and 7 in the band contrasts with the low levels in the rest of the plates and in the control plate. Note in Fig. 2 that the largest increase of Cr^{51} occurred between days 3 and 5, after which there was little further increase.

The use of nitrogen as the basis for measuring changes of hemin and Cr^{51} is not entirely valid in view of the fact that the red cells being moved contain an appreciable amount of nitrogen. However, this fact would tend to decrease the difference between the hemin and chromium per mg nitrogen in the band and that outside the band. Therefore any difference found between the two would be the more significant.

Discussion. The chromium studies provide evidence to support the thesis that red blood cells are actually attracted through agar toward a substance which diffuses from the growth of *S. aureus*. There is a true increase of hemin and Cr^{51}, which presumably measure red blood cells, in the region of the red band, *over that in any other part of the* S. aureus *plates or in the control plate.* This is especially evident when the hemin and Cr^{51} are calculated per mg total nitrogen to correct for drying of the plate.

Our hypothesis is that the substance attracting red cells diffuses from the *S. aureus* growth on agar into a well-defined area of 0.6 to 1 cm width. This diffusable substance draws red cells singly and in clumps to this area from the 0.2- to 0.4-cm wide region around it. The chemical data appear to support the hypothesis. Visually it appears that the band develops quite quickly after 4 to 5 days of incubation. Data here show that the effect develops over the course of one day, and that there is little change thereafter. The red cells seem to pack into the area of the band until there is no more room for them. In some specimens, indeed, the agar in the band bulges upward as though swollen with red cells.

Summary. *S. aureus* grown on whole blood agar produced a diffusible material which caused changes in the nitrogen, hemin and red cell content of the agar. Experiments using blood cells tagged with Cr^{51} indicated that the dense red band around the growth of the organism was due to a concentration of red blood cells which had been moved into the area of the band from the area just outside it.

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Immunologic Studies on the Mechanism of Action of Erythropoietin.* (27602)

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The ability of serum obtained from rabbits previously immunized with human urinary erythropoietin to neutralize the erythropoietic activity of human urinary erythropoietin has been demonstrated(1). Injection of such sera into normal mice markedly decreased their normal 24-hour incorporation of Fe^{59} into circulating red blood cells and their peripheral reticulocyte count. This neutralization was considered to be the result of an antigen-antibody reaction directed against either the erythropoietically active molecule or the general class of molecules to which erythropoietin belongs. The present experiments are

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concerned with the temporal changes in the relative numbers of different types of bone marrow cells and their proliferative capacities following injection of such immune sera into normal mice. Data are also presented which further support the concept that the observed changes in the marrow are the result of neutralization of endogenous erythropoietin.

Materials and methods. Serum obtained from 2 rabbits previously immunized with alum precipitates of human urinary erythropoietin according to the schedule given by Kabat and Mayer(2) was pooled and termed anti-E. The human urinary erythropoietin was prepared by ultrafiltration on colloidion as previously described(3). The potency of this serum was such that 4 daily injections of 0.25 ml depressed the 24-hour Fe^{59} incorporation into circulating red blood cells of 10 normal mice to 1.5 ± 0.2% compared to 26.9 ± 2.1% following similar injections of normal rabbit serum.

Twenty male Swiss mice weighing about 25 g were injected subcutaneously with 0.25 ml of anti-E on 4 consecutive days. A second group was injected similarly with normal rabbit serum. On the day following the first injection and for the next 9 days 2 animals were sacrificed from each group and smears made of the bone marrow contained in one femur. The other femur, sternum, spleen, kidney and liver were fixed in buffered formalin. After decalcification of the femur and sternum the tissues were dehydrated, embedded in paraffin, sectioned and stained with hematoxylin and eosin.

