Histamine deficiency promotes accumulation of immunosuppressive immature myeloid cells and growth of murine gliomas

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To elucidate mechanisms underlying epidemiological findings of decreased risk of glioma development in patients with allergies and asthma, gliomas were induced in mice deficient for histidine decarboxylase (HDC), the enzyme responsible for histamine production. These mice exhibited shortened survival and enhanced tumor growth compared to wild-type (WT) mice. Previous studies have shown a pivotal role of HDC in maturation of bone marrow (BM)-derived myeloid cells. In our glioma models, brain-infiltrating leukocytes (BIL) demonstrated an increased frequency of CD11b+Ly6C+ immature myeloid cells (IMC; both CD11b+Ly6G- and CD11b+Ly6C+ subpopulations) as well as diminished CD8+ T cell infiltration and their effector functions in HDC−/− mice compared with WT mice. Furthermore, HDC−/− IMC demonstrated a more profound immune suppression of CD8+ T cell proliferation and functions associated with increased prostaglandin E2 (PGE2) expression levels. Celecoxib, a cyclooxygenase-2 inhibitor, which is vital for PGE2 production, abrogated suppressive capabilities of HDC−/− IMC. In addition, glioma-bearing HDC-eGFP mice, in which HDC promoter drives green fluorescence protein (GFP) expression, exhibited decreased HDC promoter activities in CD11b+Gr1+ cells in the BM, spleen, and intracranial tumor site compared with non-tumor bearing HDC-eGFP mice. Additionally, in vitro culture with glioma supernatants decreased GFP expression in CD11b+Gr1− IMCs. HDC expression levels inversely correlated with suppressive functions of CD11b+Gr1+ IMCs, as GFP+CD11b+Gr1+ more profoundly inhibited CD8+ T cell proliferation compared with CD11b+Gr1+ GFP− cells. Taken together, these data show a significant role of HDC in the glioma microenvironment via maturation of myeloid cells and resulting activation of CD8+ T cells.

Introduction

Multiple epidemiological studies have consistently established a history of allergy, as well as a significant dose-response with increasing number of allergens, as a protective factor for glioma development.1-3 In addition, patients with glioma and allergies are up to 3.5 times more likely to report chronic anti-histamine use (>10 years) compared to controls, and long-term anti-histamine use is associated with increased risk of glioma.4,5 These epidemiological studies suggest unexplained roles of the histamine pathway in immunosurveillance and survival in glioma patients.

Histamine is a biogenic amine that has been studied extensively in atopic conditions such as allergy and asthma and has been shown to modulate the immune system.6 Histamine is primarily produced endogenously by L-HDC, which converts L-histidine to histamine, but can be incorporated exogenously from oral intake of histamine.7 Previously, HDC has been examined in the induction of skin and colon cancer.8 HDC is primarily expressed in BM CD11b+Gr1+ IMCs, and its expression is vital for myeloid maturation. HDC−/− mice possess increased CD11b+Gr1+ IMCs in the BM, spleen, as well as peripheral blood, and exogenous histamine promotes differentiation of IMCs. In chemical carcinogen-induced models of skin and colon cancer, HDC−/− mice exhibited accelerated growth of cancer in compared with WT mice as well as enhanced tumor-infiltration by IMCs.9 In these models, increased IMCs directly promote tumor angiogenesis.8

Multiple tumor-derived factors including growth factors and cytokines have been demonstrated to promote the development of tumor-promoting IMCs by halting their normal differentiation into mature antigen-presenting cells and inducing immunosuppressive effectors in IMCs, such as reactive oxygen species (ROS),9 inducible nitric oxide synthase,10 and arginase 1.10,11 These cells have been shown to promote tumor development by inhibiting T cell-mediated antitumor immunity as well as directly promoting tumor proliferation, survival, angiogenesis, and invasion.12,13
We have demonstrated that depletion of these cells inhibits glioma growth and prolongs survival in murine models. We have also reported a protective effect of non-steroidal anti-inflammatory drugs (NSAIDS) and cyclooxygenase 2 (COX2) blockade against the development of IMCs and glioma. In the current report, we extended our study to elucidate the bioimmunological mechanism underlying the epidemiological finding of use of histamine inhibitors as a risk factor for development of malignant glioma. We demonstrate that histamine is involved in myeloid cell maturation using histamine-deficient HDC−/− mice, and that HDC−/− IMCs suppress CD8+ T cell responses more profoundly than control IMCs. In addition, we demonstrate that glioma-derived factors can suppress HDC expression in CD11b+Gr1+ IMC, thereby potentiating immunosuppressive activities and the resulting glioma growth.

