration of male rats results in a diminution of biosynthetic enzymic activities and tissue concentrations of ascorbic acid to levels characteristic of females. The data support the hypothesis that the sexual difference in tissue concentration of ascorbic acid in intact rats is dependent upon a difference in the rate of hepatic biosynthesis. The sexual difference in hepatic enzymic activities is interpreted to be dependent upon testicular secretion of androgens.

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Enteric Bacteria as a Possible Cause of Hemolytic Antibody-Forming Cells in Normal Mouse Spleens.* (32479)

V. CHENG AND J. J. TRENTIN

Division of Experimental Biology, Baylor University College of Medicine, Houston, Texas

By the hemolytic plaque assay of Jerne and Nordin(1), 19 S hemolysin producing cells can be individually detected and enumerated (2). With the addition of anti-mouse gamma globulin before addition of complement, the 7 S antibody producing cells can also be detected(3,4). This method can be adapted to study bacterial somatic polysaccharide(5) and other antigens that adhere to sheep red blood cells (SRBC). One perplexing and bothersome aspect of this technique is the variable small number of hemolysin producing "background" plaque-forming cells (B-PFC) against SRBC in the spleens of mice(2), rabbits(5), and humans(6) without prior SRBC injection. These 19 S hemolysin producing B-PFC are also present in small numbers in lymph nodes, thymus(7) and bone marrow(8) as well as following transplantation of isogenic lymphoid tissues to lethally irradiated hosts(9). These B-PFC can be considered as belonging to a clone of cells which produce specific antibodies without prior contact with the antigen according to the clonal selection theory(10). Alternatively, the animal may be responding to other antigens which produce cross-reacting antibodies to SRBC. Naturally occurring hemagglutinins in mouse sera for SRBC and chicken RBC have been described(11). These vary with the strain and sex of mice and seasonally(11,12,

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13). SRBC agglutinins were found in serum of over 90% of C57 mice(12). This strain is generally regarded as a good antibody producer. A lower incidence of SRBC agglutinins was found in C57 mice from a specific-pathogen-free (SPF) colony than from a conventional colony (J. H. Stimpfling, personal communication). Cross-reactivity of bacterial antigens with red blood cell antigens has been described(14,15). This investigation was therefore undertaken to study erythrocyte cross-reactivity by the Jerne plaque method, and the possible role of bacterial cross-reactivity as an explanation of the B-PFC against SRBC.

Materials and methods. Male and female C57 and male (C57 × A)F_1 mice (age 5-7 months) from our conventional mouse colony and specific pathogen-free mouse colony(16, 17) were used. Sheep, goat, and calf red blood cells in Alsever solution were washed 3 times with sodium barbital buffered saline before use.

Entire mouse spleens were minced by scissors in minimal Eagle's medium (MEM) and gently passed through a 50 mesh screen. The filtrate was repeatedly aspirated to break up clumps of cells and passed through a 200 mesh screen. Spleen cells from control and bacterial antigen injected mice were centrifuged at 200 *g* for 10 minutes, and resuspended in 0.3 to 1.0 ml of MEM; 0.1 ml of the suspension was used in each of 3 plates of both groups. Spleen cells from mice receiving red blood cell challenges were prepared as above but not centrifuged. Instead, a total cell count was performed and 1×10^6 spleen cells were plated out in each of 3 plates. The plating procedure was as follows: to 2 ml of 1.4% agar at 55°C were added, in sequence, 0.1 ml of diethylaminoethyl dextran (10 mg/ml), 1.8 ml of 2 X MEM, 0.1 ml red blood cells (4×10^8) and 0.1 ml of spleen cells, with mixing after each addition. The resulting mixture was immediately poured onto a 10 cm Petri dish with a solidified 1.4% agar base. If the mixture did not solidify within about 10 seconds, the procedure was repeated using only 1.6 ml of 2 X MEM, to give more rapid solidification before hemagglutination could occur. The plate was incubated for one hour at 37°C. Two ml of complement (1:15

TABLE I. Cross-Reactivity Between Antibody Producing Cells Against Sheep, Goat and Calf Red Blood Cells in Hemolytic Plaque Formation.

No. of mice	Sensitized to	Cells plated out	Plaque-forming cells on different types of red blood cells in agar plate* SRBC	GRBC	CRBC
3	SRBC†	1×10^6	392	344	28
3	GRBC‡	1×10^6	312	295	37
3	CRBC§	1×10^6	14	19	252

* Each value represents the mean of 2 plates.
† Sheep red blood cells.
‡ Goat red blood cells.
§ Calf red blood cells.

TABLE II. Background Plaque-Forming Cells in Conventional Colony and Specific Pathogen Free Colony Mice.

Source of mice	Mouse strain and sex	Age (months)	No. of mice	Mean PFC per spleen	Mice without PFC %	Mice with 10 or less PFC (including those without PFC) %	Mice with 10 or more PFC %
Conventional mouse colony	C57 ♂ & ♀	4-5	29	18.0 (range 0-285)	10.3	58.67	41.4
Specific pathogen free mouse colony	C57 ♂ & ♀	5-7	31	7.0 (range 0-25)	16.1	74.2	25.8
Specifice pathogen free mouse colony	(C57 × A) F_1 ♂	5	15	4.0 (range 0-29)	33.3	80.0	20.0

guinea pig serum) was added and the plate further incubated for 30 minutes. The plate was cooled to room temperature and a benzidine stain(2) added for 2 to 3 minutes to facilitate plaque counting, done over a bright light so that nonspecific clear areas created by air bubbles, solidified agar, pieces of tissues and debris could be easily identified and eliminated. Questionable plaques were always verified by microscope for the identification of the center cell(2).

