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The Anemia of Thermal Injury: Mechanism of Inhibition of Erythropoiesis[1] (42236)

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Abstract. The anemia of thermal injury is a multifactorial process and includes hemorrhage and hemolysis. Much evidence suggests that a reduced rate of erythropoiesis contributes to this anemia. Prior studies show that this anemia is temporally related to the appearance in burn patients sera of a substance(s) capable of inhibiting erythropoiesis *in vitro*. Four experiments were done to elucidate the mechanism of action of this inhibitor. In all experiments sera from burn patients previously shown to be inhibitory to erythropoiesis *in vitro* were studied. In the first, inhibitory sera were exposed to erythropoietin solutions without loss of erythropoietic activity. Second, mouse marrow cells were preincubated with serum without loss of their ability to form erythroid colonies. Third, the inhibitory effect could not be overcome with increasing amounts of erythropoietin. Finally, erythroid colony formation was effected only if the inhibitory serum was present during the first 8 to 12 hr of culture. The data suggest that the erythropoietic inhibitor in these sera acts directly on erythroid stem cells *in vitro* and not by inactivating or interference with erythropoietin.

Anemia is routinely seen in patients who have been severely burned. The anemia of thermal injury (ATI) has a multifactorial etiology including hemorrhage and hemolysis (1). However, several lines of evidence suggest that the rate of erythropoiesis is inappropriately reduced after thermal injury (2–4). At the time that this hypoproliferative anemia occurs there appears a substance(s) in the serum of burn patients capable of inhibiting erythropoiesis but not granulopoiesis *in vitro*. This material is not always present immediately following burning but develops days afterward and reaches a peak of inhibitory activity 2 to 3 weeks after injury and then disappears as wounds heal (5). It has been suggested that this inhibitor plays a role in the anemia of thermal injury. The present studies were designed as a first attempt to determine the mechanism by which this substance interferes with erythropoiesis *in vitro*. The question asked was whether the inhibitor acts directly on erythroid cells or by an effect on erythropoietin.

Materials and Methods. *Patients.* Sera from 15 burn patients were collected in the University of Colorado Health Sciences Center Burn Unit. For the purposes of this study a severe burn was defined as a greater than 20% third degree thermal injury. Patients with electrical and chemical burns were not included. All burn patients were anemic at the time their sera were taken for study with a mean hematocrit of 31% (range 20–35). None had renal failure as defined as a serum creatinine over 2 mg/100 ml. All patients were cared for in a standard fashion. Eschar was usually debrided within 72 hr in the operating room and wounds were initially covered with fresh or cryopreserved cadaver homografts. Autografting was done where possible. Silver sulfadiazine was the usual topical antimicrobial agent. Dressings were soaked in neomycin–bacitracin–saline. Loose graft or burn tissue was debrided daily by nursing personnel. Nutritional status was carefully monitored including the use of total nitrogen balance studies where applicable. Enteral nutrition was preferred but occasionally peripheral vein parenteral nutrition was done. Bacterial wound counts were done at appropriate intervals, generally weekly but more often when wounds appeared to be infected. The normal sera were taken from volunteer ambulatory hospital and laboratory personnel. This study had been approved by the Veterans Administration–University of Colorado Health Sci-

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ences Center Human Subjects Committee. All blood was drawn after informed consent had been obtained and all sera were treated at 56°C for 45 min to inactivate complement and then stored in 1-ml aliquots at −90°C until studied.

Tissue culture. Erythroid colonies were grown from mouse bone marrow precursor cells (erythroid colony forming units, CFU-E) according to methods previously described (6). Briefly, cells were flushed from the tibias of CF1 mice with 1 ml of α medium (Flow Laboratories, Englewood, Calif.). The cells were cultured in a medium consisting of 50% of α medium, 40% Iscove's modified Dulbecco's medium (GIBCO, Grand Island, N.Y.) supplemented with cholesterol and egg yolk lecithin (7), asparagine, calcium chloride, penicillin, and streptomycin. The cell concentration was adjusted to 500,000/ml. The serum to be studied constituted 10% of the final medium. Medium (0.5 ml) was added to tissue culture wells (Linbro, Flow Laboratories), clotted with 50 μl of citrated bovine plasma (Colorado Serum Company, Denver, Colo.) and supplemented with 125 mU of sheep erythropoietin (EPO) Step I (Connaught Medical Research Laboratories, Ontario, Canada). The cultures were incubated for 72 hr under standard tissue culture conditions with 5% carbon dioxide. The clots were harvested from the wells, fixed with glutaraldehyde, stained with benzidine–peroxide and colonies of eight or more benzidine-positive cells counted at 100×.