Results

Histamine deficiency promotes glioma development in vivo

To address the role of histamine on glioma development, we induced de novo gliomas by intraventricular transfection of NRas, EGFRvIII, and small hairpin RNA against p53, utilizing the Sleeping Beauty (SB) transposon system in both histamine deficient HDC−/− and WT mice (both are BALB/c-background). A previous report has demonstrated the relationship between HDC activity and histamine levels in various tissues, specifically that HDC−/− mice possess reduced and/or near-zero levels of histamine compared to WT mice. Histamine levels were confirmed to be substantially lowered in peripheral circulation in HDC−/− compared to WT mice. To assess the direct effect of HDC expression and histamine on the proliferation of glioma cells in vitro, we established cell lines derived from SB-induced gliomas in WT and HDC−/− mice and observed no significant differences in their in vitro proliferation rates (Fig. 1B; left). Additionally, when we added exogenous histamine to the culture of HDC−/− glioma cell lines at varying concentrations (1, 10, and 50 μM), we did not see any effects on their proliferation rates (Fig. 1B, right). However, in vivo, HDC−/− mice bearing SB-induced de novo gliomas exhibited shorter survival compared with WT mice (Fig. 1C). To determine the role of host-derived HDC without any confounding effect of tumor cell-derived histamine, we stereotactically inoculated SB-derived HDC−/− glioma cells in WT or HDC−/− mice, and found that HDC−/−...
hosts exhibited significantly larger tumors based on bioluminescence imaging (BLI) at day 14 compared with WT mice (Fig. 1D, left). Accordingly, HDC−/− mice also exhibited significantly shorter survival compared with WT mice after these mice received inoculation of HDC−/− glioma cells (Fig. 1D, right). These data suggest a potential role of host-derived HDC as a modulator of glioma growth. Our data also suggest that the differential tumor growth between HDC−/− and WT mice is probably not due to direct effects of HDC or histamine on tumor proliferation, but more likely attributable to effects on host-derived non-tumor cells.

Accelerated glioma growth in HDC−/− mice is concomitant with decreased CD8+ T cells and increased CD11b+Gr1+ immature myeloid cells (IMC) in the tumor

Tumor-infiltrating CD8+ T cells have been shown to have a positive prognostic impact. To determine the impact of histamine production on the immunological environment of gliomas, we examined brain-infiltrating leukocytes (BIL) in glioma. While gliomas in both WT and HDC−/− mice were infiltrated by CD3+CD8+ T cells, HDC−/− mice demonstrated decreased percentages of CD8+ T cells in BILs compared with WT mice (P = 0.033) (Fig. 2A). In addition, CD8+ T cells in HDC−/− mice exhibited decreased levels of effector markers, CD107a (P = 0.043), granzyme B (P = 0.049), and perforin (P = 0.0001) compared with CD8+ T cells from WT mice (Fig. 2B). However, no differences were observed in splenic CD8+ T cells, or CD4+ T cells in BILs or spleen (data not shown). These data indicate the HDC deficiency can affect the immunological microenvironment of gliomas, specifically by suppressing numbers and functions of CD8+ T cells.

Previous reports have shown that HDC−/− mice possess an altered immune milieu, specifically increased levels of CD11b+Gr1+ IMC. Our analysis of BIL revealed increased CD11b+Gr1+ cells in HDC−/− mice compared with WT (Fig. 2C). Additionally, there was an increase of both CD11b+Ly6G+ granulocytic (G)-IMC and CD11b+Ly6C+ monocytic (M)-IMC subpopulations in BIL (Fig. 2C) and spleen (Fig. 2D).

