Identification of Albumin as the Serum Factor Essential for the Growth of Activated Human Lymphocytes*

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Albumin from human, bovine, or rabbit serum supported the growth of concanavalin A-stimulated human thymus-derived lymphocytes equally well. This activity was completely abolished by pepsin digestion. It was shown for bovine serum albumin that the albumin molecule itself, and neither an impurity nor a factor bound to albumin was essential for the growth of lymphocytes. This conclusion was based on observations that the growth-promoting activity could not be removed from albumin, and that the specific activity of albumin remained unaltered after the following procedures: molecular sieving at pH 7.5, at pH 3.0, and in 8 M urea at pH 6.6; ion exchange chromatography at pH 4.3 and in 8 M urea at pH 7.2; isoelectric focusing; charcoal treatment; acetone precipitation; and reduction with 2-mercaptoethanol in the presence of 8 M urea. Dimeric albumin was found to support growth of lymphocytes as well as monomeric albumin, and mercaptalbumin and non-mercaptalbumin were shown to have equal activity.

Thymus-derived (T) lymphocytes can be stimulated by antigen (1) or by lectins (2) to grow, i.e. to synthesize DNA and to multiply in vitro when cultured in Eagle’s basal medium supplemented with serum or plasma. Mitogenesis and proliferation of lymphocytes is an essential part of the immune response (3). Antigens stimulate only the small percentage of cells which possess specific binding sites for the particular antigen. Lectins such as concanavalin A or phytohemagglutinin, which bind to most cell surfaces, when present at low concentration, stimulate nonspecifically a large proportion of lymphocytes (4). Because it is believed that the mechanism of stimulation by antigen and by lectins is similar (4-7), lectins are frequently used as a convenient tool to study mitogenesis and growth of lymphocytes. Most cells require the presence of serum or plasma in the culture medium for in vitro growth. In only recent years have several specific factors promoting growth of different mammalian cells been partially purified from serum or from tissue (8-11).

Recently, it has been shown in this laboratory (12, 13) that Cohn Fraction V or crystallized serum albumin is essential and can completely replace serum or plasma for the growth of lymphocytes whether stimulated by antigen or by lectins. In the presence of the other plasma proteins fractionated according to Cohn, single or combined, no growth was observed. The effect of serum albumin was unaltered if a fatty acid free preparation was employed. Albumin could not be replaced by hormones, and its effect could not be altered by the addition of hormones, which are known to be carried by serum albumin as well as by other plasma proteins.

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The amino acid sequence of bovine and of human serum albumin has been reported recently, (14, 15). Both proteins consist of a single polypeptide chain. However, this does not exclude the possibility that albumin preparations contain small amounts of other proteins as impurities. For example, a proteolytic enzyme present in very small amounts has recently been isolated from bovine serum albumin (16). It can also not be excluded that a small fraction of the albumin has a factor other than fatty acids bound (covalently or noncovalently). It is well documented that serum albumin has an exceptional ability to bind other molecules such as fatty acids, bilirubin, some hormones, drugs (17), and prostaglandins (18). It was therefore of interest to know whether growth stimulation of activated lymphocytes is caused by albumin itself or by another molecule that is either present as an impurity with albumin or bound to albumin.

In this report we present data which suggest that the albumin molecule itself is required for the growth of concanavalin A-activated human blood lymphocytes.
components. The medium was made 20 mM with Hepes to maintain the pH between 7.5 and 7.5 during the incubation period. All solutions used were sterilized either by autoclaving or by passing through a Millipore filter (HA 0.45 μm). Medium was stored in the dark at 4°.

