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Chicken Serum Inhibits Lectin-Induced Proliferation of Autologous Splenic Mononuclear Cells[1] (42472)

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Abstract. Splenic mononuclear cells (SMC) proliferate well in response to phytomitogens in serum-free medium. Low concentrations of corticosterone significantly inhibit both spontaneous (1 ng/ml) and phytohemagglutinin-induced proliferation (12 ng/ml) of chicken SMC. Addition of as little as 3% normal heat-inactivated chicken serum to both autologous and heterologous suspensions of chicken SMC causes a 50% inhibition in lectin-induced proliferation. This suppression by normal chicken serum increases in a dose-dependent manner. Inhibition of DNA synthesis by chicken serum is not due to endogenous corticosterone because charcoal stripping of the serum to deplete glucocorticoids does not remove the immunosuppression. Chicken serum is not cytotoxic to lymphoid cells. These data demonstrate that low, physiologic concentrations of corticosterone inhibit proliferation of chicken SMC. Utilization of a serum-free culture system also permitted the identification of a serum factor that appears to be even more immunosuppressive than corticosterone. Since this factor inhibits the lectin-induced proliferation of both autologous and heterologous avian lymphoid cells, it may be a very important naturally occurring immunoregulatory compound.

Heat and cold exposure reduces T-cell-mediated immune reactions *in vivo* and *in vitro* (1). It is well known that several environmental stressors activate the pituitary–adrenal axis to release adrenal glucocorticoids. Pharmacologic and physiologic concentrations of glucocorticoids have a variety of effects on mammalian T lymphocytes (2–7). Inhibitory effects of glucocorticoids on lymphoid cells are due to a reduction in the synthesis of interleukin 1 (8, 9), interleukin 2 (10, 11), and the interleukin 2 receptor (12).

In birds, corticosterone is the major adrenal glucocorticoid (13). In a classic study by Sato and Glick (14), it was reported that corticosterone was not as effective in suppressing antibody production in chickens as was cortisone acetate. However, corticosterone was more effective in prolonging homograft survival than was cortisone acetate, hydrocortisone, or deoxycorticosterone. These results agree with the general concept that corticosterone inhibits T-cell rather than B-cell functions (14–16). Lymphocytes of chickens contain glucocorticoid receptors (17, 18). Both adrenocorticotrophic hormone and heat exposure increase plasma corticosterone as well as the concentration of corticosteroid bound in lymphocytes (19–21). We therefore hypothesized that physiologic concentrations of corticosterone would inhibit T-cell proliferation in chickens.

In this report we demonstrate that physiologic concentrations of corticosterone indeed can suppress lectin-induced proliferation. However, it appears that there is another factor in normal chicken serum that is even more immunosuppressive than corticosterone. This paper describes the existence of this natural, highly immunosuppressive factor.

Materials and Methods. *Cell preparation.* Chicken spleens were collected aseptically from 8- to 12-week-old male White Leghorn chickens (Cornell K strain). Spleen cells were prepared by repeatedly perfusing the organs with RPMI 1640 tissue culture medium that was supplemented with 100 units/ml of penicillin and 100 μg/ml of streptomycin. SMC were layered over 7 ml of Histopaque 1077 in sterile 50-ml polystyrene centrifuge tubes. Samples were then centrifuged at 400*g* for 40 min at room temperature. The lymphocyte-rich layer was removed and washed four times in RPMI 1640 medium (400*g*, 10 min). Cells

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were suspended in RPMI 1640 medium containing no serum. Evaluation of Wright–Giemsa-stained cytospin preparations of these cells showed that they consisted of approximately 95% small lymphocytes.

Preparation of chicken serum and corticosterone. Chicken blood was obtained by cardiac puncture and allowed to clot for 1 hr at room temperature. After clotting, blood was centrifuged at 500*g* for 10 min and the serum was collected and passed through a 0.45-μ filter. Sera were heat-inactivated for 1 hr at 57°C. A stock solution of corticosterone was prepared by dissolving 10 mg of corticosterone 21-acetate in 1 ml of ethanol and then was diluted further in 24 ml of RPMI 1640. Subsequent dilutions from this stock were made in RPMI 1640. At the highest initial concentration of corticosterone (4000 ng/ml), the maximal ethanol concentration was 0.01%. This concentration of ethanol was added to control wells that did not contain corticosterone.

