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Pharmacological control of the hormonally modulated immune response

II. BLOCKADE OF ANTIBODY PRODUCTION BY A COMBINATION OF DRUGS ACTING ON NEUROENDOCRINE FUNCTIONS. ITS PREVENTION BY GONADOTROPINS AND CORTICOTROPHIN

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Summary. Injection of a combination of three drugs, 5-hydroxytryptophan, the α-blocker phentolamine and the neuroleptic drug haloperidol into mice before or together with sheep red blood cells (SRBC) induces a complete and long-lasting inhibition of antibody production to SRBC and leads to specific unresponsiveness. The mice unresponsive to SRBC respond normally to another antigen. Treatment with a combination of luteotropic (LH), follicle stimulating (FSH) and corticotropic hormone (ACTH) before administration of drugs and antigen prevents the immune blockade.

Injection of SRBC induces an early elevation of LH in blood. This effect is prevented by previous administration of the three drugs in combination. The hormonal response to a second injection of the same antigen of mice previously made 'unresponsive' is different from that of immunized animals. The suppression of these hormonal changes which follow antigen injection by drugs acting on neuroendocrine regulation and cell membrane adrenergic receptors represents a step forward in efforts aimed at a pharmacological control of acquired immunity.

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INTRODUCTION

Recent findings have shown that rapid changes in blood levels of gonadotropins (GTH) are elicited by inoculation of allogeneic cells into mice. The results indicated that such hormonal changes represent an early endocrine response to immune interaction. This approach offered a new tool for studying the role of hormones in the initiation of the immune response (Pierpaoli, Maestroni, Kopp & Müeller, 1977). It also suggested the possibility to interfere in this early phase of immune events by intervening pharmacologically in the neuroendocrine response consequent to injection of antigens. We considered that the abolition or suppression of the elevation in level of GTH after inoculation of antigens by drugs known to act on neuroendocrine regulation might delay or block permanently the successive differentiative steps of the antigen-sensitive or -reactive immunocompetent cells. In selecting the drugs to be tested, we considered those known mechanisms of cell differentiation in which hormones are known to participate and the mechanisms of synthesis and/or release of hormones both in the central nervous system (CNS) and in the peripheral glands. In addition, the hormone recep-

tors on cell membranes and the adrenergic and possibly dopaminergic receptors on antigen-reactive cells or on their precursors were considered.

MATERIALS AND METHODS

Animals

The animals used in these experiments were male or female inbred C3H/HeJ, BALB/c and SWR mice, C57BL/6J × CBA/J F1 hybrid mice and outbred Swiss albino mice. Their ages ranged from 2 to 8 months and the body weight from 20 to 40 g. The animals were kept in groups of 4–5 per cage under conventional conditions, with free access to water and food. In order to minimize environmental stress, the mice selected for the experiments were separated and caged in groups a few days before the initiation of the experiments. Handling of the animals for the tests was also standardized (time of antigen inoculation and harvesting of cells for the tests).

Drug treatment

The drugs used in the experiments mentioned below were: L-5-hydroxytryptophan (5HTP); phentolamine (PHE, an α-blocker known as Regitine®, Ciba-Geigy AG, Basel, Switzerland); propranolol (PRO, a β-blocker); haloperidol (HAL, known as Haldol®, Janssen, Beerse, Belgium); p-chlorophenylalanine (PCPA) and dopamine (DA). With exception of PCPA which was given intraperitoneally (i.p.), the drugs were administered subcutaneously as saline suspension, or dissolved in 0·5% citric acid. The doses per kg body weight and injection schedule varied according to the different experiments.

Immunological tests for antibody production

The antigens chosen were sheep red blood cells (SRBC) suspended in 20% saline and a saline suspension of *Shigella paradysenteria* antigen (SPA) which had been prepared as described by Haran-Ghera & Peled (1967). Doses of 4×10^8 SRBC or 0·2 ml of 0·1% SPA were injected i.p. The total number of nucleated spleen cells was counted in each individual mouse. Antibody production to SRBC was measured by the haemolytic plaque assay as number of antibody forming cells (AFC) per million spleen cells or by direct haemagglutination test. Production of antibody to SPA was estimated by the direct agglutination test (Haran Ghera & Peled, 1967). When the number of direct and indirect (IgG-producing cells) plaques was measured, goat antiserum to mouse 7S γ globulin at different dilutions (1 : 10, 1 : 100 and 1 : 500) was added to the plates after 1 h incubation at 37°.

Hormones

The hormones used for inoculation of the mice were: follicle stimulating hormone (NIH-FSH-S9 ovine), luteinizing hormone (NIH-LH-B9, bovine), growth hormone (NIH-GH-B15, bovine) and thyrotropic hormone (NIH-TSH-B6, bovine). These hormones were a gift from the National Institute of Arthritis and Metabolic Diseases, Bethesda, Md, U.S.A. Adrenocorticotropic hormone (ACTH, Synacthen) was purchased from Ciba-Geigy AG, Basel, Switzerland. The hormones were generally dissolved in distilled water and administered in two daily doses by s.c. injection.

