The results of the study of the effects of temperature during the determinations indicated that the avidin-biotin complex is relativley stable with respect to heat. Further investigations of the stability of avidin-biotin complex at higher temperatures will be reported from this laboratory(3).

Summary. A method for determination of avidin by use of radioactive biotin and a gel-filtration technique has been developed. The method is based on measurement of the radioactivity of the avidin-biotin complex which separates from free biotin when a reaction solution of avidin and excess radioactive biotin is applied to a Sephadex G-25 column.

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Influence of Autoimmune Hemolytic Anemia on Susceptibility to *Salmonella* Infection.* (29486)

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Previous studies(1,2) have shown that intramuscular injection of phenylhydrazine hydrochloride or intravenous administration of anti-mouse-erythrocyte serum, heterologous erythrocytes or antibody-coated homologous erythrocytes markedly increases the susceptibility of mice to infection caused by *Salmonella typhimurium, Escherichia coli* or *Staphylococcus aureus*. The hypothesis has been advanced that phagocytosis of heterologous or altered homologous erythrocytes by reticuloendothelial cells impairs the capacity of these cells to kill ingested bacteria.

The present studies were undertaken to investigate the effect of naturally-occurring autoimmune hemolytic anemia on susceptibility to *Salmonella* infection utilizing the NZB/Bl strain of mice. This strain was developed by Bielschowsky by successive brother-sister matings(3) and has been studied by Holmes and Burnet(4). The NZB/Bl mice begin to develop autoimmune hemolytic anemia after 13 weeks of age.

Methods. Four female and 2 male litter mates of the 48th generation of the NZB/Bl strain were generously supplied by Sir Macfarlane Burnet and Dr. Margaret C. Holmes in March, 1962. By successive brother-sister matings a colony was developed. The studies to be reported were performed on mice of the 49th through 53rd generations (continuing Bielschowsky's numbering system).

Hematocrit values were determined by a micro-hematocrit method(1) in heparinized capillary tubes, 75 mm in length and 1.2 to 1.4 mm in diameter. Approximately 0.03 ml of blood was collected from the retro-orbital venous plexus and centrifuged for 3 minutes at 12,500 rpm in a microhematocrit centrifuge (Clay-Adams, Inc.). Hematocrit values were read directly from the tubes using a micro-capillary reader (International Equipment Co.).

Sera used in performing Coombs tests were prepared in rabbits. Two parts of pooled mouse serum from male Swiss mice of the

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CFl strain (Carworth Farms) were emulsified with 1 part of complete Freund's adjuvant (Difco Laboratories). One ml of the emulsion was injected subcutaneously and 0.1 ml intradermally into rabbits at weekly intervals for 4 weeks. Seven days after the last injection the rabbits were bled and the serum heated at 60°C for 5 minutes. The titer of precipitating antibody was determined in capillary tubes by drawing up aliquots of rabbit serum serially diluted in saline and adding equal volumes of undiluted mouse serum. After incubation at 37°C for 2 hours and standing at 4°C for an additional 18 hours the titer of precipitating antibody was 1/8.

The Coombs test was performed on erythrocytes from 0.02 ml blood collected from the retroorbital venous plexus; prior to use erythrocytes were washed twice in 5 ml of isotonic saline solution. Four-fold dilutions of Coombs serum were made in saline solution. One drop of each dilution was mixed with one drop of a 2% suspension of erythrocytes in saline on a glass slide. The suspensions were examined macroscopically for agglutination after 5 minutes of constant rotation of the slide. Controls were erythrocytes from NZB/Bl mice that were 4-6 weeks of age (prior to development of positive Coombs tests) and erythrocytes from male Swiss mice of the CFl strain. Erythrocytes from both groups of control mice were frequently agglutinated at a 1/16 dilution of Coombs serum but never at a 1/64 dilution. Hemagglutination at a 1/64 or greater dilution of serum was considered a positive test.

The *Salmonella typhimurium* strain KK (1) was used in all experiments. Stock cultures were maintained by storing aliquots of an 18-hour culture in trypticase soy broth at —20°C. Inocula for infecting mice were prepared by subculturing an aliquot of the stock culture in trypticase soy broth. After incubation at 37°C for 18 to 22 hours, the bacteria were washed 3 times in pyrogen-free saline solution and then diluted in saline for injection. The dilutions of bacterial suspensions were inoculated intravenously in a volume of 0.1 ml or intraperitoneally in a volume of 0.5 ml.

TABLE I. Results of Coombs Tests in NZB/Bl Mice.

