Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 2036–2040, March 1982
Medical Sciences

Glucocorticoid hormone resistance during primate evolution: Receptor-mediated mechanisms

(cortisol/protein-unbound cortisol/glucocorticoid receptors)

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Communicated by J. E. Rall, November 25, 1981

ABSTRACT **The concentrations of total and protein-unbound plasma cortisol of New World monkeys are higher than those of Old World primates and prosimians. The urinary free-cortisol excretion also is increased markedly. However, there is no physiologic evidence of increased cortisol effect. These findings suggest end-organ resistance to glucocorticoids. This was confirmed by showing that the hypothalamic-pituitary adrenal axis is resistant to suppression by dexamethasone. To study this phenomenon, glucocorticoid receptors were examined in circulating mononuclear leukocytes and cultured skin fibroblasts from both New and Old World species. The receptor content is the same in all species, but the New World monkeys have a markedly decreased binding affinity for dexamethasone. Thus, the resistance of these species to the action of cortisol is due to the decreased binding affinity of the glucocorticoid receptor. This presumed mutation must have occurred after the bifurcation of Old and New World primates ($\approx 60 \times 10^6$ yr ago) and before the diversion of the New World primates from each other ($\approx 15 \times 10^6$ yr ago).**

End-organ resistance to steroid hormones has been described for androgens (1), aldosterone (2), progesterone (3), and vitamin D (4). There are no known examples of resistance to the action of estradiol and only two examples of resistance to cortisol. First, the guinea pig has long been known as a "corticoresistant" species (5); second, there is a single example of resistance to cortisol in man (6, 7). Two New World primates, the squirrel monkey (*Saimiri sciureus*) (8) and the marmoset (*Callithrix argentatus* and *Saguinus oedipus*) (9), have been shown to have markedly elevated plasma cortisol values without any physiologic evidence of glucocorticoid hormone excess. Because these animals might be a model of glucocorticoid resistance, we examined some other aspects of cortisol secretion and transport and analyzed the properties of their glucocorticoid receptors.

MATERIALS AND METHODS

Subjects and Protocols. Animals from three New World primate species (the squirrel monkey, *S. sciureus*; the marmosets, *Callithrix jacchus*, and *Saguinus labiatus* and *fuscicollis*; and the owl monkey, *Aotus trivirgatus*), from four Old World primate species (the cynomolgus, *Macaca fascicularis*; the rhesus, *Macaca mulatta*; the baboon, *Papio papio*; and the chimpanzee, *Pan troglodytes*), and from six Old World prosimian species of the subfamilies Lemurinae or Galaginae were studied. Plasma samples for assay of total and free cortisol, aldosterone, corticosterone, glucose, and electrolytes were obtained between 7:00 and 9:00 a.m. by femoral artery puncture under ketamine anesthesia. Plasma samples were drawn during the 10- to 20-min period of anesthesia. Preliminary studies showed that the plasma cortisol concentrations measured at 2-min intervals were stable during this period of time. Human plasma samples were obtained by venipuncture at the same time of the day and, despite the differences in the sampling, have been used for comparison.

Total plasma cortisol (10) and plasma corticosterone (11), aldosterone (12), and dexamethasone (13) were measured by previously described radioimmunoassays. Plasma glucose was measured by the hexokinase method (14). The cortisol not bound to protein (i.e., free) was estimated by equilibrium dialysis at 37°C with 1:5 diluted plasma as described (15, 16). The concentration of unbound cortisol was calculated by applying the formula of Slaunwhite (17) and was corrected for the initial dilution.

Twenty-four hour urine samples were collected in metabolic cages. Urinary free cortisol was measured by radioimmunoassay as described (18), and 24-hr urinary excretion was calculated.

The suppressibility of the hypothalamic-pituitary-adrenal axis was examined by administering dexamethasone sodium phosphate (Decadron). Decadron was given intramuscularly at 8:00 p.m. to squirrel and cynomolgus monkeys at doses of 5, 10, 30, 60, 120, 460, and 1400 μg/kg of body weight ($n = 3$ at each dose level). Blood was drawn the following morning, and plasma cortisol and dexamethasone were measured.