Determination of hormones

The protein hormones LH and FSH were determined by radioimmunoassay (RIA). The kits for the determination were a gift from the National Institute of Arthritis, Metabolism and Digestive Diseases, Rat Pituitary Hormone Distribution Program, Bethesda, Md, U.S.A. (NIAMDD-Rat LH-I-3 and Rat FSH-I-3). Corticosterone was determined by the double isotope dilution derivative assay (Kliman & Peterson, 1960; Müller, 1965). The determinations were performed on duplicate samples of coded sera. The values of hormones correspond to the average radioimmunoassayable hormone content (for LH and FSH) of one serum pool deriving from groups of four or five mice. The internal variability of repeated assays did not exceed 10%.

Rationale for using distinctive combinations of drugs to suppress or block the immune response

As mentioned in our first report on the study of the effect of certain drugs on the primary immune response to SRBC (Pierpaoli & Maestroni, 1977) we considered only those substances which, given singly or in combination, have been shown to affect synthesis and/or release of GTH, GH, ACTH and TSH at the hypothalamic-pituitary level and bind to α or β-adrenergic receptors on cell membranes.

α and β-adrenergic blockers (phentolamine and propranolol). Catecholamines, that is epinephrine and norepinephrine, are the two main known

physiological adrenergic agents. They act on cell membrane receptors which, according to their molecular structure, can be divided into α and β receptors. Catecholamines have many functions in biological systems: they transmit messages between cells over long distances as hormones do, and over short distances as transmitters for neurons in the sympathetic nervous system and in certain areas of the central nervous system (Fuxe, Höckfelt, Gösta & Löfström, 1973). Norepinephrine is a mediator in the central neural system that, by an α-adrenergic mechanism, inhibits ACTH secretion, probably by acting on the ACTH-regulating hypothalamic corticotropin releasing hormone (CRH; Ganong, 1973). Blockade of α-adrenergic receptors significantly depresses secretion of GH, while blockade of β-adrenergic receptors is associated with a rise in GH (Müller, 1973). Release of LH and FSH releasing hormones (LH-RH; FSH-RH) and of prolactin inhibiting hormone (PR-IH) is linked to the α-adrenergic receptors in the hypothalamus (Kamberi, 1973; Lawson & Gala, 1975). Finally, catecholamines and dopaminergic mechanisms may also be involved in the control of synthesis and release of TSH releasing hormone (TSH-RH) and melanocyte stimulating hormone inhibiting hormone (MSH-IH; Prange, Wilson, Breese, Plotnikoff, Lara & Lipton, 1973). The sympathetic nervous system controls too, by catecholamines, the function of peripheral endocrine glands. For example, inhibition of insulin secretion occurs as a consequence of the effect of epinephrine and/or norepinephrine on the pancreatic islets. α-adrenergic blockade prevents the inhibition and induces a rise of plasma insulin as a response to hyperglycaemia. β-adrenergic blockade potentiates the inhibition. Stimulation of β-adrenergic receptors increases plasma insulin (Himms-Hgen, 1972). Most interestingly, the effects of catecholamines on leucocyte cyclic AMP, histamine release and lymphocyte cytolytic activity implies a β-adrenergic receptor on human basophils and mouse lymphocytes. It is known that an increase in lymphocyte cyclic AMP has a general inhibitory effect on the immune response (Braun & Rega, 1972; Bourne, Melmon, Weinstein & Shearer, 1974; Singh & Owen, 1976) and that early changes of cyclic AMP occur after injection of antigens (Plescia, Yamamoto & Shimamura, 1975; Yamamoto & Webb, 1975).

L-5-hydroxytryptophan (5HTP). 5HTP is the precursor of 5-hydroxytryptamine (5HT, serotonin). Tryptophan is the only amino acid which is bound to serum proteins. Free tryptophan in serum seems to be directly related to the brain tryptophan which, in turn, is directly dependent on the concentration of 5HT. 5HTP passes through the blood-brain barrier. Therefore, by increasing the brain concentration of tryptophan or 5HTP, one can increase formation of brain serotonin which is known to have, like 5HTP (Devoino, Eliseeva, Eremina, Idova & Cheido, 1975), an immunosuppressive effect. 5HT (serotonin) decreases the functional activity of the hypothalamo-pituitary-thyroid system by inhibiting the TSH-RH secretion in the hypothalamus (Mess & Peter, 1975). Serotonin exerts a negative control (inhibition) on gonadotropins (LH, FSH) and ACTH synthesis and/or release, and increases prolactin secretion (Lawson & Gala, 1975). Serotoninergic neurons are of importance in the mediation of the inhibitory feedback action of corticosterone. It has been found that a combined treatment of 5HTP and monoamine-oxidase inhibitors (MAOI) blocks spontaneous ovulation. Therefore the serotoninergic neurons may exert an inhibitory action on LH-RH as well as on C-RH secretion (Fuxe, Schubert, Höckfelt & Gösta, 1974). It has been shown that polyunsaturated fatty acids (PUFA), like arachidonic acid, are immunosuppressive (Mertin & Hunt, 1976). PUFA promote an increase of free tryptophan in serum and consequently of brain serotonin. Most PUFA are precursors of prostaglandins (PG). For example, arachidonic acid is precursor of PG-$F_2\alpha$. Prostaglandins are involved in release of gonadotropins and other pituitary hormones (Deis & Vermouth, 1975; Convey, Beal, Seguin, Tannen & Lin, 1976; Warberg, Eskay & Porter, 1976). It has been recently observed that a 20 to 80-fold increase in prostaglandin $F_2\alpha$ occurs in the spleen after intravenous inoculation of SRBC (Webb & Osheroff, 1976). This increase is not observed in T-cell deficient athymic nude and NZB mice. Cyclic AMP content of thymocytes whose basal level is very low if compared to that of lymph node, spleen and peripheral blood lymphocytes, increases rapidly after stimulation with prostaglandin E_1 and isoproterenol, a stimulant of β adrenergic receptors (Bach, 1975).