More profound suppressive function of CD11b+Gr1+ IMCs from HDC−/− mice

CD11b+Gr1+ IMCs phenotypically resemble myeloid-derived suppressor cells (MDSC), which represent a significant population of BI found in human and mice glioma. MDSC can be phenotypically heterogeneous, and the coalescing factor is their ability to suppress T cell functions. To determine whether the HDC status impacts on immunosuppressive functions of MDSC-like cells in our glioma model, CD11b+Gr1+ IMCs isolated from glioma in HDC−/− and WT mice were co-cultured with CFSE-labeled naive WT CD8+ T cells. A larger population of non-proliferating CD8+ T cell population was observed when co-cultured with HDC−/− mouse-derived IMCs compared with WT mouse-derived counterparts (Fig. 3A). As tumor-infiltrating MDSC have been shown to be BM-derived, we sought to utilize BM cells derived from WT and HDC−/− mice to gain further understanding in the role of HDC in myeloid maturation and

![Figure 2. Effects of HDC on immunological microenvironment. SB-derived HDC−/− glioma cells were inoculated into the brain of HDC−/− (KO) and WT mice. Mice were sacrificed when tumor were of similar size via BLI; i.e., day 10–12 following the inoculation. BILs were isolated and analyzed for: (A) %CD8+ T cells of total CD45+ cells (n = 10 mice/group); (B) CD8+ T cell function including CD107a, perfon, and granzyme B. (C and D) Percentages of CD11b+Gr1+ , CD11b+Ly6C+ and CD11b+Ly6G+ IMCs in CD45+ (n = 5 mice/group) BILs and (D) splenocytes. *: P < 0.05, **: P < 0.01.](image-url)
suppressive functions. When WT and $HDC^{-/-}$ BM cells were cultured under MDSC-inducing conditions (BM-MDSC), $HDC^{-/-}$ BM cells demonstrated an increased yield of CD11b$^{+}$Gr1$^{+}$ cells in comparison with WT cells, suggesting that defects of HDC signal promotes accumulation of IMCs (Fig. 3B). Furthermore, $HDC^{-/-}$ BM-IMC inhibited proliferation of CD8$^{+}$ T cells compared with WT counterparts (Fig. 3C). Additionally, we observed increased levels of PGE2 expression in $HDC^{-/-}$ BM-IMC compared to WT (Fig. 3D). To determine whether the increased PGE2 has a direct impact on the suppressive functions of $HDC^{-/-}$ IMCs, we cultured $HDC^{-/-}$ BM-IMC with COX2 inhibitor, celecoxib, which reversed the suppression and improved T cell proliferation (Fig. 3E). We have previously shown that celecoxib is capable of maturing IMCs and reducing immunosuppressive effectors in myeloid cells. Both pathways are involved in mediating atopy and regulating immune responses, and our data suggest that PGE2 may be one of mediators of the augmented suppressive function of $HDC^{-/-}$ IMC.

**Glioma-bearing conditions decrease expression of HDC in CD11b$^{+}$Gr1$^{+}$ IMC**

Gliomas are notoriously immunosuppressive. Many glioma-derived factors have been demonstrated to inhibit maturation of myeloid cells, promote development of IMCs, and induce immunosuppressive effectors including M-CSF, PGE2, TGFβ, and recruitment of regulatory T cells. We examined if glioma could affect expression of HDC and immune cell function. As previously shown, HDC is primarily expressed in CD11b$^{+}$Gr1$^{+}$ cells in the BM, which was corroborated in our own analysis (data not shown). To elucidate in vivo effects of glioma-bearing conditions on HDC.
expression, we inoculated GL261 glioma cells into the brain of HDC-eGFP mice, which express GFP under the HDC promoter. In tumor-bearing mice, CD11b\(^+\)Gr1\(^+\) IMCs isolated from the brain (Fig. 4A), spleen, and BM (Fig. 4B) revealed decreased levels of GFP expression compared with IMCs from non-tumor bearing mice. Similar results were observed with \textit{in vitro} culture of BM cells, where addition of GL261 glioma-derived culture-supernatants led to a decrease in GFP expression in CD11b\(^+\)Gr1\(^+\) cells (Fig. 4C). This change was observed in both CD11b\(^+\)Ly6C\(^+\) M-IMC and CD11b\(^+\)Ly6G\(^+\) G-IMC populations (data not shown). To determine whether the GFP expression status is associated with any changes in the suppressive function of CD11b\(^+\)Gr1\(^+\) cells, we utilized FACS-sorted GFP\(^+\) and GFP\(^-\) CD11b\(^+\)Gr1\(^+\) cells, and co-cultured them with naive CFSE-labeled CD8\(^+\) T cells. We observed an inverse association between GFP expression and suppressive capabilities. GFP\(^-\)CD11b\(^+\)Gr1\(^+\) cells exhibited a more profound suppression of T cell proliferation compared with their GFP\(^+\) counterparts (Fig. 4D). The presence of glioma is associated with reduced HDC-GFP expression in IMC, thereby inducing a more profound immunosuppression.

