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CHARACTERISTICS OF GLUCOCORTICOID-BINDING BY INFLAMMATORY TISSUE OF RATS

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Summary—Glucocorticoid-binding activities of the granuloma cytosol were compared with those of the liver cytosol and of the serum *in vitro*. The granuloma cytosol bound cortisol (HC) about 4-fold higher than dexamethasone (DX) and triamcinolone acetonide (TA); the liver cytosol bound these two synthetic agonists more than HC. The kinetic parameters of the glucocorticoid-binding components of the granuloma and the liver cytosols were studied by the Scatchard method. The binding components of the granuloma cytosol had a single class of binding sites with high affinity for these three steroids, whereas the binding site of the liver cytosol had negative cooperativity or consisted of two distinct classes, because its Scatchard plot showed a hyperbolic curve. The granuloma glucocorticoid-binding components will be protein since their binding was prevented by a trypsin treatment and completely lost by heating at 60°C for 5 min. Heating at 25 or 37°C for 30 min did not affect the HC-binding activity of the granuloma cytosol, regardless of prelabeling with the steroid. The binding activity for DX and TA were heat-labile and completely lost by heating the cytosol at 37°C for 30 min without the respective steroid. The results of thermal inactivation and ammonium sulfate fractionation show the granuloma HC-binding protein closely resembles corticosteroid-binding globulin (CBG). From enzymatic determination of hemoglobin in tissue cytosols, attribution of the contaminating blood to the HC-binding activity of the cytosol is considered to be negligible.

INTRODUCTION

The current view on the molecular mechanisms of action of glucocorticoids is that the steroids, if not all, act in cellular biological processes by controlling regulatory proteins, such as lipomodulin, like other steroid hormones: glucocorticoids have been considered to form complexes with specific recpetor proteins in the cytosol and then be transported to the nuclear acceptor sites to affect protein synthesis [1, 2].

We have already shown that the cytosol of the carrageenin granuloma, a typical model of the inflammatory tissues, binds only cortisol (HC) in the absence of sulfhydryl protecting agents such as dithiothreitol [3]. When a sulfhydryl protecting agent was added in the homogenizing medium, the granuloma cytosol could bind dexamethasone (DX) as well as HC. These findings show the glucocorticoid receptors probably differ in their characteristics between the nonphysiological target tissue granuloma and physiological target tissues. Our previous study also shows that at least two kinds of glucocorticoid-binding proteins are present in the cytosol preparation of the inflammatory tissue. Since glucocorticoids are most potent antiinflammatories, these peculiarities of the granuloma cytosol receptors must have some pathological and pharmacological significance.

The present study deals with the binding capacities of the specific glucocorticoid-binding components of the granuloma cytosol and their behavior under enzyme digestions, thermal pretreatment or ammonium sulfate fractionation, and offers some of their features in comparison with those of the glucocorticoid receptors of the liver cytosol and corticosteroid-binding globulin (CBG).

EXPERIMENTAL

Chemicals

[1,2,6,7-^{3}H]HC (92 Ci/mmol), [1,2(n)-^{3}H]DX (25 Ci/mmol), [1,2,4-^{3}H]TA (25 Ci/mmol) were purchased from the Radiochemical Centre, Amersham. Unlabeled TA and DX were purchased from Sigma Chemical Co., St Louis, Mo. Unlabeled HC and dithiothreitol was obtained from Wako Pure Chemical Industries Ltd, Tokyo, Japan. Trypsin (bovine pancreas, 185 units/mg, P-L Biochemicals Inc., Milwaukee),

ribonuclease (2500 units/mg, Worthington Biochemical Corporation, Freehold) and deoxyribonuclease (1559 units/mg, Worthington) were used for enzyme digestion experiments. Ultrapure water was prepared with Milli-Q Reagent Water System (Japan Millipore Ltd, Tokyo).

Preparation of cytosol

Male Sprague–Dawley strain rats, 6 weeks old, were purchased from Charles River Japan, Kanagawa, Japan, used after 1-week acclimatization. Carrageenin granuloma pouch was prepared on the dorsum of the rat, according to the previously described method [3]. On day 8 after the carrageenin injection, rats were bled by heart puncture under ether anesthesia and sacrificed by cervical dislocation. Immediately, the inferior vena cava was cut and the blood was remove by perfusing with ice-cold saline from the aorta, and then granuloma was removed. After removal of adhering muscle and fat, the granuloma tissue was blotted, weighed and homogenized in 4 vol of 50 mM Tris–HCl containing 1 mM EDTA and 10 mM dithiothreitol, pH 7.6, with a Vir-Tis 45 homogenizer (The VirTis Co., New York) at the speed of 45 V for 2 min with a 1-min intermission. The homogenate was centrifuged at 105,000 *g* for 60 min with a Hitachi 65P-7 ultracentrifuge (Hitachi Koki Co. Ltd, Japan) and the supernatant was used as the cytosol preparation. Pair-fed normal rats were also bled and sacrificed in the same manner to obtain the serum and the liver cytosol preparation. The serum was used after an 8-fold dilution with the buffer in order to adjust its protein concentration to the same level as that of the granuloma cytosol. Preparation of the cytosol and all subsequent procedures were carried out below 4°C, unless otherwise mentioned.