All burn patient sera were assayed in this system to be sure they were inhibitory to CFU-E before being used in any further studies. The 15 sera selected were inhibitory with less than 60% of red cell colony growth in this preliminary assay compared to cultures prepared with normal sera, Table I.

Erythropoietin assay. The exhypoxic mouse assay technique was used to measure EPO. The details of the method have been given previously (8, 9). In brief, CF1 mice were exposed to reduced oxygen concentration (approximately 0.4 atm) in hypobaric chambers (10) for 3 weeks and were then removed for 5 days. Groups of five or six were injected ip with either saline or a known amount of sheep EPO or the sample to be studied and 2 days later with 0.5 μCi of 59-iron chloride. Three days later the animals were weighed, bled by orbital puncture, and aliquots collected for hematocrit and for iron incorporation. Data from mice with an hematocrit of less than 55% were discarded. Blood volume was assumed to be 7% of body weight. Radioiron was counted in a gamma counter and total iron incorporation into red cells was calculated from this figure and the blood volume.

TABLE I. INHIBITOR LEVEL OF BURN PATIENTS SERA

Patient	% Control[a]
ESP	9
NIG	12
MAR	19
WET	21
CES	22
PHI	28
OWE	36
SAM	37
BUM	38
TEN	40
RUT	43
ORN	48
WIN	48
REY	48
LAN	58
Mean	34 ± 4

[a] % Control was calculated from the mean number of CFU-E grown in the presence of 10 different normal sera.

Data processing. Standard statistical methods were used to analyze the data. All results are reported as mean ± standard error of the mean (SEM). The lines on Figs. 1 and 2 were calculated by linear regression and their significance determined using the F test (11). Numbers of erythroid colonies grown in the presence of the normal sera varied from day to day and in order to combine results from different experiments, some data given below were "normalized" by the formula

$$\%\ \text{control} = \frac{\text{individual burned or normal serum}}{\text{mean of normal sera}} \times 100.$$

Results. Four experimental designs were used.

Erythropoietin neutralization. The effect of sera on EPO in solution was studied in two systems.

EPO assay using the exhypoxic mouse. For the experimental group 200 μl of normal saline

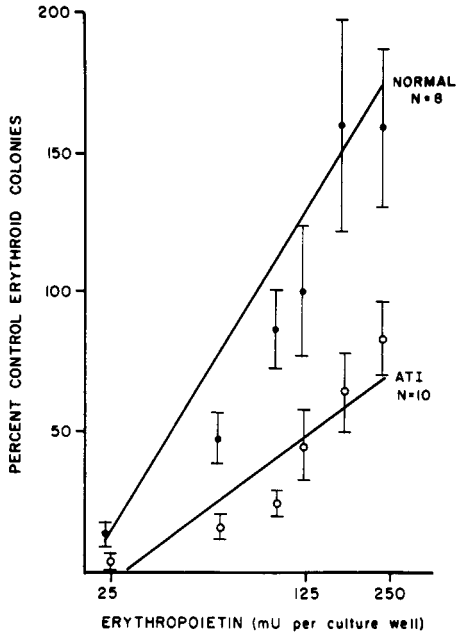

FIG. 1. EPO dose response curves in the presence of normal or burn patient sera. The horizontal axis is a log scale. The vertical bars represent the SEM. The lines were calculated by linear regression and were significant by F test (normal line, $P = 0.005$; ATI line, $P = 0.01$). The level of inhibition of the 10 ATI sera was 28–48% (median 43%) of control at 125 mU of EPO.

containing 125 mU of sheep EPO was mixed with 500 μl of either normal or burn patient serum. This mixture was incubated at 37°C for 24 hr. After the preliminary incubation, the 700 μl was injected into the previously prepared exhypoxic mice to measure EPO activity. As a control for the presence of serum, the EPO was assayed in saline alone, Table II, line 1. One normal and four burn patient sera were assayed. Two additional controls were used. First, the sera were injected into mice without additional EPO, Table II, first data column. No erythropoietic activity was detected compared to saline injection alone. For the second control, sera and EPO solutions were injected simultaneously without preincubation, Table II, second column. In the third column are the results of the preincubation of normal and burn patient sera with EPO. Preincubation of burn patient serum with EPO did not result in loss of activity.

