SERUM ALBUMIN IS ESSENTIAL FOR IN VITRO GROWTH OF ACTIVATED HUMAN LYMPHOCYTES*

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Lymphocytes, like other mammalian cells (1) freshly isolated from the body tissues, do not grow in vitro in a protein-free culture medium. Two conditions are usually required to induce mitogenesis and proliferation of lymphocytes, i.e., activation by an antigen or a nonspecific mitogen and addition of serum to the medium. Lymphocytes activated by nonspecific mitogens such as concanavalin A (Con A),[1] phytohemagglutinin (PHA), or bacterial lipopolysaccharide (LPS) are often used as a model to investigate the mechanism of antigen-induced mitogenesis because antigen-induced and nonspecifically-induced mitogenesis and proliferation of lymphocytes may be based on similar mechanisms (2–5). Nonspecific mitogens are often preferred over antigens for such investigations, because the former stimulate a much greater proportion of cells than the latter (5). The factor(s) in the serum which enables activated lymphocytes to proliferate is unknown. Isolation and identification of such a factor(s) may contribute to the elucidation of the mechanism of mitogenesis and multiplication of lymphocytes.

In the present report we compare the effect of human plasma with the effect of the various human plasma protein fractions of Cohn et al. (6) and of crystallized serum albumin (SA) on the growth of activated human lymphocytes. The results show that SA is essential for growth and can replace plasma not only for the growth of Con A-activated thymus-derived (T) lymphocytes and of LPS-activated bone marrow-derived (B) lymphocytes but also for the proliferation of antigen (purified protein derivative of tuberculin [PPD])-stimulated lymphocytes and of lymphocytes stimulated by allogeneic lymphocytes (mixed lymphocytic interaction). The immunological significance of the data is discussed.

Materials and Methods

Cultures of Lymphocytes. Human blood was collected in heparin or citrate. Red cells were precipitated with dextran (mol wt 2–3 × 10^5) added in a final concentration of 1%, for 1–1.5 h at 37°C. Granulocytes were removed by incubating the cell-rich plasma on cotton or rayon fibers loosely packed in 50 ml plastic syringes at 37°C for 30 min. The cell suspension was then pressed gently from the fibers, centrifuged, and the cells resuspended in medium. Maximum yield of pure or almost pure optimum-growing lymphocytes (2–3 × 10^8/U of blood) was most conveniently obtained by precipitating the red cells from a large volume of blood in a beaker (low column) rather

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[1] *Abbreviations used in this paper:* BSA, bovine SA; Con A, concanavalin A; EBM, Eagle's basal medium; FAF, fatty acid free; FCS, fetal calf serum; HSA, human SA; LPS, bacterial lipopolysaccharide; MLC, mixed lymphocyte culture; PHA, phytohemagglutinin; PPD, purified protein derivative of tuberculin; RSA, rabbit SA; SA, serum albumin.

than a cylinder (tall column), by reducing the cell-rich plasma volume by no more than half, by placing the plasma on the fibers so that no fluid pockets are formed, and by warming the plasma to 37°C before incubation on fibers. After one wash in medium the cells were incubated in medium containing 0.8–1 mg/ml of Con A, in amounts of $1–2 \times 10^6$ cells/ml, for 15–20 min at 37°C. The cells were washed once with medium containing 10% fetal calf serum (FCS) and once with protein-free medium to remove unbound Con A and inoculated in culture tubes already filled with medium. The magnitude of the growth response of lymphocytes, treated with Con A as described, was equivalent to the response of untreated cells incubated in the presence of 4–6 μg/ml of Con A. Con A-treated lymphocytes served as activated T cells (7). Plastic-stoppered culture tubes of 3.5 ml content (Nalge 3110-35 and 3111-12; Nalge Co., Nalgene Labware Div., Rochester, N. Y.) contained a final vol of 3 ml medium and $6–8 \times 10^5$ lymphocytes. Lymphoid cells from human adenoids and spleens were prepared as described previously (8) and were inoculated in amounts of 2×10^6 cells in 2 ml of medium containing 40–60 μg/ml of LPS from *Salmonella marcescens* and incubated in a CO_2 incubator. The latter cells served as activated B lymphocytes (7).

