Immunologic Studies in Thermal Injury: Hemagglutinating Factor in the Lymph of Burned Rats.* (31641)

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Several reports indicate the development of a state of altered immunologic responsiveness in burned patients and animals, including loss of ability speedily to reject skin homografts(1-3) and depletion of tuberculin-type hypersensitivity(4). The recent observations of McCarthy and associates(5-7) on autosensitization to erythrocytes in burned rats, as determined by direct antiglobulin tests, are of particular interest to this study.

The present report describes a hemagglutinating factor specific for rat erythrocytes that appears in thoracic duct lymph of inbred rats of the Fisher strain in response to the application of thermal injury. This hemagglutinin was absent in the serum of such burned animals.

Materials and methods. Male adult rats of the inbred Fisher strain, weighing 210 to 260 g, were used throughout this study.

Method of burning. The hair was removed by an electric clipper on the day before burning. The technique of burning has been described(3,8). Briefly, it consisted of applying a thermally regulated metal surface maintained at 250°C to 15 to 17% of the body surface area of the rat under ether anesthesia; the remainder of the animal was shielded with an asbestos frame. Electric metronome control of the duration of exposure and control of the pressure applied provided a standard, full-thickness burn of the exposed skin area.

Thoracic duct lymph. Lymph specimens were obtained from 3 groups of animals, each consisting of 14 Fisher rats subjected to thermal injury under identical experimental conditions. Prior to this operation, a polyethylene tube (Intramedic No. PE-50, Clay Adams Co., New York) was inserted into the lower *vena cava* through the femoral vein. The animals were perfused for 18 to 24 hours with 0.15 M saline containing 5 μ of heparin/ml. A syringe pump (Model 255-2, SAGE Instr., Inc., White Plains, N.Y.) was employed, which delivered 1 ml of fluid/hr. Five mg of phytonadine (Aqua-Mephyton, Merck Sharp & Dohme, West Point, Pa.) was given to each animal during the perfusion period.

The thoracic duct was cannulated following the procedure of Bollman *et al*(9) as modified by Gowans(10). The animals were operated on under pentobarbital and ether anesthesia. After a polyethylene tube was inserted into the thoracic duct, the animals were placed in a Bollman cage (Nuclear Supply Co., Washington, D.C.) and warmed with electric pads. Lymph was collected continuously for 24 hours. During this period, the animals drank 100 to 150 ml of saline containing 2 g of sucrose.

Each lymph specimen was centrifuged at 16,000 $\times$ *g* at 4°C for 1½ hours; the clear supernatant was recovered and preserved at —20°C. Before testing, the lymph was concentrated 10 times by pervaporation, and dialyzed against 0.15 M saline at 4°C for 16 hours.

Sera. Fifteen Fisher rats were exsanguinated by cardiac puncture at different times after thermal injury. The sera were separated and tested for hemagglutination.

Rabbit antiserum to pooled rat serum and goat antiserum to rat γ globulin were obtained from Hyland Laboratories, Los Angeles, Calif. The antiglobulin serum was repeatedly absorbed with normal rat erythrocytes before use. Rat antiserum to human erythrocytes was prepared by immu-

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nization of 6 rats. Each animal received 12 intraperitoneal injections of 0.5 ml of washed, packed human blood group O erythrocytes. The injections were given at 2- to 3-day intervals, with a total immunization period of 4 weeks. One week after the last injection, the animals were exsanguinated and the sera were pooled.

Rabbit antiserum to the hemagglutinating factor present in the thoracic duct lymph of burned rats was obtained in the following manner. Washed and packed erythrocytes of Fisher strain rats were mixed with an equal volume of concentrated lymph from burned Fisher rats, and the mixture was incubated for 1 hour at room temperature. Thereafter, the erythrocytes were washed 4 times and resuspended to a 10% concentration in phosphate-buffered saline, pH 7.2 (PBS). A rabbit received 9 intravenous injections of this suspension at 2- to 3-day intervals. Ten ml of the suspension were administered each time; the total immunization period lasted for 3 weeks. One week after the last injection, the animal was exsanguinated and the serum was separated. Control sera were obtained from 2 rabbits immunized in a similar way with Fisher erythrocytes incubated with the lymph of normal Fisher rats.

