The Effect of Chloramphenicol in Human Bone-marrow Cultures

By MAGDALENE R. VAS, BARBARA BAIN AND LOUIS LOWENSTEIN

THE RELATION of bone-marrow depression to chloramphenicol therapy has been well established. The depression may be reversible,[1-3] or irreversible, in some cases leading to fatal aplastic anemia.[4-5] It is not known whether these two conditions result from different mechanisms or if they represent different degrees of the same toxic reaction.

The nature of the reversible depression of hemopoiesis during chloramphenicol therapy has been studied by several authors. Krakoff et al.[6] noted that anemia is the first and most persistent finding. Saidi et al.[7] found reticulocytopenia and toxic vacuolizations in the nucleated red cells in the bone marrow, and Rubin et al.[8] demonstrated a disturbance in iron metabolism during chloramphenicol therapy.

Because of these reports it seemed logical to study the effect of chloramphenicol on hemoglobin synthesis in human bone-marrow cultures, as measured by Fe^{59} incorporation.

METHODS

Bone marrow was cultured by a modification of Osgood's cell-suspension method.[9] Cell suspension, chloramphenicol solution, and Fe^{59}-labeled serum were each prepared separately:

a) *Cell suspension:* 0.5–0.75 ml. of bone marrow was obtained by sternal puncture, and placed immediately in a centrifuge tube containing 10 ml. of Hanks' balanced salt solution* and a few drops of heparin. After centrifugation of the marrow specimen at 500 g at room temperature for 10 minutes, the supernatant was discarded and the cells were resuspended in 3 ml. of Hanks' solution. The nucleated cells were counted (using the standard white blood cell counting technic) and their number was adjusted to 8–10 x 10^6 per ml. with Hanks' solution.

b) *Chloramphenicol solutions* were prepared by dissolving crystalline chloramphenicol† in Hanks' solution, in concentrations of 500–3,300 μg. per ml.

c) *Fe^{59}-labeled serum:* Autologous serum was used throughout. Fe^{59} Cl_3‡ was diluted with physiologic saline, so that 1 ml. contained 0.1–0.2 μg. Fe, with a range of specific activity of 4.4–11.0 μc. per μg. One ml. of this solution was added to 4 ml. of serum, and the mixture allowed to equilibrate for 30 minutes at room temperature.

The iron concentration of the serum was estimated by a modification of the methods of Moore et al.[10] and Powell,[11] and the iron-binding capacity by the method of Rath and Finch.[12]

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This investigation was supported by research grant No. MA-1016 from the Medical Research Council of Canada.

Submitted Jan. 25, 1962; accepted for publication June 22, 1962.

*Obtained from Microbiological Associates, Bethesda, Md.

†Obtained through the kindness of Mr. B. H. Y. Savor, Parke Davis & Co., Ltd., Montreal.

‡Obtained from Abbott Laboratories, Ltd., Montreal.

According to the literature[13,14] and previous work by one of us,[15] the concentration of both the iron and the unsaturated iron-binding protein of the serum in the medium are important factors influencing in vitro heme synthesis. On the basis of these earlier findings, in order to secure optimal conditions, the iron concentration was always kept at more than 200 x 10^{-9} μg.Fe/cell, and the saturation of the iron-binding protein in the cultures was maintained at more than 30 per cent. This was achieved by adding nonradioactive $FeCl_3$ to those sera in which the iron concentration was below the above levels. The iron binding capacity of the serum was never exceeded.

Disposable 15 x 175 mm. plastic tubes were used as culture vessels. Penicillin and streptomycin were added to the Hanks' solution, in a concentration of 100 units per ml. and 100 μg. per ml. respectively, and filtered through a Seitz filter. Since this solution was used in both the control and the chloramphenicol-containing cultures, the presence of these antibiotics did not influence the experiments. All procedures were performed under aseptic conditions.

To set up the cultures, 0.6 ml. of cell suspension, 0.4 ml. of chloramphenicol solution, and finally 1.0 ml. of Fe^{59}-labeled serum were added to each tube to give a final volume of 2.0 ml. Control tubes, containing no chloramphenicol, were prepared from the same bone marrow and under identical conditions.

