EFFECT OF COLCHICINE ON THE ANTIBODY RESPONSE

I. Enhancement of Antibody Formation in Mice*

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In 1954, some experiments were carried out in this laboratory on the effect of colchicine (CC) on antibody formation in rabbits. At that time, we had found that antibody formation was associated with rapid and extensive cell divisions in the plasma cell and its immediate precursors which had just been associated with antibody synthesis (1). We thought, therefore, that the injection of CC would inhibit antibody formation, and Tanaka and Coons carried out some experiments to investigate this proposition. Much to our surprise, CC enhanced the antibody response in rabbits by a factor of ~8. The results of these experiments are simple to relate. We administered antigen to naive or primed rabbits with or without CC, and measured the antibody response in the serum. The enhancing effect was found to depend on the administration of CC on the same day as the antigen. It was ineffective when given 2 days before or 2 days after the injection of antigen. Indeed, it had a slightly depressive effect when given 2 days after the antigen, and an even greater effect when given on day 4 after antigen administration. But given simultaneously with the antigen, it had an increasingly pronounced effect as the dose was increased from 0.5 to 2 mg/kg. The latter dose killed three of the four rabbits tested, but the survivor had an antibody titer on the 8th day of his response 50 times higher than the control rabbits which received no CC. These facts in the rabbit were true for both the primary and secondary antibody response. At that time, we were at a loss to explain these findings and published them only in an abbreviated form (2, 3).

When Gershon discovered the existence of the suppressor cell in 1970 (4), it seemed possible that the effect of CC could be explained by the elimination of some or all of the suppressor cells which are evidently stimulated to arise during every antibody response.

It is noteworthy that in 1952, Taliaferro et al. (5) investigated the effect of X-irradiation (total body radiation with 600 or 700 rads) on hemolysin production against sheep erythrocytes in the rabbit. They found that peak titers were reached when the antigen was administered from 6 h to 10 min before irradiation took place. 10 years later, Dixon and McConahey (6) also investigated the effects of whole body radiation on the antibody response to bovine gammaglobulin in the rabbit. They found that the peak antibody titer to a primary stimulus was about four times that of the control value and appeared when the irradiation was carried out 2.5 days after the injection of antigen, although there was also a smaller elevation when the irradiation was carried out 1 day after an antigen injection.

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1 Abbreviations used in this paper: BSS, balanced salt solution; CC, colchicine; DT, diphtheria toxoid; HGG, human gamma globulin; KLH, keyhole limpet hemocyanin; LCC, lumicolchicine; LD₅₀, mean lethal dose; PFC, plaque-forming cells; SRBC, sheep erythrocytes; TKB, TNP-KLH-bentonite; TNP, 2,4,6-trinitrophenyl; VB, vinblastine.
Fourfold enhancement of diphtheria antitoxin in guinea pigs was described by Greenberg and Fleming (7) when pertussis vaccine was injected subcutaneously at the same time. Johnson et al. (8) described the enhancement of antibody formation to protein antigens by the injection of endotoxin from bacterial cell wall.

In 1963, White (9) published a review entitled "Factors affecting the antibody response" in which he described some unpublished data (Farthing and White, 1959) which demonstrated the enhancing effect of CC (1 mg/kg) on both the primary and secondary response of guinea pigs to diphtheria toxoid. They reported about a 10-fold difference in both primary and secondary responses when CC was administered 6 h before or 6 h after the antigen.

In 1976, Bash, Singer, and Waksman (10) reported that cyclophosphamide (20-50 mg/kg) given on the day of antigen injection to donor rats abrogated the suppressive effect on recipients of the T-cell fraction of rat spleen cells. Finally, Burchiel and Melmon (11) reported that the mitotic inhibitors, CC, cytosine arabinoside, and hydroxyurea, all produced dose-dependent augmentation of the antibody response in cultures of murine spleen cells.

These findings clearly predict the existence of a suppressor cell since both X-ray and CC were known to kill dividing cells. The effect of endotoxin is not so clear, but perhaps it interferes with cell division at a critical moment.

