Chloramphenicol Induces Abnormal Differentiation and Inhibits Apoptosis in Activated T Cells

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Abstract
Chloramphenicol is a broad-spectrum antibiotic used for the treatment of many infectious diseases and has become one of the major seafood contaminants. Hematologic disorders such as aplastic anemia and leukemia induced by chloramphenicol are a major concern. However, the mechanism underlying chloramphenicol-induced leukemogenesis is not known. By investigating the effects of chloramphenicol on the activation of mouse T cells stimulated with anti-CD3 antibody or staphylococcal enterotoxin B, we found that chloramphenicol induces the differentiation of activated T cells into lymphoblastic leukemia-like cells, characterized by large cell size, multiploid nuclei, and expression of CD7, a maker for immature T cells and T-cell lymphocytic leukemia, thus phenotypically indicating differentiation toward leukemogenesis. High expression of cyclin B1, but not p53, c-myc, and CDC25A, was detected in chloramphenicol-treated activated T cells, which may relate to abnormal cell differentiation. Chloramphenicol inhibited the activation-induced cell death of mouse and human T-cell receptor–activated T cells by down-regulating the expression of Fas ligand. Our findings show that abnormal cell differentiation and inhibition of apoptosis may contribute to the development of leukemia associated with clinical applications of chloramphenicol.

Introduction
Chloramphenicol is an antibiotic originally isolated from Streptomyces venezuelae in 1947. It has a bactericidal effect on a broad range of bacteria, as well as Rickettsia, Chlamydia, and Mycoplasma. Because chloramphenicol is relatively inexpensive to manufacture, highly effective in combating infections, and able to pass the blood-brain barrier, it is still widely used in developing countries (1–6). However, because of its potential toxicity to the hematopoietic system, chloramphenicol is only occasionally used in developed countries as a substitutional therapy for some infections caused by Haemophilus influenzae, Streptococcus pneumoniae, Salmonella typhi, and Neisseria species. The major side effects found in patients treated with chloramphenicol include reversible bone marrow depression, aplastic anemia, and leukemia. Epidemiologic studies indicate that 1:30,000 to 1:45,000 patients receiving chloramphenicol treatment go on to develop leukemia. Recently, chloramphenicol has become one of the major contaminants of farmed shrimps and fish, with some products containing excessive chloramphenicol residue, raising new concerns about its toxicity.

Leukemia has been linked to various risk factors, such as heredity status, chronic virus infection, radiation, chemical contaminants, and medications. Based on epidemiologic studies, chloramphenicol has been strongly correlated with leukemogenesis (7). One research group has successfully induced leukemia in chloramphenicol-treated toads (8). However, the molecular mechanism through which chloramphenicol induces leukemogenesis is still unclear. Nevertheless, nitrobenzene is a substituent chemical moiety in chloramphenicol and nitrobenzene is a long known carcinogen. It is possible that the transforming ability is related to the nitrobenzene group of this antibiotic. Pharmacologically, chloramphenicol reversibly binds to the 50S ribosomal subunit and prevents the transfer of amino acids during peptide chain elongation in bacteria. Chloramphenicol also has effects on protein synthesis in rapidly proliferating but not resting mammalian cells. Thus, it is possible that leukemia induced by chloramphenicol may directly change the expression of genes associated with cell cycle and apoptosis.

Much evidence has shown that the development of leukemia is related to gene dysregulation. c-myc is a transcriptional factor for control of cell proliferation, and high expression of c-myc has been found in leukemia (9, 10). p53 is a tumor suppressor gene, and mutations causing p53 dysfunction have been detected in patients with different types of leukemia (11–13). Cyclin B1, which plays a critical role in the regulation of cell cycle progression, was recently identified as an oncogene. Overexpression and/or unscheduled expression of cyclin B1 are always detected in the cells from leukemia and other tumors (2, 14–19). Accumulating evidence indicates that CDC25A can be oncogenic, and its overexpression is frequently shown in a large number of tumors (20–23). Moreover, leukemia is also considered a disorder of dysregulation of apoptosis because alterations in Fas and Fas ligand (FasL) expression have been found in leukemia (24, 25). These data indicate that leukemia is a multiplex disorder resulting from dysfunction in various genes, often combined with environmental causes.