Mitogenesis assays. Corticosterone (50 μl) or 50 μl of RPMI 1640 medium containing 0.01% ethanol, 50 μl of an appropriate dose of phytohemagglutinin (PHA; GIBCO, Grand Island, NY) or 50 μl of RPMI 1640 medium and 100 μl of RPMI 1640 containing 2×10^6 SMC with or without 10% fetal bovine serum were added as triplicate or duplicate cultures into 96-well microtiter plates. For studies on the direct effects of chicken and fetal bovine sera on mitogenesis, heat-inactivated sera were diluted with 0.15 *M* phosphate-buffered saline (PBS) pH 7.4. Serum (100 μl), 50 μl of the appropriate dose of mitogen (PHA, Concanavalin A (Con A), or pokeweed mitogen (PWM); Sigma Chemical Co., St. Louis, MO) or 50 μl of RPMI 1640 medium, and 50 μl of RPMI 1640 containing 2×10^6 SMC were added as duplicate cultures into 96-well microtiter plates. Cultures containing no serum were prepared by adding 100 μl of PBS, 50 μl mitogen, and 50 μl medium containing 2×10^6 cells. Cultures were incubated for 54 hr at 41°C in a 7% CO_2 atmosphere, pulsed with 1 μCi [*methyl*-^{3}H]thymidine ([^{3}H]TdR; sp act = 6.7 Ci/mmole), and then incubated an additional 18 hr. Cultures then were harvested onto glass filter strips with a 24-well PHD cell harvester. Filters were dried, 3 ml of Omnifluor scintillation cocktail was added, and [^{3}H]TdR incorporation was determined with a Beckman LS 5801 liquid scintillation counter.

Viability. The appropriate amount of autologous chicken serum (80 μl) in PBS was pipetted into 96-well microtiter plates. To these wells, 20 μl of RPMI 1640 and 50 μl of Con A (20 μg/ml) were added. Chicken SMC (50 μl) containing 2×10^6 cells then were added to each culture. This procedure resulted in final concentrations of 0, 10, and 40% serum in the cultures. Plates were incubated as described above and viabilities were determined by trypan blue exclusion (cells were diluted 1:2 with 0.04% trypan blue) at 24, 48, and 72 hr).

Charcoal stripping of serum. Charcoal stripping was accomplished by the method of Gould and Siegel (22). Heat-inactivated serum was mixed gently at room temperature for 30 min with 10 mg/ml of Norit A in 12 × 75-mm culture tubes. Tubes were then centrifuged at 800*g* for 10 min and the serum was filtered through a 0.45-μ filter and tested in a mitogenesis assay.

Dialysis of chicken serum. Pooled chicken serum was dialyzed against PBS for 72 hr in dialysis tubing with a 12,000 mol wt cutoff. Serum within the tubing was filtered (0.45 μ) and then tested against normal pooled serum for its effect on the mitogenic response of chicken SMC.

Statistical analysis. All experiments were analyzed by ANOVA as a randomized complete block design in which chickens consisted of the block effect and serum was the treatment effect. Differences between serum levels were determined by Duncan's new multiple-range test. Regression analysis, including both linear and quadratic terms, also was performed on dose-response experiments. The slopes and intercepts were tested to determine if they were statistically different from zero.

Results. *Corticosterone inhibits spontaneous and PHA-induced proliferation.* In initial experiments that were designed to determine whether corticosterone suppressed SMC proliferation to T-cell lectins, heat-inactivated fetal bovine serum was included in the tissue culture medium at a final concentration of 5%. Under these conditions, it was not possible to

demonstrate any immunosuppressive effect of corticosterone on either spontaneous or PHA-induced uptake of [^{3}H]TdR (Table I), even at pharmacologic concentrations of corticosterone (1000 ng/ml). However, other reports have suggested that chicken lymphoid cells can proliferate well in the absence of exogenous fetal bovine serum (23–27). Preliminary experiments confirmed these results, so we were then able to determine whether corticosterone would inhibit proliferation of chicken SMC in a culture system that was free of serum proteins and exogenous cortisol. This experiment demonstrated that as little as 1 ng/ml of corticosterone significantly inhibited spontaneous SMC proliferation, and 12 ng/ml significantly suppressed PHA-induced [^{3}H]TdR by these cells (Table I).

