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Splenic Leukocytes from Chickens Injected with *Salmonella pullorum* Antigen Stimulate Production of Corticosteroids by Isolated Adrenal Cells (42037)

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Abstract. Corticosteroid levels in the blood serum of White Rock pullets were significantly increased within 1 hr after an iv injection of heat-killed *Salmonella pullorum* antigen (SP-Ag), and reached levels 7 to 10 times that of PBS-injected controls within 2 hr after SP-Ag injection. Incubations with isolated adrenal cells indicated that serum from SP-Ag-injected birds had the ability to stimulate the synthesis or release of corticosteroids twice that of serum PBS-injected birds. Stripping the serum from SP-Ag-injected birds with activated charcoal and precipitated silica (Quso G-32) removed the corticosteroids and the adrenal-stimulating ability. A 2-hr incubation of isolated adrenal cells with leukocytes from spleens removed from chickens 1 hr after injection with Sp-Ag, using stripped serum as the medium, stimulated a two- to fivefold increase in corticosteroid as compared to splenic leukocytes from PBS-injected chickens incubated in the same medium. The results indicate that an "ACTH-like" substance was produced by the *S. pullorum* antigen-stimulated splenic leukocytes.

Physical and behavioral stressors are capable of suppressing antibody and cell-mediated immunity in most animal species (1, 2). Important mediators of this induced immunosuppression are adrenal corticosteroids which are secreted in greater quantities as a consequence of increased activity of the hypothalamus–pituitary–adrenal axis. Immunosuppression of corticosteroids or corticotropin (ACTH) seems to confirm this concept (3–5). In addition, direct immunosuppressive and immunostimulatory effects of peptides such as ACTH, α-melanocyte-stimulating hormone (α-MSH) or β-endorphin have been reported (6–9).

However, extrapituitary and extrahypothalamic "ACTH-like" peptides, apparently derived from the larger precursor, proopiomelanocortin (POMC), have recently been described (8). In addition, interferon (hIFN-α) preparations from Newcastle disease virus (NDV)-infected human peripheral leukocytes contain biologic and immunologic β-endorphin and ACTH activity (10). The ACTH-like activity was expressed only following pepsin or acid treatment which produced two smaller peptides from the larger molecule. Injection of hypophysectomized mice with NDV caused a rise in serum corticosterone 8 hr after injection and the splenic lymphocytes of the injected mice displayed positive immunofluorescent responses to an antibody developed against the 1–13 ACTH amide (11). Although these results strongly suggest a lymphocyte–adrenal axis (9), they do not exclude the possibility that the NDV stimulation might also be from a hypothalamic source of ACTH or β-endorphin (8). The research reported herein establishes a direct relationship between antigenically stimulated splenic leukocytes and adrenal steroid production, extends the observations to include bacterial antigen stimulation, and further extends the phenomenon previously observed in mammals to the class Aves.

Materials and Methods. *Salmonella pullorum (SP-Ag) injections.* All injections of SP-Ag were into the ulnar vein with 0.8 ml of a heat-treated (2 hr, 60°C) preparation of a 24-hr culture of strain 17 *S. pullorum.* Production of the SP-Ag, the method of determination of concentration (% packed volume), and microtitration of serum agglutinins have been described previously (5). In Experiment 1A, the SP-Ag (prepared, 1981) had a concentration of 0.006%; in all other experiments the SP-Ag concentration was 0.012% (prepared, 1983). *Krebs–Ringer Hepes buffer with glucose (KRHBG).*[1] *KS-Stock:* 4

[1] The following trivial names or acronyms are used: corticosterone (4-pregnen-11-β-21-diol-3,20-dione), Hepes (*N*-2-hydroxyethyl-piperazine-*N*'-ethanesulfonic acid).

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ml 5.75% KCl, 3 ml 6.10% $CaCl_2$, 1 ml 10.55% KH_2PO_4, 1 ml 19.10% $MgSO_3 \cdot 7H_2O$. *KRHBG buffer* (immediately before use): 9 ml KS, 20 ml 4.5% NaCl, 80 ml H_2O, 21 ml 0.15M HEPES, 260 mg glucose. NaOH (0.1 *N*) was used to adjust the pH to 7.4.

