

Coombs' (Antiglobulin) Reaction in Severely-Burned Rats *

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OBSERVATIONS made by Federov¹⁰ and Rosenthal¹⁹ and those made in our laboratory³ have implicated an autotoxic and an auto-immune response, respectively, as factors contributing to death after severe thermal injury. In the latter investigation it was noted that in severely-burned Wistar rats, positive direct Coombs' reactions were correlated with death and negative reactions with survival. The present experiments were performed to test the validity of these results. Larger numbers of animals of a different strain were used and antisera were prepared against rat gamma globulin fractionated by starch block electrophoresis instead of methanol precipitation. In addition it was our aim to ascertain whether any correlation exists between the various experimental parameters (hematocrits, circulating reticulocyte percentages, serum protein electrophoretic patterns and spleen and thymus weights) with the positive or negative direct Coombs' reactions and death or survival.

Materials and Methods

Male Sprague-Dawley rats ** (average weight 204.1 Gm., S.D. = 5.35) were used in this study. The parent stock of these animals were Caesarean-derived and foster suckled on Lobund germ-free rats.

Preliminary experiments with these animals disclosed that they were much more resistant to our standardized burn and infusion procedure than Wistar strain rats. In order to effect survival percentages similar to those previously noted, that is, 0 per cent at 12 hours postburn for uninfused

controls, and approximately 40 per cent at 21 days postburn for saline-lactate infused, it was necessary to increase the exposure in water at 90° C. from 35 to 40 seconds, and the body surface area from 32 to 37 per cent. All experimental animals received Staley's Rockland Rat Diet (complete) and water, *ad libitum*. They were housed in individual wire mesh cages in air conditioned quarters at $26 \pm 2^\circ$ C.⁷ Each infused animal received 18 per cent of his body weight consisting of 4.0 per cent of a 1/6 M sodium lactate solution and 14 per cent of a 1.4 per cent sodium chloride solution during the immediate 10-hour postburn interval according to our previously described regimen.¹⁴ The experimental animals were studied in groups of ten, including 17 burned-uninfused, 61 burned-infused, seven nonburned uninfused controls, and two nonburned, infused controls. Hematologic procedures were done on lateral tail vein blood preburn, and at 4, 7, 10, 14 and 21 days postburn.

Percentages of circulating reticulocytes were determined by the method of Brecher⁵ and hematocrits were done by the standard microcapillary technic. Serum protein electrophoretic studies were performed using the Spinco Model R paper electrophoresis system with Veronal buffer pH 8.6, ionic strength 0.075.

The rat gamma globulin was separated from whole Sprague-Dawley rat serum by the modified starch block electrophoresis method of Vannier and Campbell.²¹ After elution and lyophilization the protein was stored at -20° C. An aliquot of the reconstituted protein solution was assayed by the Biuret procedure and the remainder was combined with Difco Bacto-Adjuvant, complete (Freund) and injected into the

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toepads of rabbits according to the method of Leskowitz and Waxman.¹³ The rabbits were bled at four weeks and the anti-rat rabbit sera were absorbed twice with pooled normal rat erythrocytes, preserved with sodium azide (1:5,000) and stored in 10 ml. aliquots at -20° C.

Erythrocytes to be tested for protein coating were collected into warm physiological saline, washed four times at room temperature and resuspended to give a 2.0 to 4.0 per cent suspension. One-tenth of a ml. of the cell suspension was added to an equal volume of undiluted anti-globulin serum in an 8.0 x 75 mm. test tube. After incubation at 37° C. for 30 minutes the tube was shaken gently and the contents inspected both macroscopically and microscopically for agglutination.

Autopsies were performed within three hours on animals kept at 4.0° C. after death. The thoracic and abdominal lymph nodes, heart, lungs, gastro-intestinal tract, kidneys, adrenals and the burned area of the body wall were grossly examined for evidence of alteration. The site of the intravenous cannulation was checked for evidence of infection. The spleen and thymus were excised, trimmed of adhering tissues and weighed to the nearest milligram. Control and surviving animals were exsanguinated under ether anesthesia at 23 days postburn and examined as above.

