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A Soluble 61-kDa Protein is Associated with Inhibition of Lectin-Induced Proliferation and IL-2 Synthesis[1] (42575)

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Abstract. An inhibitor of lectin-induced splenocyte proliferation from serum of normal chickens has been characterized. This suppressive factor, found in both serum and plasma and at concentrations as low as 3%, causes a 50% inhibition in proliferative responses to T-cell lectins of autologous and heterologous lymphoid cells. The inhibitor in serum also dramatically suppresses murine IL-2 synthesis, proliferation of murine spleen cells stimulated with PHA, and synthesis of DNA in xenogeneic-transformed mammalian lymphoblastoid cell lines. Serum does not block binding of the lectin to lymphoid cells and the suppressive activity cannot be overcome by any dose of lectin. The inhibitor of DNA synthesis is destroyed by pepsin. $NH_{4(2)}SO_4$ (50%) and TCA (15%) treatments both precipitate the suppressor factor, which further indicates that the suppressive factor is a protein. A 330-fold purification of the inhibitory protein from serum was obtained when boiled serum was passed over a Sepharose 6B and then a DEAE-Sephacel column which was washed at pH 5.0 and eluted with 0.2 *M* NaCl. SDS–PAGE with silver staining revealed a nonreduced protein with an apparent molecular weight of 61 kDa. Less than 2 μg of the protein thus obtained caused a 50% inhibition in the proliferation of chicken lymphoid cells to Con A. The inhibitor of DNA synthesis is therefore not cytotoxic, does not bind to Con A or to mannose or glucose residues on lymphocytes, is acid and heat stable, and is associated with a protein that has a molecular weight of 61 kDa. Since such low concentrations of this naturally occurring, proteinaceous, immunosuppressive factor cause substantial inhibition of IL-2 synthesis and proliferative activity of T cells, this protein may be a very important immunomodulator.

We recently employed a serum-free tissue culture system to study the immunosuppressive role of glucocorticoids on T cell proliferation in chickens (1). While conducting these studies, we fortuitously discovered a factor in normal chicken serum that inhibits the proliferative response of autologous and heterologous splenic mononuclear cells (SMC) to phytohemagglutinin (PHA), concanavalin A (Con A), and pokeweed mitogen. A similar inhibitory activity was also identified in fetal bovine serum, but at three- to fourfold lower concentrations. This serum factor is a potent inhibitor of mitogenesis, for as little as 3% chicken serum causes a 50% reduction in Con A-induced proliferation of autologous SMC.

Immunoregulatory factors that are found in serum may be involved in the etiology of autoimmune diseases and also affect normal responses of lymphoid cells. Recent findings have shown that obese strain chickens, which are prone to develop autoimmune thyroiditis (2, 3), are deficient in a serum factor that suppresses IL-2 activity (4). A deficiency in serum suppressive factor could explain the enhanced T-cell proliferation, heightened IL-2 synthesis, and greater expression of IL-2 receptors that have been recently reported in obese strain chickens (5). Similarly, an IL-2 inhibitor has also been described in the serum of normal, but not nude, mice, and this suppressive factor may be absent in lupus-prone NZB mice (6). This inhibitor is also at least partly responsible for the regulation of cytotoxic T lymphocytes (CTL) *in vivo* (7).

Since chicken serum dramatically inhibits T-cell proliferation, it seemed likely that the factor in normal chicken serum (1) might act by suppressing IL-2 synthesis or activity. In this report, we tested this possibility on murine IL-2-dependent CTL. The dose-dependent

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suppression of mitogen-induced cellular proliferation in both avian and mammalian lymphoid cells by this factor, as well as suppression of DNA synthesis by transformed cell lines, are also described. We were then able to demonstrate that the suppressive activity in serum is associated with protein with an apparent molecular weight of 61 kDa. The presence of such an inhibitory protein in normal chicken sera, and perhaps mammalian sera as well, may be of physiological significance in T-cell regulation of animals.

Materials and Methods. *Animals.* Eight- to twelve-week-old White Leghorn cockerels (Cornell K strain) were used.

Medium. RPMI 1640 powdered medium (Flow Laboratories, Inc., McLean, VA) with L-glutamine was supplemented with sodium bicarbonate (2 mg/ml), 100 units/ml of penicillin, and 100 μg/ml of streptomycin. Cells, Con A (Sigma Chemical Co., St. Louis, MO), and PHA (M-form; GIBCO Laboratories, Grand Island, NY) were diluted in this medium without the addition of heat-inactivated fetal bovine serum.

Preparation of chicken splenocytes. Chicken spleens were removed aseptically and cells were flushed by medium perfusion with a 25-gauge needle. Splenocytes were layered over Ficoll–Hypaque (Sigma; density 1.077) and the tubes were centrifuged at 400*g* for 40 min at 22°C. Cells were collected from the Ficoll–Hypaque–medium interface and washed four times in RPMI 1640. Cell viabilities as judged by trypan exclusion were consistently greater that 90%. Evaluation of Wright–Giemsa-stained, cytospin preparations of these cells showed that they consisted of approximately 95% small lymphocytes.

Serum preparation. Chicken blood was obtained by cardiac puncture and allowed to clot for 1 hr at room temperature, after which it was centrifuged at 500*g* for 10 min. Serum was removed, heat-inactivated at 57°C for 1 hr, and passed through a 0.45-μm filter before use.

