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Studies on the Pathogenesis of Aplastic Anemia in Thailand: Evidence of Immune-Mediated Mechanism

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SUMMARY **The pathogenesis of aplastic anemia in Thailand was studied by using *in vitro* progenitor cells culture. In 37 patients who had active disease, the numbers of colonies derived from erythroid and granulocyte-macrophage progenitor cells (BFU-E and CFU-GM) were markedly decreased both in the blood and bone marrow as compared to normal controls. Co-culture of patients' cells with normal blood cells was performed in order to verify an immunologically mediated mechanism. In 8 of 26 patients, there were very low numbers of colonies both BFU-E and CFU-GM in the blood and bone marrow with significant suppression of colony formation in co-culture. Suppressor cells may have caused the aplasia in these patients. The rest had low colony formation and no suppression in co-culture. These patients may have absent or defective stem cells. None had normal colony formation. Therefore, aplastic anemia in Thailand may result mostly from defects involving the stem cells. Only some patients had cell mediated suppression of hematopoiesis as detected by co-culture.**

Aplastic anemia is a disease of high prevalence in Thailand.[1] The causes of this disease may be associated with exposure to drugs, chemicals, radiation and infectious agents but may be unknown in a proportion of patients. Different mechanisms accounting for marrow failure in aplastic anemia have been proposed.[2] Those include absent or defective hematopoietic stem cells, abnormalities of the marrow microenvironment and abnormalities of regulatory cells or factors.[2] Identification of the pathogenic mechanism of marrow failure is of significance since it may provide the basis for the appropriate treatment. Patients having stem cell abnormalities would be candidates for marrow transplantation whereas those with an immunologically mediated suppression of hematopoiesis may respond to immunosuppressive therapy. Several methods have been introduced in order to identify the mechanisms of marrow failure in aplastic anemia. Cell mediated suppression of hematopoiesis in aplastic anemia has been demonstrated by two types of *in vitro* experiments, inhibition of normal colony formation by addition of blood or bone marrow cells from aplastic patients and enhanced colony formation by patient cells after removal of a subpopulation of lymphocytes.[3-10]

The present study reported the pathogenesis of aplastic anemia in Thailand using *in vitro* experiments. Hematopoietic progenitors were quantitated in the blood and bone marrow. Immune-mediated mechanism was explored using co-culture study between patients' cells as effector cells and normal blood cells as target cells.

MATERIALS AND METHODS

Patients

Thirty-seven patients diagnosed as having aplastic anemia during the period between May 1983 and December 1985 were studied. Criteria for diagnosis of aplastic anemia include peripheral blood pancytopenia and bone marrow hypocellularity. Patients who had peripheral blood pancytopenia and bone marrow hypercellularity with decreased megakaryocytes as denoted as dyshemo-

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poiesis were also included. Patients ranged in age from 6 to 54 years; there were 23 men and 14 women. *In vitro* experiments have been performed immediately after diagnosis. No blood transfusions had been given prior to the study except patient No. 20. All patients received therapy with androgen or androgen plus corticosteroids. Response to therapy was defined as follows: complete recovery: return of a normal hemoglobin concentration, white cell count of more than $4 \times 10^9/1$, platelet count of more than $100 \times 10^9/1$; partial recovery: improvement in hemoglobin level but white cell and platelet counts were still decreased; no response: patients remained pancytopenic.

In vitro hematopoietic progenitor cell culture

Peripheral blood and bone marrow samples were collected in heparinized syringes before starting treatment. Mononuclear cells (MNC) were separated on a Ficoll-Hypaque gradient, washed twice and resuspended in Iscove's modified Dulbecco's medium (IMDM, GIBCO, Grand Island, NY, U.S.A.). Cultures were prepared in methylcellulose as previously described.[11] Briefly, 5×10^5 blood MNC or 1×10^5 bone marrow MNC were cultured in 1 ml IMDM containing 30% fetal bovine serum (Flow laboratory, North Ryde, N.S.W, Australia), 1% bovine serum albumin (Behringwerke, Marburg, F.R.G.), 360 μg human transferrin (Behringwerke, Marburg, F.R.G.), 10^{-4} M α thioglycerol (Sigma, St. Louis, MO, U.S.A.) and 0.8% methylcellulose. One unit of step III erythropoietin (Connaught, Canada) or 10% human-placental conditioned medium (HPCM) was added for the growth of erythroid bursts (burst forming unit-erythroid, BFU-E) and granulocyte-macrophage colonies (colony forming unit-granulocyte macrophage, CFU-GM) respectively. Colonies were scored after 14 days of incubation in a fully humidified atmosphere of 95% air and 5% CO_2 at 37° C.