Normal fetal bovine serum and chicken serum inhibits Con A-induced mitogenesis. Not only did the results presented in Table I confirm that chicken SMC proliferate well in the absence of serum factors, but the data also suggested that fetal bovine serum actually suppressed PHA-induced mitogenesis (223,000 cpm in serum-free medium and 55,000 cpm in the presence of 5% fetal bovine serum). Preliminary experiments with heat-inactivated chicken serum indicated that it was also immunosuppressive. The effects of heat-inactivated fetal bovine serum and normal, heat-inactivated chicken serum on Con A-induced mitogenesis are compared in Fig. 1. In this experiment, chicken serum was tested on autologous SMC. As little as 3% heat-inactivated chicken serum was sufficient to cause more than a 50% reduction ($P < 0.05$) in Con A-induced proliferation of autologous chicken SMC. Fetal bovine serum at a final concentration of 6% was also effective in inhibiting ($P < 0.05$) Con A-induced proliferation, but approximately three- to fourfold higher serum concentrations were required to cause the same inhibition as chicken serum. Interestingly, increasing concentrations of chicken serum caused a small but significant ($P < 0.05$) enhancement in spontaneous proliferation of autologous SMC.

Normal chicken serum inhibits Con A-induced proliferation of heterologous SMC. An experiment was designed to determine whether heat-inactivated normal chicken

TABLE I. CORTICOSTERONE INHIBITS SPONTANEOUS AND PHA-INDUCED PROLIFERATION OF CHICKEN SMC ONLY IN SERUM-FREE MEDIUM

Medium with	Number of chickens	PHA	Concentration of corticosterone (ng/ml)						
			0	0.2	1	2	3	12	50	1000
5% fetal bovine serum	12	−	2.7 ± 0.6	ND	ND	2.4 ± 0.5	ND	ND	3.0 ± 0.4	2.3 ± 0.5
	12	+	54.6 ± 15.2	ND	ND	57.7 ± 14.7	ND	ND	62.3 ± 16.5	55.2 ± 15.7
Serum-free	5	−	2.1 ± 0.4^a	1.6 ± 0.2^a	1.1 ± 0.3^b	ND	0.8 ± 0.2^b	0.6 ± 0.1^b	0.8 ± 0.2^b	ND
	5	+	222.5 ± 28.3^a	206.9 ± 17.3^{ab}	208.4 ± 24.3^{ab}	ND	174.7 ± 26.8^{ab}	126.2 ± 26.4^{b,c}	78.6 ± 20.2^c	ND

Note. Data are expressed as mean counts per minute [^{3}H]TdR ± SEM × 10^{-3}. PHA was used at a 1:40 dilution. ND indicates not determined. Means within a row with different superscripts are different ($P < 0.05$). Slope and intercept for unstimulated cells in the serum-free system were −.015 ± .007 ($P < 0.05$) and 1.33 ± 0.14 ($P < 0.01$), respectively, whereas the comparable values for PHA-stimulated cells were −2.61 ± 0.57 ($P < 0.01$) and 198.60 ± 11.76 ($P < 0.01$). Slopes of the regression lines were not significant when fetal bovine serum was included in the medium.