Cells and Cultures—The preparation of cultures of human blood lymphocytes has been described in detail (13). Briefly, lymphocytes were isolated from human blood and were incubated in medium containing 18% to 24% of concanavalin A, 6 to 8 x 10^6 cells were then inoculated into culture tubes (plastic-stoppered tubes, 3.5 ml volume: Nalgel 3110–35) already filled with medium containing the desired amount of serum albumin to a final volume of 3 ml. The cultures were incubated at 37° for 72 hours. [3H]Thymidine (2 μCi/ml) was added for the last 24 hours. The incorporation of label into DNA was determined after precipitation of the macromolecules with 10% trichloroacetic acid at 4° (20).

Determination of Specific Growth Activity of Bovine Serum Albumin—For quantitation of the growth-supporting activity of bovine serum albumin treated by various methods, the sample to be tested was assayed at 4 to 5 concentrations (between 0.1 and 0.5 mg/ml), and the curve obtained was compared with a calibration curve of untreated albumin at the same concentrations, obtained simultaneously (13). Data for untreated albumin could be reproducible within a limit of error of ±20%. Data are expressed as specific activity, i.e. activity per unit of weight of albumin in percentage of control. All albumin solutions, obtained after various treatments, were dialyzed against Hank's balanced salt solution and concentrated by ultrafiltration over an Amicon membrane PM 30 (retaining molecules of molecular weight > 10,000) since these pools could contain small proteins. The solutions were then passed through a Millipore filter (HA 0.45 μm), and the concentrations were determined from the absorption at 270 nm, using the value of 0.687 for 1 mg of bovine serum albumin/ml (21). The albumin solutions were kept frozen, and before use were diluted with Eagle's basal medium to the desired concentrations before the cells were added.

Miscellaneous Methods—Acetone precipitation of albumin was carried out by adding acetone to a final concentration of 70% to a solution of 25 mg of albumin/ml of water. The solution was left for 15 hours at pH 5.0 and 4°. The precipitate formed was dissolved and dialyzed, followed by centrifugation at 100,000 x g for 30 min. Albumin was digested with pepsin at a concentration of 1 to 2% of the total protein for 1 hour at 37° at pH 2.6, followed by neutralization to pH 7.0. A control sample was treated in the same manner but without pepsin. Reactive thiol was then measured according to the method of Ellman (22). Ellman's reagent was added to solutions containing about 2 mg of albumin/ml in 0.05 M Tris·HCl, pH 8, to a final concentration of 0.1 M (10-fold molar excess over albumin). The molecular weight of 66,000 for albumin (14) was used for the calculations.

Column Chromatography—Resins (Sephadex G-150, DEAE-Sephadex A-50, SP-Sephadex C-50, and Sepharose 6B) were prepared as outlined by Pharmacia. For chromatography at pH 3 and ion exchange chromatography the protein was dialyzed against the buffer used for preparation of the column for at least 6 hours. Further details are given in the figure legends.

Isoelectric Focusing—Isoelectric focusing was performed with 1% ampholytes, pH 3 to 6, in an LKB column (8100–1) with a linear sucrose density gradient according to the instruction manual of LKB. Thirty mg of albumin, from which fatty acids had been removed with charcoal and the dimer had been removed by chromatography on Sephadex G-150, was introduced with the less dense solution. Total focusing time was 48 hours at a final voltage of 800 V for the last 25 to 30 hours. Fractions of 2 ml were collected. The absorption at 270 nm and the pH were measured. The fractions were pooled as needed and dialyzed to remove ampholytes and sucrose.

Experiments in Urea—All experiments with urea were performed at room temperature. Cyanate was removed from urea by filtering a 9 or 10 M solution through a mixed bed ion exchange resin AG 501–X8 on a Buchner funnel. Salt and buffer were then added, and the concentration of urea was adjusted to 8 M. Albumin was dissolved in buffer without urea, and the solution was diluted 3- to 5-fold with buffered urea and dialyzed for 12 to 20 hours against the urea buffer.