To explore the mechanism by which chloramphenicol induces leukemia development, mouse primary splenocytes were used as a cell model for morphologic and molecular studies in vitro. Mouse primary splenocytes were activated with anti-CD3 in the presence or absence of chloramphenicol for a prolonged time. We found that chloramphenicol promoted abnormal T-cell differentiation and allowed the formation of leukemia-like cells, whereas in cultures without chloramphenicol all cells eventually died and no abnormal cells were found. These cells survived in culture for months. They expressed high levels of cyclin B1 and very low level of FasL. In addition, these abnormal cells also expressed high level of CD7.
a hallmark of immature lymphoblastic leukemia. Therefore, this finding for the first time provides direct evidence that chloramphenicol induces leukemogenesis in mammalian T cells.

Materials and Methods

Cell culture and stimulators in vitro. Spleens were taken from 6- to 8-week-old BALB/c mice and pulverized between two autoclaved grass slides in RPMI 1640 (Invitrogen), and a single-cell suspension was obtained by passing through a 40-μm-diameter nylon mesh (BD Biosciences). The cells were washed twice in RPMI 1640 and then cultured at 2×10⁶ to 5×10⁶ per mL density in complete RPMI 1640, containing 10% fetal bovine serum, 10% L-glutamine, penicillin, and streptomycin. Antibody against CD3 (145-2c-11) at 2.5 μg/mL or staphylococcal enterotoxin B (SEB; Sigma-Aldrich) at 5 μg/mL was used to activate T cells in the presence or absence of chloramphenicol at 500 μg/mL for 1 week. The cells were then maintained in complete RPMI 1640 with interleukin-2 (IL-2) at 50 to 100 units/mL for cell survival studies. Cell differentiation and morphologic changes were observed by light microscopy.

Cell staining. For morphologic study, cell smears were prepared by spreading of cultured cells on a glass slide and allowing them to air dry. The Giemsa reagent (Sigma-Aldrich) was used to stain the cells according to the manufacturer’s protocol. Cellular morphologic characteristics were examined under light microscopy. For immunofluorescence staining, freshly cultured cells were washed twice in ice-cold staining buffer (1× PBS with 2% fetal bovine serum and 0.01% sodium azide). Cell staining was carried out in the same staining buffer with FITC-conjugated anti-CD3 (eBioscience) and anti-CD7 plus phycoerythrin (PE)-conjugated anti-goat IgG (Santa Cruz Biotechnology) following the supplier’s protocols. After washing twice with cold staining buffer, the cells were mounted with ProLong antifade reagent (Molecular Probes) and covered with a glass coverslip. Fluorescence was analyzed under a Nikon fluorescence inverted microscope (Eclipse TE 2000-S, Nikon).

Flow cytometric analysis. Human T-cell lines Jurkat and Jcam (kind gifts of Dr. Gordon Mills, M. D. Anderson Cancer Center, Houston, TX) were activated by ionomycin at 100 nmol/L in culture medium and mouse T-cell lines A1.1 and IE5 in plates coated with anti-CD3 at 2.5 μg/mL. Different concentrations of chloramphenicol were added with the stimulators as indicated. After incubation at 37°C for overnight, the cells were harvested and washed twice with cold 1× PBS. The cell pellets were then resuspended in propidium iodide staining buffer (1× PBS with 20 μg/mL propidium iodide, 0.2% saponin, and 50 μg/mL RNase) and incubated for 30 min at room temperature. Flow cytometry analysis was carried out with a FACScan (Becton Dickinson). Cell cycle distribution is indicated by DNA content revealed by propidium iodide staining. After gating out cell fragments, the population of sub-G₀ phase on the histogram plot represents apoptotic cells. For cell surface marker testing, the mouse cell line IE5 was stimulated with and without anti-CD3 in the presence or absence of chloramphenicol at 500 μg/mL. After incubation at 37°C for 6 h, the cells were harvested and washed twice with cold staining buffer. The FITC-conjugated anti-Fas antibody and PE-conjugated anti-FasL antibodies (BD Biosciences) were used for cell surface staining and analyzed by flow cytometry.