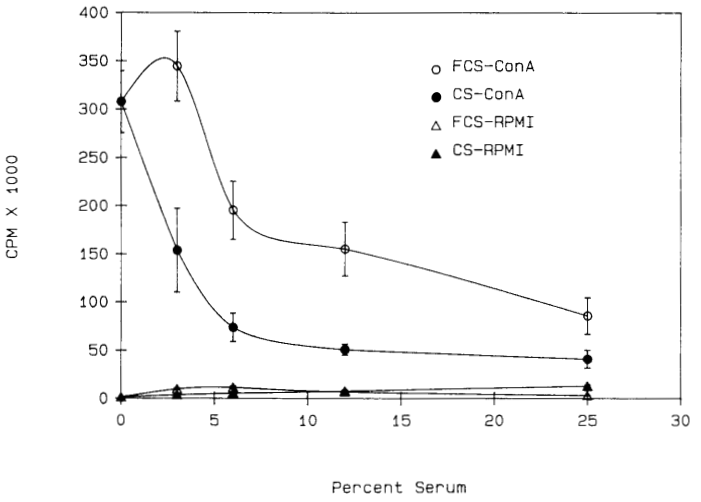

FIG. 1. Heat-inactivated autologous chicken serum and fetal bovine serum inhibit Con A-induced (5 μg/ml) [^{3}H]TdR incorporation by chicken SMC ($N = 6$). Error bars represent the SEM. Significant slopes ($P < 0.01$) and intercepts ($P < 0.01$) for regression lines were obtained for unstimulated and stimulated SMC in chicken serum and stimulated SMC in fetal bovine serum. These lines had slopes of 0.45 ± 0.05, -8.17 ± 0.17, and -9.71 ± 1.68, and intercepts of 1.86 ± 0.64, 191.73 ± 22.10, and 306.93 ± 21.44, respectively. The regression line for unstimulated SMC in fetal bovine serum was not significant ($P > 0.10$).

serum also would reduce the lectin-induced proliferation of heterologous chicken SMC. Six chickens were used, and the six sera were incubated with SMC from each of the five other chickens. Each of the six sera inhibited the Con A-induced uptake of [^{3}H]TdR of heterologous SMC from the five chickens in a dose-dependent manner (Fig. 2).

Mitogenesis induced by three different lectins is inhibited by normal chicken serum.

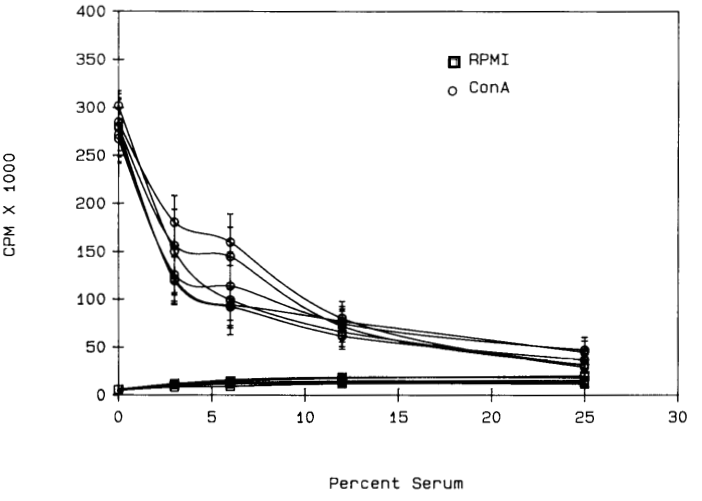

FIG. 2. The effects of six different chicken sera on Con A-induced (5 μg/ml) [^{3}H]TdR incorporation by SMC from five heterologous chickens. Each line represents one serum sample tested on five chickens. Error bars represent the SEM. Significant slopes ($P < 0.01$) and intercepts ($P < 0.01$) for composite regression lines were obtained for both unstimulated and stimulated SMC. These lines had slopes of 0.36 ± 0.05 and -7.84 ± 0.65 and intercepts of 8.44 ± 0.66 and 201.97 ± 8.31, respectively.

Heat-inactivated chicken serum at a final concentration of 25% then was added to cultures of autotologous chicken SMC that were incubated with three different final dilutions of PHA (1/40), Con A (5 μg/ml), and PWM (0.6 μg/ml) (Table II). Regardless of the lectin used, chicken serum significantly inhibited the uptake of [^{3}H]TdR. Identical responses also were observed with heterologous SMC (data not shown).

