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A LYMPHOKINE-LIKE FACTOR ISOLATED BY DEOXYCHOLATE FROM THE MEMBRANE OF HUMAN LYMPHOCYTES

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Summary

1. Membranous protein fractions containing carbohydrates were solubilized from human tonsillar lymphocytes in the presence or absence of deoxycholate. The optimal conditions for the detergent treatment, high solubilized protein yield without cell disruption, temperature and time of the treatment and concentration of the detergent were elucidated.

2. The protein fractions inhibited both the mitogen-activated and the non-activated DNA synthesis in human lymphocyte targets in vitro but did not affect the uptake of [^{3}H]thymidine. The fractions had a slight effect on the amino acid incorporation into proteins and failed to influence the uptake of amino acids.

3. It is assumed that the investigated membranous proteins are lymphokine-like materials produced continuously by lymphocytes in vivo and are incorporated into the membrane of the cells.

Introduction

Membranous glycoproteins of lymphocytes play important roles with regard to the functions of cells; they can serve as receptors for various macromolecules, e.g., antigens, hormones, lectins, immunoglobulins, etc. [1—4]. They also possess a structural role.

Membrane proteins can be readily solubilized by detergents [5—7]. Deoxycholate, a constituent of bile acids in blood plasma, is more effective in solubili-

Abbreviations: P_c, protein solubilized by Hanks' medium; P_d, protein solubilized by deoxycholate.

zation than other bile acids, probably due to its greater hydrophobic character [8]. In order to achieve the complete solubilization of membranous materials, the concentration of detergent must exceed the critical micellar concentration, i.e., 9.4 mmol/l with regard to deoxycholate [9]. In the present experiments, the concentration range of deoxycholate was much lower (24 μM—0.48 mM), exceeding the bile acid content of blood plasma by, at most, 10-fold. The same conditions were applied by Mizushima for the study of bacterial proline transport [10].

In the present experiments, membranous proteins were solubilized from human lymphocytes by a mild deoxycholate treatment without destruction of the cells. The purified fraction was studied by methods similar to those accepted for the characterization of some lymphokines which can be obtained from the medium of in vitro activated cells. The effect of the membranous fraction was studied on the synthesis of macromolecules and on the uptake of macromolecular precursors in target lymphocytes in vitro. Our purpose was to find out whether lymphokine-like factors may be obtained from the plasma membrane of lymphocytes not stimulated in vitro.

Materials and Methods

[^{3}H]Thymidine ($3.7 \cdot 10^{11}$ Bq/mmol) was a product of UVVR (Prague, Czechoslovakia). [^{14}C]Valine ($2.96 \cdot 10^8$ Bq/mmol) and $Na_2{}^{51}CrO_4$ ($1.85 \cdot 10^{12}$ Bq/mmol) were the products of the Institute of Isotopes, Hungarian Academy of Sciences. All chemicals were of reagent grade.

Human tonsillar lymphocytes were isolated from freshly removed tonsils by the method described elsewhere [11]. For the isolation of membranous proteins, $2 \cdot 10^7$ cells per ml were incubated in Eagle's or in Hanks' medium at 0, 25 and 37°C for 0, 90, 180 and 360 min and for 24 h in the presence of 0.024, 0.12, 0.24 and 0.48 mM deoxycholate. Control sera without any detergent were incubated under the same conditions. The protein content was determined by using the method of Lowry et al. [12].

Large amounts of membranous protein fractions (P_c and P_d) were isolated according to the scheme depicted in Fig. 1 in the absence and presence of 0.24 mM deoxycholate. The purified water-soluble components were studied further. The removal of deoxycholate was achieved by washing (three times) the freeze-dried protein with absolute ethanol (see Fig. 1).

Cell viability was checked by ^{51}Cr release and on the basis of [^{3}H]thymidine incorporation. ^{51}Cr-release experiments were performed by using chromium-labeled lymphocytes [13]. The labeled cells (10^7 cells/ml) were incubated with deoxycholate for different periods of time, and the chromium label was determined by a γ-counter (Gamma NK 350, Hungary) in the cell-free supernatants. DNA synthesis was measured in the following way: 10^7 lymphocytes suspended in 1.0 ml Eagle's minimal essential medium were treated with deoxycholate for different time periods. The cells were centrifuged and washed three times with Hanks' medium to remove the detergent. Then the cells were resuspended in 1.0 ml Eagle's minimal essential medium and labeled with $5.5 \cdot 10^4$ Bq [^{3}H]thymidine for 60 min at 37°C. The cells were precipitated with perchloric acid and DNA content was determined in the hydrolyzed precipitate by