Cell preparations: splenic leukocytes. Immediately after the birds were sacrificed by cervical dislocation, their spleens were excised into individual containers of ice-cold phosphate-buffered saline (PBS). Spleens were trimmed of fat and connective tissue and minced, and the cells were expressed through lens paper (Fisher Scientific, Atlanta, Ga.) into PBS (12). Leukocytes were obtained by three successive 10-min centrifugations (400*g* at 4°C) with removal of the upper (white cell) layer each time (13). All operations were performed on ice. Live–dead cells counts were made in a hemocytometer using trypan blue exclusion and cell types were enumerated using Turk's stain.

Isolated adrenal cells, modification of the Sayers (14) procedure. After sacrifice by cervical dislocation, adrenal glands were excised from non-SP-Ag stimulated male donors and placed directly in ice-cold PBS. Pooled, minced adrenals were placed in a 50-ml plastic screw-cap tube containing 2.0 to 2.5 ml KRHBG to which had been added 2.0 $mg \cdot ml^{-1}$ collagenase, 2.0 $mg \cdot ml^{-1}$ bovine serum albumin, and 0.1 $mg \cdot ml^{-1}$ lima bean trypsin inhibitor (Sigma Chemical Co., St. Louis, Mo.). The minced tissue was further dispersed by several expulsions through a plastic pipet, and then incubated with shaking by 37°C for 20 min in a Dubnoff water bath. The supernatant was poured off and held at 0°C. The above procedure was repeated 5 times, then combined supernatants were filtered through a double layer of cotton gauze and centrifuged. The supernatant was poured off and the cells were resuspended in the incubation media according to the protocol of the specific experiment. Live–dead counts were made by the trypan blue exclusion method.

Serum. Chickens used as serum donors were injected with either 1.0 ml of SP-Ag or PBS, iv. One hour later, 10 ml of blood was withdrawn by cardiac puncture. In experiments to determine effectiveness of stripping, blood samples of individuals were maintained separately; in other experiments blood from SP-Ag-injected and PBS-injected birds were collected in two separate pools. Blood was clotted at room temperature for 2 hr, then at 4°C for 3 hr before serum separated. Sera were either used unstripped or were stripped of endogenous steroids with alkaline-washed charcoal (Norit A, 50 $mg \cdot ml^{-1}$, Fisher Scientific) (15, 16), and of endogenous ACTH with precipitated silica (Quso, G-32, Philadelphia Quartz Co., Philadelphia, Pa.) (14).

Cell incubations. Concentrations of live adrenal cells, ranging from 7.3 to 11.8 $\times 10^6 \cdot ml^{-1}$, were suspended in the incubation medium and 0.1-ml aliquots were placed in 25 × 50-mm polyallomer tubes (Fisher Scientific) together with an additional 0.4 ml of incubation medium or splenic leukocyte preparations. Incubations were carried out with shaking at 37°C for 2 hr in a Dubnoff water bath in an atmosphere of 95% O_2:5% CO_2.

Corticosteroid assays: serum. After preextraction with a 10-fold volume of isooctane: ethylacetate (100:1) to remove nonpolar steroids, the corticosteroids were extracted by two 10-fold volumes of 100% ethanol and assayed in duplicate by a competitive binding assay (17), using a 0.4% solution of human plasma in phosphate buffer (pH 6.9, 0.1 *M*) as the binding protein. Extraction efficiency for [^{3}H]corticosteroid-spiked pooled serum was 96%. All assays within an experiment were in one run which avoided interassay variability; the intraassay coefficient of variability was 4.8% ($N = 10$).

Adrenal cell culture assay. The cell incubation mixtures were centrifuged at 1000*g*, and after preextraction with isooctane:ethyl acetate, the corticosteroids were extracted from the supernatant with two, 10-fold volumes of methylene chloride. The solvent was evaporated in a warm air stream and the corticosteroids were assayed as above. The extraction efficiency for this system, including evaporation and subsequent transfer into the aqueous buffer, was 86.1%.

Experimental protocol: Experiment 1A—timing of serum corticosteroid responses to SP-Ag. One-hundred-twenty-six, 10-week-old White Rock (WR) females were randomly divided, six to a cage, in aluminum batteries. At 0700 hr (time 0), three birds per cage

were injected with SP-Ag; the remaining three birds received PBS. Immediately before injection, 2-ml blood samples were removed from the ulnar vein of six birds in each of three replicate cages. At 3-hr intervals thereafter, until 18 hr postinjection (PI), three cages of birds (three SP-Ag and three PBS per cage) were randomly selected and a 2-ml blood sample was taken from each bird.