Glucocorticoid Receptor Assays. Glucocorticoid receptors were measured in circulating mononuclear leukocytes (19) and cultured skin fibroblasts (20) as described.

The circulating mononuclear leukocytes were isolated from pooled blood and incubated for 16 hr at 24°C with [^{3}H]-dexamethasone at six concentrations between 1 and 40 nM. End tubes contained 10^7 cells, and nonspecific binding was determined at each concentration by incubating the cells in the presence of 100-fold excess unlabeled dexamethasone. Viability was greater than 90% as shown by trypan blue exclusion. The data were analyzed by the method of Scatchard (21). The binding capacity is expressed as fmol of dexamethasone bound per 10^6 cells.

Fibroblast strains were grown from skin specimens obtained by punch biopsy. The biopsies were processed as described (20). Fibroblasts were grown to confluence over a period of 4–6 wk and then divided into tubes containing $0.4–1.0 \times 10^6$ cells per tube. The tubes were incubated for 60 min at room temperature with five concentrations of [^{3}H]dexamethasone between 1 and 20 nM. Nonspecific binding was assessed at each concentration by incubating in the presence of unlabeled dexamethasone. The binding capacity is expressed as the number of dexamethasone binding sites per cell.

Statistical Analysis. Unless otherwise indicated, the results are presented as means ± SEM. A two-tailed Student's *t* test

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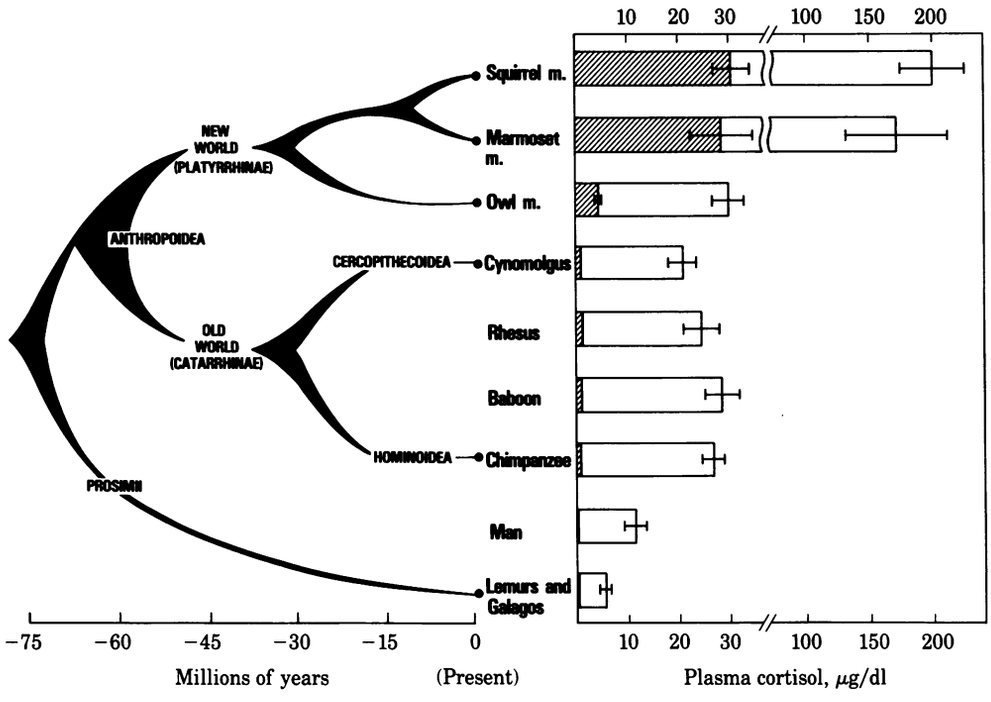

FIG. 1. Total (whole bar) and free (hatched portion) plasma cortisol concentrations in primates. An albumin and transferrin phylogeny tree for the primate taxa, in which the time scale is assigned with the assumption that their most common ancestor lived ≈60 million yr ago (25, 26), indicates phylogenetic relationships among the primate species studied in this paper. m, Monkey.