Measurement of the specific [^{3}H]glucocorticoid binding activities

A 0.2-ml portion of the cytosol or the serum was incubated with 20 nM of a [^{3}H]glucocorticoid with or without a 500-fold molar excess of the unlabeled same steroid for 90 min in an ice-water bath. Then 1 ml of a charcoal suspension (0.5% charcoal and 0.05% dextran T70 in the buffer) were added in order to remove the unbound steroid and thoroughly mixed with a vortex mixer for 3–5 s. The tubes were allowed to stand for 10 min in the ice-water bath and then centrifuged at 1500 *g* for 15 min. An aliquot of the clear supernatant (1.0 ml) was mixed with 10 ml of a scintillation cocktail, ACS-II (Amersham), and the radioactivity, standing for the bound steroid, was counted in an Aloka LSC-1000 liquid scintillation counter (Aloka Co. Ltd, Japan). Counting efficiency was about 40% as determined by the external standard method. Specifically bound steroid was calculated by subtracting the bound steroid determined with the unlabeled steroid from that determined without the competitor.

Scatchard analysis

Two series of test tubes were prepared: The first contained graduated amounts (2–50 nmol) of a [^{3}H]steroid in ethanol and the second contained the same steroid unlabeled in ethanol of a 500-fold molar excess to each amount of the hot steroid. The solvent in each tube was evaporated under N_2. Each tube of the first series containing [^{3}H]steroid was added 0.25 ml of the buffer and allowed to stand with occasional shaking at room temperature until the steroid dissolved. Then a 0.05-ml portion of the [^{3}H]steroid-buffer solution in each tube was transferred to a counting vial to determine the net radioactivity in the following incubation. Another portion (0.1 ml) of the [^{3}H]steroid-buffer solution was pipetted into the corresponding tube of the second series and the tubes were allowed to stand with occasional shaking to dissolve the unlabeled steroid. Then, the cytosol (0.1 ml) was added to all tubes. The protein concentration of the cytosol was adjusted to about 5 mg/ml. The mixture was incubated for 90 min in the ice-water bath and the bound radioactivity was determined. Concentration of unbound (free) radioactive steroid in the incubation mixture was calculated by subtracting the bound [^{3}H]steroid from the net [^{3}H]steroid. The ratio of the specifically bound steroid to the unbound steroid (B/F) was plotted against the specifically bound steroid and the results were analyzed according to Scatchard [4]. When the Scatchard plot obtained was not a straight line, the result was fitted to a theoretical curve by a program of Ishii and Kubokawa [5] based on theories of de Meytes and Roth [6] and Munck [7] with a NEC PC-9801 F2 computer of Nihon Electronics Co. Ltd (Tokyo, Japan), and the kinetic parameters were determined.

Miscellaneous

As a measure for blood contamination in the cytosol, hemoglobin content was determined

enzymatically by Marklund's method [8] using the rat serum as a source of the heptoglobin. Protein was determined by the method of Lowry *et al.* [9]. The statistical significance of differences between mean values was tested using Student's *t*-test and Cochran–Cox test.

RESULTS

Specific glucocorticoid-binding activity

Table 1 shows that the granuloma cytosol could bind HC more preferentially than DX and TA; its HC-binding activity was about 4.5-fold higher than that of the liver cytosol and slightly but significantly lower than that of the serum. On the other hand, the liver cytosol could bind DX and TA more than HC. The serum bound exclusively HC: in one experiment the amount of the [^{3}H]HC specifically bound to the serum was 0.66 pmol/mg protein, though those of [^{3}H]DX and [^{3}H]TA were 0.01 pmol/mg protein and only trace (about 0.003 pmol/mg protein), respectively. These results on the glucocorticoid-binding activity of the serum are consistent with those of the previous studies [10, 11]. As to the receptor sites concentration for glucocorticoids of cytosols, Giannopoulos has shown that the lung and the liver of fetal rabbits have a HC-binding activity about two times higher than the respective DX-binding activities [12, 13], and others have reported that the binding capacities of the corticosteroid-responsive tissue cytosols for HC, DX and TA are almost the same [10, 11]. Our present study shows that the liver cytosol binds HC less than DX and TA. These discrepancies will be probably due to differences in the degree of blood removal from the tissue.

Results in Table 1 bring forward a new example of tissues having an extremely high HC-binding activity. Then it was examined whether these glucocorticoid binding activities

Table 1. Specific glucocorticoid-binding activities of granuloma cytosol, liver cytosol and serum

	Specific binding activity		
	[^{3}H]HC	[^{3}H]DX	[^{3}H]TA
Granuloma (3)	0.453 ± 0.015*	0.114 ± 0.031**	0.121 ± 0.011
Liver (3)	0.099 ± 0.008	0.214 ± 0.014	0.164 ± 0.019
Serum (3)	0.576 ± 0.086	Trace	Trace

Specific binding activities are expressed as mean ± SD in pmol/mg protein. Number of determinations are shown in parentheses. Rat serum was used after 8-fold dilution with 50 mM Tris–HCl containing 1 mM EDTA and 10 mM dithiothreitol, pH 7.6. Abbreviations: HC = cortisol, DX = dexamethasone, TA = triamcinolone acetonide. *$P < 0.001$ compared with all the other values but the serum HC-binding activity described in the table. **$P < 0.01$ compared with the liver DX-binding activity.

of the granuloma cytosol are truly attributable to protein molecules.