Mitogenesis of chicken SMC. Chicken serum was serially diluted in 0.15 *M* PBS (phosphate-buffered saline) in 96-well microtiter plates to a final volume of 100 μl. Control wells received 100 μl of 0.15 *M* PBS instead of chicken serum. Fifty microliters of the appropriate mitogen was then added along with 50 μl of SMC at a concentration of 4×10^7/ml. Duplicate cultures were incubated for 54 hr at 41°C in a 7% CO_2 atmosphere. At 54 hr, all wells were pulsed with 1 μCi of [*methyl*-^{3}H] thymidine (^{3}H-TdR; sp act 6.7 Ci/mmole) and incubated an additional 18 hr. Cultures were then harvested onto glass filter strips using a multiple well cell harvester, scintillation fluid was added, and thymidine incorporation was determined with a Beckman LS 5801 liquid scintillation counter, as previously described (1).

Mitogenesis of murine splenocytes. Spleens from 8- to 9-week-old Balb/c mice were removed aseptically. Cells were obtained by medium perfusion, centrifuged at 400*g* for 10 min, and resuspended in 0.83% NH_4Cl in 0.1% $KHCO_3$/0.01 m*M* EDTA to lyse erythrocytes. After a 1-min incubation, splenocytes were washed four times in RPMI medium and resuspended at 1×10^7 cells/ml in the same medium containing 10% fetal bovine serum. Cells (50 μl) were plated in 96-well microtiter plates with 100 μl of the appropriate dilution of chicken serum and 50 μl of PHA (1:10 final dilution) in serum-free RPMI medium. Splenocytes were incubated for 72 hr at 39°C in 7% CO_2, and pulsing and harvesting of lymphoid cells were performed as described for chicken SMC.

Proliferation of transformed mammalian lymphoblastoid cells. The effect of chicken serum on cellular proliferation of transformed lymphoblastoid cell lines was tested. Transformed cells included the bovine lines EBL-1, BL-3, the IL-2-producing gibbon ape MLA 144 line, and an IL-2-dependent CTL line (9). The EBL-1 cell line is T-cell derived and was established from a lymph node tumor of an adult cow with lymphosarcoma (10). The BL-3 cell line originated from a bovine lymphosarcoma of uncertain origin (10). These cells are of the B-cell lineage and produce bovine leukemia virus, which is similar in structure to HTLV-1 (11). The MLA 144 line is an IL-2-secreting T-cell line established from a gibbon with spontaneous lymphosarcoma (12).

Chicken serum was diluted 1:2 in 0.15 *M* PBS and 100 μl of this preparation was serially diluted in 96-well microtiter plates. One-hundred microliters of lymphoblastoid cells (3×10^6/ml) in serum-free RPMI 1640 was then added to appropriate wells. IL-2-dependent CTL were cultured in 5% fetal bovine serum. Fifty microliters of CTL (5×10^5/ml),

along with 50 μl of IL-2-containing supernatant that was produced in RPMI 1640 from Con A-activated rat splenocytes (8), were pipetted into appropriate wells. Cultures were incubated for 54 hr at 39°C in 7% CO_2. Procedures for culturing the cells, pulsing with ^{3}H-TdR, harvesting, and counting were identical to those described for murine splenocytes.

IL-2 production and assay. Murine IL-2 was prepared and tested on CTL, as previously described (8). These CTL were derived from C57BL mice immunized against L-1210 tumor cells. In brief, 400 μl of mouse splenocytes at an initial concentration of 10^7 cells/ml in RPMI 1640 was added to 24-well plates. Six hundred microliters of the appropriate dilution of chicken serum, followed by 200 μl of RPMI 1640 or 200 μl of 10 μg/ml Con A in RPMI 1640, was then added to the wells for a final volume of 1.2 ml. Cultures were incubated at 39°C and 7% CO_2 for 48 hr, at which time the plates were centrifuged at 500*g* for 10 min and the supernatants were collected. IL-2 activity was assayed by culturing 100 μl of supernatants, 50 μl of CTL (5×10^5/ml) in medium containing 10% fetal bovine serum, and 50 μl of PBS containing various amounts of chicken serum for 48 hr (39°C; 7% CO_2), followed by an 18-hr pulse with ^{3}H-TdR. This approach ensured that all supernatants were assayed in the presence of 20% chicken serum, which permitted a direct comparison of the effects of chicken serum on synthesis of IL-2 rather than on IL-2 activity.

FITC–Con A binding. Chicken SMC were preincubated for 4 hr at 4°C with or without Con A (5 μg/ml). Cells were then washed and incubated for 1 hr with an excess of FITC–Con A (20 μg/ml), with or without chicken serum (25%). Cells were washed and the percentage of FITC–Con A binding was determined with a Zeiss Photomicroscope III equipped with dichromatic beam splitters for epifluorescence, an exciter–barrier filter and reflector combination for FITC, a high-pressure 100 W mercury lamp power supply, and phase contrast planapochromat 63X/1.4 N.A. oil and 100X/1.25 N.A. oil objectives.

Protein purification techniques. Precipitation with 15% TCA was accomplished by incubation with serum for 45 min and centrifugation at 500*g* for 10 min. The supernatant was then dialyzed and tested on a Con A-induced chicken SMC bioassay.

Ammonium sulfate fractionation was performed by adding saturated $NH_{4(2)}SO_4$ drop by drop with stirring at 4°C to an equal volume of serum. After 30 min of equilibration, serum was centrifuged at 5000*g* for 1 hr (4°C), the supernatant removed, and the pellet reconstituted in 0.15 *M* PBS and dialyzed for testing in the avian mitogenesis bioassay.

