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THE EFFECT OF SPLENECTOMY ON ISOHEMAGGLUTININ PRODUCTION
IN THE RABBIT

by

Thomas Lau

A Thesis

Presented to the Faculty of the School of Medicine of Yale University

In Candidacy for the Degree of

Doctor of Medicine

Department of Surgery

1960



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My deep and heartfelt thanks to my advisor, Dr. Mark Hayes,
and all the many others who helped me.

A great deal of research concerning the site of antibody formation has been carried out.¹ Some studies implicate the lymphoidal tissue and lymphocyte,^{2,3} others the plasma cell,⁴ still others the splenic cells that later develop into plasma cells.⁵ However, it is not likely that the capacity to produce antibodies is localized to a single type of cell, but rather is possessed by cells that are widely scattered throughout the body,⁶ and whose response is determined by the nature, quantity and route of administration of the antigen.^{7,8}

Despite the fact that the clinical reports of increased susceptibility to infections in splenectomized patients are inconclusive and conflicting,^{9,10,11,12} the evidence that the spleen is an important site of antibody formation is great,¹³⁻²⁵ and in 1950, D.A. Rowley²⁶ showed that a splenectomized man responds with very little circulating antibody to particulate erythrocytic antigen (1.0 ml. of a 2.0 percent suspension of sheep red cells) introduced intravenously. Since the hemolytic manifestations of erythroblastosis foetalis have been shown²⁷ to be due to transfer across the placenta of maternal isoantibodies that are specific for certain antigens, notably those of the Rh system, inquiries as to the role of the spleen in the production of circulating isoantibodies seemed practical.

Erythroblastosis foetalis occurs naturally in man, horses,²⁸ and donkeys,²⁹ while the hemolytic aspects of the disease have been produced experimentally in dogs³⁰ and pigs.³¹ Furthermore, doe rabbits injected first subcutaneously with incompatible rabbit red cells incorporated in Freund's adjuvant³² and then intravenously with the same saline suspended red cells develop significant isoantibody titers. If these does then mate with bucks bearing the appropriate incompatible iso-agglutigen, young will be born not only with the hematologic disturbance, but also with the main histologic features of the condition.³³ Now, if the spleen were a determining factor in the production of circulating isoantibody in this disease, then a splenectomized rabbit should produce little or none of it. In that event splenectomy might be effective therapy for some Rh negative mothers who had delivered erythroblastotic babies and desired a normal child.²⁶

However, the difficulty in producing adequate iso-hemagglutinins in the rabbit is well known and almost all workers employ non-specific antigen adjuvants such as B.C.G. or Freund's preparation of heat killed tubercle bacilli to obtain faster and higher titers.³⁴⁻³⁶

We now know that the large granulomata developed at the injection sites of the antigen-adjuvant emulsions contribute a significant part of the total resulting antibody.^{6,7} Since I planned to evaluate splenic function, these interfering

granulomatous antibody sources were unacceptable, and I chose to follow the evidence³⁶ suggesting that isohemagglutination titers run on sera drawn three times a week during and after the immunization program would demonstrate significant differences between the splenectomized and the control rabbit groups.

MATERIALS AND METHODS

Rabbits: adult male and female Cottontail, New Zealand, and mixed breed rabbits were purchased from three different dealers in and around New Haven, housed two in a cage, and fed Purina chow and water ad lib.

Blood Typing Antisera: generously supplied by Dr. J.J. Griffitts³⁷ and identified as anti-G and anti-g (following Kellner's symbols).^{38,39}

This serum, which was obtained from one of the twelve rabbits given repeated intravenous injections of red blood cells from rabbits of a presumably different strain, was found by Griffitts to contain an antibody that agglutinated the suspended red cells of some rabbits and not those of others, and is well accepted in this field.

Isotonic Alsever's Solution (Modified):

glucose	2.05 grams
sodium citrate	0.80 grams
sodium chloride	0.42 grams
distilled water	
q.s. ad.	100 ccs.
adjust to pH 6.1 with 5 per cent citric acid	

Modified Veronal Buffer Solution:

sodium chloride	83.8 grams
sodium bicarbonate	2.52 grams
sodium barbital	3.00 grams
acid barbital	4.60 grams
magnesium chloride	
solution	0.2 molar 25 ccs.

dissolve acid in 500 milliliters of hot distilled water, add the other components to this solution, cool to room temperature and dilute to ten liters for use.