was used to establish statistical significance between groups. Computer programs using linear least-squares regression were used for the routine Scatchard analyses of glucocorticoid receptor studies (22). In the case of the squirrel monkey mononuclear leukocyte receptor studies, however, total bound dexamethasone was so close to the nonspecific binding that a more sensitive analysis was required. This analysis technique, implemented in the LIGAND computer program (23, 24), uses weighted nonlinear least-squares curve fitting, with bound dexamethasone concentration as the dependent variable and total hormone concentration (labeled and unlabeled) as the independent variable. The level of nonspecific binding is regarded as an unknown parameter, subject to uncertainty, and is estimated simultaneously with the parameters for binding affinity and capacity. This approach makes more efficient use of the data than the conventional method in which nonspecific binding is first subtracted from the data before the binding parameters are estimated. Data from several experiments are pooled and analyzed simultaneously. Results of this computer analysis are used to draw a displacement curve (bound/total vs. the logarithm of the total concentration) and a Scatchard plot (bound/free vs. the concentration of bound). The computer-estimated component of specific binding is also drawn in the Scatchard coordinates.

RESULTS

Total plasma cortisol values in two New World species, the squirrel monkey and the marmoset, were 7- to 20-fold greater than those seen in the owl monkey and Old World primates and 40-fold higher than those of prosimians (Fig. 1, Table 1). Free plasma cortisol concentrations were also markedly elevated in the squirrel monkey and the marmoset. They were 25- to 160-fold higher than those of the Old World primates and the prosimians. The owl monkey, whose total plasma cortisol values were similar to those of Old World primates, had free plasma cortisol concentrations 4- to 20-fold greater than those of Old World monkeys and prosimians (Fig. 1, Table 1).

Urinary free-cortisol excretion was roughly proportional to unbound plasma cortisol concentrations: squirrel monkeys excreted 129 ± 65 μg/kg per 24 hr (mean ± SEM, $n = 6$); owl monkeys, 19.3 ± 5.4 ($n = 6$); cynomolgus monkeys, 2.28 ± 0.5 ($n = 5$); rhesus monkeys, 1.1 ± 0.3 ($n = 5$); and chimpanzees, 2.67 ± 0.73 ($n = 8$). The adult human excretes 0.70 ± 0.3 μg/kg per 24 hr ($n = 6$).

Fasting morning plasma glucose levels and serum electrolytes in squirrel monkeys were similar to those obtained from three Old World primates (rhesus, cynomolgus, and baboon). These observations (data not shown) confirm the findings of others in this species (27, 28).

Mean plasma aldosterone values (±SEM) in the squirrel monkey (21.6 ± 4.0 ng/dl, $n = 16$) were similar to those obtained from rhesus monkeys (27.5 ± 4.0, $n = 10$), cynomolgus monkeys (21.4 ± 2.7, $n = 8$), and baboons (24.7 ± 11.3, $n = 4$). Plasma corticosterone values were: squirrel monkey, 2,049 ± 350 ng/dl, $n = 14$; owl monkey, 1,196 ± 312, $n = 7$; rhesus, 884 ± 226, $n = 9$; cynomolgus, 1,384 ± 259, $n = 9$; Lemurinae, 1,095 ± 197, $n = 13$; and Galaginae, 1,221 ± 286, $n = 6$. The squirrel monkey had significantly higher values than those of the owl monkey, the rhesus, and the prosimians (P, <0.05) but

Table 1. Circulating cortisol forms in primates

Species	n	Plasma cortisol	
		Total, μg/dl	Free, μg/dl
Prosimians			
Lemurinae	13	5.7 ± 1.0	0.48 ± 0.08
Galaginae	5	5.0 ± 1.5	0.20 ± 0.06
Old World primates			
Man*	11	11.5 ± 2.0	0.29 ± 0.06
Chimpanzee	10	26.5 ± 2.0	0.76 ± 0.06
Rhesus	5	24.5 ± 3.5	1.20 ± 0.16
Cynomolgus	5	21.0 ± 2.5	0.90 ± 0.10
Baboon	5	28.5 ± 3.5	1.02 ± 0.14
New World primates			
Owl m.	8	30.0 ± 3.0	4.30 ± 0.42
Squirrel m.	10	199.0 ± 24.0	30.60 ± 3.70
Marmoset m. (*Callithrix*)	6	171.0 ± 34.0	28.40 ± 5.64
(*Saguinus*)	9	197.0 ± 30.0	ND†

m, Monkey.
* Different blood-drawing procedure.
† ND, not done.

