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Bipolar-shape response of human neutrophils to corticotropin-releasing factor

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Abstract

Human neutrophils in whole blood become bipolar in shape after exposure to chemokinetic stimuli. In normal blood, the proportion of non-spherical neutrophils was $1.2 \pm 0.07\%$ ($n = 101$). After incubation of blood samples with corticotropin-releasing hormone (CRF, 1 to 20 μM) 36 of 101 subjects exhibited a $\geq 10\%$ bipolar-shape ellipsoid response. This ellipsoid response was more frequent in female than in male subjects (32/75 vs. 4/26, $p < 0.01$). Female Caucasian subjects were more sensitive to CRF than female East Asian subjects (25/48 vs. 2/15, $p < 0.01$). Age was not a factor in sensitivity to CRF. In young female East Asian subjects (23 ± 0.4 years, $n = 8$) that did not manifest the ellipsoid response to CRF, formyl-Met-Leu-Phe (fMLP), a chemotactic peptide, 10^{-9} M increased non-spherical neutrophils to $31 \pm 0.8\%$. In these individuals, the fMLP response was inhibited in a dose-dependent manner by CRF. The pharmacological profile of the stimulatory and fMLP-inhibitory actions of CRF on neutrophil shape was consistent with that of a CRF_1-receptor mediated response. Expression of mRNA for the CRF_1-receptor was detected in hematopoietic cell lines (e.g., HL-60) using a reverse transcriptase polymerase chain-reaction method. The bipolar-shape response of human neutrophils to CRF has the potential to be a useful indicator of the functional state of this hormone-receptor system in inflammation.

Keywords: CRF (corticotropin-releasing factor); Neutrophil; Bipolar shape; Blood, human; fMLP (formyl-Met-Leu-Phe); Inflammation

1. Introduction

The key role of corticotropin-releasing factor (CRF), a 41-residue peptide, as a neuroendocrine integrator of the organism's response to stress is now well-recognized (Turnbull and Rivier, 1997). CRF is found in the mammalian brain, and in peripheral tissues such as the skin, testes, pancreas, adrenal medulla, gut, placenta, and cells of the immune system (Chalmers et al., 1996). Two types of CRF receptors have been identified: CRF_1 and $CRF_{2\alpha\beta}$. These receptors are distributed in brain, in pituitary, and in peripheral tissues such as heart and lung. Whilst the participation of CRF and its receptors in adrenocorticotropin release from pituitary is established, the consequences of peripheral release of CRF and activation of its receptors are less clear. The presence of CRF in cells of the immune system and in inflamed tissues has recently been reviewed (Karalis et al., 1997). The authors suggest that local tissue modulation of CRF levels and its receptor activities can affect the course of inflammation. Schafer et al. (1996) have shown that CRF-stimulated release of proopiomelanocortin-derived peptides from lymphocytes in inflamed tissues can produce peripheral analgesia in experimental animals. McLoon and Wirtschafter (1997) reported that local injection of CRF into the eyelids of rabbits and monkeys reduced the acute inflammatory response to doxorubicin, a toxic chemotherapeutic agent. CRF pretreatment also dramatically enhanced the rate of wound healing after doxorubicin. Recently, it has been shown that mRNA for CRF and CRF-receptor are expressed in human skin and production of CRF is stimulated by exposure to ultraviolet light (Slominski et al., 1995, 1996).

In an effort to clarify the relationship of CRF receptors to inflammatory processes, we investigated the response of

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neutrophils to CRF in whole blood samples from human volunteers. Neutrophils in whole blood react to chemoattractants by changing from a normal spherical to a bipolar ellipsoid shape (Jadwin et al., 1981; Lord and Roath, 1990) and the activity of these cells are modulated by stresses such as illnesses from infectious agents (Ader et al., 1995). A standard bacterial chemoattract such as fMLP (formyl-Met-Leu-Phe) will elicit the bipolar-shape change response in all subjects tested (Lord and Roath, 1990). Here we report that the shape change response of human neutrophils to CRF stimulation is heterogeneous. In some subjects, CRF will produce an increase in the percentage of bipolar-shaped neutrophils. In other subjects, CRF alone does not alter neutrophil shape, but it prevents the shape change induced by fMLP. The pharmacological profile of the response appears to be mediated by CRF_1 receptors.

