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Occurrence of Agglutinogens in Normoblasts.* (22261)

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It is well known that uptake of iron and hemoglobin synthesis occur in the normoblast. There is little information, however, as to the stage of development of the erythrocyte at which agglutinogens first appear. Bjorkman's(1) demonstration that normoblasts may be agglutinated by influenzal virus and a serum containing cold agglutinin suggests that receptors for agglutination make their appearance during this developmental period of the erythrocyte. In his experiments, the normoblasts were isolated from the peripheral blood of a patient with Di Guglielmo's erythro-leukemia; however, the cells were morphologically indistinguishable from myeloblasts. Inasmuch as neoplastic cells could have atypical immunologic characteristics, it seemed desirable to confirm these studies using normoblasts from a patient in which the cells were not of malignant origin.

An opportunity to study agglutinability of normoblasts arose in a patient with autoimmune hemolytic disease secondary to warm agglutinins. About a week following splenectomy, this patient had a hemolytic crisis at which time he released large numbers of nucleated red cells into the peripheral blood. During the period when the normoblast count exceeded 600 per 100 leukocytes, it was a simple matter to isolate them from the peripheral blood for agglutination experiments. A more detailed description of this case will be published elsewhere.

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Methods. To isolate the nucleated red cells, 1.0 ml of a 6% solution of dextran in 0.85% saline was added to 20 cc of the patient's freshly drawn blood in a large siliconized test tube. A 10% solution of sequestrene (disodium ethylenediamine tetracetate) was used as anticoagulant. The mixture was incubated at body temperature for 30 minutes. The supernatant plasma, rich in formed elements, was centrifuged at varying speeds, beginning at 600 rpm for 2 minutes. The residue was resuspended in saline and the supernatant plasma recentrifuged at progressively faster speeds. The resulting cell suspensions were checked for cellular content by means of the phase microscope. It was possible in this way to select test tubes which contained a reasonably pure suspension of normoblasts which were then washed 3 times in saline and prepared as a 4% suspension in 0.85% saline. Suspensions prepared in this way contained a minimal number of contaminating erythrocytes and lymphocytes, but no granulocytes or platelets were present. The normoblast suspension was brought in contact with equal portions of serial saline dilutions of serum from the same patient which contained a potent panhemagglutinin, active at 3°C and 37°C. The normoblast suspension was similarly added to serially diluted serum obtained from another patient with a high titer of cold hemagglutinins. The mixtures were incubated for 12 hours at 3°C and read macroscopically and under the phase microscope. The agglutinating effect of nor-

TABLE I. Agglutination of Normoblasts by Various Sera Obtained from Patient I.B. (Auto-Immune Hemolytic Anemia).

Anti-A serum	P*
Anti-B "	N
Anti-M "	P
Anti-N "	N
Antiglobulin serum	P
I.B. serum (patient)	P
E.S. " (cold agglutinin)	P
E.B. " (" ")	P
Normal A serum	N
B "	P
O "	P

* P = Positive; N = Negative.

mal human sera of various blood groups, in addition to typing sera of the AB and MN and Coombs' antiglobulin serum, were determined following incubation at 37°C for one hour. Siliconized glassware was used in all these experiments.

Results. The reaction of the normoblasts when brought into contact with various sera are listed in Tables I and II. It will be seen that the normoblasts in this patient were agglutinated by anti-A and anti-M serum, which corresponded to his blood type. The normoblasts were strongly agglutinated by antiglobulin serum as were his erythrocytes. Table II lists the titer of serum which resulted in agglutination of the normoblasts. The patient's serum agglutinated his normoblasts in a dilution of 1 to 16 and group O erythrocytes in a dilution of 1 to 8. The serum containing cold agglutinins agglutinated the normoblasts in a dilution of 1 to 256 and normal erythrocytes in a dilution of 1 to 1024.

TABLE II. Titration of Normoblast Agglutinins in Serum of I.B. (Patient) and E.S. (Cold Agglutinin).

	I.B.		E.S.	
Serum dilution	Normoblasts, 3°C	"O" RBC, 3°C	Normoblasts, 3°C	"O" RBC, 3°C
1:1	3+	2+	4+	3+
2	3+	2+	3+	2+
4	3+	1+	3+	2+
8	2+	1+	2+	2+
16	1+	0	2+	2+
32	±	0	1+	2+
64	0	0	1+	2+
128	0	0	1+	2+
256	0	0	1+	2+
512	0	0	±	1+
1024	0	0	0	1+
C	0	0	0	0

Comment. It has been demonstrated in this case that normoblasts derived from a patient with auto-immune hemolytic anemia have antibody receptors which are similar to those of mature erythrocytes. Apparently these receptors make their appearance relatively early in the development of the red cell.