Erythropoietin neutralization in vitro. For this work sera and EPO solutions were mixed with each other in the proportions given above. After 24 hr of incubation the ability of the EPO to stimulate the growth of erythroid colonies was measured. This experiment was performed twice and the results given in Table III. In order to combine the two experiments the data were "normalized" by the formula given above. Column 1 gives the number of colonies grown when the system contained untreated sera. Column 2 gives the colony counts when the sera were not premixed with EPO but were incubated separately at 37°C for 24 hr. The third column gives the colony counts when the sera and EPO were preincubated for 24 hr. There was no lessening of the ability of the EPO to stimulate the production of erythroid colonies when the inhibitory burn patient sera and EPO were in contact for 24 hr. Inhibitory activity was lost when the sera were incubated at 37°C for 24 hr. This is consistent with studies in progress in which it has been found that the burn inhibitor loses activity in serum at 37°C (S. Wallner, and R. Vautrin, unpublished observations).

Preincubation of cells in serum. To perform these experiments 1.25×10^6 mouse marrow cells were incubated in 250 μl of either burn patient or normal serum for 30 and 120 min at 37°C. In a control experiment no loss of inhibitory activity occurred when the burn patient sera were held at 37°C for 30 min, 45 $\pm$ 4% vs 34 $\pm$ 3%. After the preliminary incubation, the cells were centrifuged, the serum recovered, the cells washed once with α me-

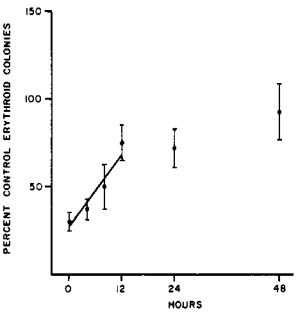

FIG. 2. Time course of the burn inhibitor. Burn patient sera were added to erythroid cultures at various times after the onset of culture. Less inhibition of colony formation was seen as sera were added at progressively longer intervals following culture up to 12 hr. The line was significant by F test, $P = 0.004$. The level of inhibition of the eight ATI sera was 12–40% (median 22%) of control at 0 time.

TABLE II. EFFECT OF NORMAL AND ATI SERUM ON EP ACTIVITY EXHYPOXEMIC MOUSE[a]

Serum group	Sera, no EP	Sera and EPO no preincubation	Sera + EPO 24-hr preincubation
Saline	0.60 ± 0.20	6.10 ± 0.80	
Normal	0.50 ± 0.05	5.90 ± 0.40	4.10 ± 1.50
ATI			
No. 1	0.90 ± 0.40	4.60 ± 1.40	4.10 ± 0.60
No. 2	0.60 ± 0.10	5.60 ± 1.50	5.80 ± 1.10
No. 3	0.50 ± 0.20	7.60 ± 2.10	9.50 ± 3.20
No. 4	0.50 ± 0.10	6.60 ± 2.60	9.00 ± 1.80
Total ATI	0.60 ± 0.10	6.10 ± 0.90	7.20 ± 0.80[b]

Note. ATI, anemia of thermal injury; EPO, erythropoietin. The level of inhibition of the four ATI sera selected was 9 to 48% (median 12%) of control in the preliminary assay.

[a] The values are RBC ^{59}Fe uptake (%). Mean ± SEM of five to six mice.

[b] *P*, nonsignificant, compared to total ATI without prior incubation.

dium to remove remaining serum, and then cultured in the absence of serum. The experiment was repeated four times. The data for the post-exposure colony counts were normalized and are given in Table IV. They show that preincubation of cells with inhibitory burned patient sera did not interfere with subsequent colony formation. Finally, the supernatant sera were assayed after being recovered from the cell buttons. The burned patient sera did not lose inhibition (after 30 min of exposure: preexposure 45 ± 4%, postexposure 35 ± 3% of colonies grown in normal sera).

Erythropoietin dose response curves. For this work, mouse bone marrow cells were cultured in the presence of sera with increasing doses of EPO from 25 to 250 mU per culture well. Ten burn patient and eight normal sera were studied. The experiment was repeated three times. The data were again normalized and 100% was defined as the mean number of colonies grown in the normal sera with 125 mU of EPO. The results are given in Fig. 1 and show that regardless of the dose of EPO fewer erythroid colonies were formed when the system contained burn patient sera. This effect could not be overcome at the highest doses of EPO. For most individual samples a plateau had been reached where the addition of further amounts of EPO did not result in formation of greater numbers of colonies.