Column chromatography was carried out on 210 ml of serum (6720 mg protein) that was held at 100°C for 5 min. The serum was centrifuged at 500*g* for 10 min and then dialyzed 3 times in 12 kDa exclusion dialysis tubing against 20 vol of 0.15 *M* PBS. The boiled serum (554 mg protein) was concentrated to a volume of 15 ml and was pumped at a rate of 15 ml/hr over a 100 × 2.6-cm column containing Sepharose 6B equilibrated in 0.15 *M* PBS, pH 7.4 maintained at 4°C. Five-milliliter fractions were collected in an LKB 2070 Ultrorac II fraction collector, as previously described (13). All tubes were read in a Gilford Response spectrophotometer at an OD of 280. Three peaks of protein were routinely obtained after Sepharose 6B chromatography, but only the third lowest molecular weight peak contained the inhibitory component. Protein in this fraction (36 mg) was dialyzed in Tris–HCl buffer, pH 7.4, and passed over a DEAE-Sephacel column maintained at room temperature (100 ml/hr) that was equilibrated with the same buffer. This column was eluted with a continuous gradient of NaCl (0 to 1.0 *M*), and 3-ml fractions were collected. The 100 ml that eluted between 0.1 and 0.2 *M* NaCl (12 mg protein) was dialyzed in Tris–HCl buffer, pH 7.4, and passed once again over the washed and equilibrated DEAE-Sephacel column. The column was then washed with a continuous pH gradient (pH 7.4–5.5) with Tris–HCl. The column was re-equilibrated to pH 7.4 and eluted with 0.1 *M* NaCl. The inhibitory factor was then eluted with 0.2 *M* NaCl (0.2 mg protein). Amount of protein in appropriate fractions was measured by a modification of the method of Lowry (14) using bovine serum albumin as the standard.

SDS–PAGE procedures utilized an LKB vertical gel electrophoresis unit. Nonreduced protein was diluted in sample buffer (0.03 *M* Tris–HCl, 4% SDS and 40% glycerol) and applied to 10% polyacrylamide gels with a 3.5% stacking gel according to a modification of

Laemmli's techniques (15). A 0.025 *M* Tris–0.2 *M* glycine buffer, pH 8.3, was used as the running buffer and bands were visualized with silver stain. A Zeineh soft laser scanning densitometer (Fullerton, CA; Model SL-TRFF) was used to quantify the proportion of protein.

Statistical analyses. Experiments were analyzed using a randomized complete block design in which individual birds were designated as blocks, and Duncan's new multiple range test was utilized to detect treatment differences.

Results. *Heat-inactivated autologous chicken serum inhibits Con A-induced proliferation of SMC.* When stimulated with Con A, chicken SMC proliferate well in the absence of exogenous serum factors (Fig. 1). When as little as 3% heat-inactivated normal chicken serum was added to the culture, there was greater than a 50% reduction in the uptake of ^{3}H-TdR ($P < .01$), and even more inhibition was observed with greater concentrations of chicken serum (Fig. 1). Another report demonstrated that this inhibition of SMC proliferation by autologous serum could not be explained by serum glucocorticoids, lysis of SMC, or competitive inhibition with thymidine (1).

Heat-inactivated plasma inhibits Con A-induced proliferation of SMC. To test the possibility that the inhibitory factor was released into serum during the process of clotting, blood from six different chickens was collected into an anticoagulant, quickly centrifuged to remove platelets and erythrocytes, and then kept in heparin to prevent further clotting. Plasma was heat-inactivated, passed through a 0.45 micron filter and tested on heterologous SMC. In the absence of plasma, average proliferation of SMC was 295,036 ± 9169 cpm (mean ± SEM). When 6% plasma from each of the six chickens was added to the SMC cultures, ^{3}H-TdR uptake averaged 52,685 ± 7261 cpm. At a concentration of 25%, proliferation of SMC was reduced even further (13,722 ± 2326 cpm). These data indicate that potent inhibitory activity is found in plasma as well as serum, and therefore the inhibitory factor is not formed upon clotting of blood.

Effect of chicken serum at different doses of Con A and PHA. The inhibitory effect of chicken serum on synthesis of DNA was not due to an artifact of shifting the dose–response curve to PHA and Con A (Figs. 2 and 3). If the dose of mitogen was sufficient to yield a proliferative response, heat-inactivated

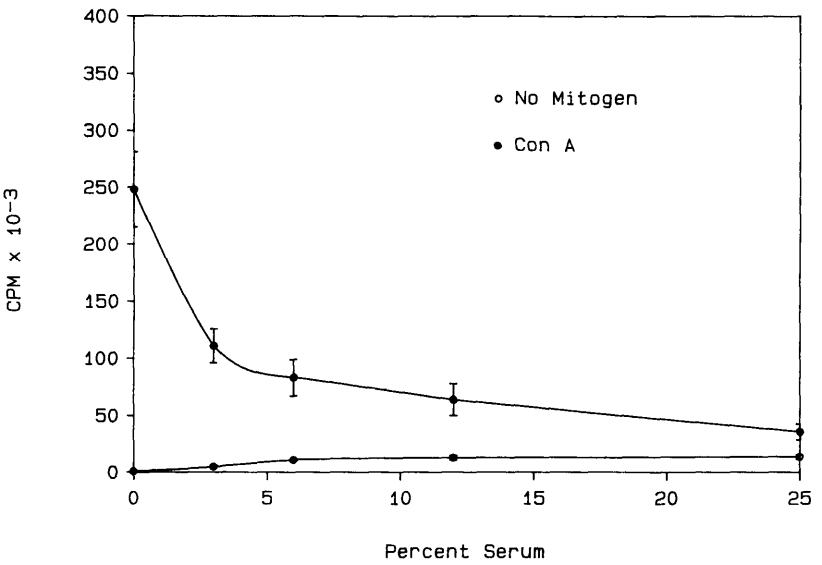

FIG. 1. Heat-inactivated chicken serum inhibits Con A-induced proliferation of autologous SMC. SMC were cultured at 2×10^6 cells/well in the presence or absence of an optimal concentration of Con A and with serial dilutions of chicken serum. After a 54-hr incubation (41°C, 7% CO_2), each well received 1 μCi of ^{3}H-TdR and cells were harvested 18 hr later. Chicken serum inhibited mitogen-induced proliferation of splenocytes in a dose-dependent manner. Error bars represent the SEM ($N = 4$).