Experiment 1B: timing of corticosteroid response to SP-Ag. One-hundred-forty 7-week WR males were randomly distributed, four per cage. At 0700 hr (0 time), two birds of each cage received 0.012% SP-Ag; two received PBS. Immediately before the injections, 2-ml blood samples were removed from four birds in each of three replicate cages. At 1-hr intervals thereafter for the first 6 hr, and for three successive 6-hr intervals thereafter, three replicate cages (two SP-Ag and two PBS) were randomly selected and a 2-ml blood sample was taken from each bird.

In both experiments sera were harvested and held at −20°C until assayed for corticosteroids. As no bird was bled more than once, complete factorial three-way analyses of variance (ANOVA) with interactions were employed for statistical analysis. At 7 days PI, all birds were bled to determine serum agglutinin response to SP-Ag.

Experiment 2: response of isolated adrenal cells to serum from SP-Ag injected birds and effect of stripping. Isolated adrenal cells were prepared from the glands of 10 non-SP-Ag-injected 5-week-old White Leghorn (WL) males. Five additional WL males were injected with SP-Ag and five more with PBS. After 1.5 hr, a 10-ml blood sample was obtained from each injected bird and the serum was harvested. The serum samples were divided into two aliquots, one stripped, the other unstripped. Duplicate adrenal cell suspensions were incubated with aliquots of stripped and unstripped serum. Corticosteroids were assayed in the sera and in the adrenal incubation supernatants. ANOVA in a factorial design was used to evaluate effects of stripping and SP-Ag injection.

Experiment 3: response of isolated adrenal cells to SP-Ag-activated splenic leukocytes. In each of two cages containing six 9-week-old female WR (Rep I and II), three birds received injections of SP-Ag and three received PBS. One hour PI, the birds were sacrificed and the spleens were excised. The leukocytes from individual spleens were diluted fivefold in PBS and four 0.5-ml aliquots were removed and centrifuged. Two of the aliquots were resuspended in stripped serum from SP-Ag-injected birds and two in stripped serum from PBS-injected birds. One of each aliquot was incubated for 2 hr in plastic tubes with shaking at 37°C under 95% O_2:5% CO_2. The cells were then separated from the supernatant by centrifugation at 400*g* and the supernatants were incubated with isolated adrenal cells. The remaining two aliquots of splenic leukocytes were suspended in stripped sera from SP-Ag-injected or PBS-injected birds and directly incubated with isolated adrenal cells. Duplicate sets of isolated adrenal cells, incubated in KRHBG, served as controls. Six non-SP-Ag-injected WR males served as adrenal donors. The results were calculated as nanogram corticosteroid · 10^6 adrenal cells^{-1} · 10^6 spleen cells^{-1} · 2 hr^{-1}, and statistically analyzed by ANOVA in a split-plot design using SP-Ag spleens vs PBS-injected spleens as main plots.

Experiment 4: response of isolated adrenal cells to SP-Ag-activated splenic leukocytes. Procedures and chickens were essentially the same as in Experiment 3, except that spleen cell supernatants were not tested. Comparisons were made among splenic leukocytes from SP-Ag-activated and nonactivated birds in KRHBG, stripped SP-Ag-activated, and stripped non-SP-Ag-activated sera. A split-plot ANOVA was used to evaluate results.

Results. *Experiments 1A, 1B.* Serum corticosteroids rose significantly ($P < 0.01$) within 1 hr PI and maximum responses were observed 2 to 3 hr PI (Figs. 1A, B). By 9 hr PI the differences between SP-Ag and PBS-injected birds were no longer significant. There was also a significant difference ($P < 0.05$) at 18 hr PI in Experiment 1A; however, a similar response was not observed in Experiment 1B which suggests that the 18-hr rise in 1A was a chance occurrence. Mean serum agglutinin titers ±SE ($\log_2$ of reciprocal of highest serum dilution showing agglutination at 7 day PI) were 6.8 ± 0.2 and 7.7 ± 0.1, respectively, for the SP-Ag-injected birds of Experiments 1A and 1B.