Time course of action of the burn inhibitor. To perform these studies culture medium was prepared without sera. At the time of culture, and at 4, 8, 12, 24, and 48 hr after the onset of culture, 50 μl of normal or inhibitory burn patient serum was layered into the clots. The total culture time was 72 hr. This experiment was repeated three times and the data are given in Fig. 2. A total of six normal and eight burn patient sera were studied. The results indicate that the burn patient serum must be present within the first 8 to 12 hr of culture for a reduction in colony number to occur. Addition of sera 24 to 48 hr after the onset of culture had no effect on colony formation. Represen-

TABLE III. EFFECT OF NORMAL AND ATI SERA ON EPO ACTIVITY ERYTHROID COLONIES[a]

Serum group	No. sera	No treatment	Sera and EPO no preincubation	Sera + EPO 24-hr preincubation
ATI	9	28 ± 7	47 ± 13[b]	49 ± 9[b]
Normal	8	100 ± 10	100 ± 9	131 ± 16

Note. ATI, anemia of thermal injury; EPO, erythropoietin. The level of inhibition of the nine ATI sera selected was 9 to 58% (median 22%) of control in the preliminary assay.

[a] Mean ± SEM.

[b] $P < 0.05$ (paired Student's *t* test) compared to no treatment.

TABLE IV. ERYTHROID COLONY FORMATION AFTER PREINCUBATION OF SERA AND CELLS AT VARIOUS TIME INTERVALS[a]

Expt. No.	30 min normal serum	Preincubation ATI serum	120 min normal serum	ATI serum
1	93%	93%		
	107	59		
		89		
		84		
2	120	116		
	65	141		
	114	114		
		134		
		78		
		70		
3	103	79		
	88	96		
	109	63		
		126		
		67		
		86		
4	137	30	111%	105%
	99	199	102	120
	64	41	87	155
Mean	100	92	100	127
SEM	7	9	7	15

Note. ATI, anemia of thermal injury. The level of inhibition of the six ATI sera selected was 12 to 58% (median 38%) of control in the preliminary assay.

[a] Marrow cells were exposed to serum and then cultured in the absence of serum. Each value represents the results of an individual subject's serum expressed as percentage of the mean of the normal sera.

tative clots from this experiment from three normal and six burn patients were examined at 1000× and the number of cells in 25 consecutive erythroid colonies was counted, Table V. There was no reduction in colony size when comparing cultures prepared with normal or burn patient sera.

Discussion. The ATI is a multifactorial process. The etiologic factors involved can be most conveniently described as those which occur early (hours) following burn, those which occur later (days), and those which are delayed (weeks) (1). Early, the most important factors are direct thermal destruction of erythrocytes at the burn site and hemorrhage, accounting for the fact that most burn victims become anemic within hours after their injury. Delayed factors include additional blood loss at the time of dressing changes and hemolysis of cells damaged but not killed by the injury and/or disrupted as they pass through the burned tissue and eschar (12). It appears that depression of the rate of red cell formation begins days following injury. Late factors include continued blood loss at the time of surgical procedures, dressing changes, and occasionally from the gastrointestinal tract. Reduction of the rate of red cell formation continues into the late burn period. Thus, the ATI is due to hemorrhage/hemolysis coupled with an inadequate rate of red cell production.

Several lines of evidence indicate that erythropoiesis is reduced postburn. Reticulocytopenia with normocellular marrows have been described despite the anemia seen in these patients (2, 3). Additional morphologic evidence in humans and the burned mouse suggests that the marrow erythroid compartment has not expanded in an attempt to repair the anemia (4, 13, 14).

The factors responsible for the reduced rate of erythropoiesis are not clear at this time. Previous studies of serum EPO have given conflicting results, some finding that EPO levels are increased postburn while others have failed to find such an increase (2, 3, 15). However, in the majority of studies, groups of patients who were anemic were found to have increased levels of EPO suggesting that the reduced rate of red cell production was not due to lack of this hormone.

