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Lymphoblastosis Inhibition and Plaque-forming Cell Response of Several Anti-inflammatory Steroids in Mice

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Abstract □ Anti-inflammatory glucocorticoid (GC) derivatives have been clinically used in immune-malfunctional diseases for their immunosuppressive activity. However, there is still a lack of knowledge on the relationship between anti-inflammatory and immunosuppressive activities. In order to compare immunosuppressive activities with the known anti-inflammatory activities of the GC derivatives, eight clinically used GC derivatives including hydrocortisone, prednisolone, 6α-methyl prednisolone, triamcinolone, dexamethasone, betamethasone, triamcinolone acetonide and fluocinolone acetonide were selected, and lymphoblastosis inhibition and plaque-forming cell (PFC) response in mice were studied as immunological parameters. In Con A-induced lymphoblastosis inhibition *in vitro*, all derivatives showed potent inhibition. IC_{50} values of the derivatives except methyl prednisolone and triamcinolone were less than 10^{-7}M and good dose dependency was obtained. This result was well correlated with that of their anti-inflammatory potencies and their receptor binding affinities. However, in PFC response, consistent results were not obtained. Total numbers of PFCs per spleen were decreased by some derivatives, but numbers of PFCs per 10^6 cells were not decreased by systemic administration of GC at the dose of 0.05 mg/mouse. Furthermore, at the dose of 0.1 mg/mouse, numbers of PFCs per 10^6 cells were found to be increased, although total PFCs per spleen were decreased.

Keywords □ Glucocorticoid, steroid, anti-inflammation, immunosuppression, receptor binding affinity, lymphoblastosis inhibition, plaque-forming cell.

Anti-inflammatory glucocorticoid (GC) derivatives have been clinically used to treat inflammatory and/or immune-malfunctional diseases. It is generally thought that anti-inflammation and immunosuppression by GC are essentially the same activities, because they exert their actions through binding to the same GC receptor in target cells and various efforts to separate these activities were not successful[1,2]. When GC are prescribed against immune-related deseases such as systemic lupus erythematosus and athma, they are conventionally used on the basis of the potenices of their anti-inflammatory activities, but not of the potencies of their immunosuppressive activities. Although anti-inflammation and immunosuppression could be regarded as the same actions and the separate experiments such as Bochner *et al.*[3] and Gilis *et al*[4] showed that the immunosuppressive activities were found to be roughly parallel to their anti-inflammatory activities of GC *in vivo*, there is still a lack of knowledge between these two activities. Therefore, it might be valuable to compare the immunosuppressive activities with their anti-inflammatory activities of the various GC derivatives.

Using one of the immune-related parameters of the GC derivatives, we have previously showed dose-dependent mice thymocyte apoptosis *in vitro* and thymus atrophogenecity *in vivo*[5]. And it was

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proposed that local steroids could be discriminated from systemic steroids with testing these thymocyte apoptotic and thymus atrophogenic activities. As a continual effort, concanavalin A (Con A) induced lymphoblastosis inhibition and inhibition of plaque forming cell (PFC) production induced by T-dependent antigen, sheep red blood cells (SRBC), have been studied for comparing with the known anti-inflammatory activities of the GC derivatives in this investigation. And GC receptor binding affinities were also compared with the anti-inflammatory/immunosuppressive activities.

EXPERIMENTAL METHODS

Materials

Hydrocortisone, TES (N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid), dextran (T70), Hepes, thioglycerol, DEAE-dextran, concanavalin A (Con A) and trypan blue were purchased from Sigma Chem. Co. (St. Louis, MO). Prednisolone was a product of Upjohn Co. (Kalamazoo, MI). Triamcinolone, triamcinolone acetonide, dexamethasone, betamethasone, methyl prednisolone and fluocinolone acetonide were obtained from Steraloids Inc. (Wilton, NH). The chemical structures of the steroids were shown in Fig. 1. [^{3}H]-Dexamethasone (44.7 Ci/mmol) and [^{3}H]-thymidine (6.7 Ci/mmol) were supplied from Dupont Co. (Boston, MA). RPMI (Rothwell Park Memorial Institute) 1640, DMEM (Dulbecco's Modified Eagles Medium), antibiotic solution (penicillin-streptomycin-amphotericin B), EBSS (Earle's balanced salt solution), DPBS (Dulbecco's phosphate buffered saline), guinea-pig complement, fetal calf serum (FCS) and glutamine were purchased from GIBCO (Grand Island, NY). Brewer's thioglycollate medium was obtained from Difco (Detroit, MI).