2. Materials and methods

2.1. Peptides

Unless specified otherwise, all CRF and CRF analogs used were based on the 41-residue human/rat amino acid sequence. CRF was obtained from American Peptide (Sunnyvale, CA) or from Dr. Nicholas Ling of Neurocrine Biosciences (San Diego, CA). Sauvagine and α-helical-CRF 9-41, a synthetic CRF receptor antagonist, were purchased from Bachem (Torrance, CA). Human urocortin, [Met (O)^{21,38}] CRF, and [D-Glu^{20}] CRF were custom synthesized by Dr. Janos Varga (California Peptide Research, Napa, CA). [Ala^{8}]ovineCRF and [Ala^{38}]ovineCRF were kindly provided by Dr. Jean Rivier (Salk Institute, La Jolla, CA). Solutions of these peptides were prepared by first dissolving the peptide in 0.1 ml of 0.1 M acetic acid and then adding 0.9 ml of saline. Peptides were generally tested at concentrations of 2.5, 6.25, and 15.6 μM. *N*-Formyl-Met-Leu-Phe (fMLP) was obtained from Sigma Chemical (St. Louis, MO) and 10^{-2} M stock solutions were prepared by first dissolving this peptide in dimethylsulfoxide.

2.2. Neutrophil shape change assay

Blood samples were obtained by venipuncture of the antecubital vein and collected into heparinized tubes (Vacutainer® Sodium Heparin 7 ml, Becton-Dickinson Systems, Franklin-Lakes, NJ). For measurement of neutrophil shape changes, blood samples were kept at room temperature and used within 1 h of collection. For 26 randomly-selected subjects, standard hematological parameters such as complete white blood cell count, blood lipids and enzyme profiles were obtained (SmithKline Beecham clinical chemistry laboratory, San Jose, CA). The age, sex and ethnic characteristics of the 101 blood donors in this study are given in Table 1. The study design and protocol were approved by the Human Subjects Research Committee of the University of California. Each subject was also given a brief questionnaire about medical history.

Table 1
Ethnic distribution of subjects manifesting an ellipsoid or a non-ellipsoid neutrophil response to CRF

Ethnic group	Male	Age	Female	Age	Total	Percent ellipsoid
African–American	1/1	48	3/6	42±4.8	4/7	57
Caucasian	3/18	40±3.5	25/48	45±1.8	28/66	42
Southwest Asian	0/2	22	2/6	22±0.8	2/8	25
East Asian	0/5	35±5.3	2/15	29±3.1	2/20	10

An ellipsoid responder has >10% non-spherical neutrophils (read from smears of whole blood samples) after incubation with CRF at 37°C for 20 min. The number of ellipsoid responders over the number of subject tested is given in the table.

The method for measuring, in whole blood, the shape-change response of human neutrophils to exogenous agents is as described by Lord and Roath (1990) and is based on the original method of Jadwin et al. (1981). Briefly, samples of 0.45 ml of heparinized blood were incubated with 0.05 ml of saline or test substances dissolved in saline and incubated at 37°C for 20 min. Three smears of each blood sample were then prepared on separate microscope slides, stained with Wright Giemsa (Ricca Chemical, Arlington, TX), dried overnight, cover-slipped with Permount (Fisher Scientific, Fair Lawn, NJ) and examined at ×40 magnification.

One hundred neutrophils from each slide were counted and classified as spherical or ellipsoid: a spherical neutrophil being the normal round neutrophil seen in standard blood smears and ellipsoid neutrophils being elongated, asymmetric and with irregular borders. A neutrophil was classed as ellipsoid if, upon visual inspection, its major axis was considered at least twice the length of the minor axis. All slides were coded and counted by two individuals unaware of the nature of treatment received by the blood samples. To assess variability in counting procedures, identical smears were coded and recounted by the two examiners and no significant differences in counts were noted. One hundred neutrophils per slide were counted for three slides and the averaged percent of bipolar neutrophils present was the measure of the shape-change response. Harkin et al. (1993) have noted that visual methods for classifying neutrophil polarization are as sensitive and reliable as computerized morphometric and flow cytometric methods.