Haloperidol. Haloperidol is a neuroleptic drug of the butyrophenones series. This drug blocks selectively cell receptors for dopamine. As a consequence

of this blockade, and by a feedback inhibition mechanism, haloperidol provokes an increase in dopamine turnover (Sandler, 1972). Haloperidol increases serum prolactin, decreases LH and FSH. The increased synthesis and release of pituitary prolactin by haloperidol is mediated by a decrease in hypothalamic PR-IH (prolactin inhibiting hormone) activity. The reduction in release of LH is due to a decrease of hypothalamic LH-RH and perhaps also by a direct inhibitory action on the pituitary (Dickerman, Kledzik & Gelato, 1974). Haloperidol is a relatively innocuous drug which has been found to have mild side effects, even at high and prolonged dosage (Man, 1973; Oberst & Crook, 1967). Also haloperidol has been found to be moderately immunosuppressive (Levy & Munson, 1976).

P-chlorophenylalanine (*PCPA*). PCPA causes a profound and long-lasting decrease in brain serotonin (Sanders-Bush, Gallager & Sulser, 1974).

Dopamine (*DA*). Dopamine is an important neurotransmitter and is the precursor of catecholamines.

RESULTS

Many preliminary experiments were performed in order to evaluate the dosage and combination of the drugs as well as the injection schedule, and thus establish a model by which so many biological and pharmacological variables could be reduced. One of the experiments which allowed us to achieve a substantial reduction of direct AFC to SRBC and thus indicated the drug combination which was most effective in suppressing the antibody response to SRBC is shown on Table 1. Groups of 3 adult male C3H/He mice aged 4–5 months were injected i.p. with 4×10^8 SRBC. The number of direct AFC was measured 4 days after antigen injection. The drugs were given s.c. once a day for 4 successive days, starting 24 h before antigen injection. The s.c. inoculation of drugs containing the α-blocker in the doses we used produced in mice a temporary condition of sleepiness and hypotonia which lasted for a few hours. Mice seem to be more sensitive to this side-effect, because it was much less evident when the same dose of the three drugs per kg body weight

Table 1. Effect of drugs on the primary immune response to sheep red blood cells in mice

Drug treatment	Dose per day (mg/kg b.w.)	Nucleated spleen cells*	AFC/10^6 spleen cells
5HTP	30	274 ± 14	204 ± 85
5HTP + PHE	30–12	239 ± 34	261 ± 104
5HTP + PRO	30–12	256 ± 49	448 ± 269
5HTP + PHE + PRO	30–12–12	236 ± 56	256 ± 114
5HTP + HAL + PHE	30–12–12	168 ± 24	48 ± 26
5HTP + HAL + PRO	30–12–12	165 ± 21	143 ± 132
5HTP + HAL + PHE + PRO	30–12–12–12	142 ± 29	148 ± 33
DA	40	200 ± 22	179 ± 30
DA + PHE	40–12	220 ± 36	302 ± 96
DA + PRO	40–12	291 ± 80	397 ± 308
DA + PHE + PRO	40–12–12	229 ± 17	410 ± 190
DA + PCPA + PHE	40–160†–12	199 ± 29	140 ± 219
DA + PCPA + PRO	40–160†–12	198 ± 9	272 ± 154
DA + PCPA + PHE + PRO	40–160†–12–12	242 ± 16	453 ± 191
Controls (SRBC only)	—	206 ± 30	452 ± 139

* Total number of nucleated spleen cells/mouse ($\times 10^6$).

† PCPA, only 1 injection i.p. 24 h before SRBC inoculation.

Groups of five, 4-month-old male C3H/He mice were injected intraperitoneally (i.p.) with 4×10^8 sheep red blood cells (SRBC). The number of direct antibody forming cells (AFC) was estimated 4 days later. The drugs were suspended in saline and given subcutaneously (s.c.) once a day for 4 days, starting 24 h before antigen injection.

5HTP, L-5-hydroxytrytophan; PHE, α-adrenergic blocker phentolamine; PRO, β-adrenergic blocker propranolol; HAL, neurolepticum haloperidol; DA, dopamine; PCPA, p-chlorophenylalanine.

Table 2. Inhibitory effect of three drugs, given singly or in combination, on the primary immune response to SRBC in mice

Drug treatment	Nucleated spleen cells (× 10^6)	AFC/10^6 spleen cells
5HTP	230 ± 44	59 ± 26
PHE	273 ± 34	166 ± 105
HAL	116 ± 39	47 ± 44
5HTP + HAL	134 ± 29	16 ± 15
HAL + PHE	116 ± 41	19 ± 8
5HTP + HAL + PHE	66 ± 21	4 ± 2
Controls (SRBC only)	201 ± 31	146 ± 48

Groups of four, 4-month-old C3H/He female mice were injected i.p. with 4 × 10^8 SRBC. The number of direct AFC was estimated 4 days later. The drugs were administered s.c. once daily for 5 days as a saline suspension, starting 12 h before antigen injection. The daily dose per kg body weight was: 5HTP, 30 mg, PHE, 12 mg and HAL, 12 mg.