Experiment 2. The results of the cortico-

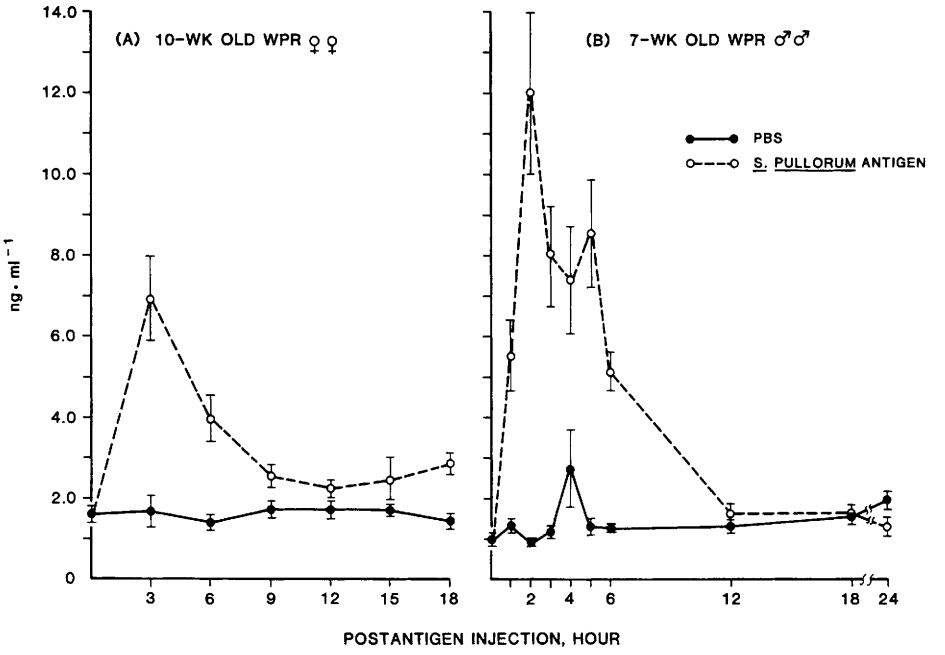

FIG. 1. Effect of injection of heat-killed *S. pullorum* antigen (SP-Ag) and phosphate-buffered saline (PBS) on serum corticosteroid concentrations of White Rock chickens. Experiment 1A: SP-Ag injected iv, 0.8 ml of 0.006% (packed volume). Each point is the mean of nine individuals ± SE. Experiment 1B: SP-Ag injected iv, 0.8 ml of 0.012%. Each point is the mean of six individuals ± SE.

steroid assay of serum taken 1.5 hr PI (Fig. 2A) confirm those of Experiments 1A and 1B, that injection with SP-Ag causes a highly significant increase in serum corticosteroid, and also that stripping effectively removes most of the corticosteroids. Unstripped serum from SP-Ag-stimulated birds caused isolated adrenal cells to produce corticosteroids at more than twice the rate of PBS-injected birds (Fig. 2B). Stripping depressed the adrenal-stimulating effects of sera of SP-Ag and PBS-injected birds.

Experiment 3. This experiment was performed to established conditions for adrenal cell stimulating response by SP-Ag activated splenic leukocyte preparations. It is shown in Fig. 3 that, overall, the leukocytes from spleens of SP-Ag-injected birds were significantly more effective in stimulating isolated adrenal cells than those from PBS-injected birds. However, the effect was almost entirely due to the activity of the leukocytes which were incubated directly with the adrenal cells with stripped serum from antigen-stimulated birds as the medium. Supernatants from the 2-hr spleen cell incubation had relatively low activity and were significantly lower than spleen cells incubated directly with adrenal cells, regardless of the serum used as the incubation medium. It should also be pointed out that all spleen cell preparations had virtually 10 times greater adrenal cell-stimulating activity than the nonspleen controls (note scale difference).

Experiment 4. In this experiment, the adrenal cell-stimulating ability of splenic leukocytes in three different incubation media was compared (Fig. 4). Confirming the results of Experiment 3, splenic leukocytes taken from birds 1 hr after SP-Ag-injection produced a highly significant ($P < 0.01$) increase in corticosteroid response from isolated adrenal cells compared to leukocytes from birds injected with PBS. Incubation with serum was significantly more effective than with KRHBG. In this experiment there were no significant differences when leukocytes were incubated in sera from SP-Ag-injected or PBS-injected birds.