In order to avoid the spontaneous agglutination which occurs about half the time when rabbit red cells are suspended in saline,³⁴⁻⁴⁰ this solution was modified to contain 5 per cent of normal serum from a Gg rabbit when finally used.

Blood Typing of Rabbits: Whole blood was drawn from a central ear vein and mixed with an equal volume of Alsever's citrate-saline solution. One drop of this erythrocytic-citrate suspension was placed on each of two clean glass slides, to one slide a single drop of anti-G typing serum was added and to the other slide one drop of anti-g serum was added. Patterns of agglutination under a hand lens were recorded immediately as zero to four plus.

Iso-Immunization: A single type GG animal (animal #110) supplied all the red cells used for the immunizations and titrations of iso-antibodies. Its blood was collected by cardiac puncture into an equal volume of Alsever's solution, centrifuged at 2,000 r.p.m. for five minutes and washed three times in the veronal buffer. After drawing blood by cardiac puncture for later antibody titers, one cubic centimeter of a 50 per cent suspension of these thrice washed red cells in buffer was injected into the central ear vein of each of the ten type gg experimental animals. This dose was repeated three times a week for three weeks.

Splenectomy: Five type gg rabbits were splenectomized under intravenous barbituate anesthesia (pentobarbital 15 mgms. per pound of body weight). A scissor cut was made through all the layers of the abdominal wall, including the peritoneum, to form a left pararectus incision of moderate length. The spleen was pulled through the wound, the gastrosplenic omentum was

doubly ligated, divided, and the spleen delivered. The abdomen was closed with interrupted catgut and the rabbits given intramuscular penicillin and streptomycin. Laparotomies (sham splenectomies) were carried out on the five control animals.

Titration of Isoantibodies by Test Tube Agglutination: 0.5 ml. of the modified veronal buffer was pipetted into all the test tubes used in a given titration. Then doubling dilutions of the unknown serum were prepared in the following way: 0.5 ml. of the undiluted serum to be tested was added to the first tube, the contents were mixed, and 0.5 ml. of this mixture was withdrawn and added to the second tube; after mixing, 0.5 ml. of this was added to the third tube, etc. The extra 0.5 ml. from the tube containing the highest dilution was discarded. The entire length of the pipette used was rinsed in buffer between each step. Then 0.2 ml. of a 5 per cent suspension of type GG erythrocytes (from animal #110) in the veronal buffer was added to all the tubes. The reagents were mixed, the tubes randomized and then incubated for one hour at 37° Centigrade. Appropriate negative (buffer rather than unknown serum) and positive (anti-GG typing serum rather than unknown serum) were run with each set of titers. The hemagglutination patterns were recorded as one to four plus by a reader who had no clue to the order of the tubes he was describing.

RESULTS

Results of Blood Typing

Table One

Red Cell Typing of 77 Rabbits with Anti-G and Anti-g Sera

Reaction of Red Cells and Antiserum		Number of Rabbits	Per Cent of Total	Possible Genotype
anti-G	anti-g			
positive	negative	3	3.9	GG
positive	positive	62	80.5	Gg
negative	positive	12	15.5	gg
negative	negative	0	0	--

Table Two

Frequency of Rabbit Blood Groups Reported by Previous Authors

Castle, 1933 ⁴¹		Fischer, 1935 ⁴²		Kellner, 1953 ³⁹		Anderson, 1955 ⁴³	
Number of Rabbits Typed		Number of Rabbits Typed		Number of Rabbits Typed		Number of Rabbits Typed	
788		100		215		87	
Blood Group	Incidence (Per Cent)	Blood Group	Incidence (Per Cent)	Blood Group	Incidence (Per Cent)	Blood Group	Incidence (Per Cent)
H ₁	28	K ₁	32	g	17.7	A	19.3
H ₂	44	K ₂	19	G	25.5	B	52
H ₁ H ₂	21	K ₁ K ₂	48	gG	57.7	AB	23
0	7	0	1	0	0	0	5.7