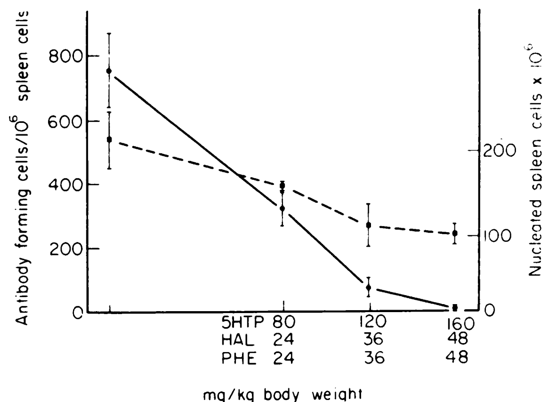

Figure 1. Dose-dependent progressive inhibition of antibody formation to SRBC obtained by a combination of three drugs.

Groups of five, 4-month-old male BALB/c mice were injected i.p. with 4 × 10^8 SRBC and the number of AFC was estimated 4 days later. The mixture of drugs was administered s.c. in one single injection as a saline suspension at the doses indicated, 2 h before antigen injection. (●) Antibody forming cells; (■) nucleated spleen cells.

was administered to rats, guinea-pigs and monkeys (Pierpaoli & Maestroni, unpublished experiments). As shown on Table 1, the combination of drugs which produced the most remarkable inhibition of AFC and a minor variability of their number was that of 5HTP, HAL and PHE. A repetition of the experiments by changing the doses of the drugs gave similar results. Therefore this combination of drugs was chosen for further experiments.

In order to establish which of the single component of the combination of the three drugs was more effective in inhibiting or blocking antibody production to SRBC, groups of four, 4-month-old female C3H/He mice were inoculated i.p. with 4 × 10^8 SRBC. The drugs were administered s.c. suspended in saline at the dosage and sequence indicated on Table 2. As shown on this Table, PHE did not affect antibody production while 5HTP and HAL given singly gave a consistent suppression of the primary immune response to SRBC. The combination of 5HTP and HAL and of HAL and PHE produced a very remarkable suppression, but only the combination of the three drugs administered together gave a virtual blockade of the response to SRBC. This experiment showed that a complete blockade of the primary response to SRBC can be achieved when the drugs are given simultaneously and at a higher dosage than in the experiment shown on Table 1.

Table 3. Effect of time and sequence of drug-antigen administration on the ability of a combination of drugs to block the primary immune response to SRBC

Drug treatment	Nucleated spleen cells (× 10^6)	AFC/10^6 spleen cells
Drugs 24 h before SRBC	100 ± 12	7 ± 5
Drugs 2 h before SRBC	90 ± 10	2 ± 2
Drugs 24 h after SRBC	124 ± 24	126 ± 48
SRBC	262 ± 33	395 ± 56

Groups of five, 4-month-old male BALB/c mice were injected i.p. with 4 × 10^8 SRBC and the number of PFC was estimated 4 days later. The mixture of drugs was given 24 or 2 h before, or 24 h after antigen injection. The drugs were administered only once s.c. as a saline suspension at the dose of: 5HTP, 160 mg; HAL, 48 mg; PHE, 48 mg per kg body weight.

In order to investigate if the degree of the suppression depends on the timing and sequence of antigen-drugs administration, an experiment was performed in which the mixture of drugs was administered before or after antigen injection. The drugs were given in one single dose as indicated on Table 3. Table 3 shows that a blockade of the response was obtained only if the drugs were given at least hours before the antigen, but that their action was readily established and then partially maintained for at least as long as 24 h after their administration.

Table 4. Effectiveness of a combination of three drugs given once as a suspension or repeatedly as a solution, to block the primary immune response to SRBC

Drug treatment	Nucleated spleen cells ($\times 10^6$)	AFC/10^6 spleen cells
A	98 ± 10	2 ± 2
B	160 ± 19	323 ± 192
C	102 ± 32	9 ± 9
D	86 ± 21	4 ± 2
E	182 ± 17	828 ± 98

Groups of four, 5-month-old C57BL/6 × CBA F1 hybrid female mice were injected i.p. with 4×10^8 SRBC and the number of AFC was estimated 4 days later. The drug treatment was: A, drugs in saline suspension given once s.c. 2 h before antigen; B, drugs solubilized in 0-5% citric acid, given once s.c. 2 h before antigen; C, drugs in saline suspension given in four aliquots once a day for 4 days, starting 2 h before antigen; D, drugs dissolved in citric acid given in four aliquots once a day for 4 days, starting 2 h before antigen injection; E, SRBC only. The total quantity of drugs given per mouse was: 5HPT, 160 mg, HAL, 48 mg and PHE, 48 mg per kg body weight.