Discussion. The purposes of the prelimi-

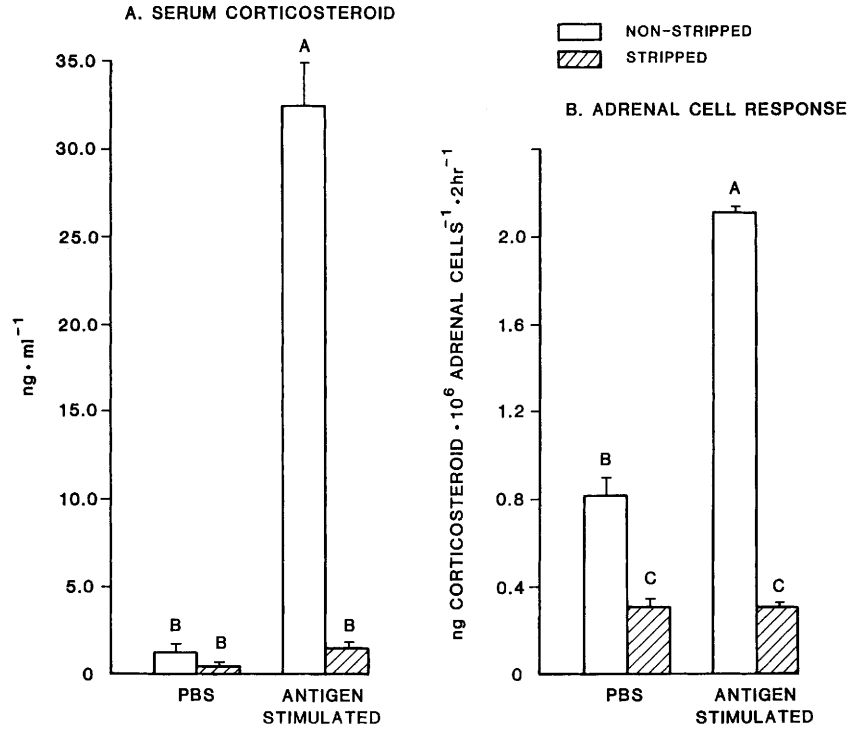

FIG. 2. Serum corticosteroid and the adrenal cell responses to sera from 4-week-old White Leghorn cockerels injected with *S. pullorum* antigen or phosphate-buffered saline (PBS). Effect of stripping the serum with activated charcoal or precipitated silica (Quso-G32), Experiment 2. Each bar represents five individuals ± SE. A,B,C differences significant ($P < 0.01$).

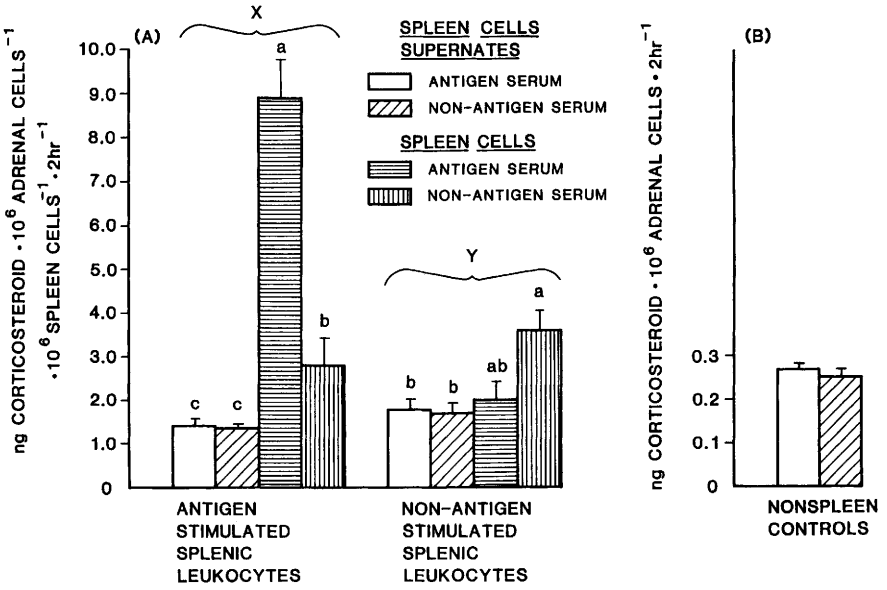

FIG. 3. Effect of 1-hr *in vivo* stimulation with *S. pullorum* antigen on the corticosteroid production of isolated adrenal cells by splenic leukocyte supernatants or direct splenic leukocyte incubations. Effects of incubation in stripped sera from antigen and nonantigen-injected chickens. Each bar is the mean of six samples ± SE. X,Y difference between antigen and nonantigen-stimulated preparations significant ($P < 0.01$), a,b,c differences among groups within antigen-stimulation groups significant ($P < 0.05$).