2.3. Reverse transcriptase-polymerase chain reaction methods (PCR) for detecting mRNA for CRF receptors

Poly(A) + RNA was isolated from human premonocyte HL-60; HL-60 cells differentiated by 0.1% dimethylsulf-

oxide treatment; human premonocyte U937; human granulocyte K562; mouse T cell line BW5145; and mouse B cell line MX279, by mRNA isolation kits (Ambion, Naugatuck, CT). Single-stranded cDNA was prepared from 1 μg of poly(A) + RNA in a 20 μl reaction containing 3.2 μg of random primer, 1 mM dNTPs, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 50 units of RNAase inhibitor, and 20 units of AMV reverse transcriptase (Boehringer, Indianapolis, IN) for 60 min at 42°C. One tenth of the reaction mixture was then subjected to 35 cycles of PCR (94°C, 1 min; 57°C, 1 min; and 72°C, 30 s) in 10 mM Tris–HCl (pH 8.5), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and 2.5 units of Taq polymerase (Boehringer, Indianapolis, IN) using 2 μM of each degenerative oligonucleotide primer (sense, 5′ TGGATGTT(T/C)G(G/T)(G/A/T/C)HGA(G/A)GG-(T/C)TG(T/C)TA(T/C)CT; antisense, 5′ AA(G/A/T/C)AC(G/A)GA(T/C)AC(G/A)AAGAA − (G/A)CC(T/C)TG(G/A)AA) for CRF_1 and CRF_2 receptors, respectively, in a MicroCycler (Eppendorf, Madison, WI).

PCR reaction products were size fractionated on 1.2% agarose gels containing 0.5 μg/ml ethidium bromide. Reaction products migrating at the predicted size (473 bp) were kinased and ligated into pCRII vector (Invitrogen, San Diego, CA) using T4 DNA ligase. Ligation products were transformed into DH5α competent cells, and individual colonies were picked up for small scale plasmid DNA preparation. Miniprep DNAs were then subjected to DNA sequence analysis using Sequenase enzyme (United States Biochemical, Cleveland, OH).

The statistical analysis included calculation of mean, standard deviation and variance. The Fisher's 2×2 exact test was used and significance level set of $P < 0.005$. The statistical analysis was conducted using standard statistical software (SPSS, Chicago IL).

3. Results

The percentage of non-spherical neutrophils in whole blood, mixed 10% v/v with heparinized-saline, of all subjects in this study averaged $1.2 \pm 0.75\%$ (S.D.), $n = 101$. None of the individual samples exceeded 4%. These background values were similar to levels reported by Jadwin et al. (1981) and Lord and Roath (1990) in studies on the shape characteristics of normal neutrophils in whole blood. The response of neutrophils to CRF in four ethnic groups are shown in Table 1. In general, the reaction of neutrophils of individuals to CRF could be classified as all-or-none, with a $\geq 10\%$ ellipsoid cells being an empirically selected quantal indicator of response. For the purposes of description, such individuals will be termed 'ellipsoid' responders. In initial range-finding studies we tested h/r CRF at concentrations ranging from 0.0256 to 97.6 μM in 7 subjects (4 female and 3 male). Sigmoidal-log

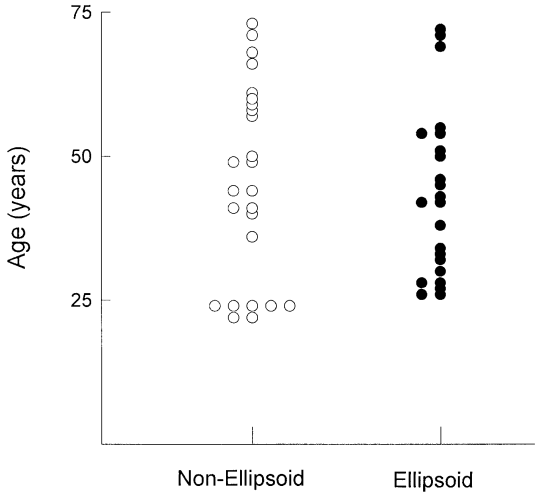

Fig. 1. Age distribution of non-ellipsoid and ellipsoid responders to CRF in female Caucasian subjects ($n = 48$).