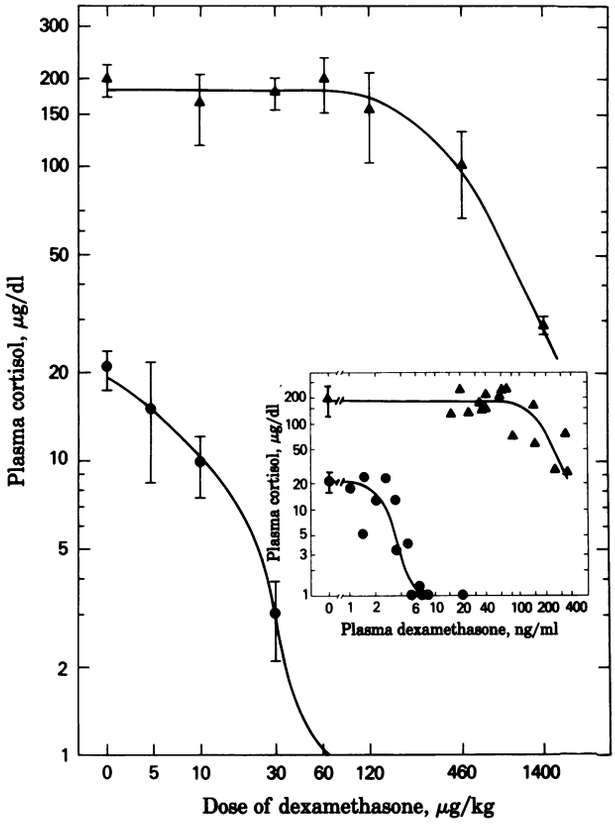

FIG. 2. Responsiveness of the hypothalamic-pituitary-adrenal axis to dexamethasone suppression in squirrel (▲) and cynomolgus (●) monkeys. Dose–response curves of total plasma cortisol (taken at 7:00 to 8:00 a.m.) to intramuscular dexamethasone given at 8:00 p.m. on the previous evening. Squirrel monkeys required 46-fold more dexamethasone for 50% suppression of plasma cortisol values when compared with cynomolgus monkeys. (*Inset*) The level of plasma dexamethasone required for 50% suppression was 66-fold higher in the squirrel monkey.

not the cynomolgus. The plasma corticosterone level of the owl monkey was the same as those of the Old World monkeys and the prosimian species.

The hypothalamic-pituitary-adrenal axis of squirrel monkeys was resistant to dexamethasone suppression. Squirrel monkeys required 460 μg of dexamethasone per kg of body weight for a 50% suppression of the 8:00 a.m. total plasma cortisol concentration. The cynomolgus monkey required only 10 μg/kg (Fig. 2). To exclude the possibility that this might be an artifact due to differences in absorption or clearance of dexamethasone between the two species, concurrent measurements of plasma dexamethasone were made (Fig. 2 *Inset*). The same doses of dexamethasone produced similar dexamethasone levels in both species, indicating no major differences in the bioavailability or rate of metabolism. The level of plasma dexamethasone required for 50% suppression of plasma cortisol was 66-fold higher in the squirrel monkey than in the cynomolgus.

The glucocorticoid receptor content of circulating mononuclear leukocytes was similar in all species examined (Table 2, Fig. 3). The apparent affinity of the receptor, however, was moderately decreased in the owl monkey (Table 2, Fig. 3) and markedly decreased in the squirrel monkey (Table 2, Figs. 3 and 4).

Because a high endogenous plasma cortisol would be expected to affect the measured K_d (29), we preincubated human mononuclear leukocytes with cortisol at 200 μg/dl for 1 hr at 37°C and then examined these cells for binding-site number and affinity. No effect of this pretreatment on number or affinity of receptors was noted (data not shown).