Cell counts were performed in duplicate and smears were made from another tube, which was prepared similarly but to which no radioactive iron was added. The nucleated cell counts varied from 2 to 4 x 10^6 per ml. of culture fluid. Smears were stained with Jenner-Giemsa. The percentage of erythroblasts* in each smear was determined by the differential count of 1,000 nucleated cells. The calculated absolute number of erythroblasts in the cultures ranged from 2 to 8 x 10^5 per ml.

Unless otherwise stated, the cultures were incubated at 37 C. and rotated at 12 rph for 24 hours. In some experiments, the chloramphenicol-containing medium was washed off the cells after four hours' incubation, and replaced by fresh medium containing no chloramphenicol. A control tube containing no chloramphenicol was treated similarly. Then both cultures were incubated for another 20 hours.

At the end of the incubation period the cells were washed three times with chilled physiologic saline solution and hemolyzed with 2 ml. of distilled water at 4 C. Hemolysis was always complete after 30 minutes. The radioactivity of the culture then was measured in a well-type scintillation counter.† The standard was prepared from the Fe^{59}-labeled serum, by dilution to 1:20 with distilled water.

Initially a count was performed upon the whole hemolysate, containing both supernatant and stroma. From this count and from the known concentration of iron in the culture, the total *iron uptake* by the cells was calculated and expressed in μg.Fe/erythroblast/hour. This value represents all of the iron removed from the medium by the cells; it includes the iron bound to the cell membrane and the intracellular nonhemoglobin iron as well as the hemoglobin iron. Uptake by cells other than erythroblasts was comparatively insignificant, and therefore was not taken into consideration in the calculations.

The tubes then were centrifuged at 3,000 g for 15 minutes, and the supernatant was assayed for radioactivity to determine the amount of *iron incorporated* into heme; this was expressed in μg.Fe/erythroblast/hour. This value may be considered to represent the heme iron, since only about 10 per cent of the supernatant iron is bound to microsomes and mitochondria.[16]

To confirm the validity of the method, heme was extracted by the method of Shemin[17] in a single experiment. Because the quantity of heme in the cultures was small, the method was modified by adding 20 ml. of bank blond, as nonradioactive carrier, to each culture. The specific activity of the heme, from both control and chloramphenicol-treated cultures, was expressed in counts/minute/mg. of dry weight of heme crystals.

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*"Erythroblasts," as used in this paper, includes all nucleated red cells.

†Atomic Instrument Co., Cambridge, Mass.

Table 1.—*Effect of 500 μg./ml. Chloramphenicol on Heme Synthesis in Bone-Marrow Suspension Culture, as Measured by Two Different Methods (24-hour cultures)*

Hemolysate Method Fe incorporation	Heme-extraction Method Fe^{59} incorporation
Control ..... 1.70 μg. x 10^{-9} Fe/cell/hr.	Control ..... 240 counts/10 min./mg. of heme
Chloramphenicol 0.79 μg. x 10^{-9} Fe/cell/hr.	Chloramphenicol 93 counts/10 min./mg. of heme
Inhibition by chloramphenicol 53.3%	Inhibition by chloramphenicol 61.2%

Results

Table 1 shows the results of the experiments in which heme was extracted from the cultures. The determination of iron from the hemolysate and the estimation of iron from the extracted heme gave closely similar values, proving that the hemolysate iron represents the iron incorporated into hemoglobin.

Initially, several chloramphenicol concentrations were used in the cultures. Figure 1 shows the results of one such experiment. It will be seen that 100 μg./ml. and 300 μg./ml. of chloramphenicol had slight effect only on the cells, whereas 500 μg./ml. decreased significantly both total Fe uptake and Fe incorporation into hemoglobin. These effects seemed to increase with higher concentration. As a result of this and similar experiments, a concentration of 500 μg./ml. was selected as the optimal concentration to consistently produce a significant measurable effect with these parameters, and this concentration was used in the following experiments.