Age (wk)	Positive Coombs tests			
	Males		Females	
		(%)		(%)
13 or less	0/42		0/41	
14-18	5/27	(19)	25/94	(27)
19-23	13/63	(21)	36/100	(36)
24-28	25/50	(50)	66/101	(65)
29-38	14/17	(82)	44/47	(94)
42-45	17/17	(100)	18/19	(95)
50-52	7/7	(100)	8/8	(100)

Induction of the Coombs-positive state in Coombs-negative mice was achieved by a modification of the method of Holmes, Gorrie and Burnet(5) in which nucleated cells from Coombs-positive donors are transferred to Coombs-negative recipients. The spleen, thymus and axillary, inguinal, mesenteric and cervical lymph nodes from individual mice were pooled and forced through wire mesh. The cells were then washed 3 times in heparinized Hanks' solution, and suspended in Hanks' solution to a final concentration of 1×10^8 nucleated cells per ml. Each suspension of nucleated cells prepared from an individual mouse was used to inject 3 mice.

Results and discussion. The results of 633 Coombs tests performed on 551 mice are shown in Table I. The Coombs test was uniformly negative in mice 13 weeks or younger. However, the incidence of positive Coombs tests gradually increased from 14 to 42 weeks of age until virtually all mice had a positive Coombs test. Table II lists hematocrit values for NZB/Bl mice with positive and negative Coombs tests. These results are similar to those reported by Holmes and Burnet(4).

Mice that were 26 to 30 weeks old with positive Coombs tests were more susceptible to infection with *S. typhimurium* than mice that were 6 to 8 weeks with negative Coombs tests. As shown in Fig. 1, after intraperitoneal inoculation with 7×10^4 *S. typhimurium,* mice with positive Coombs tests died at a faster rate and with a higher final mortality ($p<.01$) than the younger mice.

In another experiment mice with positive Coombs tests were compared with mice of the same age with negative Coombs tests. A

group of mice 15-29 weeks old with positive Coombs tests and a group matched for age with negative Coombs tests were challenged with 1.6×10^5 *S. typhimurium* by the intraperitoneal route. As shown in Fig. 2, the mice with positive Coombs tests died at a more

TABLE II. Hematocrit Values in NZB/Bl Mice.

	Negative Coombs tests			Positive Coombs tests		
Age (wk)	Number	Mean hematocrit	Hematocrit range	Number	Mean hematocrit	Hematocrit range
		(%)	(%)		(%)	(%)
13 or less	32	47.2	40–50			
14–19	28	46.7	43–57	19	47.6	43–52
23–28	8	44.7	40–50	28	43.3	35–51
29–34				7	43.1	41–46
43–45				28	36.0	22–46
50–52				13	33.9	21–39

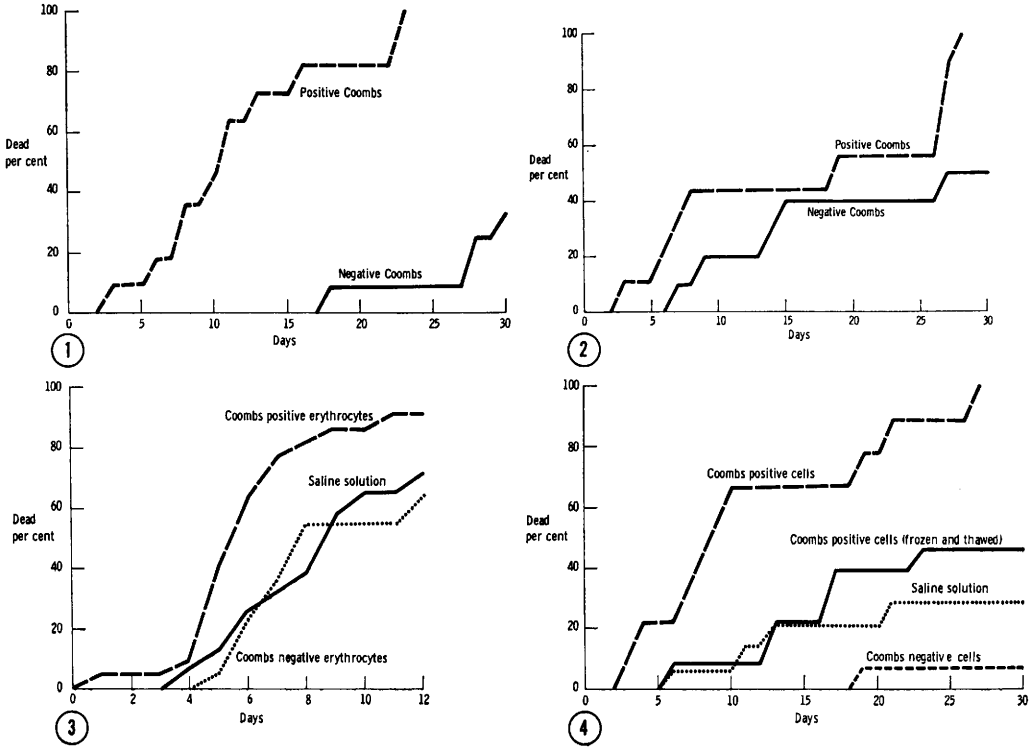

FIG. 1. Cumulative mortality after intraperitoneal inoculation of 7×10^4 *Salmonella typhimurium* in 26 to 30 week old mice with positive Coombs tests (11 mice) and 6 to 8 week old mice with negative Coombs tests (12 mice).