dose-effect relationships were obtained for 3 subjects who were ellipsoid responders, the threshold for a $> 5\%$ response in each required at least of 2.5 μM of CRF. Four of the non-ellipsoid responders did not manifest a $> 5\%$ at any of the CRF concentrations tested. In subsequent experiments, concentrations of CRF at 2.5, 6.2 and 15.6 μM were used in bioassays for detecting an ellipsoid response. Of the 101 subjects tested, 36 ellipsoid responders were detected, the proportion being higher in female (32 out of 75) than in male subjects (4 out of 26) ($P < 0.01$, Chi-square test). The number of ellipsoid responders was found more frequently in Caucasians (28 out of 66) than in East Asians (2 out of 20) ($P < 0.01$, Chi-square test). Age was not a factor which determined sensitivity to CRF (Fig. 1). For the largest homogeneous group of 48 Caucasian women, there was an almost equal distribution of non-ellipsoid vs. non-ellipsoid responders over a wide age range

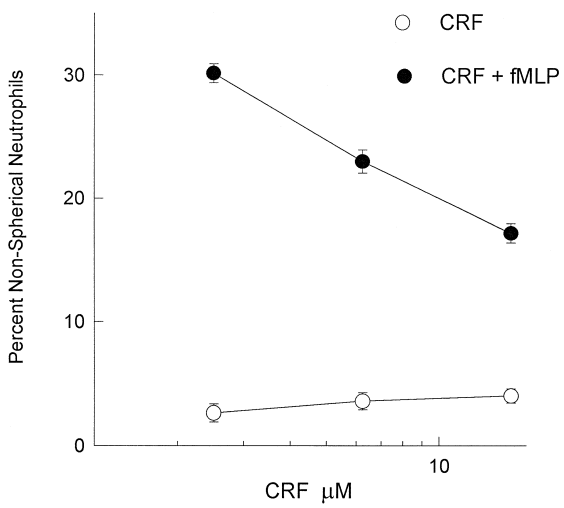

Fig. 2. Bipolar-shape response of human neutrophils to fMLP (10^{-9} M) is suppressed by CRF in non-ellipsoid East Asian subjects ($n = 8$). Vehicle, $1.2 \pm 0.2\%$; after fMLP, $31.0 \pm 0.8\%$.

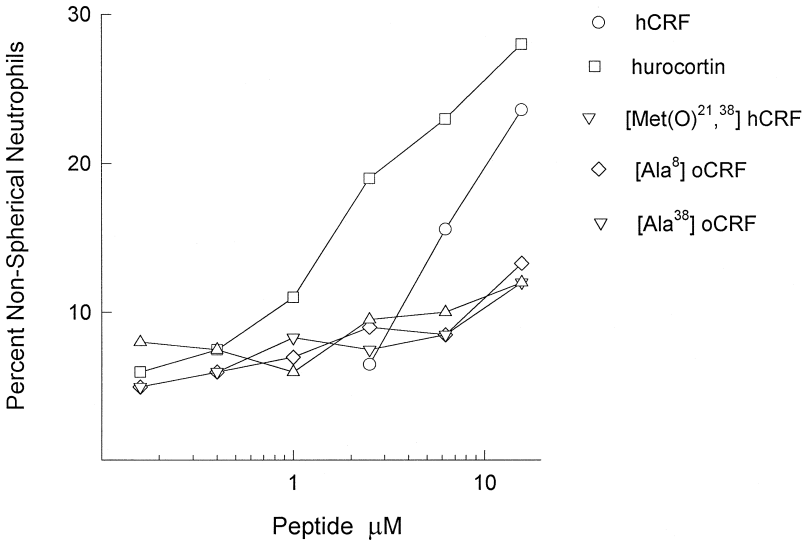

Fig. 3. Bipolar-shape response of human neutrophils to CRF analogs in an ellipsoid responder to CRF. The analogs [Ala^8]ovineCRF, [Ala^{38}]ovineCRF and [Met(O)^{21,38}]CRF are inactive on ACTH-releasing and anti-edema end-points (h = human sequence, o = ovine sequence).