Table 2. Glucocorticoid receptor characteristics of circulating mononuclear leukocytes in primates

Species	*n*	R_o*	Apparent K_d, M × 10^9
Man	7	3,793 ± 120 (6.3 ± 0.2)	2.2 ± 0.2
Rhesus	4	2,829 ± 301 (4.7 ± 0.5)	2.2 ± 0.2
Cynomolgus	4	3,612 ± 662 (6.0 ± 1.1)	3.2 ± 0.2
Owl monkey	4	3,672 ± 301 (6.1 ± 0.5)	26.6 ± 3.8†
Squirrel monkey‡		6,441 ± 2,949 (10.7 ± 4.9)	49.5 ± 20.1†

* Sites per cell; in parentheses, fmol per 10^6 cells.
† P, <0.0005 when compared to Old World primate species.
‡ Data pooled from three experiments (see legend to Fig. 4).

Studies of cultured skin fibroblasts gave similar findings. The squirrel monkey fibroblasts had the same number of glucocorticoid receptors as did the fibroblasts of Old World primates, but a decreased affinity for dexamethasone was again demonstrated (Table 3).

DISCUSSION

The free cortisol concentrations in plasma were increased in those New World primates that we were able to study, i.e., the squirrel monkey, the marmoset, and the owl monkey. The increased percentage of protein-unbound plasma cortisol, in the face of the normal serum albumin concentrations in these species (27), indicates a cortisol-binding globulin (CBG) of low concentration or low affinity for cortisol. In corroboration of these

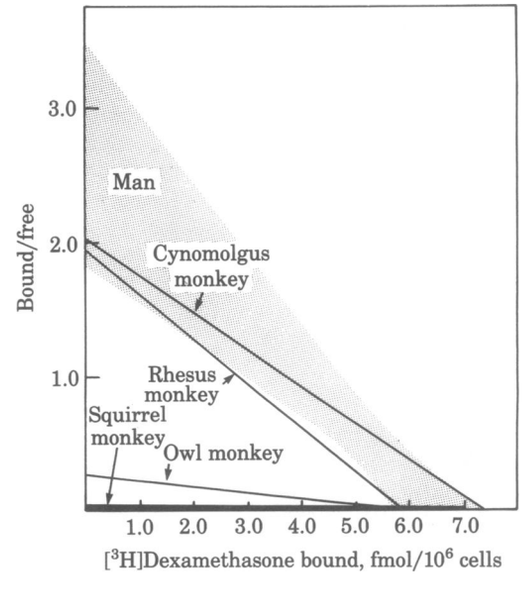

FIG. 3. Scatchard plots of representative glucocorticoid receptor studies on intact mononuclear leukocytes from various primate species (16-hr incubation at 24°C). The number of receptors per cell, indicated by the x intercept, is statistically the same in all species. The affinity of the receptor for dexamethasone, reflected by the slope of the line, is different. The shaded area indicates the human range (n = 6). The affinity for dexamethasone of the owl monkey glucocorticoid receptor is moderately decreased and that of the squirrel monkey is markedly decreased. In the latter, special analysis was required (see Fig. 4 and text) to define the affinity and number of receptors.