Rat liver glucocorticoid receptor binding affinity

From adrenalectomized Sprague-Dawley (SD) rats (♂, 200-250 g, Experimental Animal Farm, Seoul National Univ.), the rat liver receptor cytosolic fraction was obtained by ultracentrifugation (Beckman L8-70M) at 105,000g for 1 hr. Protein concentration of cytosol was measured using Lowry method[6]. *In vitro* receptor binding affinities of the GC derivatives were carried out by the competition binding assay using [^{3}H]-dexamethasone as a labeled ligand according to the previously published procedure[7].

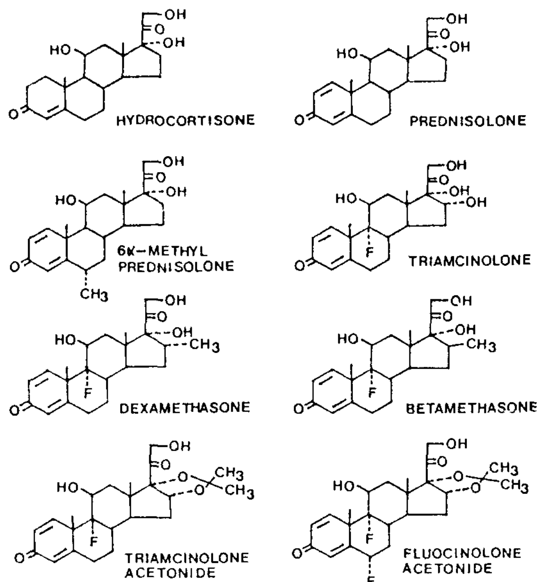

Fig. 1. Chemical structures of the steroid derivatives in this study.

Macrophage whole cell binding affinity

From C3H mice (♂ 20-25 g, Genetic Engineering Res. Institute), peritoneal macrophage was obtained by intraperitoneal injection (1 m*l*/mouse) of sodium thioglycollate medium as previously described[8]. After washing with DPBS (10 m*l*) three times at 0℃, macrophages were transferred into a 6-well cell culture plate (Nunc) in DMEM (2 mM glutamine, 10% FCS), 1×10^6 cell/2 m*l* DMEM/well. The whole cell binding procedure was the same as the previously published method of Werb *et al*[9]. After preliminary experiments to determine the appropriate concentration range of the GC derivatives, 0.3-100.0 nM of GC were used for competition binding using [^{3}H]-dexamethasone (28 nM) as a labeled ligand. Bound [^{3}H]-dexamethasone to macrophages was measured after incubation for 70 min and extraction with ethanol.

Lymphoblastosis inhibition

Lymphoblastosis inhibition by the steroid derivatives was carried out according to the procedure of Mookerjee *et al.*[10] with slight modification. Brie-

fly, spleens of C3H mice were removed and dipped in RPMI 1640 on ice. After crushing spleen with syringe plunger and removing RBC with hemolytic solution, the cells were washed with DPBS and the isolated splenocytes were transferred into a 96-well cell culture plate (Nunc) in RPMI 1640 (2 mM glutamine, 10% FCS), 2×10^5 cells/200 μl/well. And Con A (2.5 μg/well) was added to the each well with concommitant addition of the GC derivatives (DMSO). After incubation at 37℃ in 5% CO_2 incubator for 3 days, [^{3}H]-thymidine (1 uCi/well) was added to each well and incubated further for 8 hours. The cells were collected on a glass filter using multiple cell harvestor (Skatron). The filter was dried and radioactivity was measured in Aquasol-2. Stimulation Index (SI) was calculated compared to the control well treating with Con A and vehicle (DMSO) only.