Enzyme digestion

Table 2 shows that trypsin caused a marked reduction in the specific glucocorticoid-binding activities, though the nuclease digestion did not affect them. These results suggest that the glucocorticoid-binding components of the granuloma cytosol are protein. It is interesting that the DX- and the TA-binding proteins were more susceptible to the trypsin digestion than the HC-binding one (Exp. 2 in Table 2).

Thermal stability

When the granuloma cytosol was heated to 60°C without the steroid, the binding activities were almost completely lost (Table 3). This also shows the protein nature of the granuloma cytosol glucocorticoid-binding components. The cytoplasmic glucocorticoid receptor is well known to be labile to heating. The DX- and the TA-binding activity of the granuloma cytosol was also labile to heating and are completely lost by pre-heating at 37°C in the absence of the steroid (Table 3). Table 3 appears to show there may be some differences among the granuloma binding proteins for the synthetic glucocorticoid. The granuloma receptor labeled by [^{3}H]TA before heating kept more binding activity than the prelabeled DX-receptor, whereas by heating without the steroid at 25° or 37°C, both the DX- and TA-binding activities of the granuloma cytosol decreased by a similar extent. It seems, therefore, to be possible that the binding component for synthetic steroids may be able to be separated into subclasses or stabilized by TA. On the other hand, the HC-binding protein of the granuloma cytosol was completely stable to heating at 25 and 37°C and showed a similar temperature stability to that of the serum. This result also supports that the HC-binding protein of the granuloma may be different from the binding proteins for the synthetic steroids. Two separate experiments resulted in the similar tendency.

Ammonium sulfate ($(NH_4)_2SO_4$) fractionation

The HC-binding activity of the granuloma cytosol was kept for at least 7 days at −80°C, but the DX-binding activity was completely lost within 12 h at the same temperature (data are not shown). This also suggests that the HC-binding protein of the granuloma may differ from the DX-binding one. To determine if

Table 2. Effect of enzyme-treatments on the [^{3}H]glucocorticoid-binding activity of granuloma cytosol

	Enzyme		[^{3}H]glucocorticoid-binding activity (pmol/mg protein)					
			HC	RBA	DX	RBA	TA	RBA
Exp. 1	Control		0.357	100.0	0.164	100.0	0.132	100.0
	Trypsin	(150)	0.114	31.9	0.020	12.2	ND	0.0
	DNase	(150)	0.369	103.4	0.151	92.1	0.130	98.5
	RNase	(150)	0.359	100.6	0.154	93.9	0.129	97.7
Exp. 2	Control		0.379	100.0	0.105	100.0	0.120	100.0
	Trypsin	(20)	0.387	102.1	ND	0.0	ND	0.0

Aliquots (0.9 ml) of the granuloma cytosol were incubated for 1 h in an ice-water bath with 0.1 ml of an enzyme solution containing as much unit as indicated in parentheses or with 0.1 ml of the vehicle (control). Then the specific binding of each [^{3}H]glucocorticoid was determined. Relative binding activities (RBA) are expressed as % of the control value. Abbreviations: HC = cortisol; DX = dexamethasone; TA = triamcinolone acetonide; ND = non detectable.

the HC-binding protein is distinguishable from those for the synthetic agonist, we fractionated the cytoplasmic proteins by ammonium sulfate (Table 4). The precipitate obtained at 33% saturation with $(NH_4)_2SO_4$ contained 22–26% of the total cytoplasmic protein, having DX- and TA-binding activities 3–4-fold higher than those of the original cytosol, and little HC-binding activity. Another pellet, obtained by bringing the 33% $(NH_4)_2SO_4$ supernatant to 60% saturation with $(NH_4)_2SO_4$, contained 47–50% of the total cytosol protein, showing lower binding activities for DX and TA and virtually whole HC-binding activity of the original cytosol. CBG does not precipitate by the 33% $(NH_4)_2SO_4$ saturation, which is a concentration enough to precipitate the hepatic cytoplasmic glucocorticoid receptor and so on [14]. This $(NH_4)_2SO_4$ fractionation study also shows a similarity between CBG and the HC-binding protein of the granuloma cytosol. This study had good reproducibility for the two separate experiments.

