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Pregnenolone and Dehydroepiandrosterone as Precursors of Native 7-Hydroxylated Metabolites Which Increase the Immune Response in Mice

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Dehydroepiandrosterone (DHEA) and pregnenolone (PREG) were both metabolized by homogenates of brain, spleen, thymus, perianal skin, ventral skin, intestine, colon, coecum and muscle tissues from mice. The use of ^{2}H-labeled substrates and of the twin ion technique of gas chromatography-mass spectrometry permitted identification of 7α-hydroxy-DHEA and of 5-androstene-3β,17β-diol as DHEA metabolites in digests of all tissues. The extent of PREG metabolism was much lower than for DHEA with all tissues but amounts of the main transformation product were sufficient in brain, spleen and ventral skin digests for identification with 7α-hydroxy-PREG. Dimethylsulfoxide (DMSO) solutions of DHEA, PREG and of their 7-hydroxylated metabolites were injected at different doses and time intervals prior to proximal subcutaneous administration of a lysozyme antigen. Quantities of anti-lysozyme IgG were measured in the serum of treated mice and compared with that from sham-treated animals. Increase of anti-lysozyme IgG was obtained with DHEA and PREG (1 g/kg) when injected 2 h prior to lysozyme. Much lower doses (160 times less) of 7α-hydroxy-DHEA and -PREG were also found to be significantly active when administered at the moment of lysozyme injection. A larger dose of 7β-hydroxy-DHEA (50 mg/kg) was necessary for a similar effect. These results suggest that in tissues where immune response takes place, the locally-produced 7-hydroxy metabolites of PREG and DHEA are involved in a process which may participate in the physiological regulation of the body's immune response.

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INTRODUCTION

The 3β-hydroxylated precursors and metabolites of natural steroid hormones are specifically hydroxylated at the 6α, 7α and 7β positions by a cytochrome P_{450}–NADPH-dependent enzyme complex in human, canine and rat prostate [1–3], canine epididymes and perianal glands [2] and rat pituitary and brain [4–6]. This hydroxylation only occurred at the 7-position when 3β-hydroxy-5-ene steroid substrates were used [6].

Dehydroepiandrosterone (DHEA), a circulating natural 3β-hydroxy-5-ene steroid, significantly increased survival of virus-infected mice by immunostimulation only when administered either subcutaneously or *per os* [7]. Since large quantities of DHEA (1 g/kg) had to be administered hours prior to infection, we hypothesized that 7-hydroxylation of DHEA took place near the site of antigen injection and that both were necessary to trigger the immune response. This implied a natural hydroxylation process occurring on native 3β-hydroxy-5-ene precursors of steroid hormones. Since pregnenolone (PREG) and DHEA are such native circulating precursors, we investigated and compared their transformation into 7-hydroxylated metabolites by the skin and other tissues of mice. In addition, we used DHEA at the doses reported by Loria *et al.* [7] in order to compare its action with that of PREG and 7-hydroxylated metabolites on elicitation of an increased immune response in mice.

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MATERIALS AND METHODS

Animals

Six-week-old male mice of the C57BL/6 strain were purchased from Iffa-Credo (L'Arbresle, 69, France). Animals were caged for 1 week in the laboratory's animal room with food and water *ad libitum* prior to treatment or killing. Twelve hour light–dark cycles were maintained from 8.00 a.m. to 8.00 p.m. and temperature was set at 20–21°C.

Collection of tissue samples

Mice were killed by decapitation. Blood, ventral and perianal skin, intestine, colon, coecum, spleen, thymus and brain were collected. Hair was removed from skin samples. Intestine, colon and coecum were opened and freed of their content by several washes with isotonic saline. Serum was recovered from clotted blood by centrifugation at 5000*g* for 10 min. All tissue preparations were cut into small pieces with scissors and weighed before storage at −80°C.

Preparation of tissue homogenates

Each frozen tissue was pulverized in liquid nitrogen with the aid of a SPEX 6700 freezer mill (Bioblock Scientific, Illkirch, France) operated at the highest impact frequency for 3 min. The obtained powder was collected and thawed in 3 vol of 0.067 M phosphate buffer (pH 7.4) containing 1 mM EDTA. Each suspension was further homogenized at 2°C with 10 up and down strokes in a glass–Teflon homogenizer set at 600 rpm. Tissue concentrations were all expressed in mg-equiv contained in 1 ml of homogenate.

Steroids

[4-^{14}C]DHEA (51 mCi/mmol) and [4-^{14}C]PREG (50 mCi/mmol) were both purchased from NEN (Du Pont de Nemours, France). [7β-^{2}H]DHEA was a gift from Dr J. C. Orr (St John's Nfdld, Canada) and contained 99% deuterium at the 7β position. It was diluted with trace amounts of [4-^{14}C]DHEA and 50% (w/w) non-labeled DHEA. Final deuterium content was measured at 46.5 ± 1.6% in excess of natural abundance on the characteristic twin ions in the mass spectra of trimethylsilyl (TMS) ether derivatives after gas chromatography-mass spectrometry (GC-MS). [16ξ-^{2}H]PREG was a gift from Dr Mailloux (Paris 5 University, France) and contained 91% deuterium at the 16ξ position. It was diluted with [4-^{14}C]PREG and non-labeled PREG and the final deuterium content was measured as above at 43.1 ± 0.5% in excess of natural abundance.

Non-radioactive reference steroids were either purchased from Sigma (St Louis, MO, U.S.A.) and Steraloids (Wilton, NH, U.S.A.), or were generous gifts from Roussel-Uclaf (Romainville, France). The 7α- and 7β-hydroxy derivatives of DHEA, PREG and 5-androstene-3β,17β-diol were obtained or prepared as reported previously [6].