In view of our early findings with CC and the effects of X-ray and endotoxin, we decided to test the working hypothesis that these materials killed suppressor cells. To do this, it was necessary to carry out experiments with animals in which cell transfers could be carried out, namely inbred mice. This paper describes the conditions required for the effective enhancement of the antibody response by CC in mice. Our results indicate that CC is effective in promoting the antibody response of mice to protein antigens, e.g. diphtheria toxoid and human gamma globulin, as well as to a hapten, 2,4,6-trinitrophenyl (TNP). For maximal enhancement, the drug must be administered simultaneously with the antigen. In an accompanying paper, we shall present evidence which demonstrates the action of CC on suppressor cells.

Materials and Methods

**Animals.** Male or female BALB/c mice, 8- to 16-wk-old, were obtained from The Jackson Laboratory, Bar Harbor, Maine; from Charles River Breeding Laboratories, Wilmington, Mass.; or from the West Seneca Laboratory, Health Research Inc., West Seneca, N.Y. Animals were maintained in cages with free access to laboratory mouse chow and acidified, chlorinated water.

**Antigens and Immunization.** Purified diphtheria toxoid (DT) and human gamma globulin (HGG) were supplied by the Massachusetts Department of Public Health, Division of Biologic Laboratories, Boston, Mass. The hapten-carrier conjugate, trinitrophenyl-keyhole limpet hemocyanin (TNP-KLH), was prepared from 2,4,6-trinitrobenzene sulfonic acid (Eastman Kodak Co., Rochester, N.Y.) and KLH (Calbiochem, San Diego, Calif.) according to the procedure described by Rittenberg and Amkraut (12). The conjugate used in the present study had a ratio of 762 mol of TNP/mol of KLH. In some experiments, TNP-KLH was absorbed on bentonite particles by the method of Gallily and Garvey (13) for the purpose of immunization. All mice were immunized intraperitoneally. The doses of antigens used for immunization are specified in the text. CC (Sigma Chemical Co., St. Louis, Mo.) in physiological saline was administered intraperitoneally to appropriate groups of animals at doses ranging from 0.25 to 1.5 mg/kg body weight, depending upon the experimental protocol.

**Passive Hemagglutination.** Mice were bled from the ophthalmic venous plexus and the sera obtained were inactivated at 56°C for 30 min. Circulating antibody levels were measured by the hemagglutination of sheep erythrocytes, (SRBC; Colorado Serum Co., Denver, Colo.) covalently coupled to the protein antigen by bis-diazobenzidine according to the method of Stavitsky and Arquilla (14). For the detection of hapten-specific antibodies, the indicator SRBC were coupled to
TNP as described by Rittenberg and Pratt (15). Antibody titers were expressed as the reciprocal of the highest serum dilution which gave positive hemagglutinating reactions.

Preparation of Cell Suspensions and Hemolytic Plaque Assay. Mice were sacrificed by cervical dislocation. The spleens were harvested and gently teased with sharp forceps in balanced salt solution (BSS; 16). The dispersed spleen cells were filtered through stainless steel screens and were washed three times in BSS by centrifugation at 1,000 rpm for 10 min each time. The washed spleen cells were assayed for antibody-forming cells by a modified method (16) of the Jerne hemolytic plaque technique (17). Indicator SRBC were prepared according to the method of Kapp and Ingraham (18) for HGG-SRBC and of Rittenberg and Pratt (15) for TNP-SRBC. Briefly, the assay of plaque-forming cells was performed as follows. Glass culture tubes (10 × 75 mm) containing 0.3 ml of 0.7% agarose (L'Industrie Biologique Francaise S. A., Gennevilliers, France) in BSS, 50 μl of HGG-SRBC (7.5%) and 20 μl of SRBC-absorbed 0.5% bovine serum albumin solution were preincubated in a 41-42°C water-bath. 50 μl of a spleen cell suspension containing 10^5-10^6 viable nucleated cells were added to each tube. The mixture was gently but thoroughly mixed and was then spread on a microscope glass slide previously coated with 0.1% agarose. After the agarose had solidified, the slides were inverted, placed on plexiglass trays and incubated at 37°C in a humidified chamber. After an incubation period of 1.5-2.0 h, freshly reconstituted guinea pig complement (Pel-Freez Farms, Inc., Rogers, Ark.), at 1:15 dilution, was flooded under the slides. After an additional 1.5 h incubation, the number of plaque-forming cells (PFC) on each slide was enumerated under a low-power microscope. For the development of IgG PFC, a previously determined optimal dilution of a polyvalent rabbit anti-mouse IgG antiserum (kindly supplied by Dr. Carl W. Pierce of the Jewish Hospital of St. Louis, St. Louis, Mo.) was incorporated in the agarose mixture. The number of IgG PFC was calculated from the difference between the number of IgM PFC and the total number of PFC developed with the anti-IgG antiserum.