Plaque-forming cell (PFC) bioassay

From male sheep (Animal Farm, Kangweon National Univ.), blood was collected in sterile Alserver's solution (0.42% sod. chloride, 0.8% trisodium citrate, 2.05% glucose, pH 6.1) and used within 21 days. On the day of immunization, sheep red blood cell (SRBC) was washed with EBSS and 5×10^8 SRBC was intraperitoneally administered to each ICR mouse (♂, 20-25 g, Experimental Animal Farm, Seoul National Univ.). Each steroid derivative was injected i.m. to mice at 2 days before immunization. After 4 days of immunization, mice were sacrificed with head dislocation and spleens were excised, and isolated splenocytes were obtained after treatment of hemolytic solution (0.85% NH_4Cl, 0.1 M Hepes). To the final splenocyte cell pellet obtained by centrifugation at 200g for 10 min was added appropriate volume of EBSS. Following the modified procedure of Bullock and Moller[11], splenocyte (50 μl), 400 μl agar solution (250 mg agar/50 ml EBSS, pH 7.2), SRBC (25 μl) and diluted (1/3) guinea pig complement (25 μl) were mixed, poured into a bacteriological culture dish and overlayed with cover glass (45 ×50 mm). Agar plates were allowed to solidify and incubated at 37℃ in 5% CO_2 incubator for 3 hrs. PFCs were counted using a dissecting microscope.

RESULTS AND DISCUSSION

The relative anti-inflammatory activities of the glucocorticoid derivatives used in this study were reported to be 1, 4, 5, 5, 25, 25, 60 (oral)-200 (topical) and 60 (oral)-200 (topical) for hydrocortisone, prednisolone, methyl prednisolone, triamcinolone, dexamethasone, betamethasone, triamcinolone acetonide and fluocinolone acetonide, respectively[12,13]. There derivatives could be classified clinically on the basis of the potencies of the anti-inflammatory activity as potent (fluocinolone acetonide, triamcinolone acetonide), medium (dexamethasone, betamethasone, triamcinolone, methyl prednisolone, prednisolone) and weak (hydrocortisone). They could be also classified, on the basis of the route of administration used as systemic steroid (methy prednisolone, triamcinolone), local steroid (fluocinolone acetonide, triamcinolone acetonide) and both (dexamethasone, betamethasone, prednisolone, hydrocortisone). There have been numerous reports concerning immunosuppresion of glucocorticoids in experimental animals and men[14,15]. In general, GC potently inhibits cellular immunity in contrast to the conflicting results about humoral immunity (serum antibody level). Recently, major concern is focused on the regulation of various cytokine production by GC (3.4.16.17). Various GC dose-dependently inhibited IL-1 production from human lung tissue[3]. Dexamethasone inhibited IL-2 production from murine and human CTLL clones[4]. In addition to the powerful inhibition of TNF production[16], GC was also reported to inhibit interferon production[17].

When we compared the rat liver receptor binding affinity as intrinsic activity of the steroid derivatives, it was found that the order of affinity stre-

Table I. Rat liver glucocorticoid receptor binding affinities of GC

Compounds	IC_{50}(nM)	Affinity[a]
Hydrocortisone	390	19
Prednisolone	90	83
Methyl prednisolone	75	100
Triamcinolone	205	37
Dexamethasone	75	100
Betamethasone	180	42
Triamcinolone acetonide	26	288
Fluocinolone acetonide	12	625

[a]Relative affinity if dexamethasone is 100.

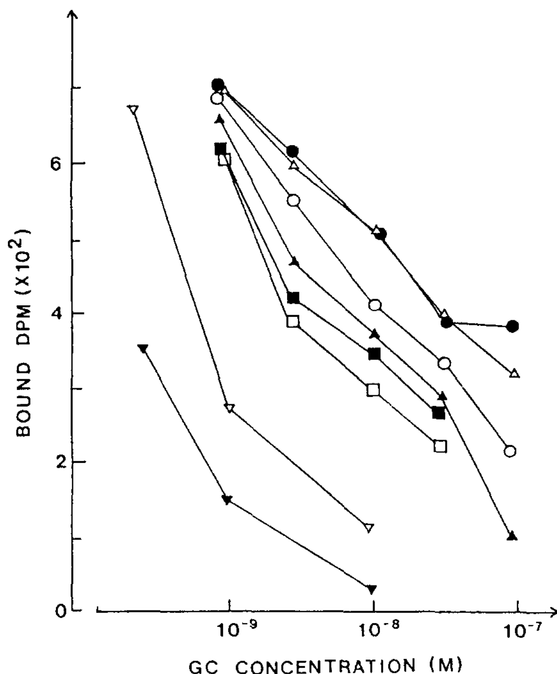

Fig. 2. Macroophage whole cell binding of GC. Hydrocortisone (△), prednisonole (▲), methyl prednisolone (○), triamcinolone (●), dexamethasone (□), betamethasone (■), triamcinolone acetonide (▿), fluocinolone acetonide (▼).