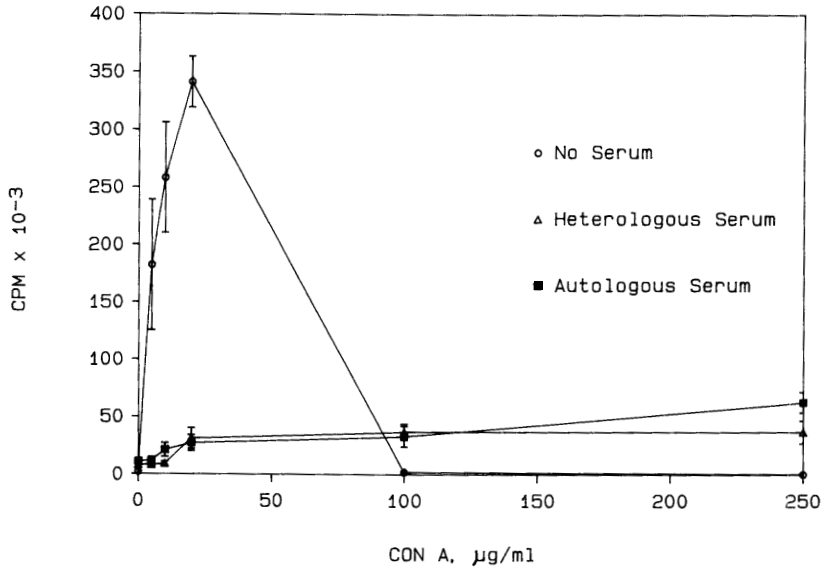

FIG. 2. Dose–response curve of SMC cultured in the presence of 10% autologous or heterologous serum. Both heterologous and autologous sera suppressed Con A-induced mitogenesis at all stimulatory doses of lectin. Error bars represent the SEM ($N = 4$).

chicken serum was able to significantly inhibit this proliferation ($P < 0.01$). Data in Figs. 2 and 3 also demonstrate that chicken serum was equally inhibitory for heterologous and autologous SMC at all concentrations of lectin.

Failure of chicken serum to block Con A binding to SMC. The possibility that serum was blocking mitogen binding was explored. Preincubation of SMC with Con A, followed by washing and addition of FITC–Con A,

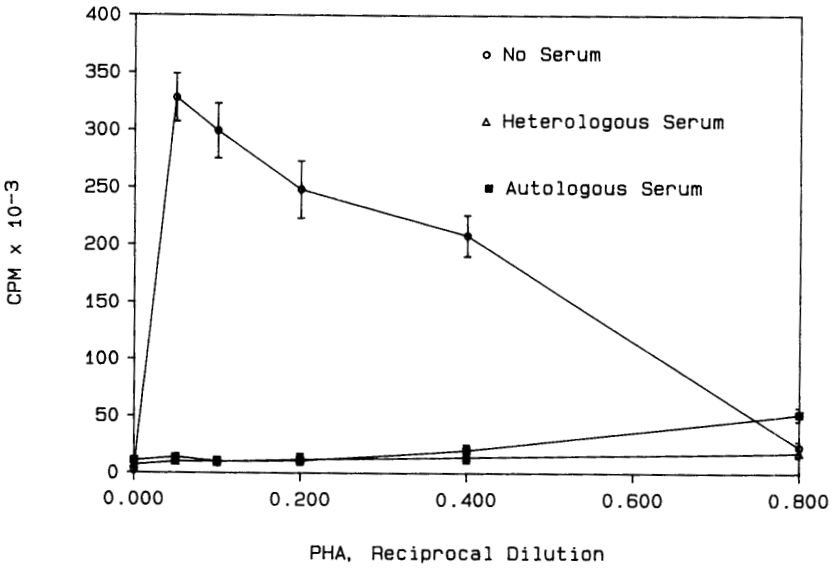

FIG. 3. PHA dose–response curve. SMC cultured in 10% autologous or heterologous sera with different dilutions of PHA showed reduced proliferation at all stimulatory doses of lectin. Error bars indicate SEM ($N = 4$).

produced significantly less fluorescence than cells not preincubated with Con A (5% positive cells vs 24% positive cells, Table I). However, there was no difference in binding to SMC when FITC–Con A was added in the presence of serum. Therefore, although the unlabeled Con A competed for Con A binding sites with FITC–Con A, the suppressive factor in chicken serum did not.

The use of FITC–Con A suggested that chicken serum did not inhibit the binding of Con A to SMC. This possibility was further tested in proliferation assays. Mills *et al.* (16) recently demonstrated that a short, 1-hr incubation with PHA was sufficient to induce proliferative activity in human T lymphocytes, and the mitogenic response of cells incubated for 1 hr with PHA was 80 to 100% of cells incubated in PHA for the entire 72-hr culture period. We adopted this approach to determine whether serum could inhibit SMC proliferation after Con A incubation. As shown in Table II, chicken SMC (2×10^6) that were preincubated with 50 μl Con A (20 μg/ml), washed, and incubated with or without serum were significantly suppressed when serum was added at the beginning of the culture (55,000 cpm vs 18,000 cpm). These results show that serum can exert its immunosuppressive effect even after lymphoid cells have been activated by a short pulse with mitogen, which supports the conclusion that serum does not block the binding of Con A to SMC.