One hour before sacrificing, the animals received an intravenous injection of 2 μc per g body weight of tritiated thymidine (360 μc/μM, Schwarz BioResearch, Inc.). Autoradiographs of the bone marrow smears were made using Kodak AR-10 stripping film. The autoradiographs were exposed 2 weeks, developed, fixed and stained with Giemsa. Bone marrow smears not used for autoradiographs were stained with Ralph's stain(4) followed by Giemsa in order more readily to identify erythroid cells. The percentage of erythroid cells, mature neutrophils, "small round cells" or lymphocyte-like cells and disrupted or unidentified cells was determined for each marrow smear. A total of at least 1000 cells was counted. Bone marrow smears of hypertransfused mice were also examined at various times after hypertransfusion.

Female Swiss mice weighing about 23 g were hypertransfused by intraperitoneal injection of 1 ml of 80% donor red blood cells on 2 consecutive days. The donor red blood cells were washed 3 times in heparinized saline and the buffy coat removed before injection into the recipient animals. Five days after the last transfusion a wave of erythropoiesis was initiated by intravenous injection of 50 μg† of human urinary erythropoietin in 0.1 ml of saline. At various times before and after injection of erythropoietin groups of 10 mice were injected intravenously with 0.1 ml of anti-E. Control groups consisted of 10 saline injected mice and 10 mice that received only erythropoietin. Two days after the erythropoietin injection 0.5 μc of Fe^{59} in 0.2 ml saline was injected intraperitoneally. Three days later the percent of the injected Fe^{59} in the total circulating red blood cells was determined assuming that blood volume was 5% of total body weight. The hematocrits of the assay animals at time of sacrifice averaged 65%. These and other aspects of this assay have been presented by DeGowin *et al.*(5).

Results and discussion. The temporal changes in percentage of erythroid cells in marrow of normal mice injected with anti-E are shown in Fig. 1. Values shown for each day are the average of 2 animals. The percentage of erythroid cells present in marrow of normal mice injected with normal rabbit serum was not significantly different during the 10 days following the first injection; therefore the mean value for all animals is indicated with the corresponding standard error. Following injection of anti-E there was a progressive daily decrease in percentage of marrow erythroid cells which became maximal on the 5th and 6th day after the first injection of anti-E or the 2nd and 3rd day after the last injection. The few erythroid cells ob-

† Direct comparison of the human urinary erythropoietin with Armour Lot No. K103-124 sheep erythropoietin in the starved rat assay indicates that this dose contains approximately 1.2 cobalt units.

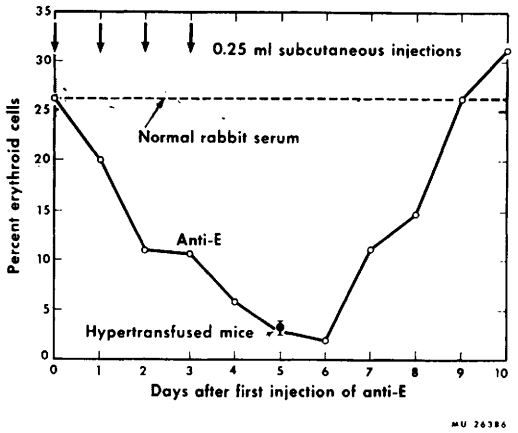

FIG. 1

served in the marrow at this time were primarily the more mature erythroid cells, although cells representing all stages of erythroid maturation could be found. The percentage of erythroid cells found in 5 mice on the 5th day after initiation of transfusion induced polycythemia is also indicated in Fig. 1 (hypertransfused mice). The cytologic and histologic appearance of the bone marrow of anti-E injected normal mice and polycythemic mice are, at this time of maximal erythroid suppression, indistinguishable, except that more mature red blood cells are observed in the polycythemic marrow.

A progressive daily increase in percentage of erythroid cells occurs after the 6th day and by the 9th day the percentage of erythroid cells has returned to that found in mice injected with normal rabbit serum. Presumably this increase in erythroid cells is due to the presence of endogenous erythropoietin. This erythropoietin may result from an increased rate of production by the mouse and/or, more likely, a decreased level of neutralizing antibody as a result of elimination of heterologous rabbit serum and stoppage of anti-E injections.