Northern blot. Mouse splenocytes were stimulated with anti-CD3 in the presence or absence of chloramphenicol at 500 μg/mL for 1 week. Total RNA was extracted using TriPure reagent and its protocol (Roche). RNA (20 μg) of each sample was separated on a 1% denaturing formaldehyde agarose gel and then transferred to a nylon membrane (Amersham...
Biosciences). The membrane was repeatedly hybridized with \[^{32}P\]dCTP-labeled probes for cyclin B1, p53, c-myc, and CDC25A. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal quantitative control.

**Regulatory T-cell population.** BALB/c mouse splenocytes were stimulated with anti-CD3 alone (2.5 \( \mu \)g/mL) or plus chloramphenicol (500 \( \mu \)g/mL) in complete RPMI 1640 for 1 week. The population of CD4^+CD25^+ cells was measured by flow cytometric analysis after staining with FITC-conjugated anti-CD4 and PE-conjugated anti-CD25 (eBioscience). FoxP3 expression in CD4^+CD25^+ cells was further analyzed by real-time quantitative PCR using SYBR Green PCR Master Mix (Applied Biosystems). The primer sequences were the following: TTCATGCATCAGCTCTCCAC (sense) and CTGGACACCCATTCCAGACT (antisense). GAPDH expression was analyzed simultaneously as an internal quantitative control.

**Results**

Chloramphenicol promotes abnormal differentiation of leukemic cells. The most suspected transformations induced by chloramphenicol are acute lymphoblastic leukemia and acute myelogenous leukemia, both of which are derived from proliferating cells. Because our preliminary experiments with in vitro culture of mouse splenocytes with chloramphenicol alone did not produce abnormal cells, no matter what concentration of chloramphenicol was used or how long the cultures were maintained, we believe that the effect of chloramphenicol is exerted on proliferating cells. Epidemiologic study of chloramphenicol-induced leukemia has shown that it is always related to a history of bacterial infection. We thus believe that some bacterial superantigens, such as SEB, may work synergistically with chloramphenicol by inducing T-cell proliferation. In these proliferating cells, chloramphenicol may promote abnormal differentiation by altering the expression of some cell cycle–related genes. By stimulating mouse splenocyte with anti-CD3 or SEB in the presence or absence of chloramphenicol for 1 week, we found that chloramphenicol at 400 to 600 \( \mu \)g/mL resulted in the appearance in culture of many large cells, which were not present in control cultures activated without chloramphenicol or treated with chloramphenicol alone (Fig. 1A). These abnormal cells were morphologically similar to leukemic cells, characterized by very large cell size and multiploid nuclei. The same cultured cells were also analyzed for DNA content with propidium iodide staining. The results show that chloramphenicol not only induces additional mitosis, as indicated by the high diploid peak, but also results in many multiploid cells, changes that were identical to those revealed by microscopy (Fig. 1B). Significantly, when we continued to culture the chloramphenicol-treated activated T cells supplemented with IL-2 for up to a month, many large cells continued to survive (Fig. 2A), whereas all cells activated in the absence of chloramphenicol died off (Fig. 2B). Furthermore, by immunofluorescence staining, we found that all of the surviving cells express CD3 and only the large cells also express CD7, a marker of immature T leukemic cells (Fig. 2C and D). This phenotypic change may represent a differentiation process toward leukemogenesis. Taken together, these results show that chloramphenicol induces abnormal cell differentiation to leukemia-like cells.

Overexpression of cyclin B causes abnormal cell differentiation. The precise execution of cell cycle events is tightly controlled by concerted activation and inhibition of the expression of various genes and the activities of their products (10, 26–28). Dysregulation of critical genes in this process leads to abnormal cell proliferation and differentiation. To understand the mechanism of chloramphenicol-mediated abnormal cell differentiation, we analyzed the expression of several cell cycle–related genes. We found that the expression of cyclin B1 is dramatically increased in cells treated with anti-CD3 and chloramphenicol, whereas the expression of other genes, such as p53, CDC25A, and c-myc, was not significantly altered (Fig. 3). Because cyclin B1 regulates the transition in the mitosis phase of the cell cycle, overexpression of...
cyclin B1 may result in excessive nuclear division and the formation of leukemic cells with multiploid nuclei. It has been shown that treatment of cancer cells with butyrolactone I causes accumulation of cyclin B1 and formation of cells with multiple nuclei (29). Even in plants, when a nondegradable cyclin B1 is expressed, it also causes defective G2-M and increase in cells with multiple nuclei (30).