The method to prepare mixed lymphocyte cultures (MLC) and to determine in vitro reactivity of lymphocytes to PPD have been described (9, 10). The culture medium was Eagle's basal medium (EBM) (11), supplemented with human plasma, FCS, SA, or plasma protein fractions of Cohn et al. (6) as described in the text. Addition of 20 mM HEPES to the medium maintained the pH between 7.30 and 7.50 during the entire incubation period, a condition necessary for optimal growth (9). Growth was determined by adding [^{3}H]thymidine, 2 μCi/culture (sp act 6.7 Ci/mmol; New England Nuclear, Boston, Mass.) for 24 h before extracting DNA as described previously (12). All experiments were performed at least three times, each point of each curve or histogram is an average of triplicate cultures.

Dextran, heparin, Con A (grade IV), bovine SA (BSA), human SA (HSA), rabbit SA (RSA), fatty acid-free (FAF) BSA, and HEPES were purchased from Sigma Chemical Co., St. Louis, Mo.; PHA-P and LPS from Difco Laboratories, Detroit, Mich.; and plasma protein fractions of Cohn from Nutritional Biochemical Corp., Cleveland, Ohio and Miles Laboratories, Inc., Miles Research Div., Elkhart, Ind. Insulin was purchased from Eli Lilly & Co., Indianapolis, Ind.; thyroxin and somatropin growth hormone from Nutritional Biochemical Corp.; and hydrocortisone sodium succinate from the Upjohn Co., Kalamazoo, Mich.

We have found that growth of lymphocytes, activated by either lectins or antigens in the presence of SA, particularly HSA, as the only supplement to protein-free medium is considerably more sensitive to the procedure of purifying the lymphocytes and to impurities in the medium than growth in the presence of plasma. Artifacts were eliminated, largely if not completely, by using the above described procedures and observing the following factors. Glassware and plastic were thoroughly cleaned; glassware was cleaned with a chromate sulfuric acid solution. All media were stored at 4°C in the dark (refrigerator) and warmed before use. Media stored at room temperature and exposed to light for a few days became highly toxic to lymphocytes, probably because of the formation of toxic photoproducts, as observed by Stoien and Wang (13). Prolonged incubation of cells on fibers, excessive numbers of contaminating granulocytes, cooling of cells (refrigerated centrifuge), and forceful pipetting of cell suspensions inhibited growth.

Results

Effect of Human Plasma, Plasma Protein Fractions, and SA on Growth of Con A- or LPS-Activated Lymphocytes. Human plasma markedly stimulates in vitro DNA synthesis in Con A-activated blood lymphocytes, while no significant synthesis occurs in cells incubated in protein-free medium (control) (Fig. 1, upper panel). From the plasma proteins fractionated into six components according to Cohn et al. (6) and added to protein-free medium, only fraction V stimulates in vitro DNA synthesis, while the other fractions, either single or in combination have no effect. HSA has a similar effect as fraction V which consists for 96–99% of SA. Fractions I–IV and VI do not enhance the growth stimulatory effect of HSA, while in two out of five experiments performed a moderate inhibition of the albumin effect was observed in the presence of