Gastrointestinal content of each of 3 mice from the conventional colony was aseptically removed into thioglycolate broth. After plating, *Escherichia coli* was isolated from each of the 3 mice and *Aerobacter aerogenes* from 2 of the mice. Each of the 5 isolates was grown in 20 ml tubes of proteose peptone broth, washed three times in sterile normal saline, heat killed at 60°C for one hour, centrifuged at 500 *g* for 15 minutes, and resuspended in 2 to 3 ml of sterile normal saline. The concentration of bacteria was estimated by nephelometer. A uniform concentration of bacteria was suspended in 0.5 ml of sterile normal saline and injected into the test mice intraperitoneally or through a lateral tail vein intravenously. Spleens were harvested 4 days later.

Results. Groups of 3 mice were injected with sheep, goat, or calf red blood cells (4×10^8 cells). Four days later, spleen cell suspensions were made and an aliquot plated against each of the 3 types of red blood cells. Much cross-reactivity was found between sheep and goat red blood cells and less with calf red blood cells. (Table I).

More B-PFC were found in the conventional colony mice than in the SPF colony mice (Table II). Each of the 5 bacterial vaccines was found to increase significantly the number of B-PFC in the spleens of SPF mice to a level much higher than that of untreated mice of either the SPF or conventional colony (Table III).

Discussion. The data of Table I indicate that the hemolytic plaque assay can readily detect erythrocyte cross-reactivity. The data of Table II indicate that a higher incidence of B-PFC exists in a higher percentage of our conventional mice than of our SPF mice. The data of Table III indicate that the occurrence of B-PFC may be related to cross-antigenicity between SRBC and enteric bacteria. It has been reported that an unusually high B-PFC

TABLE III. Increased Background Plaque-Forming Cells in Mouse Spleen, Following Immunization with Enteric Bacterial Vaccines.*

No. of mice	SRBC	Bacterial vaccine	Route of injection	Mean PFC per spleen on day 4 after bacterial vaccine
31	none	none		7.0 (range 0-25)
2	"	*E. coli* 7×10^8 (S1)†	IV	467.5
1	"	*E. coli* 7×10^8 (S1)	IP	35.0
2	"	*E. coli* 6×10^8 (S2)	IV	1484.0
1	"	*E. coli* 6×10^8 (S2)	IP	329.0
3	"	*E. coli* 7.5×10^8 (S3)	IV	568.3
1	"	*A. aerogenes* 6×10^8 (S1)	IV	1351.0
2	"	*A. aerogenes* 6×10^8 (S1)	IP	265.0
3	"	*A. aerogenes* 4.5×10^8 (S3)	IV	134.0

* All mice were C57 strain of both sexes from the SPF colony.
† S1, S2, S3 bacteria were from 3 different mice.

level exists in the appendices of rabbits(5) and that germ free piglets have no B-PFC (18). Passively administered specific antibody can inhibit further 19 S antibody synthesis induced by the same antigen(19), but Wigzell (7) was unable to eliminate B-PFC with passively administered anti-SRBC serum.

B-PFC are present in most mice at all age levels except at birth. The 90% incidence of B-PFC positive C57 mice from our conventional colony closely approximates the percentage of conventional C57 mice previously reported to have "naturally occurring" serum hemagglutinin against SRBC(11,12). Springer *et al* found that *E. coli* contains human blood group substance B. In most strains of chicken, there is a natural level of anti-B blood group antibodies. On the other hand, germ free leghorn chickens do not have these antibodies. However, when germ free chickens were fed *E. coli* O_{86} they developed the antibodies(14). When *E. coli* O_{86} was fed to human infants with diarrhea, their serum anti-B isoagglutinin titer rose. Springer further showed that 137 out of 282 strains of enteric gram negative bacteria have human A, B, or H(O) antigenic specificity(15). Humans have a constant level of isohemagglutinins. It is interesting to note that humans also have a low B-PFC against SRBC(6).

The data presented definitely suggest that B-PFC against SRBC are a product of bacterial cross-reactivity. The so-called "primary" response to SRBC may therefore represent varying degrees of primary and cross-reactive secondary response. Caution is therefore indicated in the interpretation of the early appearance by histological identification of pyroninophilic cells in spleens of mice within 24 hours after SRBC challenge(20) and of the early (18 hours) recovery of plaque-forming cells in spleens of rabbits injected with somatic polysaccharide of *S. enteritidis* (5).

Summary. 1. The Jerne hemolytic plaque assay method readily detects antigenic cross-reactivity between sheep, goat, and calf erythrocytes. 2. Naturally occurring plaque-forming cells against sheep erythrocytes are more frequent in spleens of conventional colony mice than of specific-pathogen-free mice of the same genotype. 3. Vaccines prepared from each of 5 isolates of *E. coli* and *A. aerogenes* from the gastrointestinal tracts of conventional colony mice significantly increased the number of sheep erythrocyte hemolytic plaque-forming cells in the spleens of SPF mice. 4. It is postulated that the naturally occurring hemolytic antibody-forming cells found in the lymphoid tissues are probably caused by cross-reactivity between erythrocyte and bacterial antigens.

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