Chloramphenicol inhibits activation-induced cell death. Activation-induced cell death (AICD) is a key mechanism for maintaining cellular homeostasis during lymphocyte development, immune responses, and tumorigenesis. Although mouse splenocytes at rest do not proliferate, they do exhibit dramatic proliferation potential following stimulation of anti-CD3 or SEB. Previously activated T cells will undergo apoptosis after reactivation. This apoptosis, however, can be significantly inhibited by chloramphenicol in our experiment. We found that activation of chloramphenicol-treated cells displays much less apoptosis than cells treated with anti-CD3 alone (Fig. 4A). To further confirm this finding as a universal biological phenomenon, different concentrations of chloramphenicol were also applied to inhibit AICD in the human T-cell lines Jurkat and Jcam as well as the mouse T-cell hybridomas A1.1 and IE5. As shown in Fig. 4B and C, chloramphenicol clearly blocked AICD in all four cell lines, although these cells were activated by different stimuli. Our data show that chloramphenicol not only promotes abnormal differentiation of splenocytes into leukemia-like cells but also promotes the

Figure 3. Chloramphenicol induces high cyclin B1 expression. The expression of cyclin B1, p53, c-myc, and CDC25A was analyzed by Northern blotting in splenocytes treated by anti-CD3 antibody in the presence or absence of chloramphenicol. Chloramphenicol significantly induced overexpression of cyclin B1, but not p53, c-myc, and CDC25A. GAPDH served as an internal quantitative control.

Figure 4. Chloramphenicol inhibits apoptosis in mouse primary cells and several cell lines. Mouse splenocytes were stimulated by anti-CD3 with graded concentrations of chloramphenicol, as indicated. DNA content was determined by flow cytometric analysis after staining with propidium iodide. The sub-G0 population (M1) represents apoptotic cells. A, chloramphenicol significantly inhibited AICD compared with the cells stimulated with anti-CD3 in the absence of chloramphenicol. Mouse T-cell lines A1.1 and IE5 were stimulated with anti-CD3-coated plates, and human T-cell lines Jurkat and Jcam were stimulated by ionomycin (100 nmol/L). Different concentrations of chloramphenicol were added to the culture, as indicated. Apoptosis was examined by DNA content as in A. B, the inhibition of apoptosis by chloramphenicol correlated well with its concentration. C, the effect of chloramphenicol on AICD in IE5 cells is representative.
survival of these cells by inhibiting apoptosis. We believe that these two properties of chloramphenicol could be key to its leukemia-inducing potential.

**Chloramphenicol blocks FasL expression induced by T-cell receptor activation.** AICD in T cells is mediated by induction of the expression of Fas and FasL and their subsequent interaction. Blocking this interaction with TR6, Fas fusion protein, or monoclonal antibodies against FasL can completely prevent AICD (31). Because chloramphenicol is believed to affect mitochondrial protein synthesis, we examined whether the effect of chloramphenicol on AICD is exerted through an effect on cytochrome c or the Fas pathway. We found that chloramphenicol did not change cytochrome c levels and did not inhibit apoptosis induced by anti-Fas antibody. Because AICD is largely dependent on the Fas-FasL pathway, these results indicate that chloramphenicol inhibits AICD at a step before activation of the Fas receptor. To understand the molecular mechanism by which chloramphenicol inhibits apoptosis, we examined changes in Fas and FasL expression in chloramphenicol-treated cells by immunofluorescence cell staining and flow cytometry. We found that the high expression of FasL stimulated by anti-CD3 antibody is completely inhibited by chloramphenicol. However, chloramphenicol does not seem to affect Fas expression (Fig. 5). Therefore, chloramphenicol must inhibit AICD by blocking FasL expression. Our result shows that the molecular mechanism of chloramphenicol-mediated inhibition of apoptosis is by blocking FasL expression.