Incubations

The ethanol solutions of the radiolabeled steroid substrate (nmol) were dried at the bottom of 10 ml glass tubes under a stream of nitrogen. Incubations were carried out in a total volume of 2 ml. Buffer A (0.067 M phosphate buffer, pH 7.4, containing 1 mM EDTA) was added first, followed by NADPH, final concentration 0.5 mM. Tubes were warmed at 37°C and incubations were started by addition of 100 mg-equiv of each tissue homogenate. Control incubations contained either no NADPH or 10 min-boiled homogenates or buffer in the place of homogenates. Incubations of the non-stoppered tubes were carried out in a shaking water bath (70 shakes/min) at 37°C for 60 min. Incubations were stopped by addition of 2 ml acetone followed by 5 ml ethyl acetate. The tubes were then stored at −80°C until further processing.

Extraction and separation of radiosteroids

Acetone–ethyl acetate supernatants were collected from the frozen incubation mixtures. The thawed water phase was then extracted twice with 5 ml ethyl acetate. Recoveries were in the 92–99% range. Defatting of extracts from preparative scale incubations was carried out with 70% methanol extraction as described previously [8].

The extracts were dried at 40°C under a stream of nitrogen, then taken up in 0.2 ml ethyl acetate and applied to silica F_{254} plates (Merck, Darmstadt, Germany) for thin layer chromatography (TLC). Authentic reference steroids were applied on separate lanes. The plates were developed once in ethyl acetate. In this system, the R_f of PREG, DHEA and 5-androstene-3β, 17β-diol was 0.823, 0.764 and 0.732, respectively. The 7-hydroxy derivatives of these steroids were also separated, at $R_f = 0.282$ for 7α-hydroxy-PREG, $R_f = 0.250$ for 7α-hydroxy-DHEA, $R_f = 0.376$ for 7β-hydroxy-DHEA, $R_f = 0.212$ for 5-androstene-3β,7α,17β-triol and $R_f = 0.382$ for 5-androstene-3β,7β,17β-triol.

Autoradiography of the chromatograms was carried out by exposure on Fuji X-ray films (Fuji Photo Film Co. Ltd, Japan) for 2 to 3 days. When necessary, the radioactive areas shown on the films were located on the chromatograms for recovery and elution of the silica gel with 3 × 2 ml ethyl acetate.

Quantitation of radioactive metabolites

The relative amounts of radiosteroids were measured directly on the chromatograms by scanning of the thin layer plates with an automatic TLC linear analyzer model LB 2882 (Berthold Analytical Instruments, Nashua, NH, U.S.A.). Other radioactivity measurements were carried out in Picofluor (Packard, Warrenville, RO, U.S.A.) solutions with a liquid

scintillation spectrometer Delta 300 model 6890 (Searle Analytical Inc., U.S.A.).

Formation of derivatives

TMS derivatives were prepared prior to GC-MS by reacting the dried steroids dissolved in 0.01 ml pyridin with 0.1 ml bis-(TMS)-trifluoroacetamide (Sigma) at 60°C for 30 min. Dilution with *n*-hexane was carried out as necessary.

GC-MS

A Hewlett-Packard HP 5890 series II gas chromatograph coupled with a HP 5959A mass spectrometer was used for identification of PREG, DHEA and their metabolites recovered from incubations. GC was carried out on a fused silica capillary column (12 m, 0.2 mm i.d.) coated with bonded HP-1 stationary phase. Injection of the TMS derivatives was carried out in the splitless mode with helium (5 ml/min) as a mobile gas phase. Oven temperature was programmed from 100 to 230°C with a 10°C/min increment. The column was directly coupled to the quadrupole mass spectrometer ionization chamber where the source was set at 300°C and the energy of bombarding electrons at 70 eV. Ion detection and mass spectra were recorded and processed by model B1500A # AAO Hewlett-Packard software on HP Apollo series 400 computer. When deuterium-labeled samples were processed, the percentage of deuterium in excess of natural abundance was computed from the twin ion fragments according to previously published techniques [2].

Immunization and treatment of mice

Seven-week-old C57BL/6 male mice were distributed into groups of 5 animals. One group did not receive treatment and were kept as a blank. The mice of all other groups were anesthetized with ether prior to subcutaneous (s.c.) injection in the body side of 10 mg/kg of body wt of lysozyme from chicken egg white (Sigma) dissolved in 0.2 ml sterile saline. The following steroids were tested, once dissolved into 0.1 to 0.2 ml DMSO (Merck), by s.c. injections near the site of lysozyme administration: DHEA, PREG, 7α-hydroxy-PREG, 7α-hydroxy-DHEA and 7β-hydroxy-DHEA. Quantities of administered PREG or DHEA ranged from 0.25 to 2 g/kg body wt. Injection of these steroids to different groups was carried out either at the moment of lysozyme injection, or 1, 2 or 4 h before lysozyme injection.

Quantities of administered 7-hydroxy derivatives ranged from 6.25 to 50 mg/kg body wt and were injected into the relevant groups at the moment of lysozyme injection.

One group received DMSO only near the site of lysozyme injection and were kept as sham-treated animals.

Two weeks after treatment, all but the blank group received a single s.c. boost (10 mg/kg body wt) of the same lysozyme solution. All mice were killed by decapitation 1 week after the boost. Blood was collected and serum was kept frozen at −80°C.

Serum concentrations of anti-lysozyme immunoglobulins

Concentrations of anti-lysozyme IgG in serially-diluted serum samples were measured by immunoenzymatic detection of the specific IgG–lysozyme complexes by a peroxidase-labeled goat anti-mouse IgG (Fc) (Pierce, Rockford, IL, U.S.A.). Briefly, 96-well plates were coated with lysozyme. Serum pools were made from individual samples for each group. Each plate received in duplicate from 1/60 to 1/1080 dilutions of four individual sera and one of the corresponding pool. After incubation at 37°C for 120 min and washing, the peroxidase-labeled goat anti-mouse IgG was distributed and incubated at 37°C for 60 min. After washing, the remaining peroxidase activity was detected with orthophenylene diamino dihydrochloride/hydrogen peroxide (Pierce) followed by sulfuric acid and reading at 490 nm.