Results of SplenectomyTable Three

Animal Number	Body Weight (grams)	Splenic Weight Immediately Postoperatively (grams)	Per Cent of Body Weight
180	1600	0.750	0.046
148	2500	1.630	0.065
156	1900	1.150	0.079
163	3000	1.080	0.036
71	3000	1.450	0.048

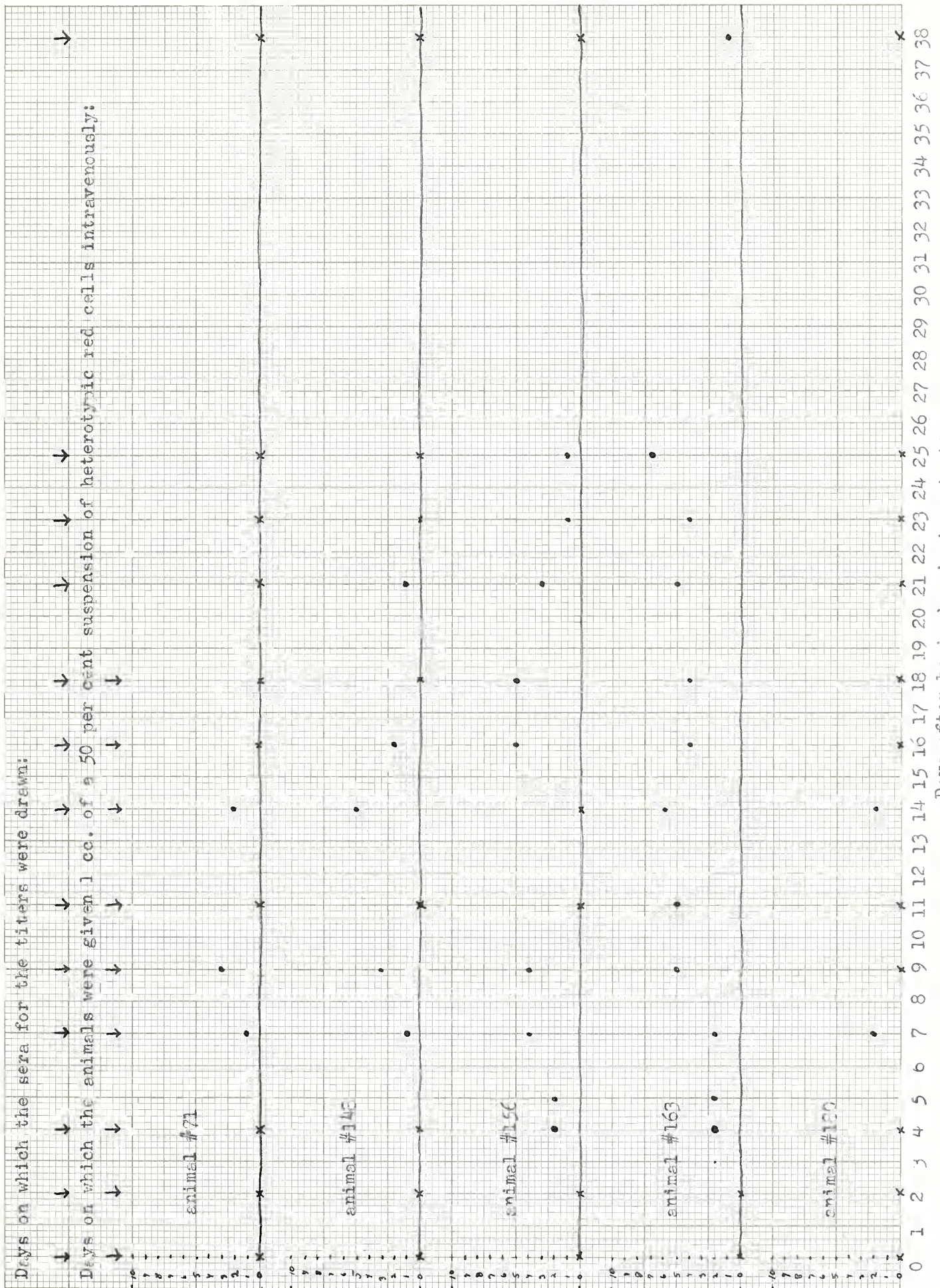
Results of Iso-Immunization

See accompanying figures

The titers for the splenectomized animals were compared with those of the control animals by the use of the Wilcoxon Rank Sum Test.⁴⁴ The differences observed between the two groups were no greater than can be satisfactorily explained by chance fluctuation.