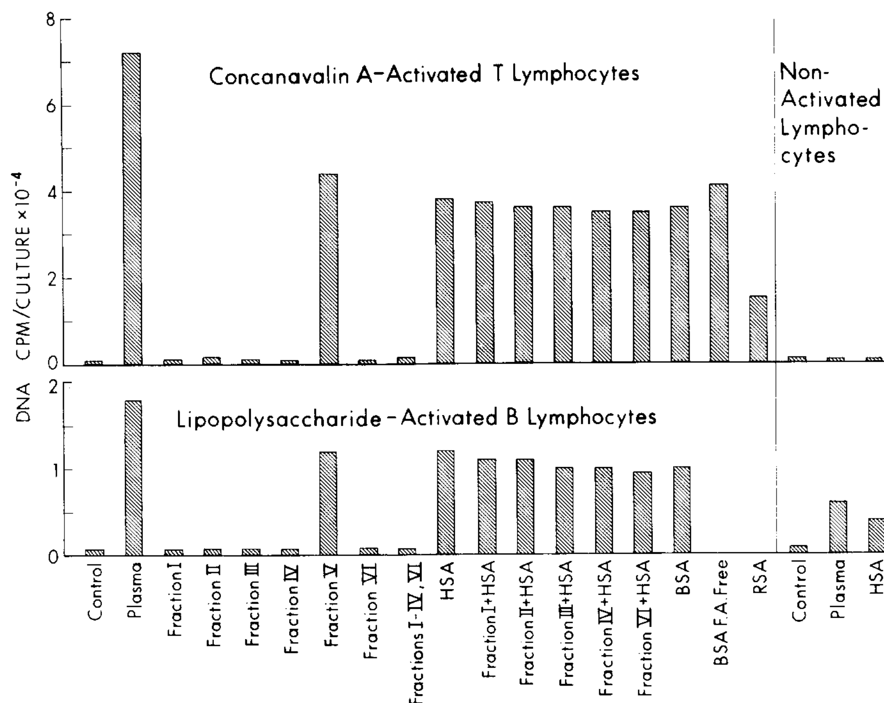

FIG. 1. Effect of human plasma, plasma protein fractions (Cohn), HSA, BSA, RSA, and FAF BSA on DNA synthesis of Con A-activated T lymphocytes (upper panel) and LPS-activated B lymphocytes (lower panel). The medium contained: 10% human plasma or 2 mg/ml of fraction V, crystallized HSA, BSA, RSA, or FAF BSA. No protein was added to control cultures. Fractions II, IV, and VI were tested in concentrations of 0.5, 1, and 2 mg/ml, and fractions I and III as saturated solutions and dilutions thereof. Incubation at 37°C lasted for 72 h. DNA was determined as described in the Materials and Methods. Data represent one of five experiments with similar results.

fractions III and IV. The results further show that BSA and RSA have similar growth stimulatory properties as HSA, indicating lack of species specificity of the effect. BSA and FAF BSA stimulate DNA synthesis to a similar degree suggesting that fatty acids are not the basis of the growth-stimulating effect.

Comparison of the effects of plasma, the Cohn fractions, HSA, and BSA on DNA synthesis in LPS-activated lymphoid cells derived from adenoids and spleens yields similar results as in Con A-activated T cells (Fig. 1, lower panel). Here again, only fraction V, HSA, and BSA promote DNA synthesis to levels similar to human plasma, while fraction I–IV and VI have no effect. The results also show that LPS is a much less potent activator of human B cells than Con A of T cells, as observed previously (14). Although the LPS effect was low, it was consistently observed in lymphoid cells from adenoids and spleens, but not in peripheral blood lymphocytes (14). DNA synthesis in nonactivated blood lymphocytes in the presence or absence of HSA or plasma equals control levels (Fig. 1, upper panel). Lymphoid cells from adenoids and spleens are slightly stimulated by either plasma or HSA, also when not activated by LPS (Fig. 1, lower panel).

Quantitation of the Growth-Stimulating Effect of SA. To quantitate the growth-stimulating effect of SA on Con A- and LPS-activated cells lymphocytes were incubated for 72 h in the presence of increasing concentrations of HSA, parallel to cultures containing increasing concentrations of fresh human plasma, the latter accounting for 40 mg HSA/ml. The results in Fig. 2 show that the growth-stimulating property of HSA for both T and B lymphocytes is concentration dependent, and levels off with increasing concentrations to reach a maximum at 6 mg/ml. The growth response to increasing concentrations of plasma is similar, however the results varied from one experiment to the other.