Reagents

Reagents and salts of analytical grade used for incubation buffer preparations were from Merck. Buffers and reagents for immunoenzymatic measurements were purchased from Pierce. NADPH was from Sigma. Solvents of RP grade were purchased from Merck and Carlo Erba (Milano, Italy).

Statistical analysis of data

Due to the lack of reference IgG, the O.D. measured from the reacted serum dilutions were taken as arbitrary units. For each serum, the measured O.D. were plotted according to the corresponding serum dilutions and the areas below the resulting curves were computed. Areas resulting from steroid treatments were statistically compared with those obtained from sham-treated animals. The χ^2 test and the Fischer test [9] were used for these comparisons in addition to a z test [9] where animals from each group were sorted into responding and non-responding individuals after comparison of their areas under the curve with mean areas obtained from the sham-treated animals. The rate of responding individuals allowed both calculation of the necessary P and estimation of significant differences.

RESULTS

Metabolism of DHEA by tissue homogenates

[4-^{14}C]DHEA (1 nmol) was incubated with a quantity of each homogenate corresponding to 100 mg of tissue, in 2 ml of buffer A supplemented with 0.5 mM NADPH, at 37°C for 30 min. Homogenates of brain, spleen, perianal skin, thymus, ventral skin, intestine, colon, coecum and leg muscle were used. TLC of the extracted radiosteroids were scanned after development in ethyl acetate. The separated radiometabolites were

located at the R_f of authentic 5-androstene-3β,17β-diol ($R_f = 0.732$), 7α-hydroxy-DHEA ($R_f = 0.250$), 7β-hydroxy-DHEA ($R_f = 0.376$) and 5-androstene-3β,7α,17β-triol ($R_f = 0.212$). Three sets of control incubations with the radiosubstrate were carried out. They included (i) intact homogenates without cofactor, (ii) 10-min boiled tissue homogenate with 0.5 mM NADPH, and (iii) 0.5 mM NADPH without homogenate. For each set, the radioactivity at the R_f of the analyzed metabolites was not significantly different from that measured after TLC in ethyl acetate of the [4-^{14}C]DHEA substrate.

The main metabolite was 7α-hydroxy-DHEA, with decreasing production rates in brain, spleen, perianal skin, thymus and ventral skin (Table 1). Other tissues showed lower production rates of this metabolite. Production of 7β-hydroxy-DHEA was observed with all tissues apart from coecum. Measured rates were much lower than for 7α-hydroxy-DHEA (Table 1). The second major metabolite of DHEA was 5-androstene-3β,17β-diol with highest and lowest production rates in spleen and intestine, respectively (Table 1). With spleen, perianal skin, coecum, colon and thymus, trace amounts of radioactivity were detected at $R_f = 0.212$ corresponding with 5-androstene-3β,7α,17β-triol. Small amounts of radioactivity corresponding with unidentified non-polar metabolites were also measured near the solvent front after TLC.

Metabolism of PREG by tissue homogenates

[4-^{14}C]PREG (1 nmol) was incubated as above for DHEA. Homogenates of brain, spleen, thymus, ventral skin, muscle, colon, coecum and intestine were used. TLC of the extracted radiosteroids were scanned after development in ethyl acetate. The separated radiometabolites were located at the R_f of authentic 7α-hydroxy-PREG ($R_f = 0.282$). Control incubations were carried out as above for DHEA, and the radioactivity measured at the R_f of analyzed metabolites was not significantly different from that measured after TLC in ethyl acetate of the [4-^{14}C]PREG substrate.

All tissue homogenates produced 7α-hydroxy-PREG with highest rates in brain and spleen and lowest rates in coecum and intestine (Table 1). In addition, small amounts of radioactivity were found near the solvent front and corresponded with unidentified non-polar metabolites. Other radiosteroids found at $R_f = 0.810$ in colon, muscle and ventral skin could correspond with reference 5-pregnene-3β,20α-diol.

Identification of 5-androstene-3β,17β-diol and 7α-hydroxy-DHEA metabolites

Preparative scale incubations were carried out in 8 tubes each containing 2 ml of buffer A with 0.5 mM NADPH, 10 nmol of [4-^{14}C,7β-^{2}H]DHEA and tissue homogenate (100 mg-equiv) at 37°C for 60 min. These sets of 8 incubations were repeated for each tissue homogenate. Once defatted, the extracted steroids of each set were pooled and separated by TLC in ethyl acetate. GC-MS of the TMS ethers of authentic 5-androstene-3β,17β-diol and 7α-hydroxy-DHEA and of the labeled extracted steroids with the same respective R_f showed nearly identical retention times (within 0.1%) and presented the same diagnostically important ions (Tables 2 and 3). The presence of deuterium in excess of natural abundance was measured from the twin ions of the characteristic fragments of the recovered untransformed DHEA substrate. No significant difference with that of the incubated substrate was noticed.

