Immunologic Abnormalities in an Animal Model of Chronic Hypoplastic Marrow Failure Induced by Busulfan

By C. A. J. Pugsley, I. J. Forbes, and A. A. Morley

The immunology of chronic hypoplastic marrow failure (CHMF, aplastic anemia) was studied in an experimental murine model of the disease induced by busulfan. B lymphocytes of peripheral blood, spleen, and bone marrow were reduced to 30%–40% and T lymphocytes of thymus, spleen, marrow, and blood were decreased to 20%–70% of control values. IgG and IgM antibody titers to sheep red blood cells were reduced to one-third of control levels, and splenic IgG, but not IgM, plaque-forming cells were fewer on day 7 after antigen stimulation. The proliferative responses to phytohemagglutinin or concanavalin A were reduced in cultures of peripheral blood lymphocytes, splenic lymphocytes, and thymocytes, and cutaneous delayed-type hypersensitivity induced by dinitrofluorobenzene was not detected in mice with CHMF. The results demonstrate disturbance of a variety of cellular and humoral functions and suggest that the disturbance was due to quantitative and possibly qualitative abnormalities of the cell types subserving these functions. The results suggest that residual cell injury, the lesion underlying experimental CHMF, is not confined to the myeloid stem cell but also involved cells of the lymphoid series.

CHRONIC HYPOPLASTIC MARROW FAILURE (CHMF, aplastic anemia) is commonly conceived of as a syndrome characterized by failure of delivery of erythrocytes, granulocytes, and platelets by a marrow that is hypoplastic but that shows no evidence of any other disease. This concept suggests that the primary disorder is restricted to the myeloid stem cell line, but there is some evidence that this is only partially true. Lymphocytopenia has been recognized for some time, deficiencies of B lymphocytes and monocytes have recently been reported, and disturbances of humoral and cellular immune function have been described.

An experimental model of CHMF can be produced in mice by treating them with a short course of busulfan. The treatment causes a type of damage to the stem cells of the marrow that has been termed “residual injury.” The characteristics of residual injury are that it is permanent, there is impairment of stem cell proliferation leading to moderate marrow failure, and complete failure of stem cell proliferation may ensue, leading to severe marrow failure and death. Studies to date indicate that this experimental model closely resembles the human disease.
The present study into the immune system in experimental CHMF was undertaken for a number of reasons: first, to test further the validity of experimental CHMF as a model for the human disease; second, to analyze the immune disturbances in more detail than would be possible in man; and third, to provide information on whether or not the lympho(myeloid) stem cell, as opposed to the myeloid stem cell, was involved in the disease. A final reason was to determine if the residual damage could affect cells other than those of the marrow, since this would have implications regarding the possible widespread biologic importance of the phenomenon.

**MATERIALS AND METHODS**

**Animals**

CHMF was produced by administration of four doses of busulfan to virgin female Balb/c mice as previously described. \(^7\) Injections of 20, 20, 10, and 10 mg/kg were given intraperitoneally at intervals of 2 wk, and the mice were then allowed to recover for at least 2 mo after the last dose before being studied. At the time of study the mice appeared well, with no obvious signs of infection, although from previous studies \(^9\) it was known that their marrows still manifested "latent" or "residual" injury.

**Preparation of Tissues**

Spleens and thymuses were teased apart using fine needles and the cells suspended in phosphate-buffered saline (PBS) supplemented with 5% fetal calf serum. Bone marrow cells were expelled from tibias and suspended in PBS. Peripheral blood lymphocytes were obtained by layering heparinized blood onto Ficoll-Hypaque gradients. \(^10\)

**Measurement of Cell Numbers**

**Nucleated cells.** These were counted with a hemocytometer.

**B lymphocytes.** B lymphocytes were enumerated by measuring the proportion of viable cells bearing surface immunoglobulin. A modification of the method of Taylor et al. \(^11\) was used. Cell suspensions were treated with Tris-buffered NH\(_4\)Cl to lyse red blood cells. \(^12\) Dead cells were removed by suspending the cells in glucose-PBS (nine parts 5\(\%\) glucose to one part PBS) and filtering the suspension through a small plug of cotton wool. Lymphocytes were incubated at 4°C with rabbit anti mouse gamma globulin conjugated with fluorescein isothiocyanate (ICN Pharmaceuticals) and were then washed four times before examination under ultraviolet light using a Leitz Ortholux II microscope. Between 150 and 500 cells per slide were examined, and the absolute number of B lymphocytes was calculated from the percentage as determined by immunofluorescence and the total cell count.