Kinetic parameters

In order to estimate the number and the affinity of the binding sites for [^{3}H]glucocorticoids in the granuloma cytosol, the specifically bound steroid was plotted as a function of the free steroid concentration in the incubation. The result was analyzed by the Scatchard method. A saturation-type binding curve was obtained for the granuloma (Fig. 1). It is noteworthy that the nonspecific binding by the granuloma cytosols accounted for about 30–60% of the total binding of [^{3}H]steroids, whereas only 20–30% by the liver cytosol. Furthermore, in contrast to the liver cytosol, the nonspecific binding by the granuloma cytosol was nonlinear in the high steroid concentration range, especially above 100 nM (data are not shown). The physiological significance of the nonspecific binding component is not known. As to the granuloma, plotting B/F against the specifically bound steroid (inset to Fig. 1) yielded a single straight line for those glucocorticoids, indicating that

Table 3. Effects of incubation temperature on the glucocorticoid-binding activities of granuloma cytosol and serum

		Specific binding activities							
	Pre-heating	Granuloma						Serum	
	(°C)	HC	RBA	DX	RBA	TA	RBA	HC	RBA
Exp. 1[a]	0	0.467	100.0	0.192	100.0	0.185	100.0	0.549	100.0
	25	0.504	107.9	0.095	49.5	0.099	53.5	0.540	98.4
	37	0.509	109.0	0.016	8.3	0.018	9.7	0.533	97.1
	60	0.035	7.5	0.013	6.7	0.012	6.5	0.013	2.4
Exp. 2[b]	0	0.500	100.0	0.176	100.0	0.120	100.0	0.520	100.0
	25	0.513	102.6	0.090	51.1	0.097	80.8	0.567	109.0
	37	0.554	110.8	0.012	6.8	0.046	38.3	0.596	114.6

[a]The granuloma cytosol and the serum were preincubated without the steroid for 5 min at 60°C and for 30 min at 25° or 37°C. After centrifugation at 10,000 *g* for 30 min to remove the insolubles, the specific glucocorticoid-binding activities were determined by incubating the clear supernatant with the [^{3}H]steroid for 90 min in an ice-water bath. Exps 1 and 2 are separate studies.
[b]The cytosol or the serum was labeled by the preincubation with 20 nM of the [^{3}H]steroid in an ice-water bath for 90 min and then treated thermally at 25° or 37°C for 30 min. The mixtures were cooled in the ice-water bath and increased for additional 90 min. Then the specific binding activities were determined.
The unit of the specific binding activities is pmol/mg protein. Relative binding activities (RBA) are expressed as % of the activities of the cytosols incubated throughout in an ice-water bath (expressed as 0°C). Abbreviation: HC = cortisol, DX = dexamethasone, TA = triamcinolone acetonide.

Table 4. Ammonium sulfate fractionation of granuloma cytosol proteins in association with the glucocorticoid-binding activity

[^{3}H]steroid		Protein fraction[a]	Specifically bound [^{3}H]steroid pmol	Recovery%[b]
HC	I	(0–33% ppt)	0.279	1.6
	II	(33–60% ppt)	19.814	112.8
		I + II	20.093	114.3
		Whole[c]	17.573	100.0
DX	I	(0–33% ppt)	3.727	44.2
	II	(33–60% ppt)	1.350	16.0
		I + II	5.077	60.2
		Whole[c]	8.432	100.0
TA	I	(0–33% ppt)	4.758	55.2
	II	(33–60% ppt)	1.207	14.0
		I + II	5.965	69.2
		Whole[c]	8.623	100.0

[a]Precipitates obtained in the indicated range of ammonium sulfate concentration: 5 ml of the granuloma cytosol were incubated with 20 nM [^{3}H]glucocorticoids with or without a 500-fold molar excess of the unlabeled steroids in an ice-water bath for 90 min. The salt was added to a final concentration of 33% saturation. After chilling in the ice-water bath for 30 min, the pellet was collected by centrifugation at 15,000 *g* for 20 min (fraction I). The remaining supernatant was brought to 60% ammonium sulfate saturation by adding the salt, and the precipitate (fraction II) was separated. After washing, each fraction was dissolved in 3 ml of the buffer, and applied to Sephadex G-25 column (1.5 × 20 cm). Radioactivities of the macromolecular fractions were determined.

[b]Percentage of the total activity found in the cytosol.

[c]The activity being present in the 5 ml of the cytosol.

Abbreviations: HC = cortisol, DX = dexamethasone, TA = triamcinolone acetonide, respectively.

only a single class of specific binding sites exists for each of these steroids under the experimental conditions. The apparent dissociation constant and the concentration of the binding sites were calculated for each radioactive steroid (Table 5). The differences in concentration of the binding sites and the dissociation constant between the binding activity for HC and that for DX or TA were statistically significant ($P < 0.05$). This probably gives the granuloma cytosol the high HC-binding capacity.

On the other hand, the glucocorticoid-binding activity of the liver cytosol as a function of the free steroid concentration showed a characteristic curve having an intermediate plateau range on it (Fig. 2). The Scatchard plots of the specific glucocorticoid-binding activity of the liver cytosol were not linear (Fig. 2). This shows the binding components of the liver cytosol for each [^{3}H]steroid have two distinct classes of the specific binding site (Fig. 3) or negative cooperativity (Fig. 4). Therefore, theoretical curves were computed according to two kinds of model, the two independent binding sites model and the negative cooperativity model: first, on the assumption that the glucocorticoid-binding components of the liver cytosol have two distinct classes of the binding sites, the number of the binding sites and the dissociation constant were calculated for both the high-affinity and the low-affinity site (Table 6A). The kinetic parameters of the glucocorticoid-binding components of the granuloma were compared with those of the liver. The glucocorticoid-binding site concentration of the granuloma was significantly higher than that of the high-affinity binding sites of the liver ($P < 0.001$) for each steroid and, except for the HC-binding protein, was lower than that of the hepatic low-affinity binding sites ($P < 0.001$). The affinity of the glucocorticoid-binding site of the granuloma cytosol was one-order lower than that of the liver high-affinity binding site, and was one-order higher than the low-affinity site of the liver, for each steroid. Second, based on the negative cooperativity model, the average dissociation constant ($\bar{K}_d$), which was defined as a reciprocal of the average affinity constant ($\bar{K}$) by de Meytes and Roth [6] of the Empty Binding Site and the Filled Binding Site, and the concentration of the binding site were calculated (Table 6B). The liver cytosol had more binding sites for the synthetic agonist than for HC. Table 6B also shows the empty binding site of the liver cytosol had an affinity higher to the synthetic glucocorticoids than to HC. These results and the similar trend of the high-affinity site (Table 6A) support the finding that the liver cytosol binds DX and TA more than HC (Table 1). Tables 5 and 6B also show that the granuloma has more HC-binding sites and less DX- and TA-binding sites than the liver, with an affinity to HC higher than that of the hepatic empty binding site for HC ($P < 0.001$).