Deuterium in excess of natural abundance was also measured from twin ions of characteristic fragments of the 7α-hydroxy-DHEA metabolite (Table 2). The most intense fragment in reference steroid was at m/z 358 (M-90), while m/z 448 (M+) and 392 (M-56) were both minor fragments. By taking these intensities into account, significant losses of deuterium were noticeable for each tissue when compared with deuterium contents of the DHEA substrate. Nevertheless, from 57.8 to 83.9% of the substrate's 7β-^{2}H was retained in the 7α-hydroxy-DHEA metabolite and this ascertained its origin from the deuterated DHEA substrate. For

Table 1. Metabolism of [4-^{14}C]DHEA or [4-^{14}C]PREG by tissue homogenates

	Substrates			
	DHEA transformed (pmol/min/100 mg tissue)			PREG transformed (pmol/min/100 mg tissue)
Tissue homogenate	7α-OH-DHEA	7β-OH-DHEA	Δ_5-Diol	7α-OH-PREG
Brain	26.9	1.8	3.5	3.3
Spleen	26.2	0.8	14.3	2.6
Perianal skin	24.1	5.3	8.6	ND
Thymus	15.9	0.7	6.2	2.3
Ventral skin	11.0	2.4	5.1	2.3
Intestine	3.8	0.3	3.1	0.2
Colon	2.4	0.7	7.6	1.1
Coecum	1.3	0.0	12.5	0.3
Muscle	1.3	0.9	6.5	2.1

7α-OH-DHEA, 7α-hydroxy-DHEA; 7β-OH-DHEA, 7β-hydroxy-DHEA; Δ_5-Diol, 5-androstene-3β,17β-diol; 7α-OH-PREG, 7α-hydroxy-PREG; ND, not done.

Table 2. Identification and per cent of deuterium content of [7β-^{2}H]7α-hydroxy-DHEA recovered from digests

Tissue homogenate	Retention times (min) reference/sample	Percent of deuterium contents in ion fragments of the samples					
		M+	M-15	M-56	M-90	M-105	mean ± SEM
Brain	11.559/11.566	54.7	29.6	ND	36.8	34.2	38.8 ± 19.0
Spleen	11.559/11.567	52.9	29.3	25.0	37.1	32.5	35.4 ± 19.2
Perianal skin	11.559/11.564	ND	30.5	ND	37.6	31.5	33.2 ± 6.3
Thymus	11.559/11.569	54.5	23.3	46.0	38.1	33.3	39.0 ± 21.3
Ventral skin	11.559/11.566	49.0	25.5	ND	37.9	33.6	36.5 ± 17.0
Intestine	11.559/11.567	ND	29.4	ND	37.2	22.6	29.7 ± 11.9
Colon	11.559/11.560	24.6	14.0	ND	35.7	33.1	26.9 ± 17.0
Coecum	11.559/11.553	42.0	11.5	37.5	29.1	23.9	28.8 ± 21.4
Muscle	11.559/11.566	ND	ND	ND	36.1	25.1	30.6 ± 11.0
DHEA substrate	13.682/13.683	45.5	47.2	45.9	47.6	46.2	46.5 ± 1.6

ND, not detected, SEM, standard error of the mean.

5-androstene-3β,17β-diol TMS ether, intensities of the characteristic fragments were relative to *m/z* 73 (100%) and ranged from 4% for *m/z* 419 (M-15) to 32% for *m/z* 344 (M-90). No loss of 7β-^{2}H was observed after analysis of the 5-androstene-3β,17β-diol recovered from the digests (Table 3) and the presence of excess deuterium found in all characteristic ion fragments ascertained its origin from the deuterated DHEA substrate. Quantities of 5-androstene-3β,17β-diol recovered from perianal skin and intestine were not sufficient to carry out deuterium content analysis. Another ^{14}C-labeled DHEA metabolite was present at the R_f and retention time of authentic 7β-hydroxy-DHEA; however, even with a consistent fragmentation pattern, low quantities and the absence of deuterium at the 7β position precluded its formal identification by the twin ion technique. The trace amounts of a metabolite at the R_f of 5-androstene-3β,7α,17β-triol were not sufficient for GC-MS identification.

Identification of 7α-hydroxy-PREG

Preparative scale incubations of [4-^{14}C,16ξ-^{2}H] PREG were carried out exactly as above for DHEA. The sets of 8 incubations were repeated for each tissue. Once defatted, the extracted steroids of each set were pooled and separated by TLC in ethyl acetate. GC-MS of the TMS ethers of authentic 7α-hydroxy-PREG and of the labeled extracted steroids with the same respective R_f showed nearly identical retention times (within 0.1%) and presented the same diagnostically important ions (Table 4).

The presence of deuterium in excess of natural abundance was measured from the twin ions of the characteristic fragments of the recovered untransformed PREG substrate. No significant difference with that of the incubated substrate was noticed. Only brain, spleen and ventral skin digests yielded enough of the 7α-hydroxy-PREG metabolite for GC-MS analysis. Twin ions at the expected retention time were only measurable at *m/z* 386–387 which corresponded with the most intense M-90 fragment. When compared with 16ξ-^{2}H contents of the substrate (43.4% based on the M-90 fragment and 43.1 ± 0.5% based on the 4 characteristic fragments), the percent of deuterium in excess of natural abundance shown in Table 4 from the M-90 ion fragment indicates that nearly 100% of the substrate's 16ξ-^{2}H was retained in the 7α-hydroxy-PREG metabolite. Small quantities of label associated with a steroid at $R_f = 0.810$ did not permit its identification with 5-pregnene-3β,20α-diol.

PREG and DHEA increase anti-lysozyme IgG concentrations in serum

DMSO solutions of PREG or DHEA were injected subcutaneously in the body side (1 g/kg body wt) of

Table 3. Identification and per cent of deuterium content of [7β-^{2}H]5-androstene-3β,17β-diol recovered from digests

Tissue homogenate	Retention times (min) reference/sample	Percent of deuterium contents in ion fragments of the samples					
		M+	M-15	M-90	M-105	M-129	mean ± SEM
Brain	15.737/15.735	47.4	46.9	39.4	40.7	52.2	41.1 ± 16.5
Spleen	15.737/15.739	45.7	47.0	40.5	41.8	51.7	45.3 ± 7.9
Thymus	15.737/15.749	48.6	46.1	41.0	42.3	46.4	44.9 ± 5.6
Ventral skin	15.737/15.737	45.7	44.8	41.6	42.1	51.4	45.1 ± 7.0
Colon	15.737/15.741	44.3	42.9	40.7	41.5	51.0	44.1 ± 7.3
Coecum	15.737/15.751	42.1	45.1	38.0	38.6	49.4	42.6 ± 8.5
Muscle	15.737/15.750	48.9	46.9	40.2	42.4	51.8	46.0 ± 8.5
DHEA substrate	13.682/13.683	45.5	47.2	47.6	46.2	ND	46.5 ± 1.6

ND, not detected; SEM, standard error of the mean.