**Discussion**

In this study, intrigued by decreased risks of glioma in asthma patients and the rather increased risks in patients who chronically use anti-histamines, we aimed to determine the role of histamine via HDC expression in glioma development. Although which anti-histamines were used was not specifically addressed in these epidemiology studies, histamine receptor 1 (H1R) antagonists are most commonly used.\(^5\) In our current study, we utilized a global blockade of histamine production via \textit{HDC}\(^/-\) mice in our established de novo and transplant glioma models. We observed a significantly shortened survival in \textit{HDC}\(^/-\) mice (Fig. 1C). Similar results were seen in our intracranial glioma cell injection model as \textit{HDC}\(^/-\) hosts exhibited more rapid tumor growth than control WT mice (Fig. 1D). A previous study has demonstrated that histamine promotes differentiation of IMC and leads to reduced tumor progression \textit{in vivo}.\(^8\) Based on our data showing histamine does not directly affect tumor growth, it is likely that histamine may influence the immune microenvironment. A previous report has shown that glioma cells can secrete histamine.\(^28\) We utilized \textit{HDC}\(^/-\) glioma cells for our cell inoculation model in WT and \textit{HDC}\(^/-\) mice to

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**Figure 4.** Effects of glioma-bearing conditions on GFP expression in CD11b\(^+\)Gr1\(^+\) IMCs of HDC-GFP mice. (A) Representative histograms of GFP expression in CD11b\(^+\)Gr1\(^+\) BILs in non-tumor-bearing mice and mice bearing intracranial GL261 glioma. (B) Percentages of GFP\(^+\) cells in CD11b\(^+\)Gr1\(^+\) populations in BILs, spleen, and BM in non-tumor-bearing and tumor-bearing mice (n = 4 mice/group). (C) BM cells were cultured for induction of BM-IMCs with or without GL261 culture-supernatants. Representative histograms of GFP expression in CD11b\(^+\)Gr1\(^+\) cells are shown (n = 3 mice/group). (D) FACS-isolated GFP\(^+\) and GFP\(^-\) CD11b\(^+\)Gr1\(^+\) were co-cultured with CFSE-labeled CD8\(^+\) T cells for 3 d at ratios of 0.5 IMC to 1 T cells and 1 IMC to 1 T cells. Percentages non-proliferating cells in total CD8\(^+\) T cells are shown (three mice/group). *, P<0.05. **, P<0.01.
eliminate any possible effect of histamine production by glioma cells. Interestingly, when we inoculated glioma cells derived from WT (HDC+/−) mice, the differential survival observed in Fig. 1D was no longer apparent (data not shown). Since there was no difference in intrinsic growth rate difference between HDC−/− and WT tumors in vitro, we presumed that these data suggest a possibly critical role of glioma cell-derived histamine activity on altering immune milieu, as multiple glioma cell lines have demonstrated ability to secrete histamine in vitro.28 The specific impact of systemic versus local histamine production on the immune environment requires further exploration.

In this study, we utilized HDC as a surrogate for histamine status. Further experiments will examine the role of exogenous histamine as a modulator of immune surveillance. Previous clinical trials have examined histamine as an adjuvant to interleukin-2 treatment for certain cancer types with modest benefits.29,30 In these referenced studies, administration of histamine resulted in decreased circulating macrophages and more robust T cell response, consistent with the decrease of IMCs in our study (Fig. 2). The possible impact of addition of exogenous histamine to other immunotherapies such as peptide vaccines or adoptive therapies is unknown and may warrant further investigation.