TABLE I. CHICKEN SERUM DOES NOT INHIBIT CON A BINDING TO AUTOLOGOUS SMC

Preincubation[a]	Number	FITC-Con A added[b]	% FITC$^+$ cells[c]
RPMI	4	With serum	19 ± 5[d]
RPMI	4	Without serum	24 ± 4[d]
Con A 5	4	With serum	8 ± 4
Con A 5	4	Without serum	5 ± 1

[a] Avian SMC were preincubated for 4 hr at 4°C in RPMI or Con A at 5 μg/ml final concentration.

[b] After preincubation, cells were washed and incubated in excess FITC–Con A (20 μg/ml final concentration) with or without serum.

[c] Percentage FITC$^+$ cells was visually assessed using a fluorescent microscope, data expressed as means ± SEM.

[d] Fluorescence of SMC cultured with RPMI is greater ($P < 0.05$) than that of SMC cultured with Con A.

TABLE II. SERUM INHIBITS PROLIFERATIVE ACTIVITY OF SMC AFTER ACTIVATION WITH CON A

Four-hour preincubation with Con A[a]	Number	Treatment	^{3}H-TdR incorporation, cpm $\times 10^{-3}$
–	3	RPMI[b]	1 ± 0.1
		serum	6 ± 1.7
+	3	RPMI	55 ± 10[c]
		serum	18 ± 8[d]

[a] Chicken SMC were preincubated for 4 hr at 4°C with or without mitogen, in the absence of serum.

[b] At 4 hr, cells were washed and incubated at 41°C, 7% CO_2, in the absence of additional mitogen, with 25% serum or RPMI. Cells were harvested at 72 hr.

[c,d] Means are different ($P < 0.05$).

Data are expressed as means ± SEM.

Chicken serum inhibits mitogenesis of murine splenocytes. Murine splenocytes were cultured with PHA or RPMI 1640 in the presence of varying dilutions of chicken serum. As observed in both the autologous and heterologous avian systems, heat-inactivated chicken serum significantly inhibited the uptake of ^{3}H-TdR by PHA-stimulated murine splenocytes in a dose-dependent manner (Fig. 4). At a concentration of 40% chicken serum, lectin-induced proliferation of murine splenocytes was reduced by more than 90%.

Suppression of IL 2 production by murine splenocytes. One mechanism of action of chicken serum on cell proliferation is suggested by data in Fig. 5. Production of IL 2 by murine splenocytes was inhibited ($P < 0.01$) in a dose-dependent manner by chicken serum, leading to almost total inhibition at a dose level of 40% serum. This inhibition of proliferative activity in CTL was not due to a direct effect of chicken serum on CTL because the IL-2 assay was conducted in the presence of identical concentrations of chicken serum (20%) in all wells.

Inhibitory effects on transformed lymphoblastoid lines. The capability of chicken serum to inhibit proliferation of transformed bovine and gibbon ape lymphoid cell lines is shown in Fig. 6, in which greater than 95% suppression of ^{3}H-TdR uptake was observed in all cell lines when 10% chicken serum was added to these cell cultures. This experiment also provides further evidence that growth suppression of normal lymphoid cells is not a result of

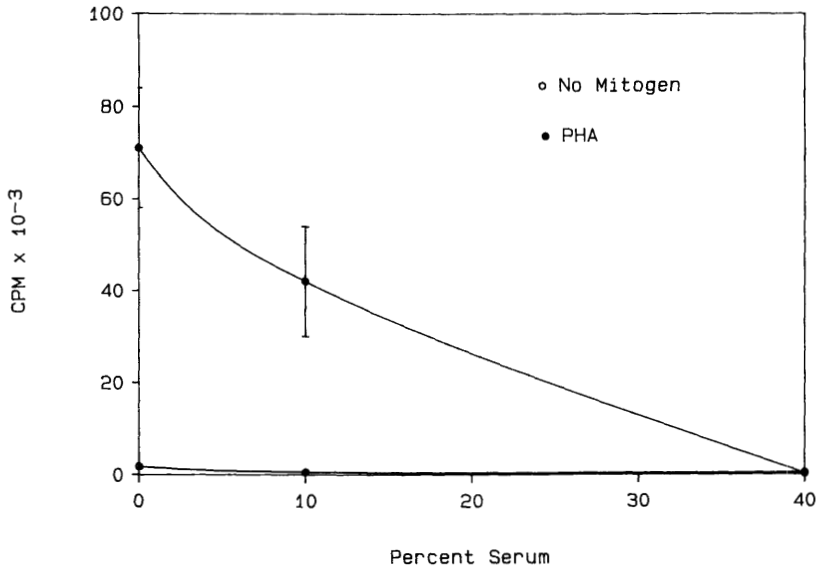

FIG. 4. Chicken serum suppresses PHA blastogenesis of murine splenocytes. Murine splenocytes were cultured with PHA and increasing concentrations of chicken serum for 72 hr at 39°C and 7% CO_2. Inhibition of mitogenesis by chicken serum was dose-dependent, with greater than 90% inhibition in the presence of 40% chicken serum. Error bars represent the SEM ($N = 4$).

blockage of mitogen binding. CTL, when cultured in the presence of exogenous IL-2, were resistant to the suppressive effects of chicken serum. Chicken serum even caused a moderate increase in proliferation of these cells at concentrations of less than 20%.