**T lymphocytes.** Thymus-derived lymphocytes were estimated by the use of anti-Thy-1.2 sera in the "two-stage" cytotoxicity test using trypan blue. \(^13\) Guinea pig serum was used as the source of complement after adsorption with agar to reduce cytotoxicity. \(^14\) Background cytotoxocity was 5\(\%\) to 10\(\%\) for all cell populations examined. Tests were performed in triplicate on mouse thymus, spleen, bone marrow, and peripheral blood.

**Immunization With Sheep Red Blood Cells (SRBC)**

Mice were injected intraperitoneally with 4 \times 10^8 SRBC on day 0 and groups of 4 or 5 mice were killed on days 3, 5, 7, 10, 14, and 21. Mice were bled by cardiac puncture, the serum was separated for antibody determination, and the spleens were assayed for absolute number of IgM and IgG plaque-forming cells (PFC). The number of splenic IgM PFC was determined by plating quadruplicate aliquots of spleen cells in the presence of SRBC. \(^13\) The number of IgG PFC was measured in quadruplicate using the method of Nordin et al. \(^16\) Briefly, plates were treated with concanavalin A (con A) (Calbiochem, grade A) and the IgG plaques developed by rabbit anti mouse IgG serum (ICN Pharmaceuticals) at a 1:100 dilution.
The IgM response to SRBC was determined by measuring the titer of hemolytic antibody.\textsuperscript{17} The antibody titer was regarded as that dilution giving 50\% lysis. The IgG response to SRBC was measured by incubating 0.1 ml serum with an equal volume of 2\% SRBC for 1 hr at 37\(^\circ\)C. The red cells were then washed four times in saline and finally resuspended in a suitable dilution of rabbit anti-mouse IgG. The titers were expressed as the last dilution of serum giving total hemagglutination. As an additional control,\textsuperscript{18} IgM antibody was inactivated by pretreatment with 0.1 \textit{M} 2-mercaptoethanol, but this had no effect on any day except day 3, when it led to a slight decrease in titer. Plaque size was determined by microscopic (\texttimes{}10) determination of the diameter of plaques formed from single cells on day 5.

**Lymphocyte Transformation**

Splenocytes or thymocytes were obtained for each experiment by removing and pooling spleens or thymuses from 3-5 mice. Whole blood lymphocyte cultures were derived from groups of 4 mice, whereas purified lymphocyte cultures were derived from groups of 8-10 control mice and 11-16 mice with CHMF. Thymus cells were cultured with con A, spleen cells and peripheral blood lymphocytes were cultured with phytohemagglutinin (PHA) (Wellcome Pharmaceuticals, HA 15 for splenocytes and HA 17 for peripheral blood lymphocytes).

Cells from thymus, spleen, and peripheral blood were cultured in RPMI-1640 medium (Commonwealth Serum Laboratories) containing bicarbonate buffer and supplemented with 6\%, heat-inactivated human serum, 2 mM glutamine, and L-arginine 150 \textmu{}g/ml.\textsuperscript{19} Cultured splenocytes (3 \times{} 10\(^6\) cells/tube) were harvested by filtration onto Whatman glass fiber paper. Each filter was washed three times with ice-cold saline, 5\%, trichloroacetic acid (TCA), water, and methanol in that order. Thymus and purified blood lymphocytes were cultured in microtiter trays (Limbro tissue culture grade). Quadruplicate 0.2-ml cultures were seeded with 4 \times{} 10\(^3\) cells that were ultimately harvested using a multiple automatic sample harvester. All cell cultures were grown for 72 hr at 37\(^\circ\)C in an atmosphere of 5\% CO\(_2\) and pulse labeled for the last 4 hr with 1 \mu{}Ci \(3^\text{H}\)-thymidine (specific activity 5 Ci/mmol; Radiochemical Centre, Amersham).