(Fig. 1). The medical history of the subjects did not reveal any correlation between CRF sensitivity and pre-existing diseases. The hematological parameters measured in 26 randomly-selected subjects (6 ellipsoid and 20 non-ellipsoid responders) were within normal values. The absolute neutrophil count of these 26 subjects averaged 3829 $\pm$ 293 (S.D.) cells/μl (normal range 1500 to 7800 cells/μl) and were not significantly different between ellipsoid and non-ellipsoid responders.

fMLP is a bacterial peptide which activates chemokinesis in neutrophils (Lord and Roath, 1990). In young female East Asian subjects ($n = 8$, age 22.7 $\pm$ 0.4 (21–24) years), previously known to be non-ellipsoid responders to CRF, fMLP (10^{-9} M) increased the number of ellipsoid neutrophils to 31 $\pm$ 0.8% (Fig. 2). In these individuals, the fMLP response was inhibited in a dose-dependent manner by CRF (Fig. 2) ($P < 0.01$ paired t-test).

The pharmacological profile of ellipsoid and non-ellipsoid responders were examined using other peptides of the CRF superfamily and CRF analogs (Turnbull and Rivier, 1997). In blood samples from several ellipsoid female Caucasian subjects, sauvagine (from the skin of *Phyllomedusa sauvegeii*) and human urocortin, which are peptides of the CRF superfamily, also elicited the ellipsoid response (Figs. 3 and 4). On the other hand, [Ala^8] ovineCRF, [Ala^{38}]ovineCRF and [Met (O)^{21,38}] CRF, modified CRF analogs which lack ACTH-releasing activities (Kornreich et al., 1992) and the ability to suppress heat-induced edema (Wei and Thomas, 1994), were inactive (Fig. 3). The synthetic CRF receptor antagonist α-helical-CRF-

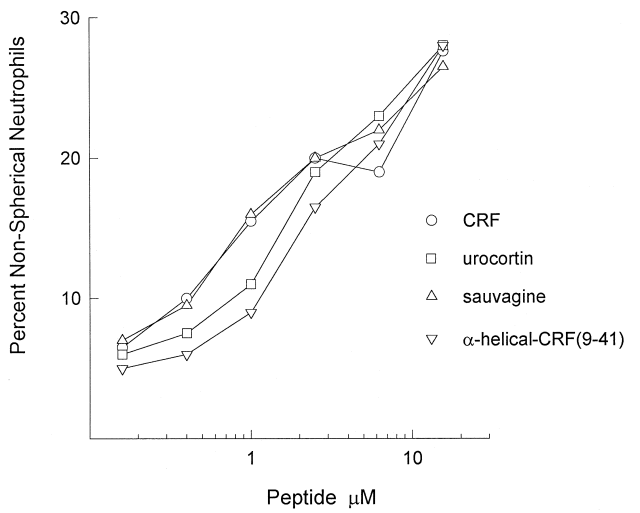

Fig. 4. Bipolar-shape response of human neutrophils to CRF, sauvagine, human urocortin, and α-helical-CRF-(9-41) in ellipsoid responders to CRF ($n = 3$ per peptide).

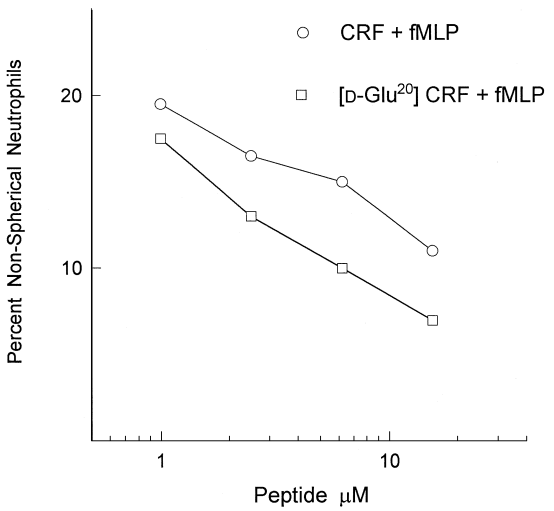

Fig. 5. Bipolar-shape response of human neutrophils to CRF and [D-Glu^{20}]CRF in a non-ellipsoid subject in the presence of fMLP (10^{-9} M). [D-Glu^{20}] CRF is a selective CRF$_1$-receptor agonist.