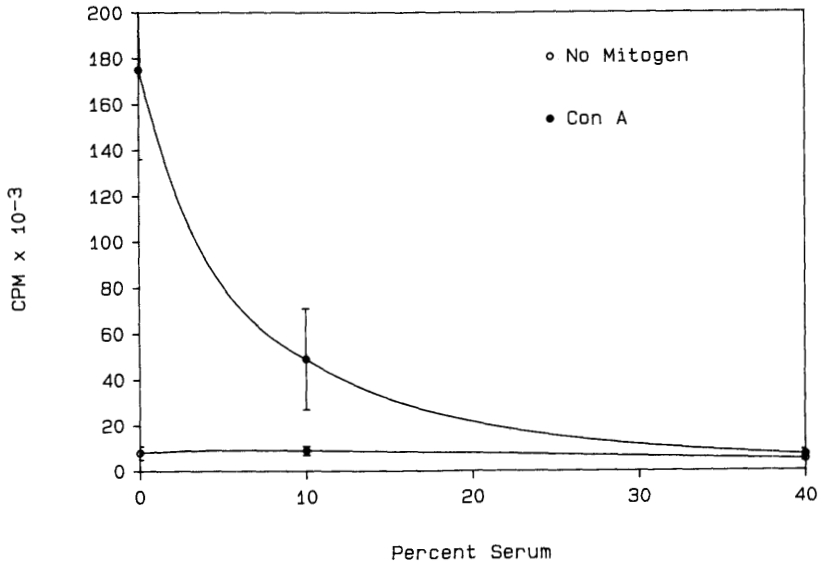

FIG. 5. Murine IL-2 production is suppressed by chicken serum. Mouse splenocytes were cultured in RPMI 1640, with or without Con A (1.67 μg/ml final concentration), in varying dilutions of chicken serum for 48 hr (39°C, 7% CO_2). Supernatants were harvested at 48 hr and tested on an IL-2-dependent CTL line (*9*) in the presence of a constant amount (20%) of chicken serum. Chicken serum inhibited IL-2 production by murine splenocytes in a dose-dependent manner. Error bars represent the SEM ($N = 4$).

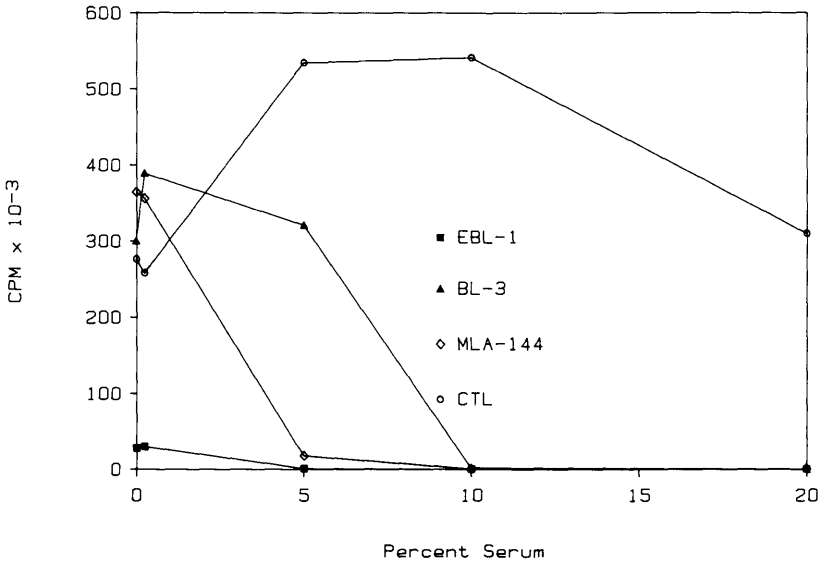

FIG. 6. DNA synthesis by transformed mammalian lymphoblastoid cell lines is inhibited by chicken serum. Bovine lines EBL-1 and BL-3, the gibbon ape IL-2-secreting T cell line (MLA-144) (3×10^5 cells/culture) and an IL-2-dependent CTL line (2.5×10^4 cells/culture) were cultured with different dilutions of chicken serum (0–20% total in culture) for 54 hr (39°C; 7% CO_2), and pulsed with 1 μCi ^{3}H-TdR for 18 hr. Exogenous IL-2 of rat splenocyte origin was added to CTL cells. Chicken serum enhanced proliferation of the CTL line at concentrations below 10%, and proliferation returned to normal at 20% serum. Chicken serum suppressed growth of all other transformed cell lines tested.

Biochemical characterization of inhibitory factor. Several techniques were used for preliminary characterization of the suppressive factor in chicken serum. Incubation of chicken serum with HCl (pH 2.0) at room temperature, followed by dialysis 3 times against 20 vol of 0.15 *M* PBS (pH 7.4) did not destroy biological activity of the suppressive factor. At a 25% level in culture, such acid-treated serum caused a 92% reduction in Con A-induced mitogenesis of chicken SMC. Chicken serum that was boiled for 5 min at 100°C also caused a 92% reduction of chicken SMC mitogenesis in the presence of Con A. Passage over a cibacron-blue column, followed by elution with 1 *M* NaCl, resulted in only 7% suppressive activity in the eluant. This finding indicated that the suppressive activity was not due to serum albumin. A Con A affinity column failed to remove the inhibitory factor, which suggested that the inhibitor did not contain adequate mannose or glucose to permit its isolation via sugar residues. A similar conclusion can be made from data in Table I. Fractionation with $NH_{4(2)}SO_4$ (50%) precipitated the inhibitory component. No inhibitory activity remained after serum was precipitated with TCA, suggesting protein or protein-coupled properties. The proteinaceous character of the inhibitory factor was confirmed by treatment with pepsin (Fig. 7), which resulted in complete loss of inhibitory activity.