Agglutinability of normoblasts by autoagglutinins and by antiglobulin serum constitutes direct evidence of the vulnerability of the bone marrow in immune varieties of acquired hemolytic anemia. The severity of anemia in auto-immune hemolytic disease may thus be attributed not only to random destruction of circulating erythrocytes but also may follow a direct attack on proliferating normoblasts. This would limit the ability of the bone marrow to compensate for hemolysis. That impaired erythropoiesis was a factor in hemolytic disease of the new born associated with anti-D antibodies was indicated by the studies of Giblett *et al.*(2). In the case she described, the presence of circulating antibody was correlated with limited erythrocyte production which was resumed following disappearance of anti-D antibodies. In their studies of auto-immune thrombocytopenia, Pisciotta, Stefanini and Dameshek(3) presented morphologic evidence to show that megakaryocytes in a normal recipient could be damaged by transfusion of plasma which contained a strong anti-platelet agglutinin. The damaging effect of the plasma on the megakaryocytes was only temporary and was correlated with a temporarily induced thrombocytopenia.

Summary and conclusions. 1. Normoblasts from a patient with auto-immune hemolytic anemia are agglutinable by type-specific sera, antiglobulin serum and sera containing cold and warm auto-hemagglutinins. 2. Agglutinogens make their appearance at the normoblast stage of development. 3. It is suggested that in acquired immune forms of hemolytic anemia, the bone marrow is also under direct attack by antibodies which may

aggravate the severity of anemia.

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Relative Growth-Promoting Activity in Tissue Culture of Co-Factors and the Parent Vitamins. (22262)

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It has been shown(1) that 2 mammalian cells in tissue culture, a human carcinoma cell (strain HeLa) and a mouse fibroblast (strain L), require nicotinamide, pyridoxal, thiamine, riboflavin, pantothenic acid, choline, and folic acid for survival and growth. The degree to which various precursors and conjugates could substitute for the corresponding vitamin in permitting the growth of the mouse fibroblast is here described.

Methods. The technics used in maintaining stock cultures, preparing replicate flasks for feeding with the experimental media, and evaluating the growth response in terms of the number of cells have been described(1-4). As in the previous vitamin experiments(1), specific deficiencies were produced by allowing the cells to grow for varying periods of time (4-14 days) in 1 liter Blake bottles, in the appropriate vitamin-free medium. During this preliminary period of vitamin depletion, the number of cells usually increased 2- to 6-fold in individual experiments; but in most of the experiments, multiplication had then stopped, and there were evidences of cell damage resulting from the vitamin deficiency (cf.(1)). The vitamin-starved cells were resuspended in the appropriate vitamin-free medium, counted, and aliquots of the suspension inoculated into a number of small replicate flasks. Twenty-four hours later, after the cells had adhered to the glass, they were fed with the experimental mixtures containing graded amounts of the vitamin or vitamin substitutes. The flasks were re-fed at 2-day intervals, and the cell count determined after 6-15 days' incubation. Since the cells at the time of the first feeding usually showed cytopathogenic evidence of vitamin depletion, it is apparent that at least 2 factors entered into the results obtained in the present experiments: the capacity of the individual compounds to revive the vitamin-depleted cells, as well as their capacity to support subsequent growth and multiplication. Accordingly, in a second type of experiment, described in the text in relation to folic acid, instead of using vitamin-depleted cells, normal cells were serially propagated in varying concentrations of the vitamin congeners. Diphosphopyridine (DPN) and triphosphopyridine (TPN) nucleotide, flavin adenine mononucleotide (FMN) and dinucleotide (FAD), and cocarboxylase were obtained from the Sigma Laboratories. The first 4 compounds were stated to be 95-98, 60-90, 100, and 65% pure, respectively; but in making stock solutions, all were handled as if they were pure material. Coenzyme A of 75% purity was obtained from the Pabst Laboratory. "Thiamin phosphate" was obtained by autoclaving a solution of cocarboxylase in 0.1 N HCl for 10 min. in a sealed tube. Crystalline pyridoxal phosphate (Palpo) was generously supplied by Dr. E. A. Peterson of the National Cancer Institute, National Institutes of Health.

Results. The results in a number of ex-

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