Table 5. Kinetic parameters of glucocorticoid-binding components of granuloma cytosol

Ligand	n	K_d ($\times 10^{-8}$ M)
HC	0.65 ± 0.29	1.88 ± 0.74
DX	0.12 ± 0.04*	1.32 ± 0.46*
TA	0.15 ± 0.05*	1.21 ± 0.21*

Results are expressed as mean ± SD of five determinations. Abbreviations: HC = cortisol, DX = dexamethasone, TA = triamcinolone acetonide, n = number of binding sites, K_d = the dissociation constant. The unit of n is pmol/mg protein. *$P < 0.05$ compared with the corresponding value for the binding of [^{3}H]HC.

Contribution of blood CBG to [^{3}H]cortisol-binding in granuloma cytosol

All results described above show that the HC-binding protein of the granuloma cytosol resembles CBG. Thus, we have studied a possible contamination of the cytosol by the blood left behind after the *in situ* perfusion of the

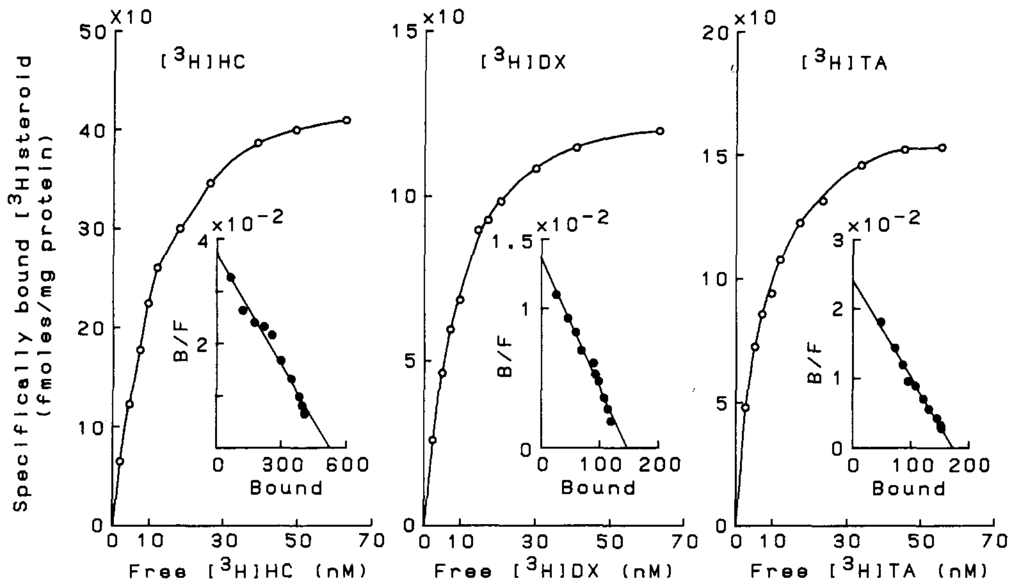

Fig. 1. Specific [^{3}H]glucocorticoid binding by granuloma cytosol. The granuloma cytosol was incubated with various concentrations of a [^{3}H]glucocorticoid. The concentration of the specifically bound steroid was depicted as a function of the free steroid concentration. The data were converted to Scatchard's plot as shown in the insets. Bound and B/F in the inset mean the specifically bound [^{3}H]steroid and ratio of the specifically bound [^{3}H]steroid to the free [^{3}H]steroid, respectively. HC, cortisol; DX, dexamethasone; TA, triamcinolone acetonide.

animals with a saline solution at sacrifice. As a measure for the blood contaminant we used the hemoglobin content, determined enzymatically [8]. Hemoglobin content of hemolyzed blood taken from the same animal was also measured. The HC-binding activity of the granuloma cytosol and the serum and hematocrit value of the blood were determined too (Table 7). Blood volume remaining in the granuloma cytosol was calculated to be $1.2 \pm 0.4\ \mu l$ per ml of the cytosol ($N = 3$). The liver cytosol has less blood contaminant, $0.4 \pm 0.1\ \mu l$ per ml of the cytosol ($N = 3$), than the granuloma cytosol in this experimental conditions. Thus, the HC-binding activity owing to the contaminated blood may account for only about 1.0% of that observed in these two cytosols (Table 7).