Depletion of corticosterone from autologous chicken serum does not remove suppressive activity. Charcoal stripping removes over 97% of the corticosterone from plasma (22, 28). However, after heat-inactivated chicken serum was incubated with Norit A, the suppression of Con A-induced mitogenesis remained and was identical to the suppressive activity that was measured in normal, heat-inactivated chicken serum (Table III). Furthermore, the charcoal-stripped serum also retained its capability to moderately but significantly enhance spontaneous proliferation of chicken SMC. These data suggest that endogenous corticosterone in normal chicken serum is not the immunosuppressive factor.

Dialyzed chicken serum retains suppressive activity. Thymidine is a small nucleotide (242 Da) that may be present in heat-inactivated chicken serum. If true, this unlabeled thymidine could be utilized by lectin-activated SMC and therefore reduce the uptake of [^{3}H]TdR. To test this possibility, heat-inactivated chicken serum was dialyzed extensively using a 12,000-Da molecular weight exclusion membrane. The dialyzed serum, in which small molecular weight compounds such as thymidine would be removed, was compared to normal, heat-inactivated chicken serum for its suppressive activity (Table IV). The dialyzed serum retained its capability to inhibit Con A-activated proliferation of SMC, which indicates that the factor that inhibits DNA synthesis has a molecular weight or is bound to a substance with a molecular weight greater than 12,000 Da.

Normal chicken serum is not cytotoxic to chicken SMC. To test whether chicken serum inhibited Con A-induced mitogenesis by directly killing SMC, various concentrations of chicken serum were incubated with autologous SMC for 24, 48, and 72 hr. Viability of the Con A-stimulated SMC ranged between 67 and 81% over the incubation period (Fig. 3). Addition of 10 or 40% heat-inactivated chicken serum to both nonstimulated (data not shown) and Con A-stimulated cultures did not increase ($P > 0.10$) the percentage of dead cells when compared to those cells cultured in serum-free medium at either 24, 48, or 72 hr of culture.

Discussion. In this paper we have confirmed that not only do chicken SMC respond well to plant lectins in serum-free medium, but also we have shown that both fetal bovine serum and normal chicken serum strongly inhibit blastogenic responses. Futhermore, these data confirm recent results (26) which demonstrate that physiologic concentrations of corticosterone inhibit lectin-induced mitogenesis in normal chickens. Absence of corticosterone-induced inhibition in medium containing fetal bovine serum was probably due to serum proteins, such as corticosteroid-binding globulin or albumin, binding to exogenous corticosterone and rendering it inactive on lymphoid cell proliferation. This interpretation is consistent with the concept that only free, unbound glucocorticoids are able to penetrate the cell membrane and affect cellular function (29–31).

Since only 1 ng/ml of corticosterone was able to significantly inhibit spontaneous proliferation of chicken SMC, and 12 ng/ml sup-

TABLE II. SMC PROLIFERATION INDUCED BY THREE DIFFERENT LECTINS IS INHIBITED BY AUTOLOGOUS NORMAL CHICKEN SERUM

	N	Serum	RPMI	Con A	PHA	PWM
Chicken SMC	4	−	2.3 ± 0.7^a	341.0 ± 22.2^a	327.9 ± 21.3^a	94.1 ± 19.3^a
	4	+	7.6 ± 0.9^b	31.3 ± 8.9^b	9.8 ± 2.0^b	13.0 ± 2.2^b

Note. Data are expressed as mean counts per minute [^{3}H]TdR ± SEM × 10^{-3}. Con A was used at 5 μg/ml, PHA at 1:40, and PWM at 0.625 μg/ml. Chicken serum was used at a final concentration of 25%. Means within a column with different superscripts are different ($P < 0.05$).