All sera were stored at —20°C and were inactivated at 56°C for 30 minutes before use.

Erythrocytes. Erythrocytes were separated from blood drawn in ACD solution, and were preserved at 4°C for not longer than one week. Rat blood was obtained in the laboratory, and human blood originated from normal blood donors. Blood from other species was purchased from the Animal Blood Bank, Syracuse, N. Y. Before being used, the erythrocytes were washed 3 times with PBS.

Hemagglutination test. Specimens tested for hemagglutinins (concentrated lymph, sera and eluates) were serially diluted in 0.1 ml volumes; to each dilution was added 0.1 ml of a 1% erythrocyte suspension. The tubes were kept at room temperature for one hour and were then centrifuged for 1 minute at 1000 rpm. The agglutination was estimated after gentle shaking of the test tubes.

Antiglobulin tests. The direct Coombs test was performed with erythrocytes obtained from burned Fisher rats. Erythrocytes were washed with PBS, and prepared as a 1% suspension. Serial dilutions of goat antiserum to rat γ globulin were prepared in 0.1 ml volumes and mixed with 0.1 ml of the erythrocyte suspension.

The indirect Coombs test was used to test for the presence of "incomplete" antibodies in lymph specimens obtained from burned rats. This was done by using a titration row from the hemagglutination test. Erythrocytes in each tube were washed 3 times with PBS. To the sediment of erythrocytes after the last washing was added 0.1 ml of goat anti-rat γ globulin serum at a dilution of 1:30. The indirect Coombs test was also used for detection of antibodies to the rat hemagglutinating factor in the serum of the rabbit injected with Fisher erythrocytes incubated with thoracic duct lymph. Serial dilutions of this serum absorbed with human O erythrocytes were prepared in 0.1 ml volumes. To each tube was added 0.1 ml of a 1% suspension of human O erythrocytes sensitized with a subagglutinating dose of rat antiserum to human erythrocytes.

Antiglobulin tests were read after one hour of incubation at room temperature, followed by centrifugation at 1000 rpm for 1 minute.

Absorption. Burn lymph specimens showing high titers of rat cell hemagglutinin were selected for absorption experiments. Rat erythrocyte stromata were obtained by hemolyzing erythrocytes with distilled water; the stromata were washed at least 10 times with distilled water and preserved in the lyophilized state. Before being used, the lyophilized stromata were washed 3 times with PBS and packed. For absorption, 1 ml of concentrated lymph was mixed with 0.5 ml of packed stromata sediment. The mixture was shaken on an electric shaker for 30 minutes and incubated for another 30 minutes at room temperature. The specimens were then centrifuged at 6,950 $\times$ g for 10 minutes. The supernates were absorbed once more in a similar fashion.

Elution. The packed sediment of rat erythrocyte stromata obtained from the first absorption of burn lymph was washed 4 times

with PBS, in order to remove unbound proteins. At each washing, stromata were throroughly resuspended with a wooden applicator; after the last washing, the sediment of sensitized stromata was resuspended in 1 ml of PBS. The test tube with this suspension was incubated at 56°C for 10 minutes under constant shaking, and centrifuged at 6,950 × *g* for 5 minutes in a container filled with water heated to 56°C. The eluate prepared in this manner was removed and tested for hemagglutinating activity and antigenic properties. As controls, similar eluates were prepared from stromata incubated with lymph specimens obtained from normal rats.

Treatment with sulfhydryl reagent(11). Concentrated lymph samples were diluted 1:4 and incubated with equal volumes of 0.2 M 2-mercaptoethanol at 37°C for 1 hour. Such samples were then immediately tested by hemagglutination and indirect antiglobulin tests.

Double-diffusion gel precipitation test (Ouchterlony). This test was performed in Petri dishes, using 1.5% Difco Noble agar with 0.01% thimerosol. The wells were 3 mm apart; they were 3 mm in diameter and 3 mm deep.