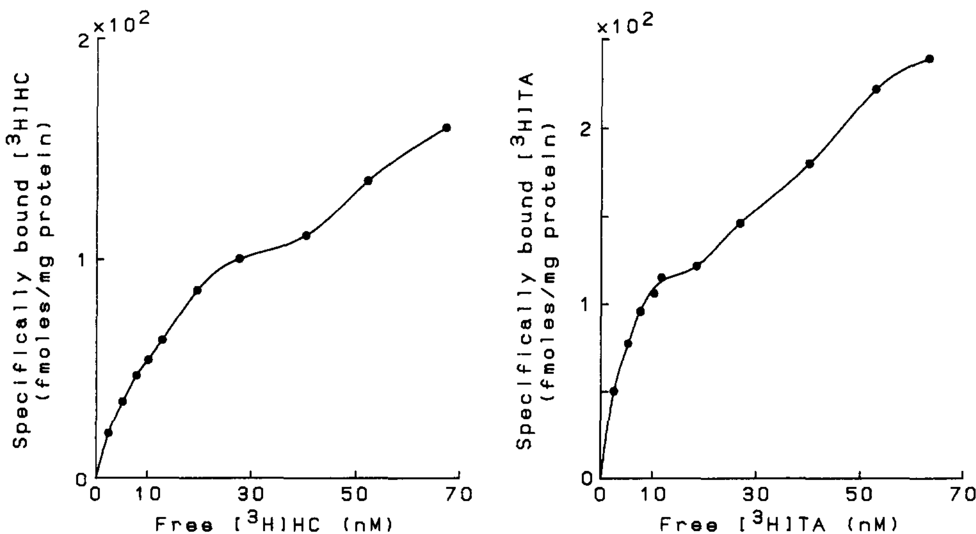

Fig. 2. Specific [^{3}H]glucocorticoid binding by liver cytosol. The liver cytosol was incubated with various concentrations of a [^{3}H]glucocorticoid and the concentration of the specifically bound steroid was depicted as a function of the free steroid concentration. HC, cortisol; DX, dexamethasone; TA, triamcinolone acetonide.

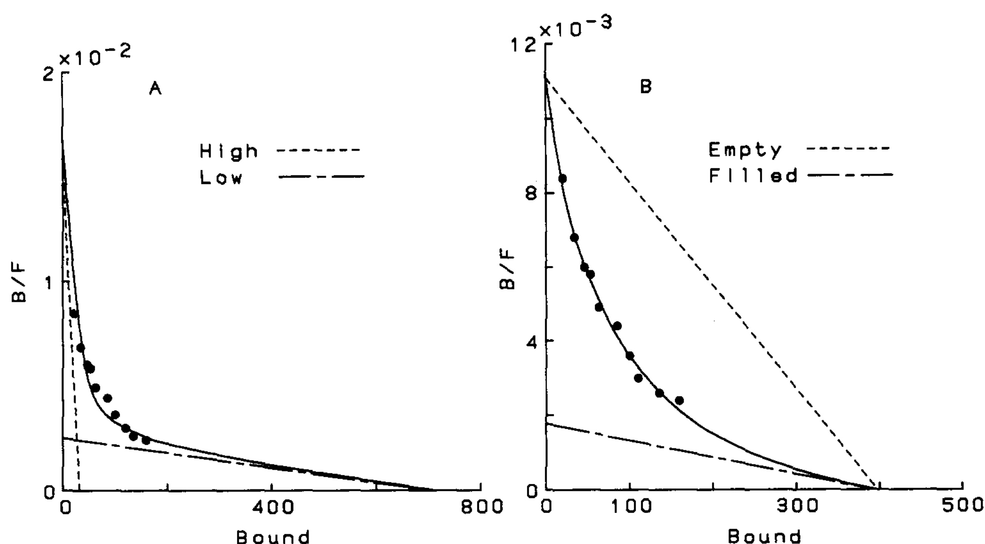

Fig. 3. Scatchard analysis of the [^{3}H]cortisol-binding activity of liver cytosol. According to the two independent binding sites model (A) and the negative cooperativity model (B), the theoretical curves for Scatchard's plotting of the specifically bound [^{3}H]cortisol were computed and the lines for the high- and the low-affinity binding sites and those for the empty binding site and the filled binding site were determined respectively.

DISCUSSION

The results demonstrate that the granuloma cytosol contains at least two kinds of binding protein, distinguishable each other by their preferential affinity for HC or for the synthetic agonist (DX and TA). Our preceding study has shown the granuloma HC-binder resembles CBG in behavior on the sulfhydryl reagents [3]. However, the previous study did not exclude a possibility that the blood remaining in the tissue after killing might be a source of CBG, to which the cytosol might owe much of its HC-binding activity through disruption of vessels during homogenization. Our present results show undoubtedly that the HC-binding activity in the cytosol is not owing to pollution by the blood during preparation of the cytosol, because determination of the blood content showed that the HC-binding activity by the blood contaminant accounted for only about 1% of the total HC-binding activity of the granuloma and the