Co-culture studies

In order to verify an immunologially mediated mechanisms, co-culture studies were set up. Healthy medical personnel served as normal controls. 4×10^5 blood MNC from normal controls were used as target cells and patients' cells both from the blood (4×10^5 MNC) or bone marrow (1×10^5 MNC) were effector cells. The numbers of colonies in co-culture studies were compared to those in control cultures of 4×10^5 blood MNC from normal controls which had no added patients' cells. The percentage colony suppression was calculated as follows:

$$\% \text{ colony suppr.} = \frac{\text{No. colonies in co-culture} \times 100}{\text{No. colonies in normal control} + \text{No. colonies in patient}}$$

Previous studies have shown that when cells from normal controls were mixed and co-cultured, the mean number of colonies observed was approximately that expected by averaging the number of colonies observed in the individual cultures. In all experiments, the percent observed/expected was above 60 percent. (Issaragrisil, unpublished observation). The mean percent observed/expected from previous studies was 103 per cent (range 61-190).[12] Significant suppression was thus defined as percent (observed/expected) values below the minimum range (61 percent)[12] observed in co-culture of cells from normal controls.

Statistical Methods

Statistical analysis was performed using the Student's *t*-test for two-group comparison for unpaired continuous variables. Fister's exact test was used to test hypothesis of no association between *in vitro* results and clinical response. P values of less than 0.05 (two tailed) were considered significant.

RESULTS

Hematopoietic progenitor cells in peripheral blood and bone marrow

The mean numbers of BFU-E and CFU-GM in the blood and bone marrow of 37 patients were compared to those of normal controls (Table 1). All patients had abnormally low numbers of colonies both BFU-E and CFU-GM in cultures of peripheral blood and bone marrow ($p < 0.001$). Virtually no colonies in the blood and bone marrow could be detected in 11 patients (Fig. 1).

To test the possibility that this deficiency in colony formation by peripheral blood and bone marrow cells might be due to the presence of inhibitory cells, either blood or bone marrow MNC from 26 patients with aplastic anemia were studied for their suppressive effect on BFU-E and CFU-GM colony formation from normal blood MNC. The results of the experiments are shown in Table 2. Significant suppression of colony formation was observed in 8 of 26 patients. In only one patient (No. 15) inhibition was observed both for BFU-E and CFU-GM. Seven of 8 patients had suppression in co-culture only for CFU-GM. Among these, 2 patients (No.16 and 21) showed suppression when patients' blood and marrow cells were co-cultured with normal blood cells. In 4 patients (No. 4, 6, 9 and 20), suppression was evident only when patients' blood and normal blood cells were mixed.

Correlation of *in vitro* tests with clinical response

In 11 of 37 patients who had no colonies observed in the blood and bone marrow, 5 patients died; one failed to respond to therapy; 2 recovered and 3 were lost to follow up. Among the remaining patients, 11 recovered, 4 failed to respond, 4 died and 7 were lost to follow up. The results are summarized in Table 3. Although, the responders in patients who had colonies (11 of 19) were proportionally higher than

Table 1. Hematopoietic progenitors in the blood and bone marrow of patients with aplastic anemia compared to normal controls.

	Peripheral blood				Bone marrow	
	BFU–E/ $5x10^5$ MNC	BFU–E/litre ($x10^4$)	CFU–GM/ $5x10^5$ MNC	CFU–GM/litre ($x10^4$)	BFU–E/ $1x10^5$ MNC	CFU–GM/ $1x10^5$ MNC
Patients						
Range	0 – 3.5	0 – 1.7	0 – 11.5	0 – 1.5	0 – 12.5	0 – 45
Mean ± SEM	0.4 ± 0.2	0.2 ± 0.1	0.7 ± 0.3	0.2 ± 0.1	0.7 ± 0.5	6.0 ± 2.1
N	33	33	35	35	28	30
Normal controls						
Range	6 – 67.5	2 1 – 32.1	5 – 42.5	0.8 – 20.2	13 – 135	11 – 144
Mean ± SEM	22.6 ± 3.1	9.3 ± 1.4	12.2 ± 1.7	5.4 ± 0.9	58 ± 6.6	60.4 ± 9.4
N	22	22	26	26	27	16