Preparation of Lumicolchicine. Lumicolchicine was prepared by the irradiation of a CC solution in a quartz cuvet placed at 12 inches from a UV lamp (Osram High Pressure Mercury Arc, HBO 200; Osram, Munich, W. Germany) for 30 min. The successful conversion of CC to predominantly beta- and gamma-lumicolchicines was monitored by the appearance of two isosbestic points at 255 and 305 nm in their UV spectra, and by a decrease of absorbancy at 350 nm (19).

Results

Primary Antibody Response. Mice injected intraperitoneally with 100 μg of TNP-KLH-bentonite (TKB) on day 0 gave a peak hapten-specific IgM PFC response on days 6 and 7 (Fig. 1 A). The simultaneous administration of CC (1 mg/kg body weight) with 100 μg of TKB to animals in the experimental group increased the IgM PFC response by about twofold. The hapten-specific IgC PFC response of CC-treated animals was also greater than that of control animals (Fig. 1 B). Significant elevation in the circulating anti-TNP antibody titers was observed in immunized animals treated with CC (Fig. 2). The enhanced antibody level appeared as early as 7 days after immunization, and the difference in antibody titer between the two groups lasted for at least 14 days. The administration of CC to animals immunized with HGG also significantly (P < .001) enhanced the serum antibody titer at 1 wk, but not 4 days, after immunization (Fig. 3). DT was found to be a relatively poor antigen for the induction of a primary response in mice even when CC was given.

Secondary Antibody Response. The immunization of mice with two injections of DT (20 Lf per injection) 20 days apart, gave a consistent response in terms of the mean circulating antibody titer 10 days after the second injection of antigen (Fig. 4). When colchicine was also administered to similarly immunized animals at the time of priming (group II), at the time of challenge (group III), or at both times (group IV), the subsequent antibody response was increased by about 15-fold. The kinetics of the secondary response in mice which received
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Fig. 1. Effect of CC on the kinetics of the TNP-specific primary PFC response. BALB/c mice were immunized intraperitoneally with 100 µg of TKB (○) or with 100 µg of TKB and CC (1 mg/kg, ●); their spleens were harvested for PFC assay at various times after immunization. Each point represents the mean ± SE of the response of 4-16 animals. SC, spleen cells.

two injections of DT and CC showed that the enhanced response occurred as early as 5 days after the second immunization (Fig. 5). The difference in antibody titers between CC-treated animals and control animals lasted for more than 15 days. The effective CC dose for maximal enhancement was between 1.0-1.5 mg/kg body weight (Fig. 6). The drug was found to be lethal to injected animals at doses above 1.5 mg/kg and the mean lethal dose (LD₅₀) of CC for BALB/c mice was 2.1 mg/kg (Fig. 7). Since a relatively large amount of purified DT would have been needed for the sensitization of indicator SRBC in a hemolytic plaque assay, the number of DT-specific antibody-forming cells was not enumerated. Instead, the effect of CC on the PFC response to another protein antigen (HGG), and to a hapten (TNP) was studied.

Mice previously primed with HGG and challenged 10 days later with the same antigen gave a relatively low HGG-specific IgG PFC response (Fig. 8 A). On the other hand, similarly immunized animals which were also given CC
showed an IgG PFC response which was about three times as large. Since very few, if any IgM PFC were detected by this system (20), we could only express our HGG-specific PFC response by the number of indirect plaques obtained. The increase in the number of antibody-forming cells was accompanied by elevated circulating antibody levels which persisted for a longer period of time (Fig. 8B). In the case of the secondary PFC response to the TNP hapten, the administration of CC to experimental animals at the time of priming enhanced both the IgM and the IgG response (Fig. 9). The IgM PFC response of the CC-treated animals was twice as large (Fig. 9A), and the IgG PFC response was about four times as large as that of the control animals (Fig. 9B).