Table 4. Identification and percent of deuterium content of [16ξ-^{2}H]7α-hydroxy-PREG recovered from digests

Tissue homogenate	Retention times (min) reference/sample	Percent deuterium content in ion fragments of the samples			
		M+	M-15	M-90	M-105
Brain	19.935/19.938	ND	ND	42.3	ND
Spleen	19.935/19.940	ND	ND	43.0	ND
Ventral skin	19.935/19.945	ND	ND	40.4	ND
PREG substrate	18.490/18.486	43.3	42.9	43.4	43.1

ND, not detected.

mice. Injections were carried out either 4, 2, 1 or 0 h prior to s.c. injections near the same site as lysozyme (10 mg/kg body wt). Each experiment was repeated with 5 mice. Further processes were carried out as described in Materials and Methods. Anti-lysozyme IgG concentrations were measured in serial dilutions of each serum and in pools of the sera from each group of 5 mice.

One example of the plots obtained after s.c. injections of PREG (1 g/kg body wt) is given in Fig. 1 where pooled sera from 5 mice were analyzed for each point. The data show that when injected 1 or 2 h prior to the lysozyme, PREG (1 g/kg body wt) induces a sharp increase in anti-lysozyme IgG concentrations. From such plots obtained from individual mice and from pools, areas under each curve were computed and were used for statistical analysis of differences between treated mice and shams.

Figure 2 shows results obtained with PREG (1 g/kg body wt) and with DHEA (1 g/kg body wt). With both steroids, injections had to be carried out 2 h prior to that of lysozyme in order to get a significant increase of serum anti-lysozyme IgG concentrations ($P < 0.05$ and 0.01, respectively).

These results led to new experiments where various quantities (from 0.25 to 2 g/kg body wt) of DHEA and PREG were subcutaneously injected 2 h prior to lysozyme administration. By using the same procedure as above, dose-dependent effects of the two steroids were measured (Fig. 3). When compared with the sham, a significant increase of anti-lysozyme IgG concentrations in sera were found with administered quantities of 0.25 and 1 g/kg body wt of DHEA ($P < 0.01$ and 0.05, respectively). In contrast, all concentrations of PREG were found to lead to IgG productions significantly higher than that of sham ($P < 0.01$). All significant increments were confirmed with the χ^2 test and with the Fischer test.

The 7-hydroxylated metabolites of PREG and DHEA increase the anti-lysozyme IgG concentrations in serum

Experiments were carried out as above, except that 7-hydroxylated steroids were subcutaneously injected at the moment of lysozyme administration and that

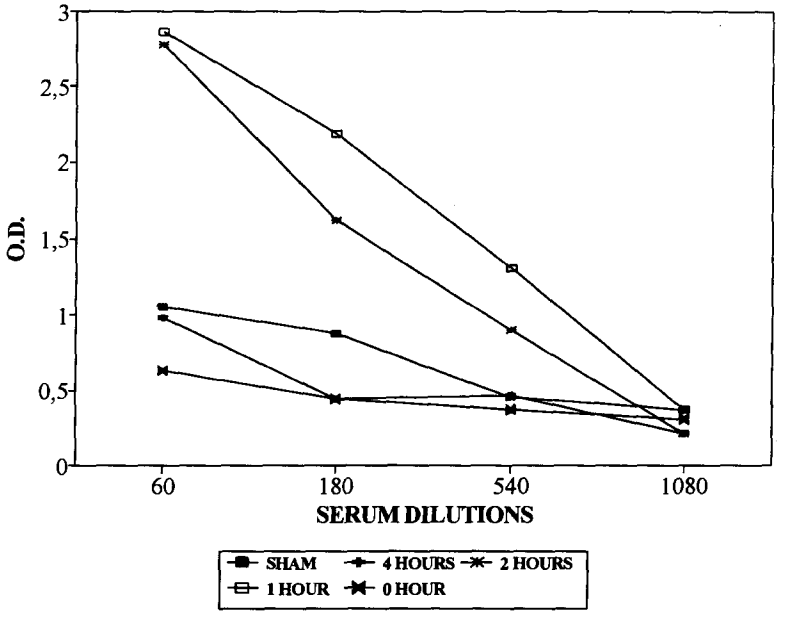

Fig. 1. PREG effect on serum anti-lysozyme IgG concentrations. Either DMSO solutions of PREG (1 g/kg body wt) or DMSO alone (sham) were injected subcutaneously 4, 2, 1 and 0 h before nearby s.c. injection of lysozyme antigen (10 mg/kg body wt). Anti-lysozyme IgG concentrations were measured in serial dilutions of the sera. Each point is the measurement obtained from pooled sera from 5 mice.