Experiments were carried out on bone marrow obtained from 23 patients

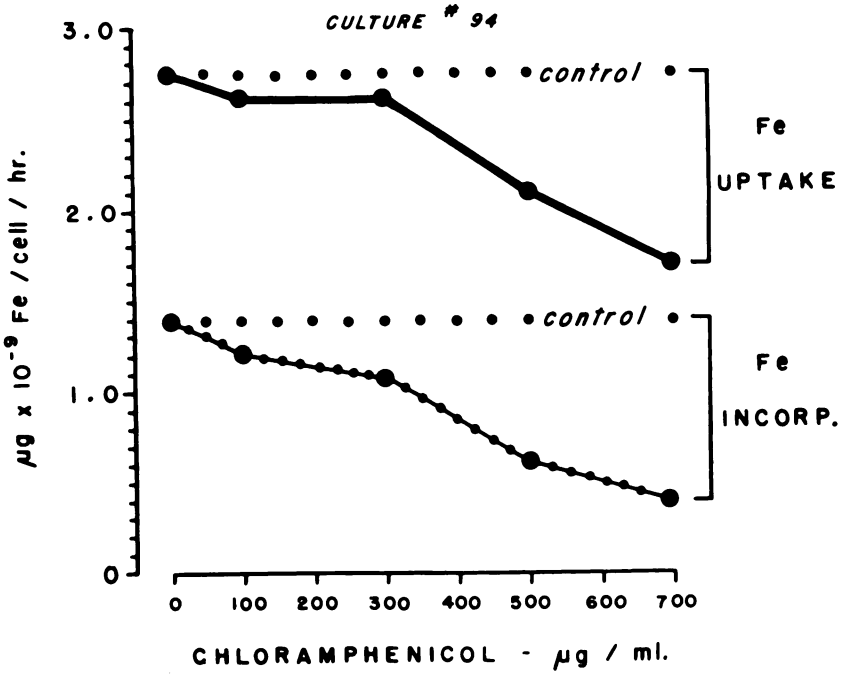

Fig. 1.—Effect of various concentrations of chloramphenicol upon iron uptake and iron incorporation by erythroblasts in bone-marrow suspension cultures. Incubation time 24 hours.

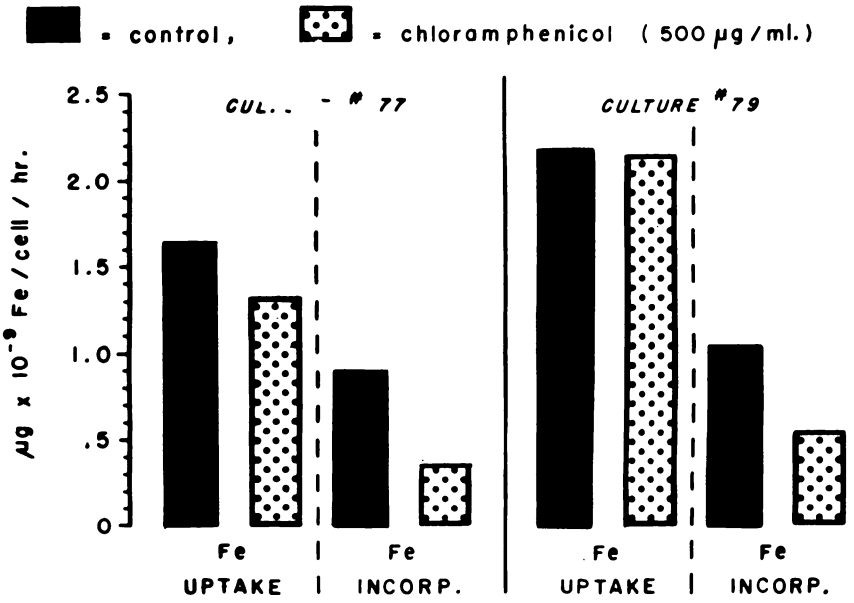

Fig. 2.—Effect of 500 μg./ml. chloramphenicol on iron uptake and iron incorporation by erythroblasts in bone-marrow suspension cultures. Incubation time 24 hours. Values of two representative experiments.

with a variety of clinical conditions, having a wide range of normal to severely disturbed hemopoiesis. None of these patients was receiving antibiotic or cytotoxic therapy when the marrow was obtained. There was no correlation of the diagnosis or of the state of the patient's bone marrow with the results obtained in the studies of their bone-marrow cultures.

Figure 2 shows the results of two representative experiments in individual cultures, and figure 3 summarizes the results of all 23 experiments. The results indicate that chloramphenicol caused a significant reduction in both uptake ($p < 0.05$) and incorporation ($p < 0.01$) of iron by the erythroid cells. It is obvious, however, that there is a marked difference in the effect of chloramphenicol on Fe uptake as compared with Fe incorporation. The uptake of iron by the erythroid cells was only slightly decreased and did not occur in every culture. On the other hand, the quantity of iron that appeared in the hemolysate was markedly reduced in the presence of chloramphenicol, and this effect was obtained in every culture.