Relationship between the Time of Administration of CC and the Enhancement of the Antibody Response. Different groups of animals were given CC at times before (day -1), during (day 0), or after (day +1 and day +2) the injection of TNP-KLH. Animals in the control groups were injected with the antigen alone. The primed animals were challenged with TNP-KLH on day 14. 3 days after the second injection, the spleens of the immunized animals were assayed for their secondary hapten-specific PFC response. Maximal enhancement of the PFC response was observed when CC was administered to animals on the same day as the injection of antigen (Fig. 10A). Although minimal enhancement was observed when the drug was given to animals 1 day before immunization, no elevation in the PFC response was noted when the drug was administered to animals 1 or 2 days after immunization. Similar results were observed in the primary response; the drug was most effective when given to animals on the same day as antigen injection (Fig. 10B).
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ANTI-HGG PRIMARY RESPONSE

Fig. 3. Effect of CC on the circulating anti-HGG antibody titers. Mice were injected intraperitoneally with 100 μg of HGG or with 100 μg of HGG and CC (1 mg/kg). Each bar represents the mean titer ± SE of 6 animals bled on day 4, and of 13 animals bled on day 7. n.s., not significant.

DAY 10 ANTI-DT SECONDARY RESPONSE

Fig. 4. CC-mediated enhancement of the secondary antibody response of mice immunized with DT. Control animals in group I were primed with 20Lf of DT on day -20 and challenged with a similar dose of antigen on day 0. Animals in the experimental groups were immunized in a similar fashion except that CC, at a dose of 1 mg/kg body weight, was also administered at the time of priming (group II), at the time of challenge (group III), or at both times (group IV). All animals were bled 10 days after the second injection. Each bar represents the mean titer ± SE of 8 animals.
FIG. 5. Effect of CC on the kinetics of the secondary response to DT. Control animals (○) were given two intraperitoneal injections of 20 Lf of DT 20 days apart. Animals in the experimental group (●) were similarly immunized except that CC, at a dose of 1.5 mg/kg, was also administered at the time of antigen injection. Each point represents the mean titer ± SE of 7-8 animals.

FIG. 6. Titration curve of the effect of different doses of CC on antibody formation. Control mice were given two intraperitoneal injections of 20 Lf of DT 20 days apart. Animals in the experimental groups were similarly immunized except that CC, at various doses as indicated in the abscissa, was also given. All animals were bled 10 days after the second injection, and their serum anti-DT hemagglutinating antibody titers were determined. Each point represents the mean titer ± SE of 5 animals.
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FIG. 7. Determination of the LD₅₀ of CC in mice. BALB/c mice (10 animals/group) were injected intraperitoneally with various doses of CC ranging from 0 to 3.0 mg/kg body weight. Each point represents the percent of animals which survived for at least 10 days after CC injection.

Comparison of the Effect of Two Different Anti-Mitotic Drugs on the Antibody Response. Vinblastine (VB), another anti-mitotic drug, was tested for its effectiveness in enhancing the antibody response. Three groups of animals were similarly immunized with TKB. Animals in group II also received CC (1 mg/kg) and those in group III were injected with a similar dose of VB on the day of immunization. 6 days later, the spleens of the treated animals were assayed for their hapten-specific PFC response. Results shown in Table I indicate that VB was as effective as CC in enhancing the anti-TNP PFC response.

Effect of Lumicolchicine on the Antibody Response. Lumicolchicine (LCC), the structural isomer of CC, was tested for its effectiveness to enhance antibody formation. Mice in the control group were injected intraperitoneally with 100 μg of TKB on day 0. Two other groups of animals were immunized in a similar manner, except that CC was also administered to 1 group and LCC to the other. The dose given in each case was 1 mg/kg body weight. The hapten-specific PFC response was assayed on day 6. Results shown in Table II indicate that whereas CC enhanced the TNP-specific PFC response by more than 100%, the administration of LCC to immunized animals had no enhancing effect.