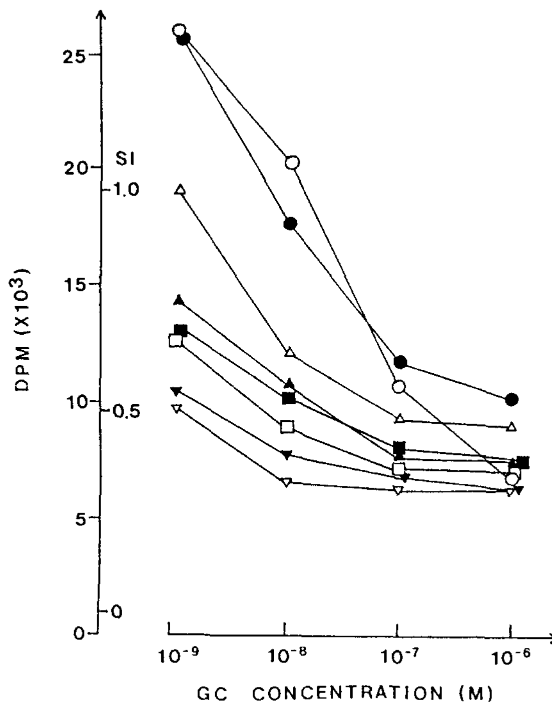

Fig. 3. Inhibition of Con A-induced lymphoblastosis by GC. Hydrocortisone (△), prednisonole (▲), methyl prednisolone (○), triamcinolone (●), dexamethasone (□), betamethasone (■), triamcinolone acetonide (▿), fluocinolone acetonide (▼).

ngth was almost equal to the anti-inflammatory activity of each derivative. Hydrocortisone was the weakest one and fluocinolone acetonide was most potent (Table I). In order to compare whole cell receptor binding affinity, mice peritoneal macrophage was obtained. As shown in Fig. 2, fluocinolone acetonide and triamcinolone acetonide were the most potent. Hydrocortisone and triamcinolone were the weakest. This same pattern of affinity relationship between rat liver receptor binding affinity and macrophage whole cell binding affinity might show that penetration rate through target cell membrane is not the rate limiting step for expressing GC activities, but simple diffusion occurred as evidenced by very rapid binding of GC to the intracellular receptors[18]. In the respect to their relative *in vivo* anti-inflammatory activities of each derivative, exception was triamcinolone which showed moderate anti-inflammatory activity *in vivo*, but showed less binding affinity in macrophage whole cell binding *in vitro*. Fig. 3 showed *in vitro* Con A-induced lymphoblastosis inhibition of the steroid derivatives. All the steroids tested significantly inhibited lymphoblastosis. These results were well correlated with the previously reported results, which showed glucocorticoids were the potent inhibitors of lymphoblastosis, especially for T-cells[4,19,20]. IC_{50} values of all the steroids tested except triamcinolone and methyl prednisolone were less than 10^{-7}M. And good dose dependent inhibition was found including good correlation with their anti-inflammatory activity. However, in this experiment, methyl prednisolone and triamcinolone showed the weakest inhibition. At less than 10^{-8}M, these two steroids did not show lymphoblastosis inhibition, in contrast to the significant inhibition of the other derivatives including hydrocortisone. These results might indicate that methyl prednisolone and triamcinolone should not be used as local steroids, but as systemic steroids, because these steroid derivatives were not potent

