Induction of Lymphomas in Mice by Busulfan and Chloramphenicol

E. Robin, M. Berman, N. Bhoopal, H. Cohen, and W. Fried

Abstract

Busulfan causes long-lasting defects in the hematopoietic stem cells and in the immune system of mice. We designed studies to determine whether chloramphenicol further damaged the already defective hematopoietic stem cells of mice that were pretreated with busulfan, and we unexpectedly observed that mice given injections of the combination of busulfan and chloramphenicol developed lymphomas in relatively high incidence. The disease is invariably associated with splenomegaly and enlargement of the thymus. Leukocytosis with lymphoblasts in the peripheral blood occurred in some affected mice. The malignant cell is a lymphoblast of thymic origin. Thirteen of 37 mice which received both busulfan and chloramphenicol developed lymphomas. An additional five of the remaining 24 mice without proven lymphoma died and were not autopsied. Twelve of the 13 proven lymphomas developed within 280 days from the start of the experiment. Four of the 35 mice treated with busulfan alone developed lymphomas, and an additional five of the remaining 31 died but were not autopsied. Two of 41 mice treated with only chloramphenicol developed lymphomas. Of the mice treated with either busulfan or chloramphenicol alone that developed lymphomas, all did so more than 280 days from the start of the experiment. None of the control mice developed lymphoma.

We conclude that both busulfan and chloramphenicol may induce lymphomas in mice that are not known to develop them spontaneously. The combination of both busulfan and chloramphenicol increased the frequency and accelerated the onset of the disease.

Introduction

Busulfan, an alkylating agent commonly used in therapy of patients with myeloproliferative disorders, is notorious for its propensity to cause long-term marrow hypoplasia. Morley and Blake (5), Morley et al. (6), and Pugsley et al. (8) have reported that the administration of busulfan to mice results in a prolonged suppression of the colony-forming unit (spleen) and colony-forming unit (cell) populations and impairment of T-lymphocyte function; yet, the peripheral blood counts remain nearly normal.

Chloramphenicol, a broad-spectrum antibiotic that impairs mitochondrial protein synthesis, has been implicated as a cause of aplastic anemia and leukemia in some patients (2, 4). However, the blood counts of the majority of patients are unaffected by moderate doses of the drug. One explanation of this phenomenon is that an occult preexisting stem cell defect predisposes some persons to the hematopoietic toxicity of chloramphenicol. Chloramphenicol does not affect the blood counts of normal mice.

We designed an experiment to determine whether the hematopoietic functions of mice, the stem cell compartments of which were damaged by exposure to busulfan, would be affected by chloramphenicol. In the course of these studies, we serendipitously observed that a significant number of mice treated with busulfan and chloramphenicol developed lymphomas. We shall report here on the incidence of lymphoma and some of the features of the disease.

Materials and Methods

Male BALB/c × A F1 (hereafter called CAF1) mice ages 6 to 8 weeks were used. One hundred mg busulfan were dissolved in 10 ml acetone and then diluted with 40 ml distilled water. One g chloramphenicol was dissolved in 100 ml 0.9% NaCl solution.

WBC, platelet counts, and hematocrits were performed on blood from the retroorbital sinus. WBC counts were performed with the Model B Coulter Counter, hematocrits were by the microcapillary method, and platelet counts were by phase microscopy.

Peripheral blood smears were air dried, fixed in methanol, and stained with Wright-Giemsa stain. Touch preparations of the spleen and smears of tibial bone marrow were air dried, fixed in methanol, and stained with Wright-Giemsa.

Organs (spleen, thymus, liver, kidney, lymph nodes) were fixed in Bouin's solution, dehydrated through ethyl alcohol and toluene, embedded in paraffin, sectioned at 4 μm thickness, and stained with hematoxylin and eosin. Bones were decalcified in EDTA after fixation with Bouin's solution and then treated as other organs.

Peripheral blood for cell surface marker determination was collected from retroorbital venous sinuses into heparinized capillary tubes. After sacrificing the mice by cervical dislocation, single cell suspensions of spleen and thymus were prepared by passing the tissue (finely cut) through a stainless steel screen and flushing through a 26-gauge needle. The cells were subjected to Ficoll-Hypaque (1) gradient separation to remove dead cells and to isolate the mononuclear cell population. Rabbit antisera to mouse T-cell antigen, IgG, IgM, and IgA, conjugated with fluorescein isothiocyanate, were obtained from Cappel Laboratories (Cochraneville, Pa.). The staining procedure used was that of Predhomme and Labaume (7). The mononuclear cell suspensions were adjusted to a concentration of 1 × 10^7 cells/ml in phosphate-buffered saline [0.01 M sodium phosphate 0.15 M sodium chloride (pH 7.2)] with 0.2% sodium azide and a solution containing 10 μg of bovine serum albumin per ml. One hundred μl of the suspension were transferred to a 10- x 75-mm test tube. One hundred μl of the antibody conjugate of appropriate dilution were added and mixed well (the dilutions were determined previously). This was incubated at room temperature for 30 min. The cell suspension was then washed 3 times with phosphate-buffered saline-bovine serum albumin. The resulting pellet was gently suspended after the final wash, transferred onto a slide, and covered with a coverslip. The cells were examined with a Zeiss microscope. At least 200 to 500 lymphocytes were counted, and the number of cells with positive fluorescence were counted.