In prior work it has been shown that burned anemic mice and humans have a substance(s)

TABLE V. EFFECT OF NORMAL AND ATI SERA ON NUMBER OF CELLS PER ERYTHROID COLONY[a]

Serum added hr after culture	Serum group	Number of colonies	Colony size
0	Normal	100 ± 28	14.5 ± 0.5
	ATI	19 ± 6[a]	13.4 ± 0.7
4	Normal	100 ± 7	19.7 ± 1.0
	ATI	19 ± 8[b]	16.5 ± 1.0
24	Normal	127 ± 15	16.3 ± 1.5
	ATI	62 ± 5	19.3 ± 0.8
48	Normal	100 ± 9	18.6 ± 1.3
	ATI	74 ± 13	17.0 ± 1.2

Note. Three normals and six ATI patients were studied. The level of inhibition of the six ATI sera selected was 12 to 43% (median 22%) of control in the preliminary.

[a] Mean ± SEM; ATI, anemia of thermal injury

[b] Student's t $P < 0.005$.

in serum capable of inhibiting erythropoiesis but not granulopoiesis *in vitro* (5, 13). This material was not always present immediately following burn, but appeared days later and reached a peak of inhibitory activity 2–3 weeks postburn and then disappeared as wounds healed. The inhibitory substance was not related to episodes of sepsis or drug or topical agent treatment.

The present studies were done to determine the mechanism by which this material inhibits erythropoiesis. In the first study, Table II, there was no evidence that inhibitory sera neutralized EPO when the EPO was subsequently assayed *in vivo*. These data must be interpreted somewhat cautiously since, to date, it has not been conclusively shown that the inhibitor is active *in vivo*.

The data in Table III are the results of serum and EPO preincubation when the EPO was subsequently assayed *in vitro*. More colonies appeared when the burned patient sera were kept at 37°C for 24 hr confirming work in progress which shows that the inhibitor is unstable in serum at 37°C. The fact that more colonies appeared argues against the formation of an irreversible EPO–inhibitor complex since one would expect about the same number of colonies as in the no treatment group. If the inhibitor were enzymatically degrading EPO, one would expect fewer colonies than in the no treatment group since the enzyme would have had an additional 24 hr to hydrolyze the EPO.

In the second experiment, bone marrow cells were preincubated with inhibitory sera, then cultured in the absence of sera, and no reduction in their ability to form erythroid colonies was found, Table IV. This suggests that the inhibitory substance does not irreversibly bind to EPO receptors on cell surfaces nor does this short period of incubation result in inhibition of erythropoiesis. The work given in Table IV was carried out for 120 min and it is possible that a longer time of incubation could be necessary for an inhibitor to irreversibly bind to EPO receptors. However, the finding that the burn patient sera do not lose activity upon exposure to marrow cells suggests that inhibitor is not binding to the cells.

The EPO dose response curves, Fig. 1, show that the inhibitory effect cannot be overcome by adding additional amounts of EPO up to the level at which the system had reached a plateau in terms of colony formation, confirming prior studies (5) and reinforcing the concept that the inhibitor does not act by neutralizing EPO. Finally, the time course experiments show that there is a temporal effect on colony formation. The inhibitory activity must be present early in order to effect colony formation, Fig. 2, but does not affect colony size, Table V. These findings suggest that the effect is on the primitive erythroid cell and that the inhibitor acts on stem cell proliferation rather than maturation.

The data presented here are the results of *in vitro* studies of erythropoiesis and their direct application to the *in vivo* situation must be somewhat speculative. However, in prior studies it has been shown that inhibitory substances detected in serum *in vitro* may, in fact, be linked with the *in vivo* situation in patients with chronic renal failure (16). To date, no correlation of inhibition with hematocrit has been found in burn patients, perhaps due to the great complexity of these patients including dressing changes, surgical procedures, transfusion, episodes of sepsis, and other infections. In one prior study an inverse relationship between the level of serum inhibitory activity and bone marrow erythroid colony forming units was found in the burned mouse; however, there was no correlation with hematocrit possibly due to the compensatory activity which occurred in the spleen of the burned animals (13). In another, the amount of erythroid tissue in the marrow of burn victims has been found to be decreased in an autopsy study of 22 patients. Of the 22, 6 had been studied before death and all 6 had sera highly inhibitory to CFU-E (14). This also suggested that the inhibitor is active *in vivo*.

In summary, these experiments indicate that serum from burn patients contains a material(s) capable of inhibiting erythropoiesis *in vitro*. They suggest that the site of inhibition is directly on erythroid target cells, perhaps the most primitive cell detected in this system, the erythroid colony forming unit (CFU-E). No evidence was found that the inhibitor interferes with or inactivates the hormone, EPO, or binds irreversibly to EPO receptors. It is proposed that this material is responsible, in part, for the reticulocytopenic anemia typically seen in severely burned humans.

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