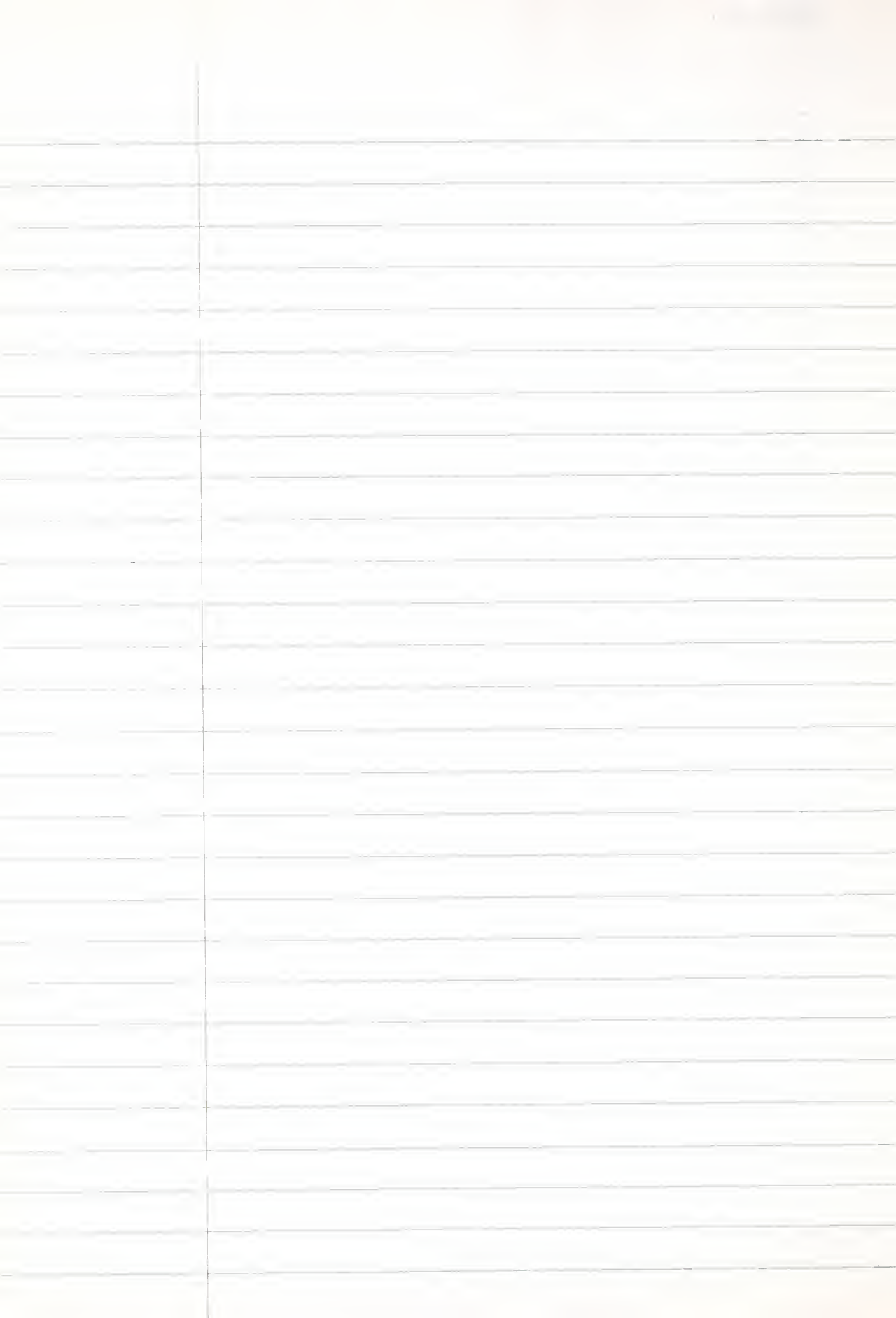
FIGURE ONE: ISOHEMAGGLUTINATION TITERS IN THE SPLENECTOMIZED ANIMALS

5780-20
20 SQUARES TO THE INCH



Reciprocal of greatest dilution giving 'plus two' agglutination expressed as a logarithm to the base two

Days after beginning isoimmunization



DISCUSSION

The distribution of blood types in my study differs significantly from those of previous workers (please refer to Tables One and Two), although my typing sera was that used by Kellner and Hedal.³⁹ Because I sought homozygous subjects, I blood typed my animals with a simple slide agglutination technique in the field before purchasing them. The other workers used an incubated test-tube agglutination coupled with either titers of trypsin-treated red cells (Anderson)³⁶ or direct Coombs testing (Kellner and Hedal).³⁹ Their more sensitive methods may account for some of this variation. However, there is a wide difference in the degree of agglutinability of g-positive cells. Some regularly give strong (four plus) reactions, while others give weak (one plus) reactions, even though the same agglutinating anti-g serum is employed. Such differences appear to be genetically determined³⁹ and are well known in the A₁-A₂ and the D-Du blood groups in man.⁴⁵ Since it is also known that g and G are the same as A and B, it follows that the figures of Anderson⁴³ and those of Kellner³⁹ (viz. Table Two) cannot both represent the true incidence of these factors in all rabbits, and it seems likely that different strains will show large variation in blood group incidence.

The immunization schedule followed in this research (see accompanying figures) was more successful in stimulating anti-erythrocytic iso-antibodies of high titer than those reported in earlier papers.³⁴⁻³⁶ Anderson, using as many as thirty-two 1 ml.

intravenous injections of a 50 per cent red cell suspension had only 15 per cent of his series show titers greater than 1 to 64³⁶, and Wright used six to ten 1 ml. doses of a 10 per cent suspension, but caused only 15 per cent of his animals to show similarly high titers.³⁴ Four of my ten rabbits had titers greater than 1 to 64; however, there was no significant difference in response between the control and the splenectomized subjects.