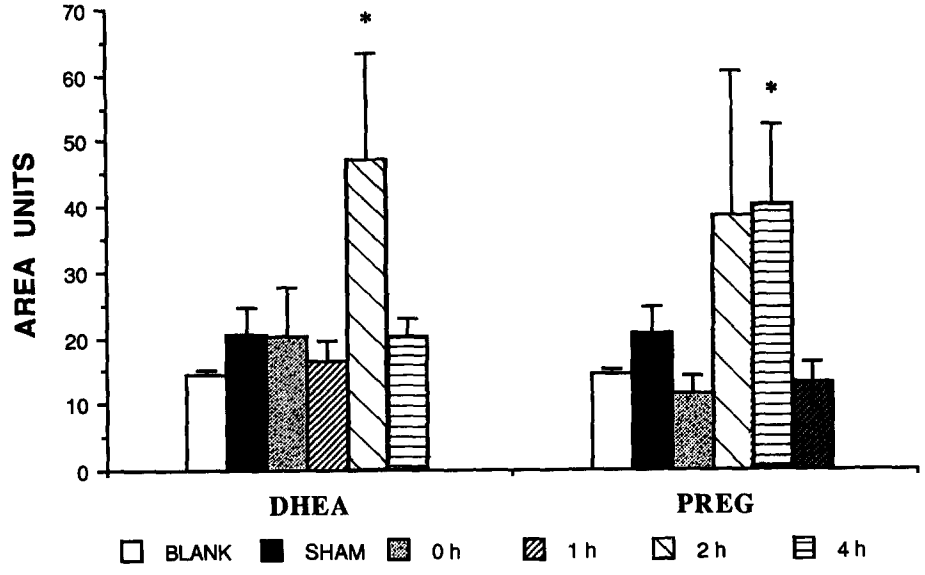

Fig. 2. Time-dependent effect of PREG and DHEA on serum anti-lysozyme IgG concentrations. Either DMSO solutions of PREG (1 g/kg body wt) or DHEA (1 g/kg body wt) or DMSO alone (sham) were injected subcutaneously 4, 2, 1 and 0 h before nearby s.c. injection of lysozyme antigen (10 mg/kg body wt). Lysozyme injections were omitted in blanks. Anti-lysozyme IgG concentrations were measured in serial dilutions of either pooled sera (as in Fig. 1) or individual sera. For the latter, each point was the mean of measurements obtained from 5 mice. Areas under each curve were computed and each one resulting from steroid treatment was statistically compared with that of the sham. $*P < 0.05$.

their doses were 20 to 160 times lower (6.25 to 50 mg/kg body wt) than for PREG and DHEA. Effects of the 7-hydroxylated steroids on anti-lysozyme IgG production are shown in Fig. 4. Both 7α-hydroxy-DHEA and -PREG administered at 6.25 mg/kg body wt were found to significantly increase the specific IgG concentrations in serum ($P < 0.05$ and 0.002, respectively). Higher doses of 7α-hydroxy-PREG led to less significant increments ($P < 0.05$ for 12.5 mg/kg body wt and $P < 0.002$ for 25 mg/kg body wt), while other

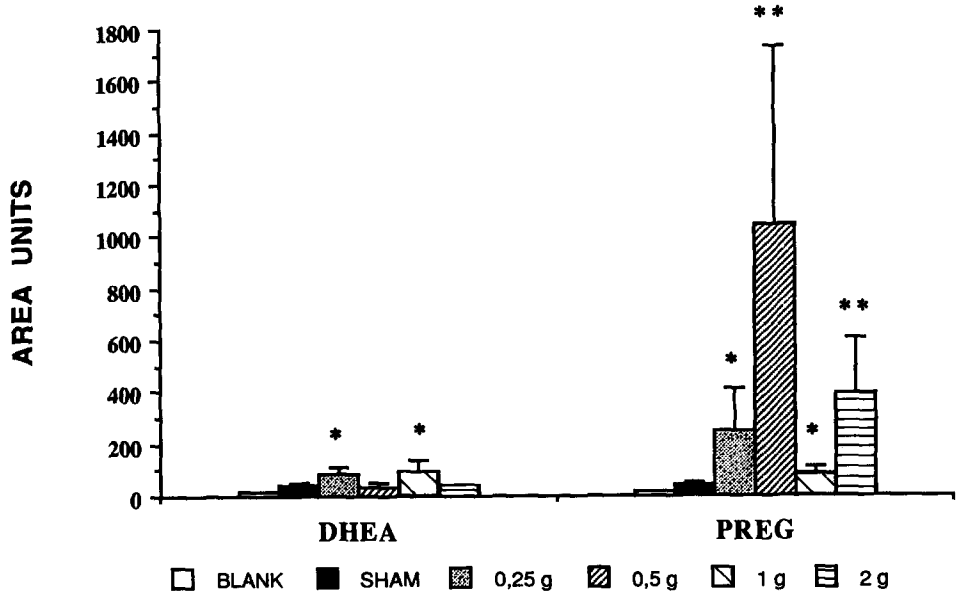

Fig. 3. Dose-dependent effect of PREG and DHEA on serum anti-lysozyme IgG concentrations. Either DMSO solutions of PREG (0.25–2 g/kg body wt) or DHEA (0.25–2 g/kg body wt) or DMSO alone (sham) were injected subcutaneously 2 h before nearby s.c. injection of lysozyme antigen (10 mg/kg body wt). Lysozyme injections were omitted in blanks. Anti-lysozyme IgG concentrations were measured in serial dilutions of either pooled (as in Fig. 1) or individual sera. For the latter, each point was the mean of measurements obtained from 5 mice. Areas under each curve were computed and each one resulting from steroid treatment was statistically compared with that of the sham. $*P < 0.05$, $**P < 0.002$.