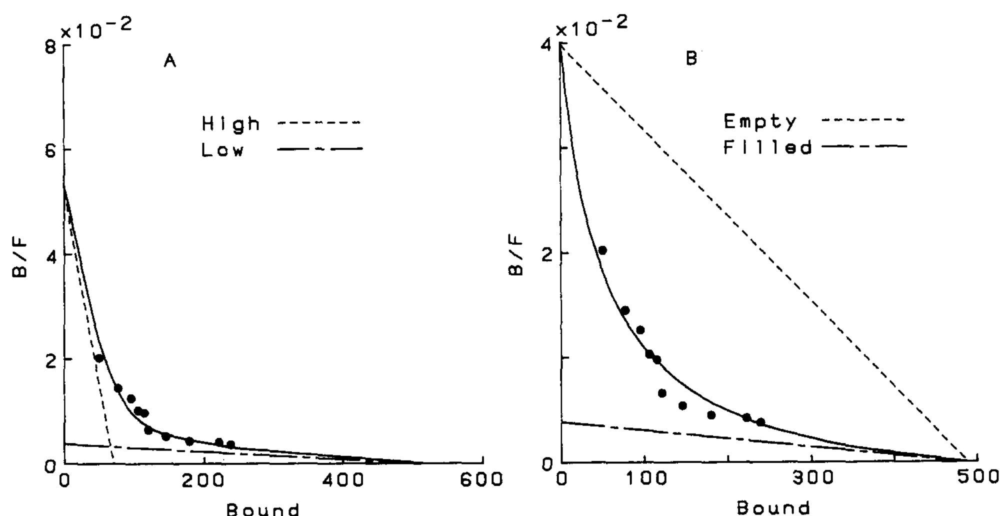

Fig. 4. Scatchard analysis of the [^{3}H]triamcinolone acetonide (TA)-binding activity of liver cytosol. According to the two independent binding sites model (A) and the negative cooperativity model (B), the theoretical curves for Scatchard's plotting of the specifically bound [^{3}H]triamcinolone acetonide were computed and the lines for the high- and the low-affinity binding sites and those for the empty binding site and the filled binding site were determined respectively.

Table 6. Kinetic parameters of glucocorticoid-binding components of liver

(A) Based on the two independent binding sites model

Ligand	High-affinity site n	High-affinity site K_d ($\times 10^{-9}$ M)	Low-affinity site n	Low-affinity site K_d ($\times 10^{-7}$ M)
HC	0.02 ± 0.01	1.88 ± 0.39	0.63 ± 0.11	1.98 ± 0.67
DX	0.06 ± 0.01**	1.47 ± 0.13	0.63 ± 0.10	1.60 ± 0.73
TA	0.05 ± 0.02*	1.79 ± 0.56	0.65 ± 0.16	1.34 ± 0.16

(B) Based on the negative cooperativity model

Ligand	n	$\bar{K}_d$ of binding site: Empty ($\times 10^{-8}$ M)	$\bar{K}_d$ of binding site: Filled ($\times 10^{-7}$ M)
HC	0.39 ± 0.02	4.10 ± 0.41	1.69 ± 0.60
DX	0.50 ± 0.06*	1.25 ± 0.14***	1.36 ± 0.23
TA	0.48 ± 0.02**	1.47 ± 0.22***	1.40 ± 0.11

Each value represents mean ± SD of three separate determinations. Abbreviations: HC = cortisol, DX = dexamethasone, TA = triamcinolone acetonide, n = number of binding sites, K_d = the dissociation constant, $\bar{K}_d$ = the average dissociation constant. The unit of n is pmol/mg protein. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$, each compared with the corresponding value of HC-binding.

liver cytosol when the blood was washed away by the whole body perfusion before removing the tissue. The similarities between the granuloma and the serum in the HC-binding reaction, therefore, will reflect the presence of an "extravasated CBG" or a "CBG-like" binding protein in the granuloma cytosol. Since an increase of the capillary permeability is invariably resulted from the inflammation, a certain amount of CBG must have leaked from capillaries *in vivo* and stayed in the inflammatory tissues. Such CBG, extravasated from blood vessels *in vivo* and remained after the perfusion for washing away the blood in the vessels, must be a member of the HC-binding proteins of the granuloma cytosol preparation. Therefore, we think that such a CBG fraction, named temporarily "extravasated CBG", should be considered as a intrinsic constituent of the inflammatory tissue and not the blood contaminant, even though it is extracellularly, and it must have a biological significance, discussed behind, under the inflammatory conditions. As to the "CBG-like" proteins, many reports have shown the presence of intracellular "CBG-like" proteins in various tissues or cells [12–18].

Thermal pretreatment showed that the DX- and TA-binding protein were much susceptible to the digestion by trypsin and to heating at 25° and 37°C (Table 3). This also supports the conclusion that most of the HC-binding activity of the granuloma cytosol will be due to other protein species different from the receptors binding the synthetic glucocorticoids.