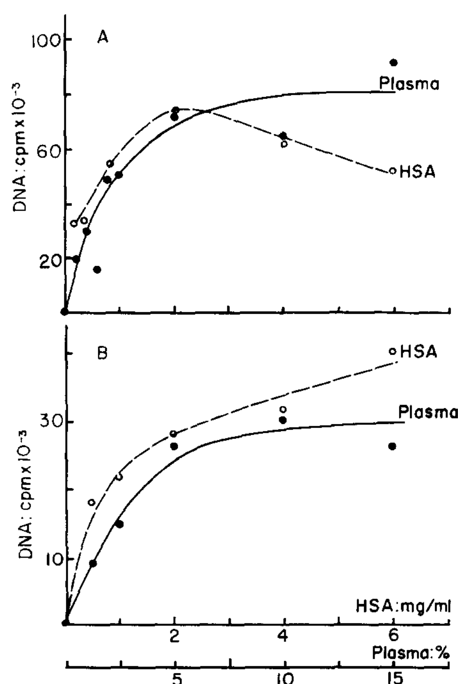

FIG. 2. Growth response of Con A-activated T lymphocytes (A) and LPS-stimulated B lymphocytes (B) to increasing concentrations of human plasma and HSA at the end of a 72 h incubation period.

In some experiments the effects of HSA and plasma were similar, in others plasma was more, but rarely less, effective (20 experiments). This variability of the results is most likely based on artifacts caused by purification of lymphocytes and impurities in the medium as described in the Materials and Methods. These results suggest but do not prove that SA is the only growth-stimulating factor in plasma for activated T and B lymphocytes.

Effect of SA-Bound Fatty Acids. From the data shown in Fig. 1 it was concluded that FAF BSA at a concentration of 2 mg/ml has a similar growth-promoting activity as normal BSA containing one or two tightly bound fatty acids. It was of interest to see in a more quantitative manner whether the bound fatty acids had an effect on the growth-promoting activity of SA. Therefore we

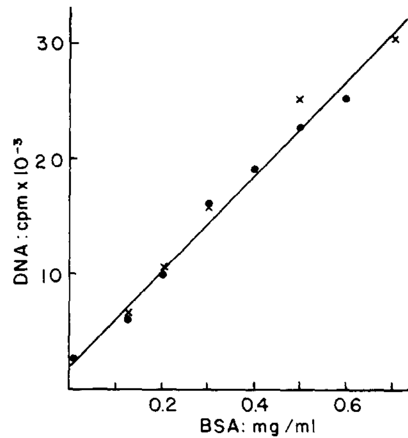

FIG. 3. Growth response of Con A-activated T lymphocytes to BSA (●) and FAF BSA (x) after 72 h of incubation.

measured growth of lymphocytes at low albumin concentrations, i.e., in the most sensitive range of the dose response curve. The results (Fig. 3) show that BSA without bound fatty acids promotes DNA synthesis to the same extent as does BSA containing one to two bound fatty acids, indicating that the fatty acids do not account for the growth-stimulating property of SA.

Effect of SA on Multiplication of Activated Lymphocytes. During the first 48–72 h of incubation lectins induce in lymphocytes mainly blasts and mitotic figures, beyond this period cellular multiplication takes place and increases with time (15). The results of Fig. 4 show that BSA as the only protein supplement to EBM is equally effective as FCS in sustaining growth of Con A- and PHA-activated human lymphocytes for 5 days. Replacement of the BSA-containing medium after 48 h by fresh medium containing the same concentration of BSA but no Con A continued to support growth, although at a reduced level. We have at the present no explanation for this reduced growth rate. However replacement of the BSA-containing medium at the same time by medium without BSA, reduces growth to control levels, indicating that the presence of SA is required not only to initiate growth but also for multiplication of Con A-activated lymphocytes.