An increased number of mature marrow neutrophils was observed following injection of both anti-E and normal rabbit serum. There was some suggestion that increased percentages of "small round cells" which resemble lymphocytes occurred in the anti-E injected animals prior to reinstitution of erythropoiesis; however, too few mice were studied to evaluate this suggestive difference. It is our impression that a similar increase in these cells occurs in polycythemic mice. Quantitative measurements of these relative changes in the marrow population are in progress. Injections of anti-E did not affect the relative numbers of megakaryocytes in sections of the femur or sternum when compared to animals injected with normal rabbit serum, nor was there any obvious difference in the histological appearance of the kidneys, spleens and livers.

Autoradiographs made of bone marrow smears one hour after injection of tritiated thymidine were examined as an adjunct to the previous study. Injection of anti-E did not obviously affect the endogenous DNA synthetic capacity of individual marrow cells. Even at the time of maximal decrease in the percent erythroid cells following anti-E injections the few immature erythroid cells found in the marrow were labeled and therefore presumably able to synthesize DNA. Similar observations have been made in polycythemic mice. Cells of the granulocytic series normally capable of DNA synthesis were labeled after anti-E injection or polycythemia. The subsequent ability of the labeled erythroid cells to divide could not be determined in the present experiments; however, granulocytic cells were apparently capable of division since no particular decrease in their relative numbers was observed.

Erythropoietin is believed to control erythropoiesis by regulating the differentiation of stem cells into the erythroid series. It has been postulated that this regulation is the result of an enzyme induction in the stem cells (6). The temporal changes seen in the erythroid population, the similarity of these changes to those observed after hypertransfusion, and the ability of immature erythroid cells to synthesize DNA after injection of anti-E support the concept that anti-E exerts its effects on erythropoiesis by neutralizing erythropoietin. The fact that anti-E injections into normal mice decrease the erythroid population also supports the concept that erythropoietin is involved in the *normal* control of erythropoiesis. Cytotoxic effects of anti-E on erythroid cells might also result in a decrease in erythroid cells. Several

observations argue against extensive cytotoxic effects. The orderly gradual decrease in erythroid cells which was observed would not be expected if anti-E simply non-discriminately lysed erythroid cells. Hemoglobinuria or hemoglobinemia which might be expected if extensive cytolysis of hemoglobin containing cells occurred, was not observed.

Support for the concept that anti-E neutralizes erythropoietin and evidence against a cytotoxic effect was obtained by determining the effect of anti-E injections on erythropoiesis in polycythemic mice. Filmanowicz and Gurney(7) have observed an orderly wave of erythropoiesis in the spleen of hypertransfused mice after injection of purified sheep plasma erythropoietin. We have observed a similar wave in the bone marrow following injections of human urinary erythropoietin. During the first 12 to 18 hours the marrow of polycythemic erythropoietin stimulated mice appears cytologically little different from non-stimulated animals, although it is our impression that an increase in the relative numbers of reticulum cells or hemocytoblasts occurs. One day after erythropoietin stimulation a peak percentage increase in proerythroblasts is observed. This is followed on the 2nd day by a peak in the percentage of basophilic erythroblasts, and on the 3rd day the peak percentage of reticulocytes is found in the peripheral blood. A day or two later the marrow is again devoid of erythroid cells.

The effect of anti-E injections on the erythropoietic response of polycythemic mice to exogenous erythropoietin as a function of the time of anti-E administration (hours) after erythropoietin stimulation is shown in Fig. 2. The amount of anti-E injected was calculated to be in excess of that required completely to neutralize all the exogenous erythropoietin injected. When this amount of anti-E was injected one hour before or at the same time as the erythropoietin no stimulation of erythropoiesis occurred; *i.e.*, Fe^{59} incorporation in the calculated blood volume of $0.10 \pm 0.01\%$ was not different from the saline injected value of $0.13 \pm 0.02\%$. The Fe^{59} incorporation observed when erythropoietin was injected alone is indicated in Fig. 2.