TABLE III. CHARCOAL STRIPPING OF AUTOLOGOUS CHICKEN SERUM DOES NOT REMOVE SUPPRESSIVE ACTIVITY

Serum	Number of chickens	Con A	Percentage serum					Intercept	Slope
			0	3	6	12	25		
Normal	4	−	1.1 ± 0.2[a]	3.6 ± 0.3[a,b]	4.9 ± 0.5[b,c]	6.3 ± 1.1[b,c]	7.6 ± 1.3[c]	2.61 ± 0.59	.23 ± 0.05
	4	+	320.4 ± 65.5[a]	157.4 ± 35.7[b,c]	162.8 ± 51.8[b]	99.1 ± 40.0[b,c]	47.0 ± 16.5[c]	222.12 ± 26.70	−7.88 ± 2.09
Stripped	4	−	1.1 ± 0.2[a]	4.7 ± 0.9[b]	6.9 ± 1.6[b,c]	8.2 ± 1.6[c]	9.1 ± 1.3[c]	3.58 ± 0.91	.27 ± 0.07
	4	+	320.4 ± 65.5[a]	175.4 ± 42.2[a,b,c]	164.4 ± 43.8[b,c]	104.2 ± 29.8[b,c]	46.0 ± 15.5[c]	228.67 ± 24.27	−8.11 ± 1.90

Note. Data are expressed as mean counts per minute [^{3}H]TdR ± SEM × 10^{-3}. Con A was used at 5 μg/ml. Means within a row with different superscripts are different ($P < 0.05$). Previous experiments demonstrated no difference ($P > 0.10$) between PBS and stripped PBS, so normal PBS was used for diluting serum samples in both the normal and the dialyzed treatments. Slopes and intercepts of the four regression lines were significant ($P < 0.01$).

pressed PHA-induced proliferation, it might be argued that endogenous corticosteroid was responsible for the serum-induced inhibition of DNA synthesis of chicken SMC. However, this argument is untenable for several reasons: (a) normal serum from 6- to 8-week-old chickens contains around 1 to 4 ng/ml of corticosterone (20, 21, 26, 32), and this concentration of corticosterone did not inhibit lectin-induced proliferation. However, as little as 3% normal heat-inactivated chicken serum, which would contain no more than 0.2 ng/ml of corticosterone, caused a 50% suppression in blastogenesis. (b) Similary, low doses of corticosterone inhibited spontaneous proliferation of SMC, but higher concentrations of heat-inactivated chicken serum actually enhanced spontaneous uptake of [^{3}H]TdR. (c) Corticosterone-depleted serum that was generated by charcoal stripping was just as effective in inhibiting mitogenesis as the control serum. (d) Unless corticosterone was bound to a serum protein and then released in the culture medium, it would not have been found in the dialyzed chicken serum that inhibited mitogenesis. (e) Preliminary experiments in which chicken serum was incubated with pepsin indicated that this protease treatment destroys the inhibitory substance, which suggests that the suppressor factor is proteinlike (manuscript in preparation).

The finding that normal chicken serum strongly inhibits the proliferation of autologous SMC suggests that some factor which naturally exists in serum serves an important immunomodulatory role. Our initial interest was to determine whether the stress-induced suppression of T-cell proliferation caused by heat and cold exposure could be explained by elevated concentrations of corticosteroid in chicken serum. The present results confirm that low levels of corticosteroids can indeed suppress T-cell proliferation, but the data also reveal that other substances in normal chicken serum are even more immunosuppressive than corticosterone. Due to the potency of this factor, small changes in its concentration could have major effects on the capability of chicken SMC to respond to plant lectins.

A recent report (33) has shown that thymidine phosphorylase is found in human blood, and this enzyme can catabolize

TABLE IV. DIALYZED CHICKEN SERUM RETAINS ITS SUPPRESSIVE ACTIVITY ON HETEROLOGOUS SMC

Serum	Number of chickens	Con A	Percentage serum					Intercept	Slope
			0	3	6	12	25		
Normal	6	−	4.0 ± 1.1^{a}	$8.7 \pm 2.5^{a,b}$	11.3 ± 3.0^{b}	12.8 ± 3.2^{b}	13.7 ± 3.6^{b}	7.16 ± 1.82	0.32 ± 0.14
	6	+	283.7 ± 11.0^{a}	207.8 ± 15.4^{b}	177.6 ± 15.1^{b}	109.5 ± 27.4^{c}	58.0 ± 14.4^{c}	243.80 ± 13.02	-8.32 ± 1.02
Dialyzed	6	−	4.0 ± 1.1^{a}	$5.9 \pm 1.2^{a,b}$	$7.2 \pm 1.4^{a,b}$	$10.2 \pm 2.7^{a,b}$	12.1 ± 3.2^{b}	4.98 ± 1.32	0.32 ± 0.10
	6	+	283.7 ± 11.0^{a}	203.9 ± 20.4^{b}	189.5 ± 22.2^{b}	168.6 ± 26.5^{b}	88.1 ± 22.3^{c}	248.40 ± 14.13	-6.69 ± 1.11