**Chloramphenicol promotes a more regulatory T-cell population.** Regulatory T cells (Treg) are potent modulators of immune responses (32–35). Various studies indicate that Tregs are immunosuppressive. Animals with depleted Tregs spontaneously develop various T-cell-mediated autoimmune diseases (36). Transfusion of Tregs inhibits lethal graft-versus-host disease (GVHD) in bone marrow transplantation (33). Because activation of the T-cell receptor (TCR) can induce T-cell differentiation, including a population of CD4+CD25+ cells, we examined what would occur in the presence of chloramphenicol. We found that splenocytes stimulated with anti-CD3 in the presence of chloramphenicol had significantly more CD4+CD25+ cells (Fig. 6A). Moreover, expression of the transcription forkhead box P3 factor (FoxP3) was also significantly higher than in the controls (Fig. 6B). Thus, chloramphenicol promotes more Treg differentiation, which may act to suppress possible immune responses against leukemic cells and thus allow the development of leukemia.

**Discussion**

It has been known for more than 15 years that there is a link between the use of chloramphenicol and the development of leukemia (37, 38). However, the molecular mechanism for leukemogenesis induced by chloramphenicol is largely unknown. Because chloramphenicol is still popularly used as an effective treatment for many bacterial infections in developing countries, or as a substitutitional therapy for drug-resistant bacteria in developed countries, a clear understanding of the mechanism of leukemogenesis induced by chloramphenicol would be of benefit to the development of new drugs and to human health worldwide. In addition, the current increase in the use of chloramphenicol in seafood farming and its associated concerns to human health also demand a better understanding of the toxicology of this antibiotic. In this report, we present evidence that chloramphenicol induces leukemia-like cells in activated T cells by promoting abnormal cell differentiation and inhibiting activation-induced apoptosis.

Leukemia is a type of blood cancer characterized by a large number of abnormal or immature cells in the bloodstream. Unlike most blood cells, the malignant cells tend to multiply and live for a longer time, thus leading to accumulation within the body. However, the mechanisms through which most leukemias develop
are still not known. It is well known that cancer cells frequently arise in the body but they normally die by suicide through apoptosis or by fratricide through immune recognition before they accumulate in significant numbers. Therefore, leukemia development is closely related to two key events: unrestrained proliferation and enhanced survival. By using mouse splenocytes as an experimental model in vitro, we showed that chloramphenicol combined with mitogenic stimuli significantly induces the formation of leukemia-like cells, characterized by large cell size, multiploid nuclei, and especially the expression of CD7, a marker for immature T cells and T-cell lymphoblastic leukemia.

The cell phenotypic changes are associated with aberrant expression of cyclin B1, which helps control cell mitosis. It has been shown in other cell systems that overexpression of cyclin B1 promotes the transformation of cells with a multiploid nuclei (39, 40), a mechanism very likely also operative in inducing the multiploid cellular phenotype in our experimental system. Another cellular modification required for leukemogenesis is that the malignant cells must escape apoptosis and avoid attack by the immune system. We show that chloramphenicol is also a potent inhibitor of activation-induced apoptosis, which is efficient in primary cells as well as cell lines of human and mouse. This property of chloramphenicol has not been revealed previously and we believe that it plays a critical role in chloramphenicol-induced leukemogenesis by allowing proliferating cells to continuously survive. Furthermore, we showed that the molecular mechanism of chloramphenicol inhibition of apoptosis is through blocking of FasL expression. Therefore, it is likely that the cellular changes associated with chloramphenicol make leukemic-like cells more likely to survive long-term and develop into clinical leukemia.

Chloramphenicol had been a popular antibiotic before realizing that it could cause aplastic anemia. It is now also believed to be a carcinogen based on limited evidence of carcinogenicity from clinical studies in humans. Three case reports have shown the development of leukemia after chloramphenicol therapy. In a case-control study in China, Shu and colleagues (37, 38) found increased risks of childhood leukemia, which correlated with the number of days chloramphenicol was administered. Two case-control studies revealed high, but nonsignificant, increases in the risk of aplastic anemia associated with the use of chloramphenicol (41, 42). However, other studies found no association between the use of chloramphenicol and the development of adult leukemia (43, 44). Interestingly, chloramphenicol also induced leukemia in toads (8). When mice were treated with busulfan and chloramphenicol, they develop transplantable T-cell leukemia, providing clear evidence that chloramphenicol can indeed induce leukemia in T cells (45). Therefore, the evidence of a link between aplastic anemia and leukemia, and the increased risk of leukemia found in some case-control studies supports the conclusion that chloramphenicol exposure is associated with an increased malignancy risk in humans.