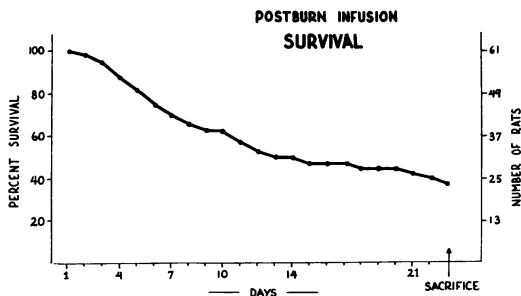


FIG. 1. Percentages of survival recorded at daily intervals postburn for 61 animals burned 37 per cent of the body surface at 90° C. for 40 seconds and infused with 4 per cent NaC₂H₃O₃ and 14 per cent NaCl (1.4% soln).

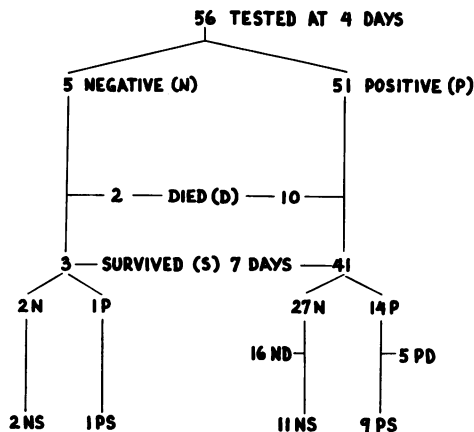


FIG. 2. Summary of results of direct anti-globulin (Coombs') tests on 56 burned-infused rats from the fourth through the twenty-first day postburn.

Results

All of the 17 burned uninfused controls died within 12 hours postburn. Twenty-three of the 61 burned-infused animals (38%) survived until sacrificed between the third and fourth week postburn (Fig. 1). It will be noted that the majority of deaths occurred prior to tenth day postburn.

Of the 56 animals tested at four days postburn (Fig. 2) five (9.0%) were found to be direct Coombs' negative. Of these two died by the seventh day postburn, one became positive at seven days and survived until sacrificed, and two remained negative and survived until sacrificed.

At four days postburn 51 animals (91%) were found to be direct Coombs' positive. Of these ten died before the seventh day. Of the remaining 41, 27 became negative upon subsequent testing; of these, 11 (41%) survived until sacrificed at 23 days postburn. Of the 14 which remained positive, nine (64%) survived until sacrificed. The 23 per cent difference in survival in favor of those animals sustaining a positive direct Coombs' reaction was not statistically significant, P = .30.

The strengths of the Coombs' reactions shown by scattergram (Fig. 3) could not be correlated with survival. Approximately

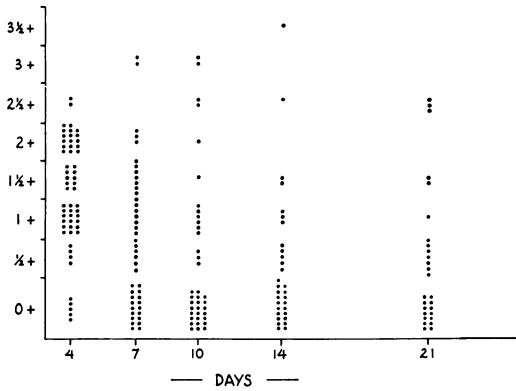


FIG. 3. Scattergram indicating the magnitude of the agglutination in the direct Coombs' tests. The ordinate shows the intensity of the reaction from the negative (0+) to strong positive (3 1/2+). Each dot above the postburn intervals on the abscissa represents one animal.

half of the positive survivors at 21 days postburn were plus one or above and half were less than plus one.

The data relating to the experimental parameters discussed below are presented as average values for those animals tested on postburn days 7, 10, 14, and 21. These animals are grouped with respect to the Coombs' reactions as follows: negative survivors, NS; negative deaths, ND; positive survivors, PS; and positive deaths, PD. The average values for the nonburned, uninfused control animals tested concomitantly are presented for comparison.

The hematocrit and reticulocyte data for the NS and PS groups (Fig. 4) were not significantly different. The hematocrits in both groups were decreased approximately 10 per cent at four days postburn. By 21 days the values for both groups were approximately equal and within 4.0 per cent of initial. The reticulocyte values were inversely related to the hematocrit values and showed a significant increase compared to initial and control levels at four days postburn. The reticulocyte values for both groups paralleled each other and were slightly elevated for the PS group at each test interval. By 21 days, the values in both groups had declined to approximately the initial levels. The hematocrit and re-

ticulocyte data for the PD and ND groups (Fig. 5) were not significantly different from each other nor from those of the NS and PS groups through seven days postburn. Hematocrit values for the PD group at 10, 14 and 21 days were considerably below the ND groups thus possibly indicating increased erythrocyte destruction in this group; however, no real compensatory rise in reticulocyte values was observed.