The Scatchard analysis of the glucocorticoid-binding activity of the granuloma and the liver cytosol showed a marked difference between these two preparations. The granuloma binders for HC and for DX and TA had a single class of specific binding site for each steroid. As for the liver cytosol, plotting the ratio B/F against the specifically bound steroid yielded a concave curve. When the Scatchard plot yields a curve, there is no fixed theory to clarify the nature of binding sites. For this case, three possibilities can be thought according to Munck [7] as follows: First, both saturable and nonsaturable binding sites are present in the liver cytosol. Second, the liver has independent binding sites with more than one association constant diminishing progressively the effective association constant as more and more sites become filled. Third, the liver cytosol has several same binding sites that interact in such a way that binding to one site reduces the affinity for the next, i.e. with negative cooperativity. A concave Scatchard plot has been shown for the HC-binding in mouse embryos [19], embryonic chick neural retina [20] and steroid-responsive tissues of adult rats including the liver [21], and for the DX-binding in macrophage [22]. The presence of negative cooperativity has been reported for DX-binding of rat thymus cytosol [23]. Munck has also suggested that failure to reach equilibrium can give rise to a nonlinear plot of Scatchard [7]. When the granuloma cytosol incubated for 4 h with [^{3}H]steroids, the Scatchard analysis gave similar results to those obtained

Table 7. Negligible contribution of blood contaminants to the [^{3}H]cortisol (HC)-binding activities of granuloma and liver cytosols

Tissue	Serum volume in cytosol (μl/ml)	Specific [^{3}H]HC-binding: Serum (fmol/μl)	Specific [^{3}H]HC-binding: Cytosol (fmol/ml)	Cytosol: Obstacle by blood (fmol)	Cytosol: Obstacle by blood (%)
Granuloma	0.6 ± 0.2	43.9 ± 13.5	3258.1 ± 337.6	27.4 ± 13.4	0.8 ± 0.4
Liver	0.3 ± 0.1	44.4 ± 3.5	1262.6 ± 123.6	12.0 ± 6.0	0.9 ± 0.4

The estimated blood volumes in the granuloma and the liver cytosols are described in Results. Serum volumes are calculated from the blood volumes using the hematocrit values (45.3 ± 2.1% for the inflamed rats and 45.7 ± 3.2% for control rats). Each value represents mean ± SD of three separate determinations.

by 90-min incubation (data are not shown). This shows that the glucocorticoid-binding reaction reached an equilibrium by after 90 min.

Present data for the saturation kinetics of the liver glucocorticoid-binding activities (Fig. 2) resemble those reported by Beato and Feigelson [14]. They speculated that a linear increase of the HC-binding, following a sigmoidal binding curve at low concentrations of HC, might be attributed to albumin. In the present study, albumin will not contribute to the saturation kinetics, because the nonspecific binding of a steroid is subtracted by addition of a large excess of the unlabeled steroid. Thus Fig. 2 will show that the liver cytosol has both saturable and nonsaturable binding sites for both natural and synthetic glucocorticoids.

There may be three possibilities as to the result that the granuloma cytosol glucocorticoid-binding activity gave a single straight line on the Scatchard analysis: First, the granuloma cytosol has a single class of the binding site for every natural and synthetic glucocorticoids. Second, the granuloma also has two sorts of binding sites for each glucocorticoid, but the difference between their dissociation constants may be small and the Scatchard plot results in a single straight line merged from the putative two lines. Third, there are two kinds of binding sites for each glucocorticoid in the granuloma cytosol, and either has so low affinity to the steroid that it could be demonstrated in the present study. Indeed, Roth has shown that, unlike HC, DX poorly binds to "low" affinity sites of steroid-responsive tissues of rats [21].

Koehler and Moscona have reported that a Scatchard plot of the HC-binding activity of adrenalectomized rat serum shows a convex curve indicating positive cooperativity [20]. This may be due to the presence of two distinct binding sites on CBG as suggested by Chader and Westphal [25]. The present study may, therefore, show that, if CBG itself (the "extravasated CBG") composes the major part of the HC-holding protein of the granuloma cytosol, some alterations may have occurred in its steroid-binding nature.

Munck suggested a possibility that the anti-inflammatory effects of the glucocorticoids might be mediated by receptors with specificities similar to and with affinities lower than the cytoplasmic receptors found in physiologic target tissues of the steroids [10]. In this view, it can be significant that the dissociation constants of the glucocorticoid receptors of the granuloma cytosol (Table 5) were one-order larger than those of the high affinity binding sites of the liver cytosol (Table 6), which were calculated on the two distinct binding sites model.

Presence of a high HC-binding capacity in the inflamed locus is probably reasonable, because the glucocorticoid bound to the nonreceptor protein CBG, is biologically inactive and, therefore, it prevents the steroid from inhibiting the inflammatory reactions until accomplishment of appropriate control of the injury. In fact, corticosteroid secretion increases under the inflammatory state [26]. In this view point, the kinetic analysis based on the negative cooperativity model (Table 6B) will be significant: the comparison of the results on Tables 5 and 6B shows the HC-binding components of the granuloma cytosol are higher in both the concentration of the binding sites and the affinity to HC than those of the liver HC-binders.

The authors reveal that the present study does not deny that a granuloma cytosol receptor for synthetic glucocorticoids also has an affinity to HC, because it may be possible that the HC-binding activity of the receptor is too weak to detect in a crude sample such as the whole cytosol. Further studies to clarify characteristics of the HC-binding protein of the granuloma is under investigation in the laboratory.

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