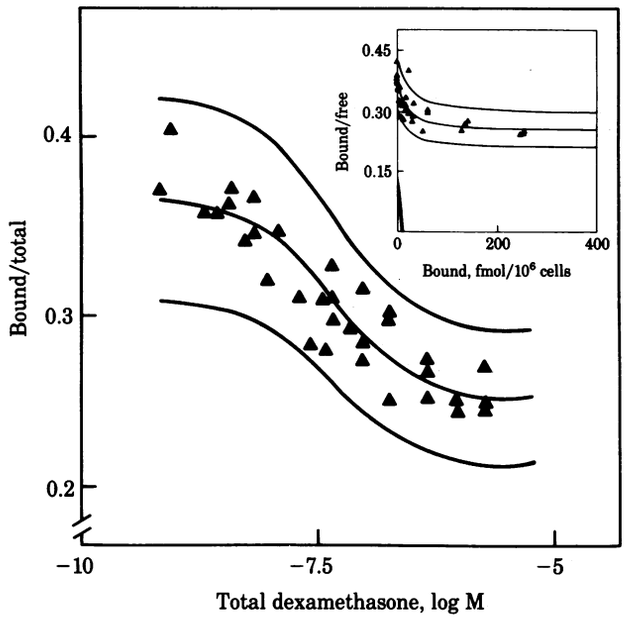

FIG. 4. Displacement curve and Scatchard plot (*Inset*) for the glucocorticoid receptor in mononuclear leukocytes from squirrel monkeys. Results from three experiments are pooled. The ratio of bound to total dexamethasone is plotted against the logarithm of the total concentration of dexamethasone (labeled and unlabeled). The displacement curve is shown ± 95% confidence limits. (*Inset*) The ratio of bound to free dexamethasone is plotted against the concentration of bound hormone before correction for nonspecific binding. The computer-estimated binding component is shown as the straight line with intercept at ≈10 fmol per 10^6 cells.

findings, urinary free cortisol, a reflection of protein-unbound plasma cortisol, was markedly increased in squirrel monkeys and moderately increased in the owl monkey. The levels seen in New World primates, adjusted for weight and body surface area, were within and above the range seen in Cushing syndrome in man (30). However, although high plasma cortisol appears to be a common trait among most New World primate species, as evidenced by this study and others (8, 9), we currently do not know if it offers some selective advantage for the survival of these species or whether it is merely the result of tolerated genetic drift (31). The former possibility is suggested because only high cortisol-producing New World primates seem to have survived.

The life expectancy of the New World primates is similar to that of Old World primates. They have normal carbohydrate metabolism and serum electrolytes (27, 28), suggesting increased resistance to cortisol. To define the degree of resistance, we tested the squirrel monkey pituitary-adrenal axis suppressibility by dexamethasone and compared the results to those obtained with cynomolgus, a representative Old World primate. We found that the degree of resistance to dexamethasone correlated with the degree of resistance predicted from the free plasma cortisol concentrations.

Table 3. Glucocorticoid receptor status in cultured skin fibroblasts obtained from primates

Species	*n*	R_o*	Apparent K_d, M × 10^9
Man	6	133,400 ± 18,500	6.0 ± 0.5
Chimpanzee	2	71,600 ± 12,400	7.2 ± 0.8
Rhesus	3	102,600 ± 15,000	6.3 ± 1.0
Cynomolgus	1	66,900	6.1
Squirrel monkey	4	94,350 ± 15,900	13.0 ± 3.5†

* Sites per cell.
† P, <0.05 when compared to Old World primates.

Glucocorticoid hormones exert their cellular actions by forming complexes with specific cytoplasmic receptors which, in turn, translocate to the nucleus and bind to specific sites on chromatin (32). Glucocorticoid receptor concentrations were similar in all species in both circulating mononuclear leukocytes and cultured skin fibroblasts. However, the apparent affinity of the mononuclear leukocyte glucocorticoid receptors for dexamethasone was markedly decreased in the squirrel monkey and moderately decreased in the owl monkey—findings that correlated with the relative free plasma cortisol concentrations. The apparent affinity of the glucocorticoid receptor for dexamethasone in the cultured skin fibroblast also was decreased significantly in the squirrel monkey but to a lesser degree. We presently are unable to explain the difference in affinity seen between the mononuclear leukocyte and cultured skin fibroblast assay systems. We controlled for possible artifacts produced by the elevated endogenous cortisol in the mononuclear leukocyte assay but found that this does not account for the difference. In addition, further evidence against interference of endogenous cortisol in the assay comes from the finding that humans with Cushing syndrome caused by high plasma cortisol levels have normal number and affinity of glucocorticoid receptors in their peripheral leukocytes (33).