Discussion

Results of experiments performed in mice and reported in the present paper confirm the original observations by Tanaka and Coons (2) that CC is effective in enhancing the antibody response. Apparently, there is no species difference in terms of the capacity of CC to promote antibody formation since the drug works well in rabbits (2, 3), guinea pigs (9), hamsters (21), and in mice as presently reported.

Previous reports on CC-induced enhancement expressed the enhancing effect only in terms of the serum antibody level, and the magnitude of the maximal enhancement varied from 3- to 50-fold (2, 9, 21, 22). In the present study with
mice, our data indicate that CC-induced enhancement of the circulating antibody titer varied from as low as 3-fold (in the primary response to HGG, Fig. 3) to as high as about 15-fold (in the secondary response to DT, Fig. 4) more than that of the control group. In addition to evaluating the serum antibody titer, this study also examined the cellular aspect through the assessment of the PFC response. The data indicate that the number of antibody-forming cells in the spleens of CC-treated animals was significantly increased. For example, the hapten-specific primary PFC response was doubled (Fig. 1) and the secondary IgG PFC response was enhanced by at least fivefold (Fig. 9). In the case of the secondary PFC response to HGG, the response of CC-treated animals was at least three times as large as that of control animals (Fig. 8 A). This
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Fig. 9. Effect of CC on the secondary TNP-specific PFC response. Mice in the control group (○) were given 2 intraperitoneal injections of 100 μg of TNP-KLH 14 days apart. Animals in the experimental group (●) were similarly immunized except CC, at a dose of 1 mg/kg, was also given at the time of priming. On each day between 2 and 6 days after challenge, spleens of the immunized animals were harvested for the assay of their hapten-specific PFC response. Each point represents the mean response ± SE of five animals. SC, spleen cells.

increase in the PFC response was accompanied by an elevation of the serum antibody titer (Fig. 8 B). These results implied that the administration of CC at the time of antigenic penetration affected certain cellular events leading to an increase in the number of antibody-forming cells which in turn increased the circulating antibody level.

For effective enhancement of the antibody response by CC, there are at least two critical factors which have to be observed; (a) the dose of CC used, and (b) the time of its administration.

Within the dose range that is not lethal for mice, maximal enhancement may be obtained by the injection of 1.0–1.5 mg CC/kg body weight (Fig. 6). An increasingly pronounced enhancing effect was also observed in the rabbit when the dose of CC was increased from 0.5 to 2.0 mg/kg (2). It is of interest to note that despite the tremendous difference in susceptibility to the lethal effect of CC between mice (LD₅₀ 2.1 mg/kg; Fig. 7) and hamsters (LD₅₀ 300 mg/kg; Ref. 22) the optimal dose of CC for the enhancement of antibody formation lies within the same range of about 1.0–1.5 mg/kg.
Fig. 10. Effect of varying the time of CC administration on the enhancement of the PFC response. Groups of mice (five animals per group) were given CC, at a dose of 1 mg/kg, on various days (as indicated on the abscissa) before, during, and after the immunization with 100 μg of TNP-KLH on day 0. Control animals received the same dose of antigen but no CC was given. 14 days after the first injection, all animals were challenged with 100 μg of TNP-KLH. The spleens of the injected animals were harvested and assayed for their TNP-specific PFC response 3 days after the second injection. Each point represents the mean response ± SE of the CC-treated animals (●) or of the control animals (○). SC, spleen cells.

The timing of the injection of CC seems to be critical. For the maximal enhancing effect, the drug has to be administered simultaneously with or on the same day as the antigen (Fig. 10). Only minimal enhancement of the PFC response was observed when CC was given 1 day before the injection of antigen. Effective enhancement had been reported in the guinea pig when CC was given as early as 6 h before antigen injection (9). On the other hand, administration of the drug 1 or 2 days after immunization was ineffective (Fig. 10). CC was also found to be incapable of enhancing the antibody response in rabbits when it was given 2 days before or 2 days after the injection of antigen (2). The failure of CC
to enhance the antibody response when it was administered as early as 1 day
after immunization suggests that certain critical regulatory cellular events
must have taken place within 24 h after contact with antigen. Indeed, Eardley
and Sercarz (23, 24) recently demonstrated in their elegant studies that
regulatory suppressor cells appeared as early as 24 h after priming with
antigen, and that cell divisions were required for the expression of suppressive
activities. Taken together, these observations strongly support our hypothesis
that CC may act on the early dividing suppressor cell or its precursors.