Table II. Plaque forming cell (IgM) response by GC

Compounds[a]	0.05 mg/mouse			0.1 mg/mouse		
	Cell no. of spleen (×10^8)	PECs/spleen (×10^3)	PFCs/10^6 cell	Cell No. of spleen (×10^8)	PFCs/spleen (×10^3)	PFCs/10^6 cell
Control	0.72±0.16[b]	0.2±0.3	2±4	0.88±0.11	0.7± 0.8	8±10
SRBC	1.05±0.11	16.2±3.7	155±36	1.15±0.18	27.7± 9.3	240±80
Hydrocortisone	1.02±0.21	20.9±5.9	212±68	0.99±0.13	25.0± 6.6	252±53
Prednisolone	0.91±0.20	19.1±1.9	229±180	0.59±0.22*	23.2± 5.6	389±128
Methyl prednisolone	0.97±0.24	15.4±6.7	175±90	0.45±0.17*	24.4± 4.1	542±102*
Triamcinlone	0.86±0.17	10.1±4.3*[c]	120±52	0.42±0.15*	18.2± 9.8	432±204
Dexamethasone	0.56±0.17*	11.4±5.4	233±29	0.35±0.27*	14.7± 4.7*	420±140*
Betamethason	0.79±0.15	20.4±8.3	269±62	0.36±0.21*	26.8±10.9	736±620
Triamcinolone acetonide	0.61±0.10*	10.5±5.0	173±76	0.32±0.10*	10.3± 3.7*	320±180
Fluocinolone acetonide	0.71±0.13*	10.5±3.0*	148±36	0.25±0.09*	15.6± 4.3*	624±348*

[a]Eight mice per group were used. [b]Mean±SD. [c]Significantly different from SRBC treated group, $p<0.01$

in vitro at local site, but they showed significant activity *in vivo* by systemic administration. And high activity expression *in vivo* by systemic administration for triamcinolone may well agree with pharmacokinetic properties of triamcinolone which has long biological half-life in the body, $T_{1/2}$: 300 min[12]. In order to elucidate the immunosuppressive activity of the GC derivatives in another kind of model system, as a parameter of *in vivo* immune response, PFC bioassay was employed. The GC derivatives were administered to mice 2 days before immunization with SRBC, from the finding that serum antibody level was the lowest in mice when GC was injected 1-4 days before immunization[21]. By treatment of GC, numbers of IgM secreting cells against SRBC per spleen were reduced in several groups (Table II). But, no meaningful dose-dependency and no significant inhibition of PFCs per 10^6 cells were found. At the dose of 0.05 mg/mouse, total PFCs per spleen against SRBC were reduced in the triamcinolone and fluocinolone acetonide treated groups. However, even in these groups, PFCs/10^6 cells were not reduced, which indicated that involution of spleen (spleen atrophy) might be a main phenomenon. Actually, total cell numbers of splenocytes were found to be reduced. And reduction of cell number was similarly matched with potencies of the GC derivatives. At the dose of 0.1 mg/mouse, total PFCs were significantly decreased in the dexamethasone, triamcinolone acetonide and fluocinolone acetonide treated groups. But again, PFCs/10^6 cells were revealed to be not reduced, but significantly increased in the methyl prednisolone, dexamethasone and fluocinolone acetonide treated groups. This phenomenon could not be explained exactly at present. However, these results might be interpreted in several ways. First, as T cell was found to be more sensitive to GC treatment than B cell in thymus atrophy[5] and lymphocytophenia[22], T-cell may be also more susceptible than B-cell in spleen atrophy. Therefore, remaining splenocytes might be largely B-cells which may lead to the increase of PFCs per 10^6 cells. The more resonable results could be obtained if we count only B-cells instead of total cells. Unfortunatly, we did not try the differential counts and it remains to be elucidated. Second, Bradly *et al*[23] found that GC inhibited suppressor cells more profoundly than helper T-cells *in vitro*, which might increase PFC response of T-dependent antigen, SRBC. Third, there have been several reports to find the increased Ig production by GC. In *in vitro* human mononuclear cell culture without no stimulant, GC induced Ig synthesis detected by reverse hemolytic plaque assay[24,25]. And recent report found that glucocorticoid did not reduce serum IgG level, but redistributed antibody in the body[26], although older references[21,27] described reduced total PFCs and serum level of IgG and IgM in mice. Therefore, it needs more research to establish the PFC response of GC clearly.

In conclusion, GC was found to be a powerful inhibitor of Con A-induced lymphoblastosis, which

was well correlated with their anti-inflammatory potencies including *in vitro* GC receptor binding affinities and macrophage whole cell binding affinities. But, inconsistent results were obtained in PFC responce by the GC treatment. And more research is needed to elucidate PFC response of GC.

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