Whole blood cultures were performed by the method of Han and Pauly.\textsuperscript{20} Quadruplicate cultures were established in RPMI-1640 supplemented with 1\% bovine serum albumin (Cohn Fraction V, Sigma Chemicals Lot 55C-0074), glutamine, arginine, and antibiotics as above. Cultures containing 0.075 ml of whole blood were harvested on the third day, when the peak mitogenic response had been obtained.

**Delayed-type Hypersensitivity (DH)**

This phenomenon was measured in two experiments using the radioisotopic method of Vadas et al.\textsuperscript{21} The DH response was measured isotopically in the ears, and the severity of the response was assessed by the magnitude of the ratio of the radioactivity between the left and right ears. For each experiment, normal mice and mice with CHMF were divided into one test and two control groups, each containing 4-6 mice. The antigen used was dinitrofluorobenzene (DNFB) in a carrier solution of acetone:olive oil (1:1). The test group was challenged and sensitized with DNFB. The first control group was sensitized with DNFB but challenged with carrier solution, and the second control group was sensitized with carrier solution but challenged with DNFB. The method of sensitization and challenge and the time of injection of \(\text{\textsuperscript{125}}\)Iodo-2-deoxyuridine (specific activity 90-110 mCi/mg; Radiochemical Centre, Amersham) and of ear removal were as described by Vadas et al.\textsuperscript{21}

**Significance Testing**

The statistical techniques used were the Wilcoxon rank sum and \(x^2\) tests and analysis of variance.\textsuperscript{22}

**RESULTS**

**Cell Numbers**

In Table 1 are shown the number of total nucleated cells in spleen, thymus, and marrow; Fig. 1 shows the number of B lymphocytes in spleen, marrow, and
Fig. 4. Three views of three-dimensional scale model of bone marrow from hypertransfused rat; m, megakaryocytes.

Table 1. Number of Nucleated Cells/Organ

<table>
<thead>
<tr>
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<th>CHMF</th>
<th>Control</th>
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<tr>
<td>Spleen (x 10^{-8})</td>
<td>1.30 (1.39-1.21)</td>
<td>2.03 (2.11-1.94)</td>
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<tr>
<td>Thymus (x 10^{-7})</td>
<td>1.03 (1.32-0.80)</td>
<td>4.18 (5.07-3.45)</td>
</tr>
<tr>
<td>Marrow (x 10^{-6})</td>
<td>2.7 (3.3-2.3)</td>
<td>9.8 (10.3-9.3)</td>
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The results are based on 20 mice in each group and are expressed as the logarithmic mean with the mean plus 1 SE and the mean minus 1 SE in parentheses. For each organ there were significantly (p < 0.01) fewer cells in mice with CHMF.

blood and T lymphocytes in thymus, spleen, marrow, and blood. The number of B lymphocytes in thymus was too low to score with accuracy. In Fig. 1 each point for thymus, spleen, and marrow refers to the results from one mouse, whereas each point for B lymphocytes in blood is derived from blood pooled from two or three mice and each point for T lymphocytes in blood is derived from blood pooled from six to eight mice. The logarithmic mean number of B lymphocytes in mice with CHMF was 30\% - 40\% of control values, and the logarithmic mean number of T lymphocytes was 20\% - 70\%.

All of the low results for mice with CHMF were statistically significantly less than results for control mice, except for the number of T lymphocytes in peripheral blood. In this instance, although the observed logarithmic mean in mice with CHMF was 58\% of the mean observed for control mice, the difference did not quite reach statistical significance (0.1 > p > 0.05, two-tailed Wilcoxon test).