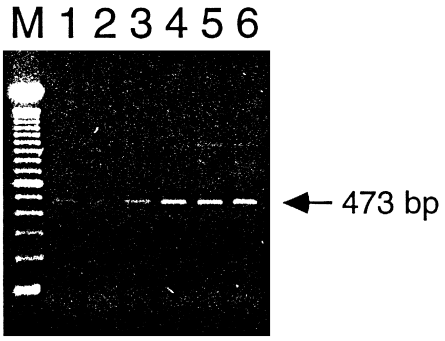

Fig. 6. Expression of CRF receptor mRNA by reverse transcriptase polymerase chain reaction in hematopoietic cell lines. Lane M, 100 base-pair ladder markers; lane 1, human premonocyte HL-60; lane 2, differentiated HL-60 cells treated by 0.1% DMSO; lane 3, human premonocyte U937; lane 4, human granulocyte K562; lane 5, mouse T cell line BW5145; lane 6, mouse B cell line MX279.

(9-41), which has partial agonist actions on CRF receptors (Chalmers et al., 1996), also elicited the bipolar-shape response in ellipsoid subjects but was less potent than CRF (Fig. 4). In a non-ellipsoid male, [D-Glu^{20}] CRF, a CRF analog selective for the CRF_1-receptor (Wei et al., 1996) was at least as potent as CRF in suppression of the fMLP effect (Fig. 5). This pattern and chemical specificity of the ellipsoid response to CRF peptides suggested that CRF_1 receptors mediated these effects (Chalmers et al., 1996). Messenger RNA code for the expression of CRF_1 receptors were detected by reverse transcriptase polymerase-chain reaction in a variety of mammalian hematopoietic cell lines (Fig. 6).

The neutrophil shape change assay in response to CRF was repeated in several subjects in order to gain insight into factors that may affect sensitivity. One ellipsoid Caucasian female subject was tested seven times over a period of a year and half and the ellipsoid response was detected on each occasion. Two out of six young female East Asian subjects, previously non-ellipsoid responders to CRF, became ellipsoid responders in a second test 2 months later. On the day before the second test, all six had been subjected to the stress of taking Medical College Admissions Tests or being interviewed for admission to medical school.

4. Discussion

The response of neutrophils to CRF in whole blood samples divide subjects as 'ellipsoid' or 'non-ellipsoid' responders to this hormone. Ellipsoid responders may be operationally defined as those individuals whose whole blood samples, after 20 min of incubation with CRF, respond with a $\geq 10\%$ bipolar neutrophils at tested concentrations of 1 to 20 μM of CRF. The percentage of bipolar neutrophils in non-ellipsoid responders remain at $< 5\%$ at these test concentrations. The fMLP response of neutrophils in non-ellipsoid responders is attenuated by CRF, indicating that this hormone has dual actions on neutrophils. Age is not a determinant of ellipticity, but it appears that gender and ethnicity may be factors which influence individual response. In our limited sample, the ellipsoid response was found more frequently in females and in Caucasians when compared to East Asians.

The precise location of the CRF receptors mediating the neutrophil shape-change response is not known at this time. The pharmacological profile of the response (Chalmers et al., 1996) and the PCR data in hematopoietic cells implicate CRF_1 receptors as the responding elements. Recently, mRNA coding for the CRF_1 receptor gene was found to be present in human neutrophils (Elizabeth Linton, pers. communication). However, CRF receptors are also present on monocytes in human blood (Leu and Singh, 1993) and it is possible that secondary release of cytokines from monocytes can influence neutrophil shape (Hagan et al., 1992). The application of sensitive and rapid flow cytometric techniques to measurement of white blood cell responses to CRF may help resolve the question of the primary target sites for the CRF effect on neutrophil shape.

The bi-directional nature of the interaction of CRF with human white blood cells indicate that this hormone has subtle effects on the conditioning of cells which participate in inflammation. Current attempts to categorize CRF as either a pro-inflammatory (Karalis et al., 1997) or an anti-inflammatory substance (Wei et al., 1993) in peripheral tissues may be an over-simplified description of a complex process. The neutrophil-shape test characterizes individual responses to CRF and, when used in conjunction with clinical studies, may be a tool to offer clues on how this hormone-receptor system influences the course of inflammation in human subjects.

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