FIG. 2. Cumulative mortality after intraperitoneal inoculation of 1.6×10^5 *Salmonella typhimurium* in 15 to 29 week old mice with positive Coombs tests (9 mice) or negative Coombs tests (10 mice).

FIG. 3. Cumulative mortality after intraperitoneal inoculation of 2×10^6 *Salmonella typhimurium* in 5 to 13 week old mice 1 hour after intravenous injection of 0.5 ml of Coombs positive erythrocytes (22 mice), 0.5 ml of Coombs negative erythrocytes (22 mice) or 0.5 ml of saline solution (31 mice).

FIG. 4. Cumulative mortality after intravenous inoculation of 4×10^5 *S. typhimurium* in mice with positive Coombs tests transferred by previous intraperitoneal injection of nucleated cells from Coombs positive mice (9 mice) or in mice with negative Coombs tests previously injected with saline (14 mice), nucleated cells from Coombs negative mice (14 mice), or frozen and thawed nucleated cells from Coombs positive mice (13 mice).

rapid rate and with a higher final mortality ($p<.05$) than the mice with negative Coombs tests.

Mice with negative Coombs tests transfused with Coombs positive erythrocytes were more susceptible to *Salmonella* infection than Coombs negative mice transfused with Coombs negative erythrocytes. Erythrocytes were prepared for transfusion by removing the plasma, washing 3 times in saline solution and making a 50% suspension in saline. On microscopy, erythrocytes from Coombs positive mice were not clumped. Groups of mice 5-13 weeks old with negative Coombs tests were inoculated intravenously with 0.5 ml of saline solution or 0.5 ml of a 50% suspension in saline solution of erythrocytes from Coombs positive or Coombs negative mice. One hour later 2×10^6 *S. typhimurium* were injected intraperitoneally. The mice that had been transfused with Coombs positive erythrocytes died at a more rapid rate than the mice transfused with Coombs negative erythrocytes or infused with saline. As shown in Fig. 3, 77% of mice transfused with Coombs positive erythrocytes had died by the seventh day after challenge with *Salmonella*; in contrast only 36% of the mice transfused with Coombs negative erythrocytes were dead at this time ($p<.01$). There were no deaths in mice that were transfused but not infected.

Four groups of Coombs negative mice 4-8 weeks old were injected intraperitoneally with 1 ml of (a) saline solution or 1 ml of saline solution containing 1×10^8 nucleated splenic, thymic and lymph node cells from (b) Coombs negative mice, from (c) Coombs positive mice, or from (d) Coombs positive mice after freezing and thawing the cells twice. Two weeks after these injections, Coombs tests were positive in 40% of mice in (c) but were negative in mice in (a), (b) and (d). Coombs positive mice in (c) and the mice in (a), (b) and (d) were challenged with 4×10^5 *S. typhimurium* by the intravenous route. As shown in Fig. 4, the Coombs positive mice died at a much faster rate after challenge with *Salmonella* than the mice in the other 3 groups ($p<.02$). There were no deaths in Coombs positive mice in (c) that were not infected.

These studies demonstrate that mice with naturally occurring autoimmune hemolytic anemia are more susceptible to infection with *S. typhimurium* than mice of the same strain with negative Coombs tests. Furthermore, susceptibility to *Salmonella* infection was increased after transfusion of Coombs positive erythrocytes or following induction of the autoimmune state by transfer of nucleated cells.

Summary. Mice of the NZB/Bl strain with naturally occurring autoimmune hemolytic anemia are more susceptible to infection with *S. typhimurium* than NZB/Bl mice without autoimmune hemolysis. The increase in susceptibility can be demonstrated by comparing the response of the following groups of mice to challenge with *Salmonella*: 1. Old mice with positive Coombs tests compared with young mice with negative Coombs tests. 2. Mice with positive Coombs tests compared with mice of the same age with negative Coombs tests. 3. Young mice with negative Coombs tests transfused with erythrocytes from Coombs positive mice compared with young mice with negative Coombs tests transfused with Coombs negative erythrocytes. 4. Young mice with positive Coombs tests resulting from transfer of nucleated cells from Coombs positive donors compared with young mice with negative Coombs tests following transfer of nucleated cells from Coombs negative donors or following transfer of frozen and thawed cells from Coombs positive donors.

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