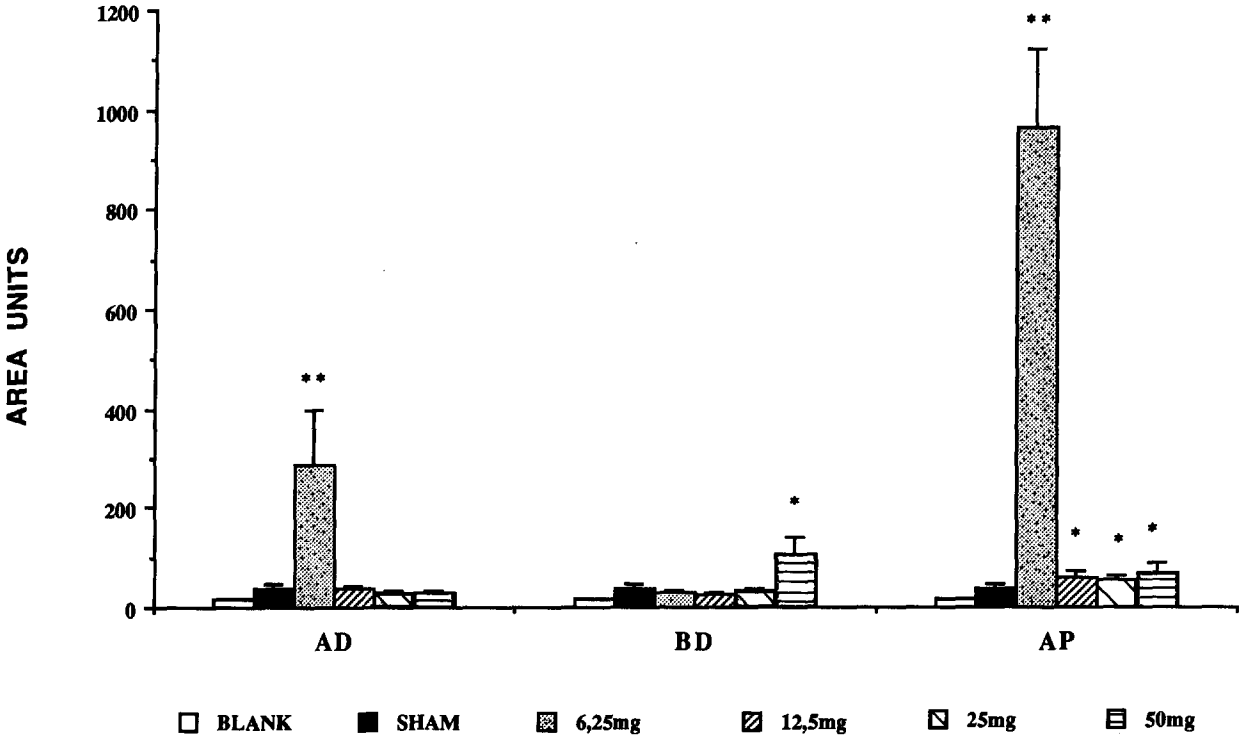

Fig. 4. Dose-dependent effect of 7α-hydroxy-DHEA (AD) and 7β-hydroxy-DHEA (BD) and 7α-hydroxy-PREG (AP) on serum anti-lysozyme IgG concentrations. Either DMSO solutions of AD (6.25–50 mg/kg body wt) or BD (6.25–50 mg/kg body wt) or AP (6.25–50 mg/kg body wt) or DMSO alone (Sham) were injected subcutaneously at the moment of nearby s.c. injection of lysozyme antigen (10 mg/kg body wt). Lysozyme injections were omitted in blanks. Anti-lysozyme IgG concentrations were measured in serial dilutions of either pooled (as in Fig. 1) or individual sera. For the latter, each point was the mean of measurements obtained with 5 mice. Areas under each curve were computed and each one resulting from steroid treatment statistically compared with that of the sham. $*P < 0.05$, $**P < 0.002$.

doses of 7α-hydroxy-DHEA were without significant effect. All significant increments were confirmed with the use of the χ^2 and Fischer test. The tested 7β-hydroxy-DHEA when administered at 50 mg/kg body wt was found to be significantly effective only with the z test ($P < 0.05$).

DISCUSSION

The present study describes the characterization of PREG and DHEA metabolites formed after incubation with mouse brain, spleen, thymus, perianal and ventral skin, intestine, colon, coecum and leg muscle tissue homogenates. We have applied the twin ion technique of GC-MS [2] with deuterated substrates to establish identity of the metabolites.

Identifications of the [7β-^{2}H]7α-hydroxy-DHEA, [7β-^{2}H]5-androstene-3β,17β-diol and [16ξ-^{2}H]7α-hydroxy-PREG metabolites formed in most tissues were based on (i) an identical R_f with that of reference steroids on TLC, (ii) nearly identical retention times of the diTMS ethers, (iii) identical fragmentation patterns in mass spectra of each deuterated metabolite and respective reference steroids, and (iv) retention of either the 7β-^{2}H-label or the 16ξ-^{2}H-label in the produced metabolites.

In contrast to [16ξ-^{2}H]7α-hydroxy-PREG and [7β-^{2}H]5-androstene-3β,17β-diol, partial loss of 7β-^{2}H (16.1 to 42.2%) occurred in [7β-^{2}H]7α-hydroxy-DHEA. This loss may be explained by a deuterium isotope effect [10] during 7α-hydroxylation.

Quantities of the 7α-hydroxy-DHEA metabolite produced by each tissue preparation were sufficient for a positive identification by the GC-MS twin ion technique. Much smaller quantities of 7α-hydroxy-PREG were obtained and they made identification possible only in brain, spleen and ventral skin digests. Lack of identification of the 5-androstene-3β,17β-diol from intestine was due to the same reason except for perianal skin where the sample was lost.

Small amounts of putative 7β-hydroxy-DHEA and 7β-hydroxy-PREG have been detected, but total loss of 7β-^{2}H and lack of large enough quantities did not permit their definitive characterization.

Our findings with mice complete those of Šulcová and Stárka [11] who reported 7α-hydroxylation of DHEA in several tissues from female rats, rabbit and calves. Other work with normal human skin showed 5-androstene-3β,17β-diol to be thoroughly transformed into DHEA and related 7α/β-hydroxy metabolites [12]. Several investigators reported 7α-hydroxylation of DHEA and of other 3β-hydroxy-

steroid substrates in rat brain, pituitary and prostate [3, 5, 6, 13], canine prostate, epididymes and skin [2] and human prostate [1].