Column chromatography. The best purification of suppressive activity was obtained when boiled serum was subjected to a variety of chromatography columns as described under Materials and Methods. Table III summarizes the efficiency of purification and biological activity of the protein during fractionation. Appropriate chromatography fractions of nonreduced protein were subjected to SDS-PAGE as described under Materials and Methods. A single protein (apparent mol wt 61 kDa) comprising about 90% of the total protein applied to the lane was obtained from the boiled serum fraction after separation on a Sepharose 6B column and passage over a DEAE-Sephacel column that was pH washed and then eluted with a 0.1–0.2 *M* NaCl buffer (Fig. 8). The protein was purified by a factor

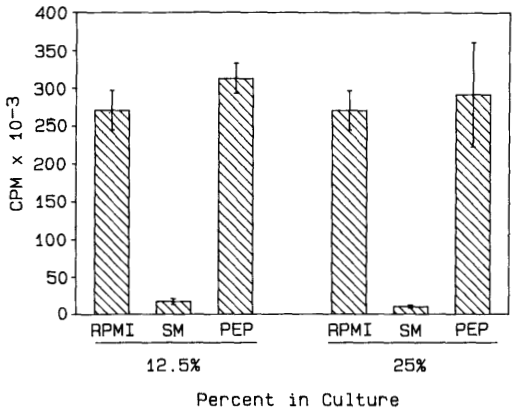

FIG. 7. Pepsin destroys inhibitory activity in chicken serum. Pooled chicken serum was incubated for 24 hr with pepsin (2.5 μg/ml; 37°C). Pepsin-treated serum (PEP), pooled serum that had not been treated (starting material; SM), or RPMI 1640 were incubated at concentrations of 12.5 and 25% with 2×10^6 Con A-stimulated (5 μg/ml final concentration) chicken SMC. Cells were cultured for 56 hr (41°C; 7% CO_2), followed by an 18 hr pulse with 1 μCi ^{3}H-TdR. Nonenzyme-treated serum caused greater than 90% suppression of mitogenesis, while pepsin-treated serum had no effect. Error bars represent the SEM ($N = 4$).

of 336-fold from serum and produced an inhibition of Con A-induced proliferation of chicken SMC by approximately 50% at less than 2 μg/10^6 cells. The amount needed to cause 50% inhibition is probably even smaller, because the concentration of purified protein was slightly less than the lower limit of our Lowry assay (4 μg).

Discussion. This report describes the characterization of an inhibitor of DNA synthesis that is normally found in chicken serum. Suppressive activity is associated with a protein with an apparent molecular weight of 61 kDa that causes a 50% inhibition in lectin-induced proliferation of lymphoid cells at concentrations of less than 2 μg per 10^6 SMC. The suppressive factor is effective on both autologous and heterologous SMC, and it also inhibits the synthesis of IL-2 in murine lymphoid cells. Earlier findings have clearly shown that this factor is not cytotoxic to chicken SMC (1). Inhibition of proliferation of autologous SMC suggests that this factor plays an important role in T-cell-mediated immune events *in vivo*. Furthermore, since this proteinaceous factor exhibits suppressive effects on a wide variety of both normal and transformed lymphoid cells at very low concentrations, it may also be of potential clinical usefulness.

Preliminary characterization of the suppressive factor suggests that the normal serum suppressor that is deficient in obese strain chickens is not the suppressive factor that we have isolated. This conclusion is based on the finding that chicken serum did not inhibit the proliferation of CTL that were cultured with exogenous IL-2, which contrasts with the IL-2 inhibitor that is found in both normal chick-

TABLE III. PURIFICATION AND BIOLOGICAL ACTIVITY OF SERUM FRACTIONS

Fraction	Total protein (mg)	μg Protein/ 10^6 cells[a]	Biological activity[b]	Units activity/ μg protein[c]	Percent purification[d]
Whole serum	6720	800	55	0.07	—
Boiled serum	554	192	85	0.4	6
Boiled Seph 6B	36	25	99	4.0	56
6B DEAE NaCl	12	6	63	10.5	150
6B DEAE pH NaCl	<0.2	<2	47	23.5	336

[a] Fractions containing the indicated μg of protein were added to 10^6 chicken SMC in culture.

[b] Biological activity indicates percent suppression of Con A-induced mitogenesis when compared to control cultures containing no serum or serum fractions.

[c] Units of activity were derived by dividing biological activity by μg protein/10^6 cells.

[d] Percent purification was calculated by dividing units of activity of all serum fractions by whole serum activity units (0.07).

Note. Fractions tested included whole serum; boiled serum; peak 3 obtained after passing boiled serum over a Sepharose 6B column; peak 2 of a gradient salt elution of a DEAE-Sephacel column over which peak 3 of the Sepharose 6B column was passed; and a 0.2 *M* NaCl elution of a DEAE-Sephacel column over which was passed peak 3 of the Sepharose 6B column, followed by a pH gradient wash (pH 7.4–5.5) in Tris-HCl before salt elution.