Our examinations focused on the impact of HDC on induction of immunosuppression. A previous study has indicated aberrant maturation of BM-derived myeloid cells, specifically increased CD11b−Gr1+ IMC derived from HDC−/− mice.31 Our analysis corroborated this previous study by demonstrating increased systemic and glioma-infiltrating populations of CD11b+Gr1+ IMCs in HDC−/− hosts (Figs. 2C and D). These cells have been shown to promote tumor growth via expression of interleukin-6 (IL-6), which can promote tumor growth and angiogenesis in colorectal cancer models.8 These cells phenotypically resemble MDSC, which can negate an adaptive T cell response.22 The definition of MDSC is a functional one, so we sought that these cells could suppress T cell response. HDC−/− CD11b+Gr1+ IMC derived from gliomas (Fig. 3A) as well as BM-IMC from HDC−/− mice (Fig. 3C) exhibited more profound suppression of CD8+ T cells in vitro compared with WT counterparts.

HDC−/− myeloid cells demonstrated increased activation of PGE2, which has known immunosuppressive and tumor-promoting effects.14,31,32 Addition of celexoib, an inhibitor of PGE2, at least partially reversed immunosuppression by HDC−/− BM-IMC (Fig. 3E). However, the moderate degree of reversal by celexoib strongly suggests that there are other mechanisms underlying the effects of HDC. Nonetheless, based on these data, we can surmise that HDC expression is involved in myeloid cell maturation, and that deficiency promotes the accumulation of CD11b−Gr1+ IMCs as well as induction of immunosuppressive function at least partially via increased production of PGE2.

Similarly, ex vivo examination of CD8+ BIL populations corroborated the in vitro results of T cell inhibition, as HDC−/− BIL demonstrated reduced CD8+ T cell populations and functions (Figs. 2A and B). Whereas our co-culture results revealed a role for IMCs as a regulator of T cell function, histamine may also influence T cells in other manners. T cells were not shown to express HDC,8 but they do express histamine receptors. Histamine has been shown to affect differentiation toward type 1 CD4+ and CD8+ T cells, primarily via histamine 1 receptor signaling (H1R).33-35 HDC−/− CD8+BILs showed no differences in IFNγ production levels compared with WT counterparts in our study (data not shown), which may be due to the impact of global histamine deficiency rather than knockdown of a single receptor (Fig. 2B). Additionally, histamine deficiency has been shown to reduce antigen presentation by dendritic cells, resulting in reduced CD8+ T cell priming and function.34 This was also mediated primarily by H1R signaling on dendritic cells.34 In our studies, HDC−/− mice showed increased IMC in both BILs and the spleen (Figs. 2D and E). Although we did not specifically examine antigen-presentation capabilities, it is possible that HDC deficiency may also affect T cell priming and modulate T cell function via altered antigen-presentation efficiencies in addition to influencing regulatory populations. A more comprehensive analysis of the effects of lack of histamine on T cell function is required.

Gliomas are notorious for potentiating immunosuppression to promote their own development. In glioma-bearing conditions, we observed a decrease in HDC expression, specifically in CD11b+Gr1+ IMCs not only in the tumor tissue but also in the spleen and BM (Fig. 4B). In vitro, addition of glioma culture-supernatants led to a decrease in the HDC transcription in both G-IMC and M-IMC subtypes of CD11b+Gr1+ BM cells (Fig. 4C). Functionally, the GFP− cells demonstrated more profound immune suppression (Fig. 4C), suggesting that loss of HDC is linked to induction of immunosuppressive effectors. These findings of tumor-induced downregulation of HDC are consistent with previous observations with colorectal cancer cells.8 The specific glioma-derived factors that can possibly suppress expression of HDC is currently unknown. Many tumor-derived cytokines and growth factors have been shown to induce the MDSC phenotype, but none have been previously shown to act via the HDC pathway. Based on our data with glioma cell culture supernatants, it can be hypothesized that at least one of those can be a secreted factor. As demonstrated in Fig. 3D and E, PGE2 was upregulated in HDC−/− IMC and blockade of the COX-2 pathway partially abrogated the T cell suppression. Therefore, PGE2 may be regulated by HDC. Future studies will examine the specific secreted factors that can regulate HDC, leading to immaturity of myeloid cells as well as other mechanisms, such as preferential recruitment and/or survival of HDC−/− IMC in the glioma microenvironment.