Effect of SA on Growth of Lymphocytes Stimulated by Allogeneic Lymphocytes or PPD. Con A and LPS have no immunological specificity as activators of lymphocytes, therefore we compared the capacity of plasma and BSA to stimulate DNA synthesis in two immunologically specific situations, i.e., the mixed lymphocyte interaction (MLC) and activation of sensitized lymphocytes by PPD; in both instances T lymphocytes are activated (16, 17) and multiply (17, 18). The results in Table I show clearly that BSA can effectively replace plasma to stimulate DNA synthesis in lymphocytes activated by allogeneic lymphocytes or PPD. Similar results were obtained with HSA. The stimulation index (ratio) in the presence of plasma as compared to BSA is variable. DNA synthesis is lower in the presence of BSA than in the presence of plasma, however, DNA synthesis in protein-free medium or in the presence of a mixture of the Cohn

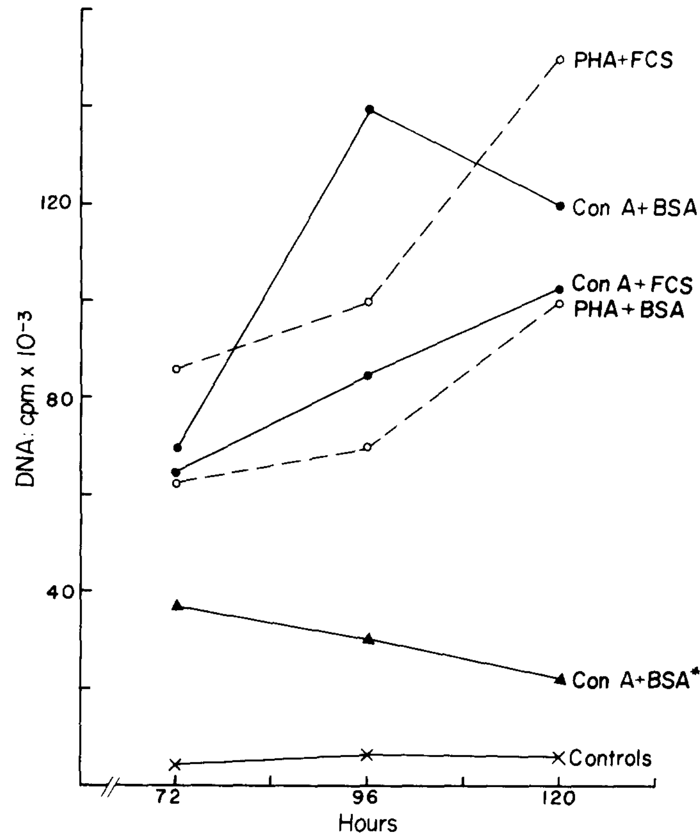

FIG. 4. Growth of Con A- and PHA-activated lymphocytes during a 5 day incubation period. Con A was used as described in the Materials and Methods. PHA was added to the medium in amounts of 3×10^{-3} ml/ml. The concentration of FCS was 10% and of BSA 2 mg/ml. Controls include cultures with normal lymphocytes and lymphocytes treated with Con A and PHA in protein-free medium, and normal lymphocytes in FCS- or BSA-containing medium. Controls are represented by a single line since the differences among them at each time interval were very small. The curve labeled BSA* represents growth of Con A-activated cells in BSA-containing medium which was replaced at 48 h with fresh BSA-containing medium without Con A. Cultures of which the BSA-containing medium was replaced by protein-free medium at 48 h are included in the controls. [^{3}H]thymidine incorporation lasted for 24 h preceding determination of DNA at each time interval.

fractions without fraction V was consistently below or similar to controls in the presence of BSA. DNA synthesis in the MLC could not be increased by increasing the concentration of BSA. Only MLC responses strong in the presence of plasma could be reproduced in the presence of BSA and MLC response weak in the presence of plasma could not be detected in the presence of BSA.