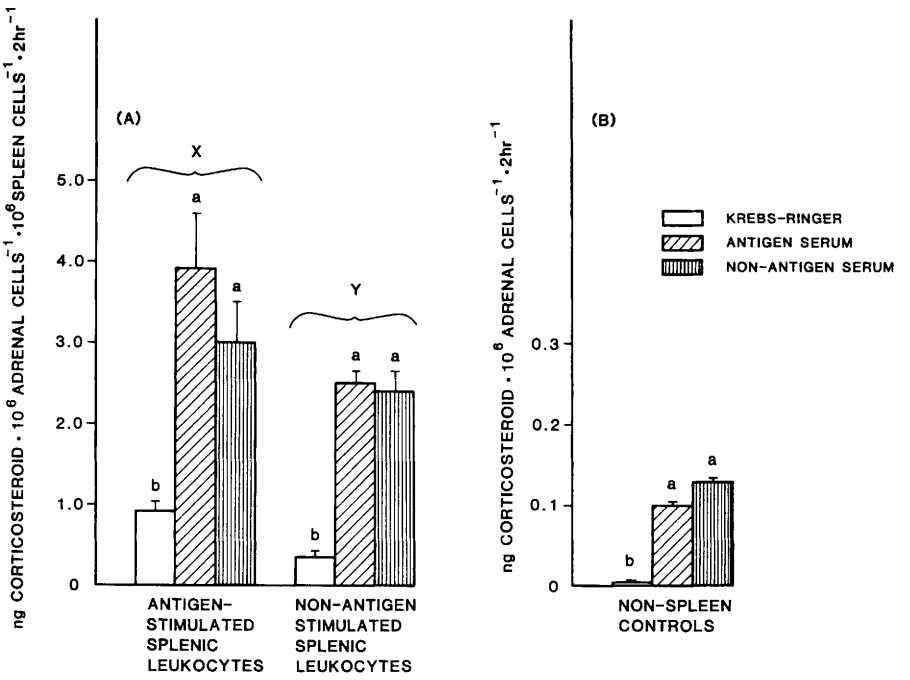

FIG. 4. Effect of 1-hr *in vivo* stimulation with *S. pullorum* antigen on the corticosteroid production of isolated adrenal cells by splenic leukocytes. Effect of incubation in KRHBG and stripped sera from antigen- and nonantigen-injected chickens. Each bar is the mean of six samples ± SE. X,Y difference between antigen- and nonantigen-stimulated splenic preparations significant ($P < 0.01$). a,b differences among incubation media within antigen stimulation groups significant ($P < 0.01$).

nary experiments (1A, B) were to determine whether injection of a bacterial, "O"-type antigen would stimulate a significant rise in serum corticosteroids and to estimate the timing of the event. The serum corticosteroid responses by chickens injected with SP-Ag (Experiments 1A and 1B) were similar to the results obtained with mice injected with NDV-antigen (11), differing only in the chronology of the response. Whereas peak responses in the mouse experiments were 8 hr PI, in our experiments with chickens, the peak was 2 hr PI. At the present, it is not known whether the difference was due to a difference in recipient species response or to the nature of the antigen.

The purposes of Experiment 2 were to determine whether there was an increase in an adrenal-stimulating "ACTH-like" substance present in the serum of SP-Ag-stimulated birds and to show that stripping with activated charcoal and precipitated silica would essentially remove the corticosteroids and the ACTH-like material. The results (Fig. 2) show that not only were the corticosteroid levels in the serum increased following SP-Ag activation, but also that there was an increase in a substance which has the ACTH-like capability to stimulate adrenal cells to produce corticosteroids. The corticosteroids were effectively adsorbed on activated charcoal (15, 16) and the adrenal-stimulating substance was adsorbed on Quso. It has been previously reported that Quso adsorbs up to 87% of the ACTH added to the plasma of hypophysectomized rats (14).