Injection of anti-E one or 2 days after initiation of erythropoiesis, when identifiable erythroid cells are present in the marrow, had no significant effect upon the subsequent ability of these erythroid cells to develop into mature red blood cells as measured by Fe^{59} incorporation. This indicates that anti-E did not exert a cytotoxic effect on these erythroid cells and that anti-E did not alter the ability of these erythroid cells to synthesize DNA, to divide, or to utilize Fe^{59} for hemoglobin synthesis. We have found that the iron levels and iron-combining capacities of plasma obtained from normal mice injected with anti-E are within normal limits and that absorption of anti-E with transferrin (Cohn Fraction IV-4, human) does not alter its neutralizing ability suggesting that anti-E does not disturb the transport of iron to the marrow. These observations support the concept that erythropoietin is not concerned with the maturation of erythroid cells, but only with the differentiation of stem cells into the erythroid series.

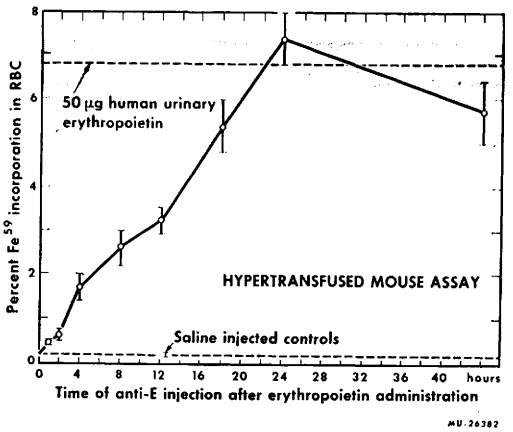

FIG. 2

Any interpretation of the effects of anti-E on the wave of erythropoiesis during the first 12 to 18 hours after injection of erythropoietin must be speculative. It seems reasonable to assume, although direct experimental evidence is lacking, that during these times a certain amount of the erythropoietin has acted in some fashion on receptive bone marrow stem cells, some may be destroyed in a non-selective manner without exerting any erythropoietic stimulation, and some free erythropoietin remains to stimulate stem cells

or be destroyed at a later time. Injection of anti-E apparently neutralizes the free erythropoietin which remains in the animal and further erythropoietic stimulation by this erythropoietin cannot occur. The fact that the erythropoietin-stimulated cells continue to differentiate and mature in the presence of anti-E provides further evidence that anti-E does not exert a cytotoxic effect even at earliest stages of erythroid maturation. The absence of any effect on these early stimulated erythroid cells suggests that the combining sites on the erythropoietin molecule are no longer available for an antigen-antibody reaction once erythropoietin has stimulated the cell. Possibly erythropoietin exerts its stimulatory effect within the stem cell rather than on the cell surface.

When erythropoietin is allowed to act for 12 hours before injection of anti-E, a sufficient number of stem cells have been stimulated so that their subsequent proliferation results in formation of enough red cells to give an Fe^{59} incorporation approximately equal to half of what would have been obtained if the erythropoietin had been allowed to act for 24 hours or if no anti-E had been injected. Reference to a dose response curve for human urinary erythropoietin obtained by using this same assay indicates that less than half the injected erythropoietin had stimulated erythropoiesis at this time. Thus, the rate of disappearance of erythropoietin in these hypertransfused mice is much slower than has been observed in rats(8). Erythropoietic stimulation becomes less and less as the time between erythropoietin and anti-E injections decreases, but even 0.5 hour exposure of the animal to erythropoietin is sufficient to stimulate some stem cells.

Why does erythropoietin require at least 24 hours to stimulate erythropoiesis maximally in these animals? The ability of erythropoietin to stimulate erythropoiesis and the magnitude of this stimulation during any time interval may depend upon a variety of factors, such as 1) size of the stem cell compartment, 2) availability of erythropoietin to cells within this compartment, 3) ability of a particular stem cell to respond when exposed to erythropoietin, and 4) competition for stem cells between erythropoietin and factors stimulating the differentiation of stem cells into other types of marrow cells. It seems reasonable to assume that within an hour or two after intravenous injection erythropoietin is available to all potential stem cells. The generation times of 8 to 12 hours generally found for rapidly dividing cells suggest that an increase in the stem cell compartment as the result of cell proliferation could not occur before 8 to 12 hours; however, after this time such a proliferation may be of importance. We are inclined, therefore, to attach more importance to the last two factors listed above in explaining the difference in the erythropoietic response observed when the same dose of erythropoietin is allowed to act for 2 and 8 hours.