Immunoelectrophoresis. The modification described by Scheidegger(12) was followed, using 1.5% agar in barbital buffer of pH 8.6 and ionic strength of 0.075.

Results. Table I illustrates the results obtained in 42 burned Fisher rats. The thoracic duct of these rats was cannulated at different time intervals after thermal injury. The times listed in the Table refer to the beginning of a 24-hour cannulation. Collection of lymph in the first 3 animals (170, 270, 296) was started immediately after burning. In this connection, these rats could not be perfused through the *vena cava* after the injury as the other rats were. To keep experimental conditions as consistent as possible, these rats were perfused for 18 hours preceding the thermal injury. The lymph obtained from each animal was tested against erythrocytes of the Fisher, Sprague-Dawley, Lewis, and Wistar rats.

Lymph specimens obtained immediately after injury gave weakly positive results.

TABLE I. Hemagglutinating Activity of Thoracic Duct Lymph of Fisher Rats After Thermal Injury.

Rat No.	Days after burning	Titer with erythrocytes of: Fisher	Sprague-Dawley	Lewis	Wistar
170		16	16	8	4
270	0	4	4	4	<4
296		8	<4	4	<4
162		8	4	<4	4
283	1	8	16	32	8
317		16	8	16	16
169		16	16	8	<4
201	2	32	256	64	32
318		64	32	32	8
149		256	256	256	256
163	3	16	32	8	8
167		64	128	128	32
166		128	32	16	4
168	4	512	128	64	32
194		512	1024	256	64
156		256	512	256	64
172	5	512	512	128	64
288		512	512	32	32
157		256	256	128	64
174	7	512	128	128	32
264		512	512	32	32
160		512	512	64	128
178	14	256	128	128	32
272		128	32	32	64
146		512	128	128	8
165	21	256	128	64	32
185		256	256	256	16
180		128	32	16	16
206	28	256	256	128	32
262		128	128	64	64
182		128	64	16	32
186	35	256	64	32	32
200		256	64	64	16
199		64	16	16	4
205	42	64	128	32	32
274		8	<4	8	<4
187		16	16	8	4
191	49	32	8	32	16
209		128	32	16	16
188		<4	<4	<4	<4
189	56	8	4	<4	4
190		16	8	8	<4

These results were quite similar to those obtained when lymph specimens from normal rats were examined. Such normal lymph also very frequently contained rat cell hemagglutinins at low titers (1:4 to 1:8). The titer of the hemagglutinins in burn lymph specimens was significantly higher by the 3rd and 4th days after injury, and strong positive results persisted for at least 5 weeks. A definite decline in titer was not noted until 6 to 7

TABLE II. Indirect Antiglobulin Tests with Thoracic Duct Lymph Obtained from Burned Rats.

	Lymph obtained from burned rats				Normal lymph
	Sample 1		Sample 2		Sample 3
Rat erythrocytes used	Untreated	ME*	Untreated	ME	Untreated
Fisher strain	1024 (512)†	512 (256)	512 (256)	512 (128)	8 (8)
Sprague-Dawley strain	1024 (512)	1024 (512)	1024 (256)	1024 (256)	n.d.
Lewis strain	256 (128)	512 (128)	256 (128)	128 (128)	n.d.

* Lymph treated with 2-mercaptoethanol.
† Numbers in parentheses indicate titers obtained by hemagglutination tests.

TABLE III. Effects of Absorption with Erythrocytes upon Hemagglutinating Activity of Lymph Obtained from Burned Rats.

	Agglutination titer against erythrocytes of	
	Fisher strain	Sprague-Dawley strain
Unabsorbed lymph	512	512
1) Fisher erythrocyte stromata	<1	<1
Lymph absorbed with:		
2) Sprague-Dawley erythrocyte stromata	32	<1
3) Human erythrocyte stromata	512	512

weeks after burning. High hemagglutinating titers were observed with syngeneic erythrocytes of the Fisher strain as well as with allogeneic erythrocytes of the Sprague-Dawley and Lewis strains. On the other hand, erythrocytes of the Wistar strain were agglutinated at considerably lower titers. Lymph specimens were also tested against erythrocytes of foreign species: man, ox, sheep, pig, rabbit, guinea pig, mouse, chicken and turkey. Consistently negative results were obtained with such erythrocytes. Serum samples of burned Fisher rats were also studied for agglutination of erythrocytes from rats and other species. In all instances, negative results were obtained.