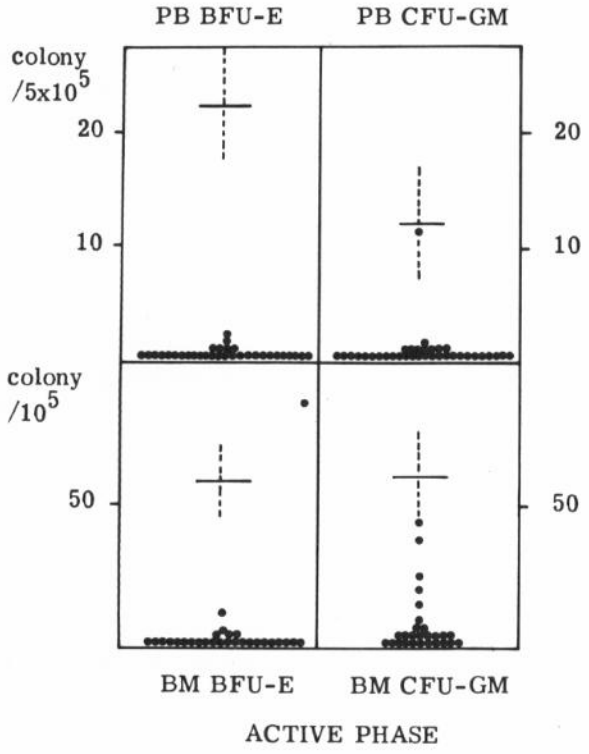

Fig. 1 The numbers of BFU–E and CFU–GM in the blood and bone marrow of patients with aplastic anemia compared to normal controls.

responders who had no colonies (2 of 8), Fisher's exact test showed no correlation between the *in vitro* test and clinical response ($p = 0.13$).

When the results of co-culture studies were correlated with the response to therapy in 8 patients who had inhibitory effect in co-culture, only 2 ptients (No. 15 and 22) recovered after treatment with androgen and corticosteroid. Two patients (No. 4 and 20) died of septicemia. One patient (No. 9) had a recovery after androgen therapy. Two patients (No. 16 and 21) were lost to follow up. One patient (No. 6) failed to respond to androgen and corticosteroid.

Of 18 patients who had low colony numbers without suppression in the co-culture assay, half of these received androgen therapy whereas the others received a combination of androgen and corticosteroid. In the first group, 4 patients (No. 11, 12, 23 and 25) recovered, 3 (No. 10, 24 and 26) died and 2 (No. 1 and 19) were lost to follow up. In the second group, 5 (No. 2, 5, 7, 17 and 18) had a recovery, 3 (No. 3, 8 and 13) failed to therapy and one (No. 14) died.

DISCUSSION

Theoretically the pathogenesis of aplastic anemia may be divided into three different types of defects based on *in vitro* experiments. They are defects of stem cells, the differentiative environment or suppressors. Patients with stem cell defects have low colony numbers without suppression in the co-culture assay. Since the progenitor cells assay does provide a differentiative environment *in vitro* capable of supporting normal hematopoiesis, it is unlikely that aplasia in those patients was due to an environmental defect *in vivo*. The absence of suppression in the co-culture assay suggests that some of these patients may be exclusion result from a stem cell defect.

Table 2. Results of co–culture of patients' cells and normal blood cells

Patient	Percent observed/expected			
	BFU–E		CFU–GM	
	+patient blood MNC	+patient marrow MNC	+patient blood MNC	+patient marrow MNC
1	530	600	125	175
2	156	119	97	111
3	ND	ND	220	200
4	91	132	57*	107
5	73	95	109	120
6	87	125	57*	138
7	133	ND	69	ND
8	ND	91	ND	86
9	ND	200	52*	ND
10	ND	150	ND	90
11	ND	ND	ND	140
12	ND	ND	192	154
13	ND	ND	ND	105
14	75	75	138	184
15	54*	37*	60*	ND
16	240	ND	57*	47
17	220	210	103	129
18	183	ND	113	65
19	91	ND	187	104
20	ND	ND	60*	83
21	ND	ND	20*	33*
22	ND	ND	183	20*
23	ND	125	ND	ND
24	ND	ND	220	ND
25	ND	ND	400	117
26	241	241	200	114

* = Significant suppression in co–culture
ND = Not done

Table 3. Outcome of the 37 patients with aplastic anemia based on the presence or absence of colonies in the blood and bone marrow.