Changes in the percentages of the serum proteins (Fig. 6) of animals showing either positive (PS, PD) or a negative (NS, ND) direct Coombs' reactions were similar. However, marked differences in these serum protein values were noted when comparisons were made between the values of the survivors (NS, PS) and those which died

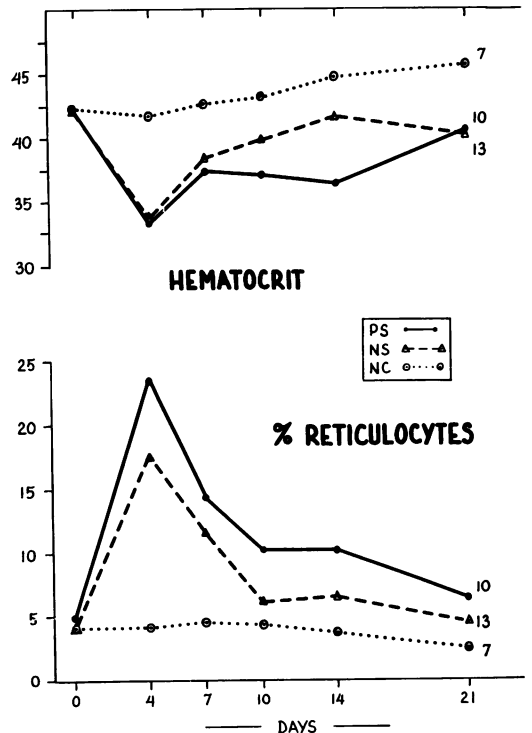


FIG. 4. Average hematocrit and reticulocyte values of animals surviving through 21 days postburn. Dotted lines connect normal control values (NC), solid lines connect values of animals having sustained positive direct Coombs' tests (PS) and broken lines connect values of animals having negative direct Coombs' tests (NS). The numbers of animals in each group are recorded after the 21 day value.

(ND, PD). The percentages of albumin declined after burn in both survivor groups reaching their lowest point (25%) at 10 days. These values then increased to an average of 30 per cent at 21 days or approximately half the preburn level. By contrast the albumin values in the death groups declined at each successive test period reaching an average value of 7.5 per cent for the four animals surviving to 21 days postburn, or 12 per cent of the preburn level. The α_1 -globulin percentages in both survivor and death groups increased through the seventh day postburn. The values for the survivors then declined approaching the control levels at 21 days; the values for the death groups remained elevated throughout the test period. The α_2 -globulin values followed the same pattern as those of the α_1 -globulins;

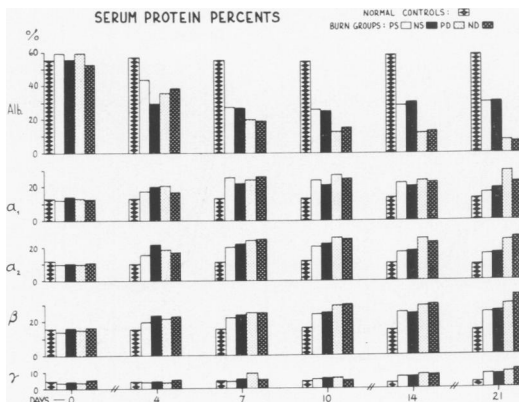


FIG. 6. Average albumin and globulin per cents pre- and postburn (p.b). The numbers of animals in each survivor group were: 7—normal control, 10—positive direct Coombs' test (PS), 13—negative direct Coombs' test (NS). In the group of p.b. animals showing a positive Coombs' test at death (PD) there were: 5 at 7 days, 4 at 10 days, 2 at 14 days and 2 at 21 days. In the group of animals showing a negative Coombs' test at death (ND) there were: 16 at 7 days, 11 at 10 days, 6 at 14 days, and 2 at 21 days.