Table 5. Maintenance of immune unresponsiveness or lack of memory response to SRBC in mice whose primary response to SRBC has been blocked by a combination of three drugs

Drug treatment	Nucleated spleen cells ($\times 10^6$)	IgM and IgG forming cells/10^6 spleen cells
Drugs + SRBC	200 ± 25	15 ± 9
SRBC	250 ± 32	148 ± 86

Two groups of ten, 4-month-old female C57BL/6 × CBA F1 hybrid mice were injected i.p. with 4×10^8 SRBC. The drugs were administered s.c. in saline suspension once a day for 4 successive days, at the dosage given on Table 2, starting 2 h before antigen injection. Twelve days after the first antigen injection, the two groups of mice were injected again with the same amount of SRBC, and the number of direct (IgM-producing) and indirect (IgG-producing) AFC was evaluated 2 days later.

Another experiment was planned in order to establish a correlation between the dose of the drugs and the extent of the inhibition of antibody production. Various doses of the drugs were injected once s.c. as a saline suspension into groups of mice 2 h before antigen injection. Fig. 1 shows the dosage which produced a complete blockade of antibody production to SRBC. One single injection of the three drugs in combination did not result in complete inhibition of the immune response when the total dosage was decreased below 160 mg 5HTP, 48 mg HAL and 48 mg PHE per kg body weight respectively. However, this dosage seems to be valid only in these specific experimental conditions and for the strain of mice used (BALB/c, males).

The experiment shown on Table 3 had demonstrated that a blockade of the immune response to SRBC is achieved when the drugs are given before the antigen. This suggested that the concentration of the single components in the mixture at the moment of antigen injection might be critical for obtaining a complete blockade. In fact, it is known that both 5HTP and PHE are rapidly absorbed and that their half-life is only a few hours. Therefore a further experiment was planned in which the drugs were either solubilized in 0·5% citric acid and injected once a day starting 2 h before antigen injection, or the drug mixture was given as a saline suspension in one single dose or divided into four aliquots over 4 days. The experiment illustrated in Table 4 shows that a complete inhibition of the response to SRBC could be achieved if the drugs were administered undissolved as a suspension or dissolved, but that one single injection of the whole 4-day dose of the dissolved drugs given 2 h before antigen injection did not allow a complete block of antibody production and that a higher variability in number of AFC was present when the drugs were given solubilized on 0·5% citric acid (Table 4).

The experiment reported on Table 5 shows that the complete inhibition of antibody production obtained by the administration of our combination of drugs and of an antigen is long-lasting. In fact, a second injection of 4×10^8 SRBC at 12 days after the first antigen challenge evoked formation of very few IgM (direct plaques) and IgG (indirect plaques) forming cells. This persistence of the unresponsiveness to the second injection of antigen in the 'blocked' mice was confirmed further by the experiment shown in Fig. 2. This experiment also demonstrated that the same mice, which were unresponsive to SRBC when injected with this antigen a second

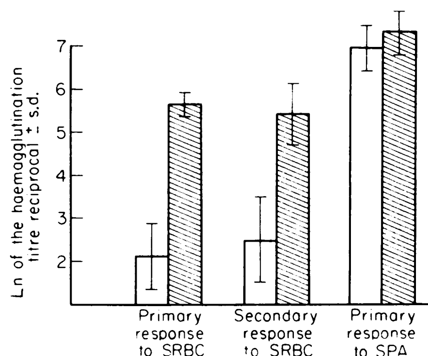

Figure 2. Antigen-specific blockade of the primary and secondary immune response to SRBC in mice by a combination of three drugs. Normal response to a second antigen.

One group of ten, 3-month-old SWR female mice was injected s.c. with a saline suspension of the three drugs in combination 2 h (40 mg 5HTP, 12 mg PHE and 12 mg HAL per kg body weight) and 15 min (80 mg 5HTP, 24 mg PHE and 24 mg HAL per kg body weight) before injection of the antigen (0·1 ml of 20% SRBC in saline suspension i.p.). The drugs were injected again at 20, 40 and 50 h after injection of the antigen (40 mg 5HTP, 12 mg PHE and 12 mg HAL per kg body weight each time). One group of 6 mice was used as control and injected only with SRBC. The mice were bled 10 days after antigen injection and serum agglutinins were measured by the direct agglutination test. The same mice were injected again with the same dose of SRBC 67 days after the first antigen injection and the serum agglutinin titre determined 3 days later. Ten days after the second injection of SRBC, the same mice were injected i.p. with 0·2 ml of *Shigella paradysenteria* antigen (SPA) and the agglutinin titre was measured 7 days later. Open columns, drug-treated; hatched columns, controls.

time 67 days after the first antigen and drug administration, were able to respond to SPA almost as efficiently as mice primed with that antigen (Fig. 2). Therefore the long-lasting unresponsiveness induced to SRBC did not impair at all the ability of the same mice to respond to a second antigen.