The route of CC administration does not seem to be important. In this study,
we injected CC and the antigen separately into the peritoneal cavity. However,
effective enhancement had also been observed when CC and the antigen were
injected via different routes. For example, CC had been administered subcuta-
neously to rabbits immunized in the foot-pad (2), and intraperitoneally to
hamsters immunized intravenously (21, 22); in both cases, antibody formation
was increased.
If the enhancement of the antibody response by CC is dependent on the anti-mitotic action of the drug, then any anti-mitotic drug, e.g. VB, should also be capable of mediating the enhancement. Indeed, when VB was given to animals at the time of antigen injection, it was found to be as effective as CC in elevating the antibody response (Table I).

These observations with both CC and VB strongly suggest that there is a connection between the anti-mitotic action of the drug and its enhancement of antibody formation. The dose of CC (1-1.5 mg/kg) that we used to enhance the antibody response has been shown to be effective in inhibiting mitosis in vivo (25, 26). If the mitosis-blocking property is crucial for the enhancing effect of CC, deprivation of its anti-mitotic capacity should render it inactive in this regard. Indeed, we were able to show that the conversion of CC to its non-anti-mitotic structural isomer, LCC, abolished the enhancing effect (Table II). Thus, it seems justifiable to say that the anti-mitotic action of CC is an essential element for successful enhancement of the antibody response. The critical timing in the administration of CC necessary for effective enhancement, a feature of the effect of X-irradiation, indicates that these agents are most likely acting upon rapidly dividing cells. It is noteworthy that X-irradiation which also predominantly causes damage to dividing cells (27) also enhances the antibody response in vivo (28).

In contrast to the enhancing effect reported here, the mitosis-blocking capacity of CC has been employed to suppress the antibody response in rats by the administration of the drug a few days after immunization, but within several hours before the treated animals were sacrificed for the PFC assay (26). The effective suppressing dose (1.3 mg/kg) of CC used lies within our enhancing dose range (1.0-1.5 mg/kg). Thus, approximately the same dose of CC administered to animals may mediate either enhancement or suppression of antibody formation depending upon the timing of drug administration in relation to the injection of antigen. The suppressive effect of CC, observed when the drug is injected in the later phase of the antibody response, has been suggested to be the result of the mitotic blocking action of CC on cellular divisions of the antibody-forming cell and its precursors (26). On the other hand, we postulate that the enhancing effect of CC, observed when it is injected at the same time as antigen, is the consequence of its interruption of mitosis that prevents the generation of antigen-stimulated, early dividing suppressor cells.

It is clear, from the general effect of such anti-mitotic agents in rabbits, mice, guinea pigs, and hamsters on both the primary and secondary antibody responses to several protein antigens, that the early development of suppressor cells usually, if not always, accompanies antigenic stimulation. The accompanying paper presents evidence that CC does indeed prevent the development of suppressor cells.

Summary

Colchicine (CC) enhances the antibody response in mice to protein antigens, like diphtheria toxoid and human gamma globulin, as well as to the 2,4,6-trinitrophenyl hapten. Maximal enhancement was observed when CC was administered to animals on the same day as the injection of antigen. The
optimal dose of CC was in the range of 1.0–1.5 mg/kg body weight. The enhanced antibody formation was evident from elevated circulating antibody titers and from an increased number of antibody plaque-forming cells (PFC) of the spleen. The circulating antibody titer of CC-treated animals was higher than that of control animals by a factor of about 3–7 in the primary response, and by a factor of at least 15 in the secondary response. In terms of the number of antibody forming cells, CC enhanced the primary PFC response by ≈100%, and the secondary PFC response by as high as fivefold. The enhancing effect of CC seemed to be related to its mitosis-blocking capacity since (a) vinblastine, another antimitotic drug, was found to be as effective as CC and (b) lumicolchicine, the non-anti-mitotic structural isomer of CC, was ineffective in potentiating antibody responses. The critical timing in the administration of CC on the same day as antigen suggests that most likely, the mitotic poison was acting on antigen-stimulated early dividing suppressor cells.

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