**Methods**

**Animals**

WT (BALB/c) mice were obtained from Jackson Laboratory. Histamine-deficient HDC−/− BALB/c mice have been previously described (Columbia University).8 Animals were handled in Animal Facility in the University of Pittsburgh per an Institutional Animal Care and Use Committee-approved protocol.
Mice were bled from their submandibular veins. Sera were collected and separated by Microtainer tube with serum separator (BD Biosciences). Histamine in serum samples was measured by using histamine enzyme immunoassay per manufacturer’s directions (Bechman Coulter).

Sleeping Beauty de novo glioma model

The procedure was performed as described previously.\textsuperscript{36,37} In vivo compatible DNA transfection (In vivo-JetPEI) was obtained from Polyplus Transfection. The following plasmids were used for glioma induction: T2/C-Luc/PGK-SB13 (0.125 μg), pT/CAGGS-NRASV12 (0.125 μg), pT2/shP53 (0.125 μg), and PT3.5/CMV-EGFRvIII (0.125 μg). Tumor size was monitored via BLI using IVIS200 (Caliper Life Sciences). Glioma cell lines from both WT and HDC\textsuperscript{-/-} mice were established from de novo gliomas as described above and subsequent in vitro culture and cloning in our laboratory.

Intracranial Glioma Cell Inoculation

The procedure to establish glioma cell lines from SB transposon-mediated de novo murine gliomas has been described previously.\textsuperscript{14,36} Mice were intracranially inoculated with glioma cells in 2 μL of PBS at the bregma 3 mm to the right side of sagital suture and 3.5 mm below the surface of skull using stereotactic frame (David Kopf Instruments, Tujunga, CA), stereotaxic injector (Stoelting Co., Wood Dale, IL) and using stereotactic frame (David Kopf Instruments, Tujunga, Los Angeles, CA).

Isolation of brain-infiltrating leukocytes (BIL)

The procedure for isolation of BIL via Percoll density gradient has been described by us previously.\textsuperscript{14,36}

Bone marrow (BM)-IMC bone marrow culture

A similar procedure has been previously described.\textsuperscript{15,24} Briefly, red blood cell (RBC)-depleted BM cells were isolated from WT and HDC\textsuperscript{-/-} mice. At day 0, GM-CSF (100 ng/mL) and G-CSF (100 ng/mL) were added to cultures. At day 3 and day 9, GM-CSF (100 ng/mL), G-CSF (100 ng/mL), and IL-13 (80 ng/mL) were added. All cytokines were purchased from Peprotech. At day 10, BM cells were cultured isolated for co-culture with CD8\textsuperscript{+} T cells. Celecoxib was purchased from Sigma and used at 50 μm/mL.

IMC-mediated T cell inhibition

Naïve CD8\textsuperscript{+} T cells were isolated WT BALB/c splenocytes using magnetic bead-negative selection (Miltenyi Biotec), labeled with 1nM CFDA-SE (Invitrogen), and incubated with varying numbers of cultured or FACS-sorted CD11b\textsuperscript{+}Gr1\textsuperscript{-} IMCs from gliomas in the presence of anti-CD3/CD28 Dynabeads (Invitrogen) and 30 U/mL of hIL-2 (Peprotech). Cells were analyzed via flow cytometry on an AccuriC6 (BD Biosciences).

Real-time PCR

The procedure was described previously.\textsuperscript{15,36} The following primers were obtained from Applied Biosystems: GAPDH (Mm99999915_g1) and PROSTAGLANDINE2 (Mm00478374_m1).

Flow Cytometry

Samples were analyzed on Accuri C6 (BD Biosciences) via CFlow Plus (BD Biosciences). Antibodies utilized: anti-CD4\textsuperscript{+} (VH129.19), anti-CD8\textsuperscript{+} (53-6.7), and anti-Ly6C (AL-21) from BD Biosciences; anti-CD11b (M1/70), anti-CD107a (1D4B), anti-FoxP3 (NRRF-30), and anti–Gr-1 (RB6-8C5) from eBioScience; anti-Ly6G (1A8) from BioLegend. FACS-sorting was performed on BD MoFlo Astrios.

Statistical Analysis

Student’s t test was carried out to analyze differences between two groups. Log-rank tests were done to analyze survival of mice with glioma. Value of P < 0.05 were considered statistically significant. All data were analyzed by Graphpad Prism.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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