In order to clarify whether LH, FSH and other pituitary hormones are involved in the initiation of the immune response, a number of protein hormones were tested for their capacity to prevent the drug-induced inhibition of antibody formation to SRBC and thus to protect them from the action of the drugs. The experiment shown on Table 6 demonstrates that GH, TSH and LH given singly or in combination did not prevent the drug-induced inhibition of antibody production. The experiment in Table 7 shows that a significant protection from the action of the drugs could only be obtained when

Table 6. Inability of luteotropic hormone (LH), thyrotropic hormone (TSH) and growth hormone (GH) given singly or in combination to prevent the drug-induced inhibition of the immune response to SRBC

Drug treatment	Nucleated spleen cells ($\times 10^6$)	AFC/10^6 spleen cells
GH + Drugs + SRBC	95 ± 9	14 ± 7
LH + Drugs + SRBC	125 ± 28	35 ± 18
TSH + Drugs + SRBC	93 ± 16	37 ± 27
GH + LH + TSH + Drugs + SRBC	117 ± 59	50 ± 17
Drugs + SRBC	98 ± 39	15 ± 10
SRBC	203 ± 35	382 ± 80

Groups of four, 4-month-old C3H/He male mice were injected i.p. with 4×10^8 SRBC. The number of AFC was estimated 4 days later. The mice were injected s.c. with the mixture of the three drugs (5HTP, HAL and PHE) at the dosage given on Table 2 2 h before antigen injection and once daily for 3 successive days.

The hormones were injected s.c. twice a day, in the morning 1 h before administration of the drugs and in the evening. The total daily dose of hormones was: GH, 200 μg; TSH, 100 μg and LH, 200 μg.

the mice were injected daily with a combination of ACTH, LH and FSH.

The experiment shown in Fig. 3 was devised to see whether the inoculation of a non-replicating antigen, namely SRBC into normal, untreated mice does also induce, as allogeneic cells do, an early increase of LH, and whether the treatment with the combination of drugs does indeed prevent specifically the LH response to inoculation of antigen while blocking the immune response. Four groups of male C57BL/6 × CBA/J F1 hybrid mice (4 animals each group) were injected respectively with the drugs in combination and SRBC, only drugs, only SRBC, and one group with the suspension medium (Gey's solution). The mice were killed by exsanguination at 0·5, 1, 2, 3 and 4 h after inoculation of the SRBC. Fig. 3 shows that i.p. injection of 4×10^8 SRBC into mice induced a sharp elevation of LH level at 3 h after antigen injection. Injection of drugs only induced a sharp decrease of LH at 1 h after drug inoculation. Finally, i.p. inoculation of SRBC in drug-treated mice induced a deep and protracted depression of LH in blood. The level of LH returned to normal values at 4 h after i.p. SRBC injection. The difference in level of LH between the antigen-treated and the drug- and antigen-treated mice was 100 ng/ml (Fig. 3).

Table 7. Protection from and prevention of drug-induced blockade of the immune response to SRBC by a combination of luteotropic hormone (LH), follicle stimulation hormone (FSH), and adrenocorticotropic hormone (ACTH)

Drug treatment	Nucleated spleen cells ($\times 10^6$)	AFC/10^6 spleen cells
ACTH + Drugs + SRBC	120 ± 27	30 ± 29
FSH + Drugs + SRBC	133 ± 46	47 ± 19
ACTH + LH + Drugs + SRBC	99 ± 10	35 ± 23
ACTH + FSH + Drugs + SRBC	119 ± 10	50 ± 39
ACTH + FSH + LH + Drugs + SRBC	123 ± 27	174 ± 69
SRBC + Drugs	104 ± 26	10 ± 4
SRBC	178 ± 29	424 ± 102

Groups of five, 8-month-old female C3H/He × C57BL/6 Fl hybrid mice were injected i.p. with 4×10^8 SRBC. The number of AFC was estimated 4 days later. The mixture of the three drugs was injected s.c. once a day for 4 days as a saline suspension at the dose given on Table 4 (group C). The hormones alone on in combination were injected s.c. ½ h before the drugs. The total quantity of hormones injected daily was: LH, 200 μg; FSH, 200 μg, ACTH, 5 μg.

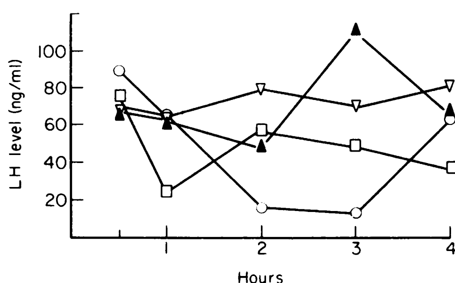

Figure 3. Increase of LH level in blood of mice after injection of SRBC. Its suppression by a combination of three drugs.

Four groups of 4-month-old male C57BL/6 × CBA/J F1 hybrid mice were injected with (a) one single dose of a combination of three drugs s.c. (5HTP, 160 mg, HAL, 48 mg and PHE, 48 mg/kg body weight) suspended in saline. The same group was injected i.p. with 4×10^8 SRBC 1 h after injection of the drugs; (b) the combination of drugs; (c) only SRBC and (d) the suspension medium (Gey's solution). Groups of four mice each time were exsanguinated at 0·5, 1, 2, 3 and 4 h after the inoculation of the SRBC. Sera from each time and group were pooled and frozen until determination of LH. (○) SRBC + drugs; (□) Drugs; (▲) SRBC; (▽) medium.

Another experiment was carried out to further confirm if (a) inoculation of SRBC into mice results in changes of hormone levels in peripheral blood; (b) if these changes are similar to those obtained by inoculation of allogeneic cells and (c) if pre-immunization of the recipient mice with SRBC or previous treatment with the combination of drugs and SRBC (to render them tolerant or unresponsive to SRBC) modifies the pattern of the hormonal response to a second inoculation of SRBC.