	Number of patients		
	Death + nonresponders	Responders	Loss to follow up
No. colonies in the blood and bone marrow (N = 11)	6	2	3
Presence of colonies in the blood and bone marrow (N = 26)	8	11	7

It is still possible that some of these patients of low colony formation without suppression in the co-culture assay may in fact be due to suppressor cells that recognize surface antigens expressed only on the patients' cells and not on normal cells. These suppressor cells would only inhibit colony formation by the patients' cells and not by normal cells.

In patients who have normal colony formation without evidence of suppression in the co-culture assay, the pathogenesis of the disease should be due to an environmental defect. Marrow aplasia in this instance cannot be ascribed to absent progenitor cells since these cells are present and undergo normal proliferation and differentiation *in vitro*. Similarly a suppressor cell mechanism is unlikely since patients' cells are not capable of suppressing normal cells. In this study none had normal colony formation. Therefore, a microenvironmental defect as a cause of aplasia in our patients is rare.

Patients who have a combination of low colony formation and suppression in the co-culture assay are considered to have suppressor cells of hematopoiesis. From this study, 8 of 26 patients (37%) had a significant suppression of colony numbers in co-culture. By using these *in vitro* experiments, a proportion of patients who might respond to immunosuppressive therapy are identified. However interpreting the significance of *in vitro* colony inhibition by patients' cells is limited. Co-culture may be not sensitive enough to detect immune-mediated mechanisms in some patients. The mononuclear cell fraction which is cultured contains a heterogeneous cell population comprising a small fraction of stem cells and progenitor cells, lymphocytes and monocytes. Lymphocytes/monocytes and their lymphokines/monokines may affect colony formation. Added cells may act directly on progenitor cells or they could act via auxiliary cells present in the culture. Therefore in such a culture it is difficult to determine how exogenous factors or co-cultured cells are modulating colony growth.

Furthermore, it has been suggested that activated suppressor cells may be a consequence rather than a cause of aplasia. Study in untransfused aplastic patients before

and at various time intervals after their first transfusion indicated that within 24 hours of transfusion, T lymphocytes acquired the ability to inhibit colony growth.[6,13,14] This inhibiting activity was attributed to activated lymphocytes which are HLA-DR positive and express the IL-2 receptor. Activated or suppressor cells in aplastic anemia may thus result from allosensitization via blood transfusions. In this study, all patients (except patient (No. 20) had not received blood transfusions prior to the *in vitro* experiments. Therefore, inhibition in co-culture is not due to allosensitization.

We also set up autologous systems to explore immune mediated mechanisms in aplastic patients.[10] The experiments were designed to determine whether the numbers of detectable progenitor cells grown from patients' blood and bone marrow could be increased by removing various subpopulations of cells using specific monoclonal antibodies and complement. Although we observed higher numbers of colonies in some patients, the numbers of colonies were still very low and no correlation with the response to methylprednisolone therapy was detected.[10] Of more interest is that responders to methylprednisolone therapy had higher numbers of suppressor T cells and natural killer cells.[10] It is thus possible that these cells play a role in the pathogenesis of the disease and the patients recover following immunosuppressive therapy because these cells are destroyed. In this study response to immunosuppressive therapy was correlated with co-culture results. Two of the 3 patients who had inhibition in co-culture recovered after treatment with androgen and corticosteroid. One patient responded to androgen therapy alone.

IN vitro study prompts us to explore the various mechanisms of aplastic anemia. Most patients had very low numbers of hematopoietic progenitors both in the blood and bone marrow. By using the co-culture technique, the cause of the disease could be traced to a cell-mediated mechanism in 37% of the patients, whereas the rest had a stem cell defect.

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