Response to Antigenic Stimulation by SRBC

The IgM and IgG responses to SRBC were measured in three separate experiments. The pooled results are shown in Fig. 2. The numbers of IgM and IgG plaque-forming cells (PFC) were measured in two separate experiments;

![Figure 1](image-url) Fig. 1. Number of T and B lymphocytes in mice with CHMF and in control mice.
the results are shown in Fig. 3. The IgM and IgG antibody responses in mice with CHMF were consistently and significantly (each $p < 0.005$) lower than those observed in normal mice, with the mean values being between one and two dilutions less, i.e., the titers were $\frac{1}{2}$ of those in control mice. There was no significant difference in the number of IgM PFC between mice with CHMF...
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hypertransfused rat marrow contained only one class of erythroblasts; i.e., all the erythroblasts in the cluster were at the same stage of maturation, as shown in Fig. 6. In contrast only 5% of the isolated erythroblastic islands from normal rat marrow contained only one class of erythroblast; 50% contained two classes, and 45% contained three classes of erythroblasts (Fig. 6).

DISCUSSION

The three-dimensional model of bone marrow described above reinforces the earlier hypothesis of Bessis, with respect to erythroblastic islands and their role in erythropoiesis and also demonstrates the special societal relationships in bone marrow proposed by Weiss and Chen. In addition it provides new information regarding these concepts.

The finding of individual clusters of erythroblasts in the marrow of hypertransfused rats (Fig. 4) supports the concept that erythropoiesis occurs in erythroblastic islands in situ. The geometric progression of the number of erythroblasts per cluster and the finding that all the erythroblasts in an individual cluster are at the same stage of maturation suggests that these groups all originate from a single precursor cell and that erythroid maturation occurs synchronously in individual clusters. The findings also support the general assumption that there are four or five divisions in the morphologically recognizable erythroid compartments. The finding of mixed clusters in normal marrow (Fig. 5) is presumably due to the following: (1) the eightfold higher number of erythroblasts that exists in the normal marrow places severe spatial constraints on the available space for individual clusters to develop without mixing with their neighbors; and (2) dispersion of developing erythroblasts in the marrow space adds to the spatial constraint and contributes to the mixing among neighboring groups.

A majority of isolated erythroblastic islands from marrow of hypertransfused rats contains erythroblasts at the same stage of maturation (Fig. 6), and these can be used for studies in vitro of clonal development. However, in spite of careful preparation of isolated islands, some of the erythroblasts are lost from the clusters, and one rarely finds the expected 2, 4, 8, 16, or 32 erythroblasts. In contrast, we as well as others have found erythroblasts at two or three stages of development in isolated erythroblastic islands from the marrow of normal rats (Fig. 6) and have interpreted this finding to indicate that many generations of erythroblasts develop in one cluster. However, in view of the high density of erythroblasts in the normal marrow and the consequent intermingling of erythroblasts from neighboring groups, it is difficult to ascertain whether the presence of two or three classes of erythroblasts in an isolated island truly represents the production of a multiple generation from a single precursor cell as proposed by Bessis, or is the result of a breakup along random lines of fracture.

The macrophage is invariably present in close association with all the erythroblasts, as previously shown by Bessis; however, its role remains a mystery. Its function in the removal of expelled erythroblastic nuclei by phagocytosis is certain, although its involvement in ferritin transport remains unclear. The recent speculations concerning the role of the macrophage in erythropoietin synthesis are attractive but have not yet been confirmed experimentally.

Lymphocyte Transformation

Preliminary experiments established that normal mice and mice with CHMF produced peak mitogen responses after 72 hr of culture.

and normal mice, but there were small but significant differences ($p < 0.025$) in the number of IgG plaques on days 7 and 10 in one experiment and days 5 and 7 in the other.

The diameter of splenic IgM plaques obtained on day 5 was measured as an index of the ability of single cells to produce antibody, but no significant difference was observed between normal mice and mice with CHMF.

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The dispersal of erythroblasts as maturation proceeds is one of the new findings of this study. The close packing of immature erythroblasts and their subsequent spreading suggests that significant changes must be taking place in the membrane surface properties with maturation that would lead to the breaking up of close physical contact. The observation of Bessis'7 that erythroblasts acquire motility but not locomotion with maturation suggests that the erythroblasts cannot move through the marrow on their own to enter the circulation. However, the possible significance of the observed motility in aiding the cells to disperse themselves in the marrow space to begin their migration toward the sinuses for eventual entry into the circulation must now be reconsidered.