The results illustrated in Table 8 show that i.v. inoculation of SRBC into mice pre-immunized or 'unresponsive' to SRBC did not affect the levels of LH at 1, 2 or 3 h after inoculation of the antigen. The levels of corticosterone were significantly increased by inoculation of antigen, but no differences were present when the recipient mice were 'unresponsive' or pre-immunized. The FSH-response to inoculation of SRBC followed a completely different pattern when the recipient mice were unresponsive or immunized. Therefore, although two main different components of this experiment (pre-immunization of the recipients and i.v. route of inoculation) do not allow us to compare these data with those of the experiment illustrated above (Fig. 3), these findings confirm that (a) an early hormonal response is elicited by inoculation of a non-replicating antigen into mice; (b) also the levels of corticosterone and of the gonadotropin FSH are affected; (c) the levels of FSH are completely different if the recipients mice are pre-immunized or unresponsive to SRBC and finally (d) the character and pattern of the hormonal variations change completely in time (Table 8).

DISCUSSION

In a preceding paper (Pierpaoli *et al.*, 1977) we had demonstrated that a rapid increase in blood levels of luteotropic hormone (LH) and, to a minor extent, of follicle stimulating hormone (FSH) occurs after inoculation of allogeneic cells from normal or allo-immunized donors into normal mice. The interpretation of those endocrine changes was that gonadotropins (GTH) might play a crucial role in initiating the immune response (Pierpaoli *et al.*, 1977). The drugs used in the experiments reported here are known to interfere with synthesis and/or release of GTH and, most probably, other protein hormones of the adenohypophysis. Former work on the role of corticosteroids in the initiation of the immune response (Ambrose, 1970) and our own data had also suggested that the pituitary adrenocorticotropic hormone (ACTH) might participate, with GTH, in

Table 8. Hormonal changes in blood of immunized (to SRBC) or drug-treated and unresponsive (to SRBC) mice elicited by a second administration of SRBC

Injection	Exsanguination time (h)	Recipients	LH (ng/ml)	FSH (ng/ml)	Corticosterone (μg/100 ml)
SRBC	1	Immunized	16	47	46·4
SRBC	1	Unresponsive	17	145	50·0
Medium	1	Immunized	19	268	15·6
SRBC	2	Immunized	13	177	12·5
SRBC	2	Unresponsive	14	234	12·4
Medium	2	Immunized	24	100	9·0
SRBC	3	Immunized	20	120	21·6
SRBC	3	Unresponsive	23	66	25·3
Medium	3	Immunized	16	304	8·3

Groups of five, 3-month-old female C57BL/6 × CBA/J F1 hybrid mice, immunized with one i.p. injection of 4×10^8 SRBC 5 weeks before, were inoculated i.v. with 4×10^8 SRBC or the suspension medium (PBS). The mice were exsanguinated 1, 2 and 3 h after inoculation of SRBC or medium. The sera from the different groups were pooled.

the first differentiative steps of the antigen-stimulated cells.

The information emerging from the experiments reported here demonstrates that a specific and long-lasting unresponsiveness to antigens (SRBC) or a blockade of antibody production can be achieved in mice by treatment with a combination of drugs, namely the precursor of serotonin 5-hydroxytryptophan, the α-blocker phentolamine and the neuroleptic drug haloperidol (Tables 1 and 2). An identical blockade of antibody production can be achieved in rats and in monkeys (*Macaca fascicularis*) similarly treated (Pierpaoli & Maestroni, unpublished experiments). A complete blockade of antibody production is achieved only when the mixture of drugs is administered before antigen, while a consistent reduction but no blockade is obtained if the drugs are injected after the antigen (Table 3). A correlation exists between the concentration of the three drugs and the extent of immune inhibition. By decreasing the three components of the mixture simultaneously, the animals rapidly escape the blockade (Fig. 1). Likewise, the concentration of the single components of the mixture is critical for achieving a complete blockade (Pierpaoli & Maestroni, unpublished experiments). The maintenance of a certain concentration of the drugs at the moment of antigen injection seems to be necessary for achieving the blockade of antibody production. This is clearly shown on Table 4. The completely solubilized drugs, given in one single injection, produced a moderate inhibition of antibody production while the same dose given as a saline suspension induced a complete blockade. However, the same amount of solubilized drugs induced a complete blockade when given in four aliquots within 4 days (Table 4). Most interestingly, the immune blockade so induced seems to be persistent. In fact, the appearance of both IgM- and IgG-producing cells was completely abolished when the 'blocked' mice were injected again with the same antigen (Table 5). In addition, the immune unresponsiveness achieved was specific for the antigen administered together with the drugs; the same mice which were completely unresponsive to SRBC, could respond normally to a second unrelated antigen (Fig. 2). This indicates that the overall immune capacity of the animals has not been impaired by the drugs administered and has no long-lasting effects on the immune system in general.