This body of evidence points towards a 7-hydroxylating enzyme widely distributed in organs of the studied mammalian species. At present, we know of no proved homologies between this enzyme system which appears to specifically 7-hydroxylate most 3β-hydroxysteroids, and the characterized testicular and hepatic testosterone/androstenedione 7α-hydroxylase [14, 15] or the known hepatic cholesterol 7α-hydroxylases [16].

Several workers have reported the absence of endocrine effect by 7-hydroxy derivatives of 3β-hydrosteroids and their absence of retention in prostatic tissue [3, 13, 17–19]. This conclusively led us to consider them as terminal steroid metabolites fated to excretion. We now report a positive effect of these native 7-hydroxy metabolites of PREG and DHEA on the production of IgG in mice.

In order to validate our experimental approach, we have first confirmed the findings of Loria *et al.* [7] with DHEA and extended them to PREG. Our results show that large doses (1 g/kg body wt) of DHEA or PREG must be injected 2 h prior to antigen administration in order to get a significantly increased immune response. Subcutaneous injections of the steroids were chosen because DHEA was found to be inactive when injected intraperitoneally [7]. Immediate proximity of steroids and lysozyme injections was selected because when DHEA and antigen administrations were separately carried out on mice opposite foot pads instead of a single foot pad, no change in interleukin patterns was detected in aged mice [20]. Our last consideration involved recent findings with rat astrocyte cultures where intensities of DHEA and PREG 7-hydroxylations were shown to increase with cell densities and cell to cell contacts [21].

Taken together, these findings led us to assume that 7-hydroxylation occurred at the site of antigen injection and that DHEA and PREG were substrates for the production of active 7-hydroxy metabolites when administered near that site. If such events were true, s.c. injections of smaller quantities of the 7-hydroxy metabolites of DHEA or PREG, when carried out at the moment of antigen injection and near the same site, should lead to an activation of immune processes larger than with their parent steroids.

We found that the smallest doses of administered 7α-hydroxy derivatives (160 times less than DHEA or PREG) led to a sharp increase of anti-lysozyme IgG. A larger dose of the 7β-hydroxy derivative of DHEA (but 20 times less than DHEA) was necessary for the same effect. We have also tested the 7-hydroxy derivatives of 5-androstene-3β,17β-diol as in Fig. 4 without any significant response (data not shown). Absence and variations of the significant responses with other doses of the tested 7-hydroxylated steroids are puzzling. Since the exact biological mechanism involved in their immunoactivating action is as yet unknown, only general considerations may be proposed for suggested explanations. First, the injected quantities range well above physiological levels since the circulating free DHEA precursor in murine is well under 6 mg/kg [8]. Whether large enough concentrations of 7-hydroxylated steroids have cytotoxic or adverse physiological effects is not yet known. Second, different 7-hydroxylated steroids may result in different availabilities to their target cells. Thus, steroid solubilities, transportations and rapid elimination by the liver should be taken into account. Third, the variety of responses to 7-hydroxylated steroids may involve the intensity of individual stress which is known to result in an increased production of immunosuppressing glucocorticoids. Lastly, injections were carried out near the site of lysozyme administration. It is possible that small variations in the proximity of such injections may result in widely different responses. We actually think that the selected steroid should be administered as an adjuvant to the antigen in order to get more reproducible responses. Nevertheless, a paracrine effect of the locally-produced 7-hydroxy metabolites of circulating PREG and DHEA is suggested both by our findings and by the consideration of results from others [20, 21]. DHEA had to be administered with antigen on the same foot pad of mice in order to get a significant change in interleukin patterns [20]. This leads us to suspect that only the locally-produced 7-hydroxy metabolites were active. Other recent work with rat astrocyte cells cultures showed that increased 7-hydroxylation of PREG and DHEA was related to increased cell densities and cell to cell contacts [21]. If these findings apply to other cells, the increased cell to cell contacts which occur during inflammation could lead to the increased production of immune-prone 7-hydroxy metabolites of circulating PREG and DHEA.

Our findings ascertain a key role for 7-hydroxylated metabolites of native PREG and DHEA in the triggering of the immune response. Irreversible formation of these derivatives occurred in all tested tissues but to a lesser extent in muscle than in tissues such as brain, skin and digestive track barriers where intense immune processes are required, or in organs such as spleen and thymus as reservoirs of competent cells for immunity.

Both PREG and DHEA circulate in large quantities as sulfate esters which commonly give rise to the free steroids after hydrolysis by a sulfatase already detected in peripheral lymph nodes and spleen [22]. As a precursor of very active 7-hydroxy derivatives and of both DHEA and immunosuppressing glucocorticosteroids, PREG appears as a key steroid. Circulating amounts are quite stable throughout age and do not depend upon adrenarche [23]. Circulating DHEA appears after adrenarche [23] but quantities decrease with age [24, 25] or with pathologies where the immune system is at stake [26, 27].

If 7-hydroxylated steroid metabolites are directly involved in the physiological balance which controls the immune response, quantities of circulating DHEA and PREG and the activity of the 7-hydroxylating enzyme will both be the limiting factors for the production of the 7-hydroxylated steroid metabolites which could act against the immunosuppressive action of glucocorticosteroids. Our evidence for this new aspect of steroid metabolism and action on immune processes should contribute to the understanding of several pathologies related to DHEA deficiency or with age [26, 27]. Further work will be necessary to investigate the detailed biochemical and biological mechanisms of production and of immune-prone action of the described native 7-hydroxylated steroids. Our other findings indicating that 7-hydroxylated derivatives of PREG and DHEA decrease the nuclear binding of dexamethasone–glucocorticoid receptor complexes will be reported elsewhere.

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