The observation that erythropoiesis is not spatially restricted to the immediate vicinity of the marrow sinuses is in contrast to hypotheses of other investigators.6 A significant portion of the marrow space is occupied by the sinuses. Random sections would show a number of erythroblasts developing next to the sinuses, and might lead the observer to the inappropriate conclusion that erythropoiesis is restricted to the immediate vicinity of the sinuses. In both normal and hypertransfused marrow, however, we found some erythroblasts as far as 50 μm from the nearest sinus. This observation raises some interesting questions on how reticulocytes reach sinuses from their distant maturation sites.

Granulopoiesis has always been thought to occur in the marrow spaces away from the sinuses, with the granulocytes delivered into the circulation as a consequence of their intrinsic ability to move. Bone marrow reticulocytes have also been shown to be motile but presumably with no locomotion. Whether or not this motility nevertheless plays any role in the transport of reticulocytes to the marrow sinuses is now an open question. In contrast to the erythroblasts, every megakaryocyte found in the marrow studied was in direct physical contact with the sinus, reinforcing the hypothesis that the nonmotile platelets are released directly from these maturation sites into the marrow sinuses.

**ACKNOWLEDGMENT**

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antibody response to SRBC, and cellular immunity was abnormal as assessed by proliferative response to mitogens and delayed intradermal hypersensitivity to DNFB. Although the impairment of humoral immunity could have been due solely to the demonstrated decrease in number of B lymphocytes, it is known that the antibody response to SRBC is dependent on T lymphocytes and macrophages in addition to B lymphocytes. Abnormalities of T lymphocyte number and possibly function were shown in the present study. Decreases in blood monocytes were shown in a previous study, and preliminary studies from our laboratory suggest that tissue macrophages may be decreased in some but not all sites. Thus it is possible that the observed impairment of antibody response was due to deficiency and/or abnormality of several cell classes rather than being solely due to deficiency of B lymphocytes.

T lymphocytes were shown to be depleted in thymus, spleen, marrow, and possibly blood, but the abnormal proliferative response to mitogens cannot be explained solely on this basis, since all cultures with mitogens other than whole blood cultures were set up to contain the same number of test and control cells. The most straightforward explanation for the abnormal responses observed was that T lymphocytes from mice with CHMF were also qualitatively abnormal and showed an impaired proliferative response to stimulation. A similar abnormality of proliferation has already been demonstrated for the myeloid stem cell and the granulocytic progenitor cell.

However, the proliferative response to mitogens may also involve a population of adherent mononuclear cells, and it is conceivable that the abnormal responses observed in the present study were partly or wholly due to an abnormality of this population. The delayed intradermal hypersensitivity response is known to involve both T lymphocytes and macrophages, and the absent response in our experiments could have been due to quantitative or qualitative abnormality of one or both of these cell types. Data from studies of CHMF in man suggest that a macrophage defect was most probably the principal cause of the abnormality, since the degree of abnormality of DH in man correlates with the degree of monocytopenia and not with the degree of lymphocytopenia.

It is now recognized that suppressor lymphocytes may modulate the immune response to SRBC and to mitogens. There are also data from studies of CHMF in man that could be interpreted as indicating an overactivity of suppressor cells in the disease. These considerations raise the question of the possible etiologic role of suppressor lymphocytes in experimental CHMF. It seems unlikely that these lymphocytes, if increased, would be exerting a primary role, since the experimental disease is produced by treatment with busulfan. It is possible, however, that their presence might perpetuate part or all of the immunologic disturbance. Such a situation would be similar to the experimental model of agammaglobulinemia in chickens developed by Blaese et al., who showed that a population of suppressor cells developed secondary to immunodeficiency produced by early bursectomy. From present knowledge of the actions of suppressor cells it seems unlikely that an increase in their number could explain all of the numerical and functional immunologic abnormalities detected, although it might explain the impaired antibody response to SRBC and
impaired proliferative response to mitogens. We have not as yet examined immunologic function in experimental CHMF, but our studies on myeloid function have failed to provide evidence of enhanced activity of suppressor cells leading to marrow depression.