Indirect Coombs tests were performed with selected lymph specimens (Table II). The antibody titers against erythrocytes of the Fisher, Sprague-Dawley and Lewis strains were usually twice as high in the indirect Coombs test than in the hemagglutination test. As may be noted in Table II, treatment with 2-mercaptoethanol had no significant effect on the hemagglutinating activity of the lymph.

There was no difference between the hemagglutinating titers of untreated lymph specimens and the titers of specimens maintained for 30 minutes at 56°C. Heating at 65°C caused decreases in the agglutinating titers, and heating at 70°C resulted in complete loss of the hemagglutinating activity of all lymph specimens tested.

Additional information concerning the serologic specificity of the hemagglutinating factor was sought by absorption experiments. Selected burn lymph specimens were absorbed with rat erythrocyte stromata of the Fisher and Sprague-Dawley strains and were tested against erythrocyte samples of these two strains (Table III). Agglutinating activity for erythrocytes of both strains was completely removed from a positive lymph sample by erythrocyte stromata of the Fisher strain. Absorption with Sprague-Dawley erythrocyte stromata removed the agglutinin against Sprague-Dawley erythrocytes, but left some residual agglutitnating activity against Fisher erythrocytes. Human erythrocyte stromata failed to affect the hemagglutinating activity of burn lymph against either rat strain.

The antigenic properties of the hemagglutinating factor were studied by using eluates from Fisher erythrocyte stromata incubated with positive lymph specimens. Fig. 1 illustrates an experiment in which such an eluate was tested against rabbit antiserum to rat serum. One distinct line of precipitation was formed by an eluate merged in an identity reaction with a line formed by Fraction II of

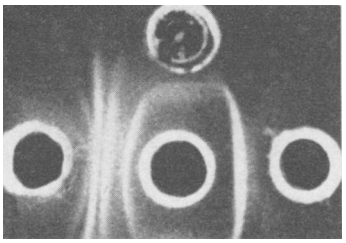

FIG. 1. Double diffusion gel precipitation test. Central well: Rabbit antiserum to pooled rat serum. Pheripheral wells: Top, eluate from Fisher erythrocyte stromata sensitized with lymph of burned Fisher rat; right, 0.5% Fraction II of pooled rat serum; left, lymph of burned Fisher rat.

pooled rat serum. Eluates obtained from rat erythrocyte stromata incubated with normal rat lymph gave consistently negative results in precipitation tests.

To study further the antigenic characteristics of the hemagglutinating factor, a rabbit was immunized with rat erythrocytes agglutinated by burn lymph. The resulting antiserum was titrated against human erythrocytes sensitized with a subagglutinating dose of rat antiserum to human erythrocytes. It agglutinated such sensitized erythrocytes up to a dilution of 1:2430. In contrast, rabbit antisera to rat erythrocytes incubated with normal rat lymph showed no agglutinating activity.

The antiserum obtained in the rabbit injected with rat erythrocytes agglutinated by burn lymph was studied further by immunoelectrophoresis against rat serum. As noted in Fig. 2, this antiserum formed a major precipitation line in the IgG region with rat serum separated by electrophoresis. Another minor line visible in this figure was probably formed with β1A globulin.

Direct antiglobulin tests were performed with erythrocytes of 6 Fisher rats bled at different time intervals after thermal injury.

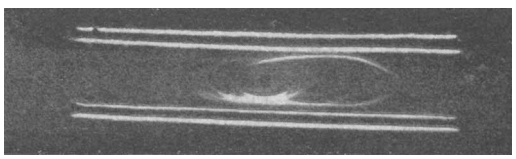

FIG. 2. Immunoelectrophoresis. Central well: Normal rat serum. Upper trough: Rabbit antiserum to Fisher erythrocytes agglutinated by lymph of burned Fisher rats. Lower trough: Rabbit antiserum to pooled rat serum.