Figure 4 shows the mean results of five experiments in which the chloramphenicol-containing medium was washed off the cells after four hours' incubation and replaced by fresh medium containing no chloramphenicol. During the first four hours the chloramphenicol had no effect on the uptake of iron by the erythroblasts but had already caused a significant inhibition in the incorporation of iron into heme. During the 20-hour incubation period following removal of chloramphenicol from the medium, the rates of iron uptake and incorporation were the same as in the control tubes. In the tubes left undisturbed for the entire 24 hours, the chloramphenicol caused a significant inhibition of both uptake and incorporation of iron by the erythroblasts.

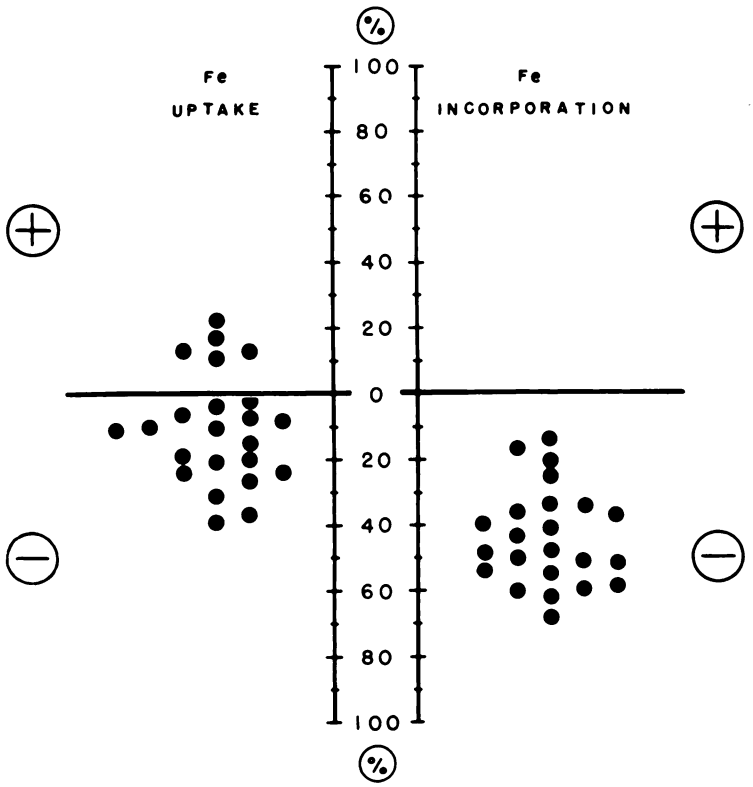

Fig. 3.—Effect of 500 μg./ml. chloramphenicol on iron uptake and iron incorporation by erythroblasts in bone-marrow suspension cultures. Incubation time 24 hours. The figure shows the results of 23 experiments; the inhibition in the chloramphenicol-treated cultures expressed as percentage of the control values. In (−) sections: values lower than the controls. In (+) sections: values higher than the controls. Mean value of inhibition by chloramphenicol for iron uptake = 10 per cent; for iron incorporation = 44 per cent.

Discussion

The ability of immature red cells to take up and incorporate iron in vitro provides a method of estimating their metabolic activity.[18-21] Three steps are involved in this process: the uptake of iron on to the cell surface; its entry into the cell; and its incorporation into heme.[14]

Our results suggest that, under the conditions of these experiments, the significant action of chloramphenicol takes place during the final step, and interferes with the incorporation of iron into heme. Lead[14] and potassium cyanide[22] seem to act similarly.

Recently, Erslev and Iossifides[23] reported a decreased uptake and incorporation of iron in chloramphenicol-treated cultures of normal bone marrow and blood. Contrary to our observations, they did not note a significant difference between the effect on uptake as compared with the effect on incorporation.

In our experiments the toxic effect of chloramphenicol became significant at a concentration of 500 μg. per ml. This represents a 10–15 times higher level than is found in vivo in the blood during chloramphenicol therapy.