RESULTS

Growth-promoting Effect of Serum Albumin of Different Species—A comparison of the growth stimulatory effect on concanavalin A-activated human lymphocytes of human plasma with human serum albumin has been presented in our previous publication (13). The data suggested that albumin is the only factor present in serum or plasma which affects growth of activated lymphocytes. The growth-promoting effect of serum albumin from three different species, human, bovine, and rabbit, as a function of their concentration in the culture medium is shown in Fig. 1. At low albumin concentrations, the effect of various albumins and of plasma was the same, suggesting lack of species specificity. The effects at high concentrations differed. However, these differences varied from experiment to another, and can probably be explained as artifacts, since growth in the presence of albumin was considerably more sensitive than in the presence of plasma. This has been discussed at length in our previous publication (13).

Various Treatments of Albumin—Bovine serum albumin, after various treatments, was assayed at low concentrations for growth stimulation of concanavalin A-treated lymphocytes as described under "Experimental Procedure." It was shown earlier (13) that fatty acid-free albumin (Sigma) also promotes growth of lymphocytes. No growth-promoting activity could be removed by precipitation of albumin with 70% acetone, indicating that no dissociable acetone-soluble molecule is involved in the growth effect (Table I).

The activity of albumin, heated for 30 min at 65°, remained unaltered. After exposure to pepsin, however, all growth
activity was completely abolished, suggesting the active factor to be a protein or peptide (Table I).

Extensive dialysis at pH 7 or at pH 3 did not alter the growth-promoting activity. Albumin was also dialyzed in 8 M urea. The specific activity of the renatured protein was, after removal of some precipitate, the same as that of the starting material (Table I). Because neither the native nor the urea-unfolded protein lost any activity after dialysis, it was concluded that the growth-promoting activity was not due to a small noncovalently bound factor, but was attributable either to a molecule too large to pass through the dialysis membrane or to a factor covalently bound to albumin.

**Table I**

Recovery of growth-promoting activity of bovine serum albumin after various treatments

The specific activity of the treated albumin was determined as described under "Experimental Procedure." The results are the averages of two experiments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Recovery of specific activity in % of control</th>
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<tbody>
<tr>
<td>Dialysis</td>
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<tr>
<td>At pH 7</td>
<td>100</td>
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<tr>
<td>At pH 3</td>
<td>100</td>
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<tr>
<td>In 8 M urea</td>
<td>110</td>
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<tr>
<td>Acetone precipitation</td>
<td>110</td>
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<tr>
<td>Heating 30 min, 65°</td>
<td>96</td>
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<tr>
<td>Reduction with 2-mercaptoethanol in 8 M urea</td>
<td>105</td>
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<tr>
<td>Isoelectric focusing</td>
<td>90</td>
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<tr>
<td>Pepsin digestion</td>
<td>0</td>
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**Chromatography on Sephadex G-150—** If albumin is chromatographed on Sephadex G-150, the small amount of dimeric albumin present in most albumin preparations is separated from the monomer (17). Monomeric and dimeric albumin were found to be equally effective in promoting the growth of lymphocytes, and no variation in specific activity was observed within the main albumin peak (Fig. 2A). The results indicated that if the effective molecule is a protein different from albumin and not bound to it, it must have a molecular weight similar to that of albumin and it must have the same tendency to dimerize. If on the other hand some growth factor is bound to a small fraction of the albumin molecules, such a factor must be small enough not to change the molecular weight to a noticeable extent, and it must be equally distributed among the dimeric and the monomeric form of the protein.

To test further for a noncovalently bound growth factor, we chromatographed albumin at pH 3 (Fig. 2B). Again no difference in activity was found between dimeric and monomeric albumin, or between various subfractions. Also, no activity could be detected in fractions eluted after the albumin peak. The possibility that a factor present in very small amounts, once dissociated from albumin, was inactive and could no longer have been detected, could be excluded because all pooled fractions had the same specific activity as the starting material.