Mice were initially divided into 4 groups, each with 45 mice. Two groups (B/C and B/O) were given injections of 0.5 mg (0.25 ml) of busulfan i.p. every 2 weeks for 4 doses, i.e., on Days 1, 15, 29, and...
43 of the experiment. The other 2 groups (O/C and O/O) received injections only of the diluent, i.e., acetone plus distilled water. After a 20-week rest period, on Day 183 of the experiment, there were 78 mice left in the B/C and B/O groups and 88 mice left in the O/C and O/O groups. The remaining mice died within 24 hr of injection, apparently of complications of the injections. The groups designated B/C and O/C then received 2.5 mg (0.25 ml) of chloramphenicol 5 days per week for 5 weeks, i.e., from Day 183 to Day 218 of the experiment. Groups B/O and O/O received injections of only 0.9% NaCl solution in the same frequency. Prior to the development of lymphoma, 3 mice from each group were removed for cell surface marker determinations of peripheral blood, thymus, and spleen, leaving the following number of mice in each group: B/C, 37; B/O, 35; O/C, 41; and O/O, 41.

Blood was to be obtained from the retroorbital sinus of 5 mice per group every week for determination of hematocrit, WBC, platelet, and differential counts (no single mouse was to be sampled more than 2 times during the study). The original plan was to continue the study for a total of 20 weeks after the initial injection of chloramphenicol, i.e., Day 323 of the experiment. However, because of the development of lymphomas, this plan was slightly modified. After the initial observation of lymphoma on Day 235 of the experiment in one mouse of the B/C group, we continued the initial protocol. However, when 2 more mice in the B/C group developed lymphomas on Day 240 of the experiment, we introduced the following modifications. All mice were inspected daily (Monday through Friday). Mice that appeared obviously ill or had distinctly protuberant abdomens and/or palpably enlarged spleens had blood counts performed, were sacrificed, and were autopsied. The spleens were weighed, touch preparations were made, and the following tissues were submitted for histological sections: spleen; thymus; bone marrow; axillary lymph nodes; liver; and kidney. Histological sections of spleen were also obtained from randomly selected mice that did not apparently have lymphomas. The T- and B-lymphocyte content of the peripheral blood, thymus, and spleen of 6 lymphomatous mice (3 B/C and 3 B/O) as well as 6 control mice (O/O) was determined after the onset of lymphomas. As indicated above, the T- and B-lymphocyte content of peripheral blood on nonlymphomatous mice (3 B/C and 3 B/O) as well as 6 control mice (O/O) was determined after the onset of lymphomas.

In a total of 8 mice with lymphomas, one-half of the spleen was removed immediately postmortem; suspensions were made, and $10^9$ or $10^8$ nucleated cells in 0.5 ml Hanks’ solution were injected into each of 10 male CAF, mice, ages 6 to 8 weeks, to determine whether the lymphoma was transplantable.

On Day 350 of the experiment, all surviving mice were sacrificed after obtaining blood for determination of the hematocrit and WBC. The spleens were weighed, and any suspicious organs were fixed, sectioned, and stained for histological examination.

As indicated in “Results,” although an attempt was made to identify moribund mice and to determine whether they had developed lymphomas, a total of 10 mice (5 in B/C and 5 in B/O) died and were not autopsied.

**RESULTS**

Table 1 shows the results of hematocrit, WBC, and platelet counts prior to treatment with chloramphenicol, immediately after chloramphenicol, and 6 weeks after completing treatment with chloramphenicol (mice in B/O and O/O received diluent instead of chloramphenicol). There was considerable variability of the WBC in the 4 groups, with no statistically significant differences among the experimental groups. There were no consistent differences in the hematocrits or platelet counts of mice in the various experimental groups.

Table 2 shows the blood counts of lymphomatous mice. All had significant decrease in hematocrits and platelet counts.
E. Robin et al.

Fig. 2 shows the morphology of lymphoma cells. The neoplastic cells are larger than the lymphocytes in normal splenic white pulp. In tissue section, the nuclei of lymphoma cells are leptochromatic, while in smear preparations, the chromatin is delicate and finely distributed. The nucleoli generally are not evident; however, when seen, they are usually single and small to medium in size. The ratio of nucleus to cytoplasm is high, and the cytoplasm is basophilic and agranular.

All mice with lymphomatous spleens had enlarged thymus and lymph nodes, which histologically were diffusely invaded by neoplastic cells resulting in effacement of normal architecture.