Two different series of experiments were devised to clarify the mechanism of action of the drugs in specifically abolishing the immune response. One series of experiments (Tables 6 and 7) considered the pituitary hormones which are most probably involved in the initiation of the immune response, and their capacity to prevent the action of the drugs. A significant protection was achieved only by a combination of LH, FSH and ACTH (Tables 6 and 7). Therefore these three hormones in combination seem to be needed for counteracting the effects of the drugs. It seems quite possible that the timing of administration and the dosage of the hormones and

drugs can be so adjusted as to obtain a complete protection from the inhibitory action of the drugs. These experiments also show that the protection exerted by the hormones does not depend on a general 'trophic' or mitogenic stimulus of the hormones on the number of the precursor cells in the spleen. In fact, the prevention of the drug-induced inhibition of the number of AFC by the hormonal treatment is not correlated to the number of nucleated spleen cells although the drugs, when given in combination, definitely reduced the number of nucleated cells in the spleen (Tables 1–7). This indicates that ACTH, LH and FSH act not only on proliferation of those spleen cells whose number is reduced by a possible direct cytotoxic action of the drugs, but rather on the differentiation of the antigen-stimulated cells (Tables 6 and 7).

A further proof of the rather specific action of ACTH, LH and FSH on the antigen-reactive cells is given by experiments reported elsewhere (Pierpaoli & Maestroni, 1977) in which the pool of the three hormones was given before or after drug administration. That experiment showed that the hormones act most probably by a competitive mechanism at the central (CNS) or peripheral (lymphocyte membrane) level and that they can properly antagonize the action of the drugs only if given before or at the time of antigen injection (Pierpaoli & Maestroni, 1977).

The second series of experiments is of special value (Fig. 3 and Table 8). Inoculation of the non-replicating T-cell dependent antigen (SRBC) which has been used in all the immunological tests reported above, also induces elevation of LH in blood, with a peak at 3 h after antigen injection. Administration of the combination of drugs before injection of the antigen maintains the levels of LH at extremely low values. At 4 h after inoculation of SRBC and/or drugs, the level of LH returns to baseline thus showing that the relevant changes of LH consequent to antigen injection are rapid, temporary, and *linked to the presence of the antigen* (Fig. 3). In fact, the drugs alone induce only an earlier and shorter decrease of LH level in blood (Fig. 3). The experiment illustrated in Table 8 demonstrates that the endocrine response is quite different in mice pre-immunized or made 'unresponsive' to SRBC. It is not surprising that, in these conditions of pre-sensitization of the recipient mice, no variation of LH is noticed among the different groups, while differences in levels of FSH are present. It is known that release of LH, spontaneous or following injection of LH-releasing hormone, is asynchronous and in peaks, while that of FSH occurs later and follows a different pattern (Franchimont *et al.*, 1974; Hashimoto *et al.*, 1973; Millet *et al.*, 1973; Mortimer *et al.*, 1974; Naftolin & Yen, 1972; Rebar *et al.*, 1973). Therefore it is quite probable that the intravenous inoculation of the antigen into the pre-immunized or unresponsive mice elicits very early (before 1 h) changes in level of LH in the pre-immunized or no changes in the unresponsive recipients (Table 8). This is quite feasible considering the very rapid elevation in splenic cyclic AMP and prostaglandins after i.v. inoculation of SRBC into mice (Plescia *et al.*, 1975; Webb & Osheroff, 1976). The changes in levels of FSH seem to follow quite a different pattern when the recipient mice are immunized or unresponsive to the antigen. The variations of FSH levels shown on Table 8 are almost identical to those observed in earlier experiments in which cross-tolerant or normal mice were inoculated with allogeneic cells from allo-immunized or cross-tolerant donors (Pierpaoli *et al.*, 1977). In that case also, as in the experiment illustrated on Table 8, the highest value of FSH in the groups of mice inoculated with either syngeneic or allogeneic cells was observed at 2 h after inoculation of allogeneic cells from and into cross-tolerant animals.

These findings together demonstrate the relevant role of GTH in the initiation of the immune response and the possibility of inducing a persistent, specific condition of immune unresponsiveness in mice by administration of a combination of drugs which act on neuroendocrine regulation of gonadotropin synthesis and/or release. As suggested in our preceding paper (Pierpaoli *et al.*, 1977), it is likely that a release of prostaglandins ($PG\text{-}F_{2\alpha}$) from the antigen-activated T cells after injection of SRBC in mice (Webb & Osheroff, 1976) determines the variations of GTH which are needed for the further differentiation of the antigen-activated cells. However, more studies are needed to determine whether prostaglandins act directly on the antigen-activated cells or if they modulate the immune response by regulating GTH levels and the action of these protein hormones on cyclic AMP level in the antigen-activated, prostaglandin-producing T cells (Pierpaoli *et al.*, 1977).

Many natural models indicate a crucial role for GTH in the regulation of the immune response. The most common model is the mother-foetus com-

patibility in which a specific hormonal balance helps to block the mother-versus-foetus immune reactions. Also the generally higher immune responsiveness in the female can be considered as related to a different hormonal response of females to immunization. SJL/J mice whose gonadal activity is congenitally impaired in the sense of progressive age-dependent hyperfunction, besides developing systemic neoplasms, show a striking tendency to immune sensitization (Pierpaoli *et al.*, 1974).

The data obtained so far indicate that this pharmacological method for controlling neuroendocrine functions may find therapeutic applications in immunology and in those endocrine derangements which precede onset of tumours like leukaemia (Pierpaoli, Haran Ghera & Kopp, 1977) and mammary cancer (Christakos, Shina & Dao, 1976).

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