Erythrocyte samples from 4 animals were negative. In 2 remaining animals some reactions were observed 36 days after injury. These reactions were weak, however, and the observed titers did not exceed 1:50 in spite of the fact that a very potent antiglobulin serum was used for the tests.

Discussion. Results of this study indicate that thoracic duct lymph obtained from Fisher rats at various times after thermal injury contains an agglutinating factor directed against rat erythrocytes. Absorption studies suggest that this factor is mainly directed against syngeneic Fisher erythrocytes and that reactions with allogeneic erythrocytes of other rat strains (Sprague-Dawley, Lewis) may be cross-reactions.

The immunoglobulin nature of this factor was investigated by two different approaches. In the first, eluates from rat erythrocyte stromata sensitized with lymph of burned rats have been shown to exhibit the serologic properties of rat γ globulin. In the second, Fisher erythrocytes agglutinated with the lymph of burned Fisher rats have been shown to elicit in the rabbit a serum antibody response directed against rat γ globulin.

It is of interest that thoracic duct lymph samples obtained from normal rats have also been shown to contain low titers of hemagglutinins for syngeneic erythrocytes. This finding is in agreement with a previous report of the occurrence of autohemagglutinins in blister fluid of normal human beings(13).

It would therefore appear possible that thermal injury may simply result in an increase in titer of such naturally occurring hemagglutinins. This possibility may also provide an explanation for the fact that the peak titers of such hemagglutinating activity are reached as early as 5 days after the injury.

The consistent absence of hemagglutinating activity in the serum of burned rats suggests that the agglutinating factor may be absorbed by host erythrocytes upon release into the blood circulation. Positive direct Coombs tests obtained with erythrocytes of burned rats in the present study, as well as the more definitive results obtained in this regard by McCarthy and associates(6,7) in rats subjected to more extensive thermal injury, tend to support that possibility. The effects of absorption

of the hemagglutinating factor upon erythrocytes of the burned host are not clear. They may, however, provide an experimental approach to the study of hematologic complications of the burn syndrome.

Summary. Thermal injury is associated in the rat with the development of an agglutinin directed against syngeneic erythrocytes. This hemagglutinin is present in the *lymphatic* but *not* in the *blood* circulation of the burned host, and has the characteristics of an immunoglobulin.

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Elongation of the Interpubic Ligament in the Little Brown Bat *(Myotis lucifugus).** (31642)

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Separation of the pubic symphysis during late pregnancy and after treatment with relaxin in an estrogenized animal has been described in only 5 species: the guinea pig(1), the mouse *(Mus musculus)*(2), the deermouse *(Peromyscus maniculatus* sp. *bairdii* and *Peromyscus maniculatus* sp. *gracilis)*(3), and the Skomer bank vole *(Clethrionomys skomerensis)*(4). This phenomenon occurs also in the monkey *(Macaca mulatta)* although it differs from the above species in requiring pretreatment with progesterone in addition to estrogen(5,6).

The present study is concerned with the elongation of the pubic ligament of the little brown bat *(Myotis lucifugus)* during pregnancy and after treatment with relaxin.

Materials and methods. Female little brown bats *(Myotis lucifugus)* were obtained from Ray's Cave, Greene County, Indiana, and Grotto Cave, Monroe County, Indiana. These hibernating animals were collected in 2 groups, one group in late November, 1965, the second group in early April, 1966. The bats were maintained in the laboratory at 92°F, housed 2 per cage and fed 10-20 mealworms daily. A constant supply of water was available within the cages. Bats collected in April, 1966, that were thought to be pregnant were observed daily. Immediately after delivery or abortion the bats were killed and their pubic symphyses examined.

The non-pregnant bats were primed with a single dose of 5 μg estradiol cyclopentylpropionate (ECP) administered into the pectoral muscles. Seven days later the bats received a single injection of relaxin† in-

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