The liver and kidney were found to be involved in some lymphomatous mice. The liver contained a diffuse infiltration of neoplastic cells, that was most marked in the portal areas and less prominent in the sinuses of the hepatic lobules. In the kidney, the interstitial tissue was diffusely infiltrated by neoplastic cells.

Table 4 shows the results of cell surface marker determinations on lymphomatous and control mice. In the mice with lymphomas, 87 to 100% of the lymphocytes in the blood, spleen, and thymus had cell surface markers, indicative of T-cell lineage. T- and B-cell markers done on peripheral blood of treated, nonlymphomatous mice revealed markers similar to those of control mice.

Transplantation of spleen cell suspensions from 8 of 8 mice with lymphoma resulted in clinically evident lymphomas in 100% of the recipients within 3 weeks when injected with 10^8 cells and within 4 weeks when injected with 10^6 cells. All mice that received 10^6 cells were dead within 5 weeks, and all mice that received 10^8 cells were dead within 7 weeks.

DISCUSSION

The studies reported in this paper were undertaken initially to determine whether in mice chloramphenicol further influences hematopoiesis already altered by busulfan. Serendipitously, we observed that mice treated with both chloramphenicol and busulfan developed lymphomas earlier and more frequently than did those treated with either agent alone. No lymphomas were detected in control CAFt mice, and this hybrid strain is not known to develop any tumor in particularly high incidence.

Morley and Blake (5) noted that mice treated with busulfan alone developed lymphomas but did not describe these neoplasms in detail. It should also be noted that their report concerned studies done with Swiss Webster mice, a totally unrelated strain to the CAFt mice used in our studies. The lymphomas which developed in CAFt mice treated with busulfan and chloramphenicol were of T-cell type and almost invariably presented with splenomegaly. Lymphocytosis with a leukemic peripheral blood picture was present in some but not all mice. The thymus, lymph nodes, liver, kidney, and bone marrow were infiltrated to varying extents in many autopsied animals with lymphoma. The mechanism by which busulfan and chloramphenicol exert their lymphomagenic effects on CAFt mice is not known. Possibly, by interfering with immune function, they permit the propagation of virally transformed cells (8). We are currently evaluating this possibility.

Another possible mode of action is that these agents directly cause or facilitate the transformation of lymphocytes to a malignant growth pattern.

Yunis et al. (10) demonstrated that conversion of chloramphenicol to a nitroso derivative creates cell cycle changes similar to those of nitrosoureas. They then speculate that this compound may be responsible for the chloramphenicol-induced aplastic anemia and acute leukemia seen in some patients (2, 4). That busulfan predisposes a host to the conversion of chloramphenicol to its nitroso derivative is intriguing in light of our findings but not demonstrated as of now.

The protocol described here for induction of lymphoma in mice may prove to be an excellent model for studying spontaneously occurring lymphomas, since the incidence of lymphoma in our treated mice is relatively high. Also, since patients treated with chemotherapeutic agents have been shown to develop lymphomas several years after induction of remission (3, 9), it is possible that further studies of the mechanism by which busulfan and chloramphenicol induced lymphomas will help to improve our understanding of this phenomenon. Finally, chloramphenicol has been shown to produce not only aplastic anemia but, in some patients, also an acute nonlymphocytic leukemia (2, 4). Perhaps, further study of this phenomenon in mice will shed some light on the occurrence of this event in humans.

Table 4

<table>
<thead>
<tr>
<th>Source of lymphocytes</th>
<th>T/B^a lymphoma (%)</th>
<th>T/B control (%)</th>
</tr>
</thead>
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<tr>
<td>Peripheral blood</td>
<td>87/11</td>
<td>68/16</td>
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<tr>
<td></td>
<td>96/3</td>
<td>80/12</td>
</tr>
<tr>
<td></td>
<td>100/0</td>
<td>78/14</td>
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<tr>
<td></td>
<td></td>
<td>80/14</td>
</tr>
<tr>
<td>Spleen</td>
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<td>67/31</td>
</tr>
<tr>
<td></td>
<td>100/1</td>
<td>53/46</td>
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<td></td>
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<td>60/57</td>
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<tr>
<td></td>
<td></td>
<td>66/57</td>
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<tr>
<td>Thymus</td>
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<td></td>
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^a T/B, percentage of T-cells/percentage of B-cells.
REFERENCES


Fig. 1. Photomicrograph of normal (A) and lymphomatous (B) spleens. A, normal spleen showing red pulp (RP) with its megakaryocytes (arrows) and other myeloid cells. Lymphoid nodule of white pulp (WP). B, red and white pulp diffusely occupied by lymphomatous cells; some megakaryocytes (arrows) remain. CAP, capsule. × 100.

Fig. 2. Photomicrograph of lymphocytes of white pulp of normal spleen (A) and lymphoma cells of involved spleen (B). × 1000.
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