Immune surveillance also has an important role in killing malignant cells. Recent studies have shown that T-cell leukemia, such as that induced by human T-cell lymphotrophic virus, possesses a Treg phenotype. Tregs are potent modulators of immune responses (32–35). Various studies indicate that Tregs are hypo-responsive and suppressive. Animals with depleted Tregs spontaneously develop variable T-cell–mediated autoimmune diseases (36). Transfusion of Tregs inhibits lethal GVHD in bone marrow transplantation (33). Because activation of the TCR can induce cell differentiation, including populations of CD4+CD25+ cell, we examined what would occur in the presence of chloramphenicol. We found that splenocytes stimulated with anti-CD3 in the presence of chloramphenicol had significantly more CD4+CD25+ cells. Moreover, expression of the transcription

Figure 6. Chloramphenicol induces the expansion of Tregs. The induction of Tregs from splenocytes of BALB/c mouse was done as described in Materials and Methods. A, CD4+CD25+ cells were detected by flow cytometric analysis following two-color staining. B, FoxP3 expression in CD4+CD25+ cells was evaluated by real-time quantitative PCR. Chloramphenicol was found to favor the outgrowth of the Treg population.
forkhead box P3 factor FoxP3 was also significantly higher than in the controls. Thus, chloramphenicol promotes more Treg differentiation, which may act to suppress possible immune responses against leukemic cells.

In conclusion, we have shown a molecular mechanism of chloramphenicol-induced leukemia, resulting from overexpression of cyclin B1 during cell differentiation and down-regulation of Fasl expression, thus preventing apoptosis. Disruption of normal immune responses by developing a Treg phenotype may also promote leukemogenesis by down-regulating the immune surveillance mechanisms.

References


Announcements

MEETING OF THE RADIATION RESEARCH SOCIETY

The annual meeting of the Radiation Research Society will be held at the State University of Iowa, Iowa City, on June 22-24, 1953. The Society will be the guest of the University, and all meetings will be held on the campus. The program will consist of: (1) Two symposia, one on "The Effects of Radiation on Aqueous Solutions," which includes the following speakers: E. S. G. Barron, Edwin J. Hart, Warren Garrison, J. L. Magee, and A. O. Allen. The second is "Physical Measurements for Radiobiology" and companion talks by Ugo Fano, Burton J. Moyer, G. Failla, L. D. Marinelli, and Payne S. Harris. (2) On Monday night, June 22, a lecture by Dr. L. W. Alvarez on meson physics has been tentatively scheduled. On Tuesday night, June 23, Dr. L. H. Gray of the Hammersmith Hospital, London, will speak on a topic to be announced. Dr. Gray's lecture is sponsored by the Iowa Branch of the American Cancer Society. Those desiring to report original research in radiation effects, or interested in attending or desiring additional information, please contact the Secretary of the Society, Dr. A. Edelmann, Biology Department, Brookhaven National Laboratory, Upton, L.I., New York.

ERRATUM

The following correction should be made in the article by Beck and Valentine, "The Aerobic Carbohydrate Metabolism of Leukocytes in Health and Leukemia. I. Glycolysis and Respiration," November, 1952, page 891; substitute for the last paragraph:

The data in Table 8 permit several interesting calculations. If one compares the amount of glucose actually disappearing with the sum of the amount equivalent to lactic acid produced plus that equivalent to O₂ consumption, it is seen that the amount of glucose "cleavage products" exceeds the amount of glucose utilized by 12 per cent in N and 27 per cent in CML and is exceeded by the glucose utilized by 16 per cent in CLL. If the assumption is made that, in this respect, the myeloid and lymphoid cells of leukemia are similar to those of normal blood, it may be that the computed normal figure represents a summation of the myeloid (M) and lymphoid (L) cells that make up the normal leukocyte population. Thus, if M = +0.27 and L = −0.16 and the normal differential is 65 per cent M and 35 per cent L, then

\[ 0.65 (+0.27) + 0.35 (-0.16) = +0.12 \]

a figure identical to the observed +0.12 for normal leukocytes.
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