Effect of Hormones on Lymphocytic Growth. It has been shown that the hormones insulin, thyroxin, growth hormone, and hydrocortisone are transported in the plasma being bound to specific carrier proteins and to SA (19–21). The first three hormones have been shown to stimulate in vitro growth of certain strains of mammalian cells (22), while Gospodarowicz observed that

TABLE I

Effect of Plasma and BSA on the In Vitro Response of Lymphocytes to Allogeneic Lymphocytes (MLC) and Antigen (PPD) as Determined by [^{3}H]Thymidine Incorporation in DNA

Cells	Plasma, 10–20%	Ratio	BSA, 2 mg/ml	Ratio
	cpm		*cpm*	
Response of lymphocytes to allogeneic lymphocytes				
ABx	40,285	7.5	21,777	14
AAx	5,324		1,527	
CDx	150,840	15	14,503	14
CCx	10,046		1,050	
EFx	26,305	5	28,304	20
EEx	5,233		1,422	
Response of lymphocytes to PPD				
K + PPD	33,152	11	16,922	8
K	2,984		2,077	
K + PPD	40,093	12	21,938	7.5
K	3,299		2,895	
L + PPD	24,835	18	8,490	5.5
L	1,376		1,555	
L + PPD	30,070	6	16,993	5
L	4,990		3,491	

MLC were prepared and used as described (9). ABx refers to mixtures of allogeneic cells and AAx to autologous controls. A represents 1.5×10^5 responding, and Bx and Ax 6×10^5 stimulating cells exposed to 5,000 rads (from ^{60}Co) for 21 s. PPD stimulation (1 μg/ml) was carried out as described (10), using 10^6 lymphocytes from PPD-positive donors (K and L) and incubated in 3 ml of medium for 5 days, the last in the presence of [^{3}H]thymidine. Results are expressed in counts per minute per culture. The ratio of experimental over control cultures indicates the stimulation index.

hydrocortisone potentiates markedly growth of 3T3 cells by a growth factor isolated from brain and pituitary (23). The effect of the above hormones on 72 h growth of Con A-activated lymphocytes was determined in protein-free medium and medium containing 2 mg/ml BSA. The concentrations of hormones were used as follows: 0.8, 2, and 4 U/ml insulin; 3, 6, and 9 μg/ml thyroxine; 0.1, 5, and 10 IU/ml somatropin growth hormone (porcine); and 0.1, 1, and 10 μg/ml hydrocortisone. The physiological concentrations in the serum of these hormones are respectively: 0.1–3 U/ml, 3–6 μg/ml, 0.2–5 IU/ml, and 0.1 μg/ml. In three experiments performed it was consistently observed that insulin, thyroxine, and growth hormone had no effect on growth of Con A-activated lymphocytes in either the presence or absence of BSA. Hydrocortisone had no effect in physiological concentrations but inhibited growth in concentrations 10 and 100 times higher.

These data indicate that the growth-stimulating effect of SA is not due to and not affected by the hormones investigated. It was also found that BSA dialyzed

at pH 3 or 8 for 24 h against 100 volumes of saline or after chromatography on Sephadex G 150 had similar growth-stimulating properties for Con A-activated lymphocytes as untreated BSA. Comparison of treated and untreated BSA on lymphocytic growth was assayed at low concentrations as shown in Fig. 3.