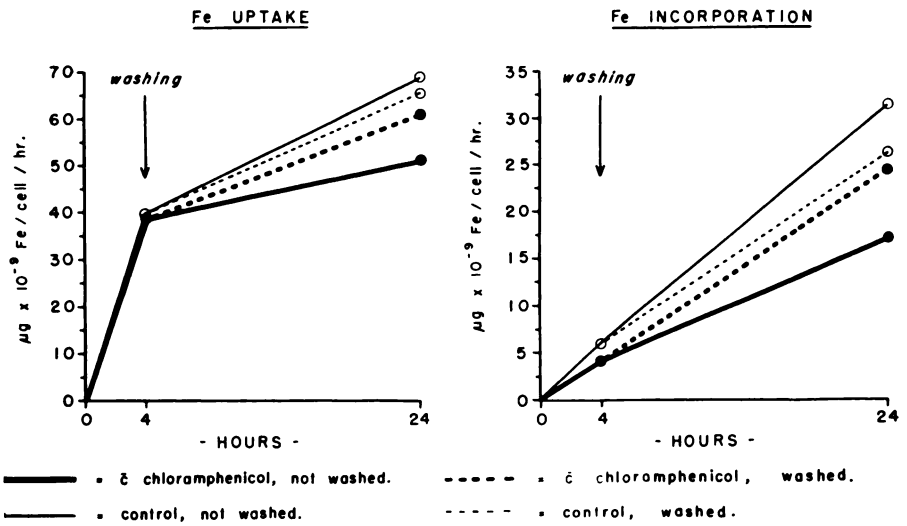

Fig. 4.—Reversibility of the toxic effect of 500 μg./ml. chloramphenicol on iron uptake and iron incorporation by erythroblasts in bone-marrow suspension cultures. *Fe uptake*: Inhibition by chloramphenicol at 4 hours = 2.2 per cent; at 24 hours = 25.4 per cent. For "washed" cultures, inhibition at 24 hours = 6.9 per cent. *Fe incorporation*: Inhibition by chloramphenicol at 4 hours = 28.3 per cent; at 24 hours = 45.3 per cent. For "washed" cultures, inhibition at 24 hours = 6.9 per cent.

Other authors noted similar discrepancies between the effective in vivo therapeutic concentrations and those required to produce toxic effects in vitro. According to Follette et al.[24] a selective concentration of the drug in the hemopoietic tissue can account for these differences. Yunis and Harrington[25] state that, of the several factors that may be responsible, the longer exposure to the drug in vivo probably is the most important. The findings by Erslev and Iossifides that a similar effect may be produced in vitro by high concentrations of tetracycline raises the question as to whether this effect is specific to chloramphenicol or is shared by a number of substances in appropriate concentrations. At present there is no adequate explanation for the variable sensitivity of cultures from different bone marrows to the toxic effect of chloramphenicol. When, after incubation for four hours, chloramphenicol was removed from the cultures, the cells recovered from its toxic effect. This would suggest that, after this short exposure to the drug, the cells did not suffer irreversible damage.

The results of our experiments suggest that chloramphenicol acts directly on heme synthesis. It has been suggested that it also inhibits protein synthesis in vitro.[26-28] Whether chloramphenicol interferes primarily with globin synthesis, causing secondary impairment in the synthesis of the heme molecule, or whether disturbances of other aspects of erythroblast metabolism are responsible for this inhibition of heme synthesis, has not been determined.

Summary

1. The effect of chloramphenicol on Fe uptake and Fe incorporation, using Fe^{59}, was studied in human bone-marrow cultures.

2. Choramphenicol, in a concentration of 500 μg. per ml., resulted in a slight depression of uptake of iron by the erythroid cells and in a marked depression of the incorporation of iron into heme.

3. The results suggest that one toxic effect of chloramphenicol upon the bone marrow may involve direct interference with heme synthesis.

4. Under certain experimental conditions the inhibitory effect of chloramphenicol upon iron uptake and heme synthesis by the erythroblast is reversible.

Summario in Interlingua

1. Le effecto de chloramphenicol super le acceptation e le incorporation de Fe in culturas de medulla ossee human esseva studiate per medio de un technica utlisante Fe^{59}.

2. Chloramphenicol—in un concentration de 500 μg per ml—resultava in un leve depression del acceptation de ferro per le cellulas erythroide e in un marcate depression del incorporation de ferro ad in le hem.

3. Le resultatos suggere que un del effectos toxic de chloramphenicol super le medulla ossee resulta de un directe interferentia in le synthese de hem.

4. Sub certe conditiones experimental le effecto inhibitori de chloramphenicol super le acceptation de ferro e le synthese de hem per le erythroblastos es reversibile.

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