Note. Data are expressed as mean counts per minute [^{3}H]TdR ± SEM × 10^{-3}. Con A was used at 5 μg/ml. Means within a row with different superscripts are different ($P < 0.05$). Previous experiments demonstrated no difference ($P > 0.10$) between PBS and stripped PBS, so normal PBS was used for diluting serum samples in both the normal and the dialyzed treatments. Slopes and intercepts of the four regression lines were significant ($P < 0.01$).

[^{3}H]TdR to unincorporable [^{3}H]thymine. Therefore, [^{3}H]TdR may not be useful for measuring lymphoid cell proliferation in whole blood cultures containing plasma that has not been heat inactivated. However, it is unlikely that this enzyme is responsible for the serum-induced decrease in [^{3}H]TdR uptake by stimulated SMC for three reasons: (a) Over 95% of the activity of thymidine phosphorylase is destroyed by heating at 60 C for 30 min (34). In our studies, all serum samples were maintained at 57°C for 1 hr before they were analyzed in proliferation assays. Indeed, suppressive activity remains after chicken serum is boiled, and we have exploited this property to isolate the suppressive protein (manuscript in preparation). (b) Higher concentrations of chicken serum, which would contain more thymidine phosphorylase, actually enhanced spontaneous uptake of [^{3}H]TdR. (c) Thymidine phosphorylase is a 110,000-Da protein that is not biologically active at a molecular weight of 55,000 (34, 35). Recent evidence from our laboratory has shown that active size of the suppressive protein in chicken serum is 61,000 Da.

It remains unknown as to whether heat and cold exposure, or other factors such as hormones, age, or nutritional status, affects the concentration of this inhibitory factor in serum. Given the emerging evidence of reciprocal systems of communication between the endocrine, immune, and nervous systems (3, 36–39), elucidation of the physiochemical and physiological properties of this inhibitory compound will be most interesting. Although inhibitors of DNA synthesis have been reported (40), there is a paucity of information in avian systems. Perhaps the most well-characterized factor(s) that inhibits PHA-stimulated mitogenesis is derived from thymic and bursal lymphocytes of chickens that have been sensitized with *Mycobacterium tuberculosis* (23). It is unknown whether the inhibitory factor in serum is produced by avian lymphocytes, but data from experiments with thymectomized and bursectomized chickens might reveal the answer. Our current efforts are concentrating on purifying the factor and characterizing the biochemical properties of this inhibitory compound with the goal of developing a specific immunological assay to

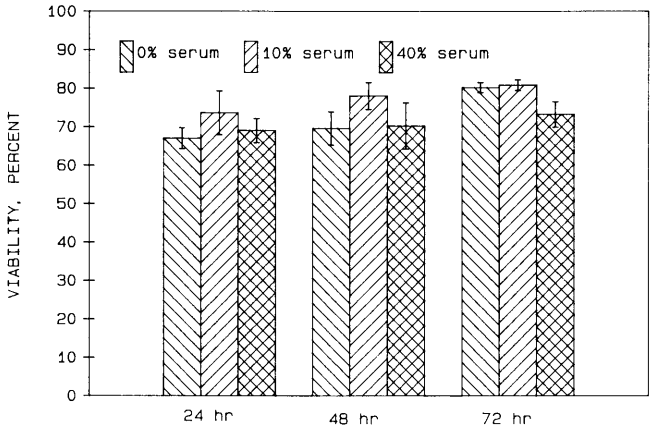

FIG. 3. Viability of Con A-stimulated chicken SMC cultured at three different levels of chicken serum ($N = 4$). Error bars represent the SEM. Slopes of the three regression lines were not significant at any time period ($P > 0.10$).

measure this factor directly. It then will be possible to determine the physiological events that modulate the concentration of this naturally occurring immunosuppressive factor, as well as to determine its immunomodulatory activities in both birds and mammals.

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