The most important points to emerge from the present study concern CHMF and the phenomenon of residual damage. The close parallel between the abnormalities detected in CHMF in man and in the experimental model of CHMF in the mouse provides further evidence for the validity of the model. The detection of abnormalities involving cells of the lymphoid system in addition to cells of the myeloid system indicates that the primary disturbance in CHMF involves either the common lymphomyeloid stem cell or cells of both differentiation pathways. Both alternatives may be correct, since they are not necessarily mutually exclusive.

Residual damage to the myeloid system may represent a persistent cellular lesion produced by some alkylating agents.\(^3\) The present study has shown that one of these, busulfan, may also produce residual damage to the lymphoid system. Insofar as acute effects are concerned busulfan is known to have a relatively greater effect on the myeloid system than on the lymphoid system, whereas other agents such as chlorambucil are known to have a relatively greater effect on the lymphoid system.\(^4\) Therefore it seems possible that some agents might produce a residual lesion of the immune system of such magnitude that immune failure might be the predominant clinical manifestation; if so, some cases of acquired immunologic failure might actually be due to residual damage produced by an unrecognized toxic agent.

The biologic importance of residual damage as a distinct form of cell injury remains to be determined. Nevertheless, the fact that two different cell lines have now been shown to be affected suggests that this type of cell damage may be more important and widespread than heretofore realized. Clearly, the question can be resolved only by study of other classes of cells.

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REFERENCES

Osmotic Fragility of Peripheral Blood Lymphocytes in Chronic Lymphatic Leukemia and Malignant Lymphoma

By P. Resnitzky and N. Reichman

The osmotic fragility (OF) of peripheral lymphocyte OF compared with those of the blood lymphocytes from patients with two control groups, and the difference was chronic lymphatic leukemia (CLL) and non-statistically significant (p < 0.001). In Hodgkin malignant lymphoma (ML) was CLL the increase in OF was more pronounced than in ML, and the shape of the curve was different from that in the other groups. The employment of peripheral blood lymphocyte OF as an additional diagnostic parameter in the diagnosis of CLL and ML is suggested.

RELATIVELY LITTLE INFORMATION is available concerning the osmotic behavior of lymphocytes in malignant lymphatic diseases. Moreover, evaluation of the published data is complicated by the marked lack of any standardized procedure. Nir et al. described a method in which a Fragiligraph was used to record curves of the osmotic fragility (OF) of leukocytes in a continuously decreasing salt concentration, achieved through dialysis of the cell suspension against water. Using this technique we have studied the OF of peripheral blood lymphocytes from patients suffering from chronic lymphatic leukemia (CLL) and malignant lymphoma (ML).

MATERIALS AND METHODS

Subjects
Lymphocytes from four groups of subjects were tested: (1) 15 healthy volunteers; (2) 10 patients suffering from nonhematologic nonneoplastic diseases; (3) 11 patients with CLL; (4) 10 patients suffering from non-Hodgkin malignant lymphomas. Tables 1-3 record clinical and laboratory details of the patients in the different groups.

Lymphocyte Separation
Peripheral blood was drawn into a heparinized syringe and the lymphocytes were isolated by a Ficoll-Metrizoat gradient centrifugation method. After three washings with veronal-saline buffer (0.85 NaCl, 0.1 M Na veronal, 0.1 M HCl, pH 7.4, 264 mosm), the cells were suspended in the buffer at a concentration of 10 x 10^6 cells/mL.

Osmotic Fragility
Lymphocyte OF curves were automatically recorded with the Fragiligraph according to the method described by Nir et al. Immediately before the tests were performed, the instrument was calibrated to 100% light transmission. A dialyzing microcuvette containing 40 μl cell suspension was held in a distilled water bath placed in the light path of the instrument.

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