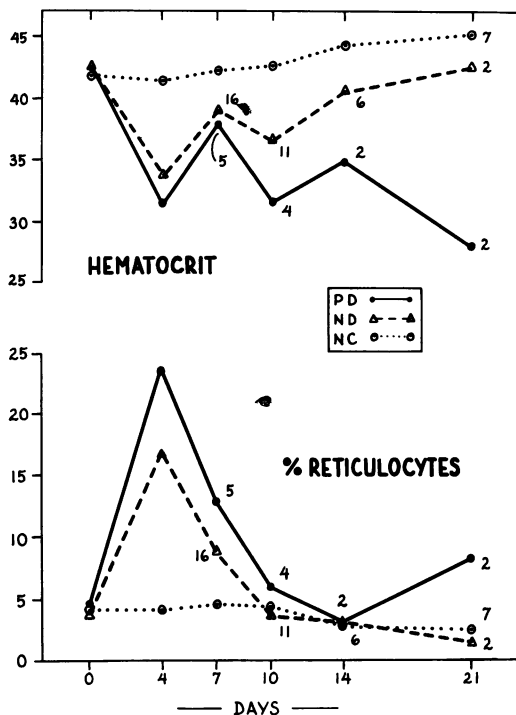


FIG. 5. Average hematocrit and reticulocyte values of animals which died within 22 days postburn. Dotted lines connect control values (NC), solid lines connect values of animals having sustained positive direct Coombs' tests (PD) and broken lines connect values from negative direct Coombs' test animals (ND). The numbers of survivors are recorded at the successive test points.

the terminal values for ND and PD groups at 21 days postburn were approximately 65 per cent greater than those of the NS and PS groups. Beta-globulin values in both survivor and death groups increased through the tenth day postburn attaining levels of approximately 150 and 180 per cent, respectively, of the control values. These elevated beta-globulin percentages persisted through 21 days in all burn groups. The gamma-globulin percentages for the survivors showed progressive increases on subsequent test days attaining levels approximately twice those of the controls at 21 days postburn. The gamma-globulin values for the ND and PD groups showed similar increases.

Albumin-globulin ratios (Fig. 7) for the surviving animals and for those which died were found to decline at similar rates through the seventh day postburn. The ratios at 21 days postburn for the respective groups (PS, NS) and (PD, ND) were identical. Ratio for survivor groups was notably higher (0.46) than the four animals in the death groups (0.08) at 21 days.

Comparisons of the average body weights

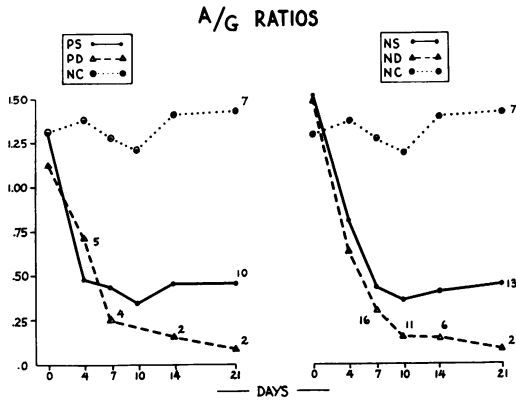


FIG. 7. Albumin-globulin ratios. Values for those animals that sustained a positive direct Coombs' test are shown on the left; values for those that were Coombs' negative are shown on the right. The dotted lines connect control values (NC), solid lines connect values for the survivor groups (NS and PS) and broken lines connect values for the death groups (PD and ND). Numerals at the test points represent numbers of animals.

(in Gm.) of the NS, PS and controls at sacrifice and those of the ND and PD animals at death, together with the spleen and thymus weights (in Gm. per 100 Gm. of rat) are shown in Table 1. The average body weight of the NS and PS animals was 67 per cent of that of the untreated controls whereas the average body weight of ND and PD animals was 48 per cent of that of the controls. The average spleen weight of the NS and PS animals was approximately 240 per cent greater than that of the controls; the average spleen weight of the ND and PD animals was 140 per cent greater than that of the controls. The average thymus weight of the surviving animals was 75 per cent of that of the controls whereas the average thymus weight of those that died was 22 per cent of that of the controls.

Data from the nonburned, infused controls for all parameters showed no difference from those recorded for the nonburned, uninfused controls.

Discussion

In seeking to explain the occurrence of the postburn positive direct Coombs' re-

action and its relationship if any to death or survival we have tried to evaluate our data in relation to those of others to determine whether such findings could indicate an immune or autoimmune response. In these experiments the appearance of positive Coombs' reactions was noted at four and seven days postburn; 44 per cent remained positive until death or sacrifice. A single attempt to recover an eluate from Coombs' positive cells capable of giving a positive indirect Coombs' reaction was successful. Although these observations suggested an immune response the following facts made such an interpretation suspect: 1) incidence of positive direct Coombs' reaction was extremely high (91%); 2) 66 per cent of positive animals surviving to seventh day postburn became and remained negative until death or sacrifice; and 3) attempts to demonstrate a circulating red-cell specific factor by indirect Coombs' reaction were uniformly unsuccessful.