The present experiments show that the splenic leukocytes from chickens injected with the heat-killed *S. pullorum* antigen have a greater ability to stimulate corticosteroid production from adrenal cells than those from birds injected with PBS. These results with a bacterial antigen parallel those in which viral antigen (NDV) induced an immunofluorescent response to ACTH (1-13 ACTH amide) in murine splenic lymphocytes 8 hr after the injection of the antigen (11). It has been suggested that the immune system

has a sensory function, mimicking the neuroendocrine system by the elicitation of peptide hormones which produce a physiological response (9). This concept is supported by the work of Smith and Blalock and Smith *et al.* (10, 11) which showed that hIFN-α preparations contain β-endorphin and ACTH-like activities. However, the work with mice, although performed with hypophysectomized animals, does not preclude the possibility of an extrapituitary, hypothalamic source of tropic substances (8). In Experiments 3 and 4 of the present research, the potential hypothalamic sources were circumvented by directly reacting splenic leukocytes with the isolated adrenal cells.

The adrenal-stimulating capability of the leukocytes was significantly increased by using serum as the incubation medium when compared to incubation with KRHBG. This result is probably due to the fact that most of the precursor cholesterol for steroid biosynthesis in adrenal cortical cells is derived from the serum low-density lipoprotein (LDL) cholesterol that is taken up endocytotically by specific receptors in cellular plasma membranes (18). ACTH acts to stimulate LDL metabolism in cortical cells via an increase in the LDL binding sites on the plasma membranes (19). Although, in Experiment 3, it appeared that the serum from SP-Ag-injected birds was stimulatory, suggesting that the serum was incompletely stripped, the same effect was not apparent in Experiment 4.

Adrenal cortical cell synthetic activity may not have been the only cellular activity influenced by serum lipids. DNA synthesis and blastogenesis in lymphocytes have been shown to be correlated with cholesterol and phospholipid synthesis (20, 21). Because lymphocytes obtain most of the membrane cholesterol from plasma LDL (22), it is possible that normal membrane permeability, and thus synthesis of ACTH-like peptides, were optimized in the serum medium.

The splenic leukocytes of SP-Ag-injected birds had a greater stimulating effect when coincubated with isolated adrenal cells than spleen cell supernatants separated after a 2-hr incubation. This result suggests that some of the ACTH-like substance may have been inactivated during the 2-hr incubation period. ACTH is rapidly destroyed by proteolytic enzymes in plasma (23, 24), but the effect is retarded at temperatures below 4°C (25). The results of spleen cell–adrenal cell coincubation are also similar to those in which progesterone production was increased by luteotropic peptides only when monocytes were cocultured with luteinized murine granulosa cells (26).

Besedovsky *et al.* (27) have shown that an increase in plasma corticosterone follows 1 day after immunization of mice with equine erythrocytes or 4 days after immunization of rats with ovine erythrocytes (SRBC). This response was concomitant with a decline in thyroxine and was followed in 1 day by an increase in splenic plaque-forming cells. It was shown subsequently that immunization of rats with SRBC brought about significant declines in splenic norepinephrine (28). An increase in metabolic heat production has been reported to occur 4 days after immunizing chickens with SRBC and was related to the appearance of 2-mercaptoethanol-resistant (IgG) antibodies (29). These responses have been related to lymphokine production by antigenically-stimulated lymphocyte (30).

How the present results relate to those of Besedovsky *et al.* (27, 30) is not clear. In the present experiments, and in those of Smith *et al.* (11), the response to the antigen was rapid; in our birds a significant response was observed within the first hour after immunization, and had declined to control levels within 9 hr. In the results reported by Besedovsky *et al.* the onset of corticosteroid response was from 1 to 4 days following immunization depending on species and antigen; however, injection of the purified lymphokine preparation resulted in a plasma cortisol response within 0.5 hr (30).

Thus, we have found that the previous work showing increases in ACTH-like activity in mouse lymphocytes by virus infection can be extended to include the effects of a bacterial antigen in the avian species. Furthermore, this research shows that there is a direct response to antigenically stimulated leukocytes which become activated within 1 hour of immunization.

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