Decreased glucocorticoid binding affinity has been reported in the guinea pig (34, 35). Interestingly, cytoplasmic preparations from guinea pigs appeared to inhibit the binding of tritiated dexamethasone to rat glucocorticoid receptors. The authors proposed that this inhibitory activity may reflect a system of glucocorticoid hormone sequestration, perhaps by metabolizing enzymes with a high affinity for glucocorticoids. Low specific binding of [^{3}H]dexamethasone persisted, however, when the cytoplasmic inhibitory activity was almost totally eliminated after adrenalectomy 24-hr before the studies. Thus, there appears to be two mechanisms responsible for decreased binding of glucocorticoids to guinea pig receptors—a binding inhibitor and a low-affinity receptor. Both mechanisms, low affinity of the glucocorticoid receptor, and intracellular inhibitions of binding, also may be present in New World monkeys.

Although we would postulate that the receptor defect found in the New World primates is primary and led to a compensatory increase of cortisol levels (primary resistance), it is possible that other regulatory mechanism derangements produced high plasma cortisol levels, and the change in receptor affinity is a protective mechanism (secondary resistance). This seems unlikely because it would require two mutational events, whereas a decrease in receptor affinity would be automatically compensated for by the response of the hypothalamic-pituitary-adrenal feedback axis. This response would ensure an appropriate number of nuclear steroid receptor complexes and, thus, a system with normal functional characteristics except for the high plasma cortisol concentration.

The fact that prosimian species have free plasma cortisol levels in the same range as Old World primates and at least four New World primate species have high free plasma cortisol concentrations suggests that alterations in glucocorticoid receptor affinity must have occurred after the bifurcation of the New World from the Old World primates (≈60 × 10^6 yr ago) and before the diversion of the New World monkeys from each other (≈15 × 10^6 yr ago) (25, 26). Interestingly, the owl monkey that appears to have diverted from the other New World primates before marmosets and squirrel monkeys separated from each other (25, 26) (Fig. 1) has an intermediate affinity defect. The molecular basis of the differences in receptor affinity between

these New World species is unknown. We believe that a first mutation of the glucocorticoid receptor that occurred before the diversion of the owl monkey was followed either by a second one that took place before the squirrel monkeys and the marmosets diverted from each other or by a continuous adaptive change in receptor affinity with different rates between the owl and the common ancestor of the other New World species.

Glucocorticoid hormone resistance has been described recently in the human (6, 7). High levels of cortisol (without stigmata of Cushing syndrome), resistance of the hypothalamic-pituitary-adrenal axis to dexamethasone, and a glucocorticoid receptor affinity defect characterize this syndrome. However, a significant difference between the human disease and the condition in the New World primates is that in the severe form of the human disease, sodium-retaining corticoids (corticosterone and deoxycorticosterone) are elevated many fold, producing hypertension and hypokalemic alkalosis; this does not occur in the New World monkeys. The mineralocorticoid overproduction in man appears to be due to corticotropin hyperstimulation of the adrenal cortex. In the New World monkeys, evolution and selective pressure seem to have favored the development of a *zona fasciculata* that can hypersecrete cortisol without "spilling" steroid precursors with sodium-retaining activity.

Thus, the New World monkeys and the patients with the syndrome described above may represent affinity mutants of the glucocorticoid receptor. Although glucocorticoid resistance of various mutant lymphoma cell lines cultured *in vitro* has been described (36–38), the defect has been shown to be decreased numbers of receptors or defective nuclear translocation. An affinity defect has not been found. Similarly, the other steroid hormone resistance syndromes described in the human are due to changes in receptor number and not affinity (39). For example, vitamin D-dependent rickets type II is associated with reduced nuclear uptake of the vitamin D–receptor complex in skin fibroblasts cultured from affected individuals (40), and progesterone resistance in the human also has been shown to be associated with a decreased number of receptors (3). Only in the androgen-resistance syndrome (testicular feminization) have qualitative changes in the androgen receptor, such as thermolability and lack of receptor stabilization with sodium molybdate, been described (41, 42).

We are indebted to Drs. Peter Munson and David Rodbard for their help in analyzing the binding studies and for the use of their computer program that made the determination of glucocorticoid receptor affinity possible in the squirrel monkey. We are grateful to Dr. E. Brad Thompson for his advice and for reviewing the manuscript and to Dr. Joseph Bruton for measuring plasma dexamethasone. We also thank Mr. Dennis Barnard for technical assistance.

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