Discussion

The observation (Fig. 1) that SA is the only plasma protein capable of stimulating growth of Con A-activated T lymphocytes and of LPS-activated B cells and that no growth occurs in a medium containing all essential and nonessential amino acids, salts and vitamins, and plasma proteins other than SA, proves that SA is essential for growth of Con A-activated T and LPS-activated human B lymphocytes. The observation that the growth response of activated T and B lymphocytes to increasing concentrations of plasma is similar to increasing equivalent concentrations of SA (Fig. 3) suggests that SA is the only growth-stimulating factor in plasma. It is however possible that fresh plasma contains additional factors which enhance growth and are not present in the Cohn fractions; such factors may have become inactivated during preparation and storage of the fractions. The latter factors could explain the higher rate of DNA synthesis particularly when the MLC is performed with plasma as compared with BSA (Table I). However, an alternative and more likely explanation is that the stimulating cells, after exposure to 5,000 rads to abolish their proliferative response, release factors (perhaps lysosomal enzymes) which may inhibit growth and the activity of which is neutralized by plasma but not by SA. Such an explanation is consistent with our observation, discussed in the Materials and Methods, that lymphocyte growth in the presence of SA is considerably more sensitive to toxic factors in the medium or factors released by excessive numbers of granulocytes, than growth in the presence of plasma. It is well established that SA has a great capacity to bind a variety of small molecules (24). It is therefore of interest to know whether growth stimulation is caused by SA itself or by another molecule either present as an impurity or bound to SA. The results of the present report exclude fatty acids and hormones bound to SA as growth stimulants. Extensive dialysis and chromatography on Sephadex G 150 failed to reduce the growth-promoting activity of SA for Con A-activated T lymphocytes, suggesting that either SA itself or a factor tightly bound to SA is the growth stimulant. Experiments in our laboratory are in progress attempting to separate a growth factor from BSA.[2]

The mechanism of the growth-stimulating effect of SA on activated lymphocytes is presently unknown. A few possible modes of its action may be considered here. It is possible that SA, although essential for lymphocytic growth, is not the actual stimulant of cellular DNA synthesis and proliferation but that the effect is a protective or permissive one. In such a case the nature of the effect would appear to be quite specific for SA since the other plasma proteins do not support growth (Fig. 1), neither could we demonstrate any such action for ovalbumin. Alternatively, SA could act by determining lymphocyte viability. Using the proliferative response of lymphocytes to lectins as a criterium of viability our

[2] Spieker-Polet, H. and H. Polet. Manuscript in preparation.

preliminary observations suggest that SA may influence the viability of activated lymphocytes, while its effect on the viability of nonactivated lymphocytes appear much less significant. Other hypotheses on the mode of action of SA will undoubtedly arise in the course of investigations to elucidate its effect on lymphocytic growth. According to the clonal selection theory, proliferation of lymphocytes, i.e., those lymphocytes responsive to the challenging antigen is an essential part of the immune response (25). The observation that SA is essential for growth of activated lymphocytes raises the question as to the role of the large quantities of SA in the body fluids for the immune system, besides its well established function in regulating the colloid osmotic pressure of blood and as a carrier of small molecules (24).

In recent years an increasing number of factors promoting in vitro growth of various strains of mammalian cells have been isolated from serum, tissue culture supernates, and various tissues (1, 23, 26, 27). The study of the mechanism of action of these factors on the growth of mammalian cells will hopefully contribute to the understanding of growth control of normal and malignant mammalian cells. The identification of SA as a factor essential for growth of activated lymphocytes will provide a useful model to study the mechanism of mitogenesis and growth control of lymphocytes. The ready availability of SA compared to the relative scarcity of the above mentioned growth factors is advantageous. Albumin-supplemented medium may further provide a useful chemically defined medium for investigations on the factors determining the in vitro immune response.

Summary

The effect of human plasma, the plasma protein fractions of Cohn, and crystallized serum albumin on the in vitro growth of human lymphocytes activated by concanavalin A (Con A) or bacterial lipopolysaccharide was compared. It was found that fraction V or serum albumin (SA) is essential for growth of activated T and B lymphocytes. The other plasma proteins have no effect. The growth response of Con A-activated T lymphocytes to increasing concentrations of SA is similar to the response to increasing equivalent concentrations of plasma suggesting but not proving that SA is the only growth-stimulating factor in plasma when added to a protein-free culture medium. The growth-promoting effect of SA is not due to the fatty acids or hormones bound to SA but is attributed to the albumin molecule itself or to a factor tightly bound to it. SA can also effectively replace plasma to stimulate proliferation of lymphocytes activated by allogeneic lymphocytes or purified protein derivative of tuberculin.

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