**Ion Exchange Chromatography and Isoelectric Focusing**—It is well known that albumin is not a homogeneous protein. This heterogeneity has been studied by many investigators (23) and is not fully understood. In part, it has been attributed to the presence of different kinds of albumin molecules, mercaptalbumin (with 1 free thiol/molecule) and non-mercaptalbumin (in which the thiol is blocked by cysteine or glutathione of the

![Fig. 2. Growth-promoting activity of bovine serum albumin fractionated by chromatography on Sephadex G-150 at pH 7.5 (A) and at pH 3 (B). Upper panels, absorption of the eluant; lower panels, specific activity of the pooled fractions determined as described under "Experimental Procedure." Arrow indicates the limit of error. Upper part of B shows in addition the absolute activity of the fractions (activity of 0.5 ml of 5 times concentrated pools diluted to 10 ml with culture medium) (right ordinate). Chromatography was performed at 4°. A, a sample of 950 mg of albumin in 1 ml of buffer (0.1 M Tris-HCl/1 M NaCl, pH 7.5) was applied to the column (90 x 5 cm) and eluted in upward flow at a flow rate of 12 ml/hour. The eluant was collected in fractions of 17 ml. B, a sample of 200 mg of albumin in 4 ml of buffer (0.05 M glycine-HCl/0.05 M NaCl, pH 3.0) was applied to the column (90 x 2.6 cm) and eluted in upward flow at a rate of 8 ml/hour. The eluant was collected in fractions of 6 ml.
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blood, and to the presence of molecules which have undergone thiol-disulfide or disulfide-disulfide interchange (24). It was of interest to investigate the distribution of growth-supporting activity among these different species of albumin. Mercaptalbumin was separated from non-mercaptalbumin by cation exchange chromatography as described by Hagenmaier and Foster (25). The method was used at the same time in an attempt to separate a growth-promoting protein differing in net charge from the albumin molecule.

The results are shown in Fig. 3. Peak 1 is mercaptalbumin with 0.94 titrable thiol/molecule. Peak 2 is mostly non-mercaptoplbumin with 0.16 thiol/molecule. These results are in good agreement with published data (25). Our data, however, differed in that a third peak was eluted from the column with 0.59 thiol/molecule, which was the same as determined for the starting material. The same results were obtained with two different albumin preparations (both fatty acid-free). We did not investigate what the third peak represented. Of greater interest for our studies was the fact that the protein of all three peaks had the same ability to support lectin-stimulated growth of lymphocytes, and that no growth factor could be separated from albumin. Hagenmaier and Foster (25) noticed very small amounts of protein in several fractions before bovine mercaptalbumin, and a proteolytic enzyme was detected in one of these bands (16). We did not investigate any fractions other than those shown. Because the specific activity of albumin in all three pools was the same as that of the starting material, the growth-promoting activity could not have been enhanced in these small bands. The amount of protein present in these fractions was too low to determine specific activity.

We also did not succeed in removing growth-promoting activity from albumin by isoelectric focusing (Table I). With this method, Kaplan and Foster (26) have demonstrated that bovine serum albumin (monomeric and free of fatty acids) can be resolved into one main peak, focusing at pH 5.28, and small shoulders on either side. Our results obtained with isoelectric focusing were very similar to those described (26). Albumin pooled from the main peak, containing very little of the material present in the shoulders, did promote growth of lymphocytes as well as the starting material (Table I), implying that the growth factor was not enhanced in the shoulders. This latter material was not tested for growth-promoting activity since the protein concentration was too low to assay for specific activity.