The explanation for this immune response despite splenectomy is most reasonably a complex one and our considerations should include the following:

First: removal of the spleen is probably equivalent to the removal of a large number of the cells of the reticulo-endothelial system and the effect would relate to both the size of the animals total reticulo-endothelial system and the size and composition of its spleen. In the rabbit, the spleen is a small organ (see Table Three), and there is much accessory splenic and hemolymph tissue. Perhaps splenic resection in the rabbit should not be expected to work major changes in antibody response, particularly to a weak antigen like the heterotypic homologous red cell. An adult human spleen averages 0.23 per cent of the body weight (Krumbhaar)⁴⁶ three to six times that of a rabbit, a fact that emphasizes the dangers of closely relating human and animal studies.

Second: this was a quantitative study of the isohemagglutinin formation, and we can speculate that splenectomy might induce as yet immeasurable qualitative changes in the gamma

globulins.⁴⁷

Third: there may, in fact, be a depressed response in the splenectomized animals that was not detected in my small study. Statistical machinations reveal that significance would be reached if the sample were doubled, all other factors remaining equal.

SUMMARY

1. Blood typing seventy-seven rabbits revealed notable differences in type frequency from other series. This variation was felt to be compatible with the genetic variations between strains.

2. Splenectomized and control animals were given repeated intravenous injections of heterotypic homologous rabbit red cells. Frequent isohemagglutination titers showed that the isoantibody response of the splenectomized animals was equal to that of the controls. This immune response despite splenectomy was thought due to the small size of the rabbit spleen and the weakness of the rabbit erythrocyte as an isohemagglutinin.

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