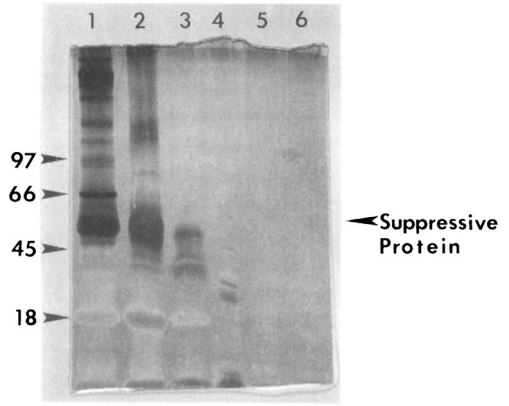

FIG. 8. SDS–PAGE analysis (nonreduced) of the putative suppressive protein in chicken serum. Band in lane 6 comprises 87% of the protein in that lane as determined by laser densitometry. Lane designations are as follows: (1) whole chicken serum (3.2 μg); (2) boiled (100°C, 5 min) whole serum (5.6 μg); (3) boiled serum after Sepharose 6B chromatography, peak 3 (2.2 μg); (4) material from lane 3 after passage over a DEAE-Sephacel column and elution between 0.1 and 0.2 *M* NaCl (3.6 μg); (5) material from lane 3 after passage over a DEAE-Sephacel column which was then washed with a continuous pH gradient (pH 7.4–5.5), re-equilibrated to pH 7.4, and eluted with 0.1–0.2 *M* NaCl (7.2 μg); and (6) same as lane 5, 2× (14.4 μg). Apparent molecular weight of the suppressive protein is 61 kDa which can be easily visualized after boiled serum is passed through a Sepharose 6B column.

ens (5) and mice (6, 7). Furthermore, the inhibitor of DNA synthesis is associated with a protein with a putative molecular weight of 61 kDa, whereas the molecular weight of the IL-2 inhibitor in chickens is less than 10 kDa and in mice it is 50 kDa (6) and 12 kDa (17). It therefore appears that normal chicken serum contains a protein that inhibits DNA synthesis and is neither antigen specific nor H-2 restricted. This protein inhibitor is similar to the nonspecific inhibitor of IL-2 activity that is secreted by Ly-2^+ cells and is also found in normal mouse serum (6, 17, 18). However, it is unknown whether this inhibitor of DNA synthesis is also deficient in obese strain and aged chickens, as is true for the inhibitor of IL-2 activity.

Origin of the chicken serum suppressor protein is unknown; however, production by immune cells is possible. Martin and Glick (19) reported the synthesis by *Mycobacterium tuberculosis*-activated T and B chicken lymphocytes of a 10- to 50-kDa inhibitor of DNA synthesis in peripheral blood leukocytes. However, this factor was not found in the serum. In mice, Fujiwara and Ellner (20) reported the production by the macrophage-like cell line (U937) of an 85-kDa factor that suppressed IL-1-, IL-2-, and PHA-induced blastogenesis of murine thymocytes. The factor was precipitable by $NH_{4(2)}SO_4$, resistant to freeze-thawing, sensitive to pH 2.5, and only partially inactivated by heat treatment. Horowitz *et al.* (21) also observed that the failure of a cloned line of helper T cells to respond to IL-2 was a result of the production by the same cells of an inhibitory substance. Okai (22) recently reported a factor that inhibited the growth of murine splenocytes and thymocytes and some transformed cell lines. The serum component was a noncytotoxic protein that acted early in the cell cycle and was heat-, freeze-thaw-, and pH-stable. The molecular weight as determined by gel filtration was approximately 100 kDa. The similarities of this inhibitory component of fetal bovine serum to the protein that is associated with inhibitory activity in chicken serum, as well as the abilities of both factors to inhibit the growth of xenogeneic cells, suggest a highly conserved protein that may have important immunoregulatory functions *in vivo*.

Lipoproteins (23, 24), α-globulins (25, 26), β-globulins (27), gangliosides (28), fatty acids (29, 30), and a variety of other poorly characterized substances (31–35) also inhibit DNA synthesis, but very few of these inhibitory compounds have been purified. Identity of the apparent 61-kDa protein described in this paper that is associated with inhibiting DNA synthesis is unknown. It may be a lipoprotein, but this is somewhat unlikely because the protein is quite hydrophilic (easily dissolved in distilled water, PBS, and RPMI medium). It could be a glycoprotein because it is apparently secreted into blood, although it does not readily bind to a Con A affinity column under the conditions that we employed. In reports that used normal rather than heat-inactivated serum, the inhibition might be explained by thymidine phosphorlyase catabolizing ^{3}H-TdR to unincorporable [^{3}H]thymine (36). However, this explanation is unlikely in our experiments (1) because thymidine phosphorylase is destroyed by heat inactivation, and we

boiled serum to isolate that inhibitory protein. This protein that is associated with inhibitory activity is similar in size to chicken albumin (65 kDa). However, passage of serum over a cibacron-blue column failed to remove the inhibitory component, which suggests a lack of association between inhibitor activity and serum albumin. There is also no evidence to show that albumin inhibits the proliferation of autologous lymphocytes. Another similar-sized protein in serum is corticosteroid binding globulin (63 kDa). However, recent experiments have shown that this protein also does not suppress proliferative responses of lymphocytes (37, 38), so it is unlikely that the inhibitor of DNA synthesis reported in this paper is corticosteroid-binding globulin.

Total abolition of inhibitor activity by pepsin clearly indicates that the suppressive factor is a protein. Definitive proof that the 61-kDa protein that we have identified is the true inhibitor of DNA synthesis will require that this protein be eluted from the SDS–PAGE gel and exhibit inhibitory activity on SMC. However, association of substantial inhibitory activity with a single protein on silver-stained, polyacrylamide gels can now lead to development of antibodies and perhaps amino-end sequencing of the protein which are needed to more completely assess the biochemical and immunosuppressive activities of the naturally occurring protein.

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