Separation Procedures in Presence of 8 M Urea—Further studies were performed in the presence of urea, which promotes dissociation and unfolding of protein molecules. Molecular sieving was performed with Sepharose 6B (Fig. 4), and ion exchange chromatography at pH 4.3 on sulfoethyl (SE)-Sephadex before bovine mercaptalbumin, and a sample of 160 mg of albumin in 4 ml of urea buffer (8 M urea/0.05 M sodium phosphate/0.1 M NaCl, pH 6.6) was applied to the column (90 x 2.6 cm) and eluted with the same buffer at a rate of 6 ml/hour. The eluant was collected in fractions of 5 ml.
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exchange chromatography with DEAE-Sephadex (Fig. 5), both in 8 M urea. The protein was eluted from Sepharose in at least four peaks, indicating that albumin was to a large extent aggregated in the presence of urea. Aggregation of albumin has also been observed in the presence of other dissociating agents, and is attributed to intermolecular thiol-disulfide interchange of the unfolded albumin (27). The pattern of the eluting protein varied somewhat from one experiment to the other, probably because of variation in the degree of aggregation. All fractions contained, after removal of urea and concentration by ultrfiltration, some highly aggregated material rendering the solutions turbid. Since it was possible that aggregation would continue slowly over longer time periods, even after urea had been removed, the assay was performed as soon as possible, and aggregated material was always removed shortly before assaying. The growth-promoting activity of albumin exposed to urea showed variations between wider limits (70 to 130%) than that of untreated albumin (80 to 120%). Within these wider limits, however, all protein-containing fractions, pooled from either method of chromatography, had the same specific activity as the untreated albumin, and no activity could be detected in pools which did not contain protein. Because the protein was eluted from Sepharose (Fig. 4) in at least four peaks, the albumin with the highest molecular weight, eluted first, must be at least tetrameric, and even this aggregated protein supported growth as well as monomeric albumin. Whether the different peaks eluted from DEAE-Sephadex in 8 M urea (Fig. 5) represented different states of aggregation or differences between mercaptoalbumin and non-mercaptoalbumin, or both, has not been investigated, but all three pools were equally active in supporting growth of lymphocytes.

Albumin was also reduced with 2-mercaptoethanol in the presence of 8 M urea. When slowly renatured and oxidized by dialysis, the majority of the protein precipitated at the concentrations employed here. The small amount of protein which remained in solution (only about 5%) had the same specific activity as the starting material (Table I). The growth-promoting activity was neither enhanced nor decreased in this small protein fraction. Instead, 5% of the protein was recovered together with 5% of the original activity.

These results showed that from denatured albumin also no growth-promoting activity could be removed by either of the methods used. They further showed that the degree of recovery of growth-promoting activity was always quantitatively the same as the degree of recovery of soluble albumin, suggesting that the albumin molecule itself is the growth-promoting factor.

**DISCUSSION**

The identification of serum albumin as a growth stimulant may provide a useful tool to study mitogenesis of lymphocytes in a chemically defined culture medium. The mechanism of its action remains to be elucidated.

Ryser (28) and Ryser et al. (29) have shown that albumin is bound to the cell surface of two different cell lines (Ehrlich ascites tumor cells and sarcoma 180). The authors reported that more albumin is bound than would be necessary to cover the entire cell surface, and they proposed that albumin is adsorbed partially in plurimolecular layers. Whether albumin is adsorbed to the cell surface of lymphocytes in a similar manner is not known. It could, however, explain why polymerized albumin is as active in promoting growth as the monomer.

Serum albumin is essential for the growth of lectin- or antigen-stimulated lymphocytes. Other factors, essential for the growth of various strains of mammalian cells, have been identified and purified from serum and from tissues. These factors have been reported to be present in minute concentrations only. Therefore, the observation that albumin, the most abundant serum protein, is essential for the growth of lymphocytes came as a surprise. Serum albumin is, of course, not the only molecule needed for growth of lymphocytes. The other essential molecule is the antigen, which is specific for a particular cell, and which is present at very low concentrations only. Therefore, the role of albumin must be different from that of the more specific factors. Possibly albumin plays a "helper" role. It has been shown that bovine serum albumin increases pinocytosis in macrophages (30), indicating that it can affect membrane functions. Perhaps albumin could alter the membrane of the lymphocytes so that binding of the antigen can occur, or, alternatively, it may change the binding of the antigen so that only then can the antigen exert its effect.

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Serum Albumin and Lymphocyte Growth

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