Direct positive Coombs' reactions have been noted by others which do not appear to be related to immunity. Muirhead *et al.*¹⁵ noted positive Coombs' tests following phenylhydrazine injections and Brown *et al.*⁶ noted positive tests coincident with trypan blue induced anemia. It has been shown by other investigators that false positive tests may be given by reticulocytes¹⁶ and that in certain disease states a strong correlation exists between the positive Coombs' test and the presence of red cells of abnormal plaque size.⁸ Evidence has also been presented indicating that positive direct Coombs' reactions in some instances may be due to bacteria, bacterial enzymes or bacteria produced hemagglutinins.²²

Since in these experiments the burn damage involved subcutaneous musculature as well as skin, tissue degradation was extensive and could be expected to have far reaching effects. Rosenthal¹⁸ has stated that "burn toxins" are released from burned skin. Toxic agents may be the cause of

TABLE 1. Average Body Weights in Grams and Average Weights of the Spleen and Thymus Glands Expressed in Grams per 100 Grams of Body Weight. The Survivors and Controls Were Sacrificed at 23 Days Postburn

	No. Rats	Body Wt. Gm.	SD	Spleen Wt. in Gm./100 Gm. Body Wt.	SD	Thymus Wt. in Gm./100 Gm. Body Wt.	SD
At sacrifice							
Positive Survivors	10	229.4	2.23	0.64	0.137	0.17	0.057
Negative Survivors	13	228.4	4.76	0.60	0.214	0.16	0.037
Controls, Unburned, Uninfused	7	340.3	10.44	0.18	0.045	0.22	0.055
Controls, Unburned, Infused	2	357.5		0.18		0.24	
At Death							
Negative Deaths	16	161.8	22.42	0.35	0.214	0.06	0.109
Positive Deaths	5	167.6	16.80	0.68	0.473	0.04	0.016

severe postburn alterations in a large portion of the erythrocyte population, such as those noted by de Hemptinne and Gauthier.⁹ Distortions of the red cell membrane could lead to the adsorption of gamma globulin or substances cross reacting with gamma globulin as evinced by the occurrence of the positive Coombs' reaction after burn. Our hematocrit data support the findings of others with regard to decreased erythrocyte volume following burns.^{12, 20} We have also noted a postburn splenomegaly, which is more pronounced in animals surviving through 21 days postburn than in those that died. These observations may be related to the action of burned tissue toxins, which may cause anomalies leading to increased sequestration and destruction of erythrocytes by the spleen.

Our serum protein data corroborated the work of others^{2, 11} which has shown that high alpha- and beta-globulin levels occur after severe burns. These persisted in those animals that died, whereas in surviving animals the alpha-globulins more nearly approached normal values by 21 days postburn. We have also noted a sharp postburn decrease in percent albumin; this decrease was found to be far greater in those animals which died late after burn than in those

that survived. Normal levels were not regained by 21 days postburn in any experimental group. Damage to the liver has been observed to give rise to an increase in serum alpha- and beta-globulins.¹⁷ Liver damage has also been cited in relation to a decrease in serum albumin;² factors such as transcapillary loss and increased albumin catabolism may also be involved.⁴ Such damage could be effected by the action (direct or indirect) of "toxic agents" released from the burn site, as suggested by Arturson.¹

Evidence presented above has not discounted the possibility that autoimmune or autotoxic phenomena are involved in severe burn sequelae. Further studies are needed to elucidate the nature and specificity of the substance(s) causing agglutination of rat erythrocytes by antiglobulin sera following severe thermal burns.

Summary

1. Fifty-one of the 56 (91%) male Sprague-Dawley rats burned 37 ± 2.0 per cent of body area developed positive direct Coombs' reactions at four days postburn. By 21 days postburn 13 survivors were negative and ten were positive.

2. Hematocrit depression, reticulocytosis,

reduced serum albumin levels and increased globulin levels were noted in all animals. These parameters could not be correlated with the Coombs' test and death or survival.

3. Sustained depression of serum albumin levels was correlated with death.

4. Decreased body weight and thymic atrophy were correlated with death.

5. Splenomegaly was found in all late deaths but was of a lesser magnitude than splenomegaly of majority of survivors.

6. The relationship of these findings to other work is discussed.

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