Summary. Injections into normal mice of serum obtained from rabbits previously immunized with human urinary erythropoietin results in a progressive daily decrease in the relative numbers of erythroid cells in the bone marrow. Injections of this serum into polycythemic mice 24 to 44 hours after initiation of a wave of erythropoiesis with erythropoietin has no effect on the magnitude of the erythropoietic response, although injections of the serum one hour before or at the same time that exogenous erythropoietin is administered completely abolish the erythropoietic response. These results support the concepts that erythropoietin is involved in normal regulation of red cell production and that this regulation is the result of the effect of erythropoietin on the differentiation of stem cells. Some speculative interpretations of the effect of injections of the immune serum in polycythemic mice during the first 18 hours after injection of exogenous erythropoietin are also presented.

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Correction of Radiation-Induced Thrombocytopenia and Bleeding Tendency by Preserved Platelets.* (27603)

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The hemorrhagic tendency associated with thrombocytopenia is susceptible to modification only by administration of freshly collected platelets in suitable amount(1,2). Pharmaceutical compounds with alleged hemostatic properties have been found ineffective in *in vivo* experiments with thrombocytopenic animals(2). Processing of concentrated platelets by various methods for the purpose of storage has been associated with loss of the property of these blood elements to correct the hemorrhagic diathesis of thrombocytopenic patients(1) or animals(2,3). On the other hand, long-term storage of glycerol-suspended frozen RBC is possible(4,5) and this technic has been applied to preservation of concentrated platelets which, reconstituted and administered to dogs, remained present in the circulating blood in sizable number for at least 24 hours(6,7). The activity of these platelets against a bleeding tendency, however, has not been demonstrated(2,7). The practicality of preservation of blood elements by suspension in glycerol is also limited by the fact that the alcohol has to be separated from the cells by cumbersome technics before administration(5). The purpose of this report is to describe a technic by means of which platelets can be preserved indefinitely by quick freezing at liquid nitrogen temperature. The suspension fluid described by Meryman(4), which can be safely injected in the recipient, is used to minimize freezing injury to the cells. Sterility is maintained by processing the cellular suspension in a special receptacle. Platelets thus preserved, upon reconstitution, maintain their morphologic integrity and antihemorrhagic activity.

Materials and methods. The technics for quantitative estimation of bleeding tendency of irradiated thrombocytopenic rats have been described(1,2) and have been used to evaluate the efficacy of allegedly hemostatic agents in thrombocytopenic patients(8) or animals(2).

Blood for platelet separation was collected from the abdominal aorta of ether-anesthetized 400 to 500 g rats (discarded breeders). The blood was mixed with 3.8% sodium citrate in proportion of 1 to 10 and centrifuged immediately in a refrigerated International Centrifuge, Model PR-11. Brief (25 to 120 seconds) spurts of centrifugation at 3,000 r.p.m. yield plasma containing more platelets with less WBC and RBC contamination than plasma obtained after the usual 10-minute centrifugation at the moderate speed of 1000 r.p.m. The platelet-rich plasma was separated from the RBC, transferred to graduate centrifuge tubes and spun down for 30 minutes at 3500 r.p.m. The clear supernatant plasma was removed and 2 volumes of it mixed with one volume of the Meryman fluid (dextrose 29.2%, sodium citrate 1.28%, citric acid 0.4%)(4). An appropriate amount of this mixture was then added to the sediment to obtain a 20% concentration (v/v) of platelets. A homogeneous suspension was made by gentle mixing with a waxed applicator. No clumping was noted. The suspension was introduced into polyethylene tubing 0.125 inch in internal diameter.† The tub-

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† Intramedic Polyethylene Tubing PE 350, Clay-Adams, Inc., New York.