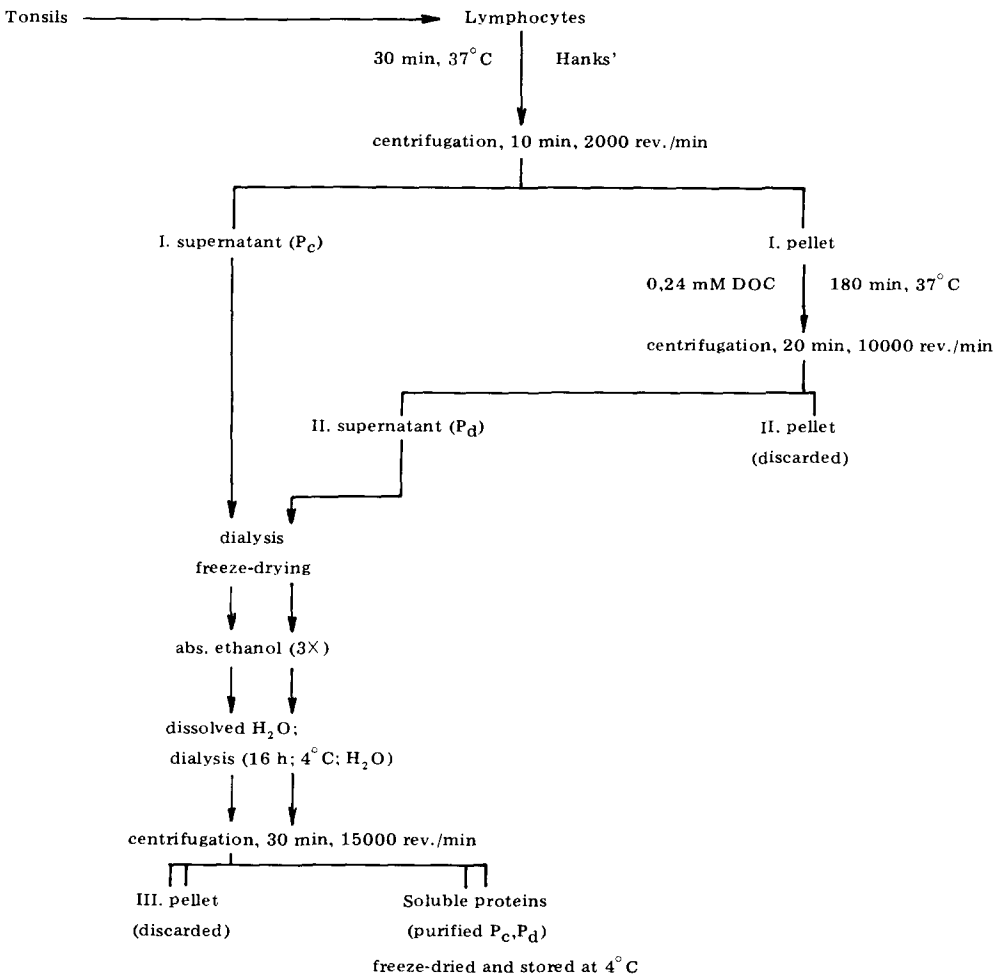

Fig. 1. Scheme of isolation and purification of membranous protein fractions from human tonsillar lymphocytes. DOC, deoxycholate.

using the method of Burton [14]. The radioactivity was measured in a toluene-based cocktail with a Beckman LS 300 liquid scintillation counter.

The effect of solubilized proteins on the mitogenactivated DNA synthesis was studied by use of the method described earlier [15]. $2 \cdot 10^6$ cells were cultured for 60 h in the presence of 10—100 μg solubilized and purified P_c and P_d fractions. 10 μg phytohemagglutinin and 20 μg concanavalin A were used as mitogens per $2 \cdot 10^6$ cells, and at the end of the incubation a 60 min pulse label was applied with [^{3}H]thymidine. DNA content and radioactivity were determined as mentioned above.

The non-activated (spontaneous) DNA synthesis and thymidine uptake were studied in short-time experiments. 10^7 cells and 10—100 μg protein fractions were incubated in 1.0 ml Eagle's minimal essential medium at 37°C for 30 and 60 min with [^{3}H]thymidine. In order to determine the uptake of labeled precursor, the reaction was stopped by cooling to 4°C, the cells were washed

twice with Hanks' medium and after precipitation by 1.0 ml 0.5 N perchloric acid, the radioactivity was measured in the supernatants. The pellet was washed and hydrolyzed, DNA content and radioactivity were determined in the hydrolyzate.

The effect of solubilized proteins on the amino acid uptake and incorporation was studied in a similar way, but Eagle's minimal essential medium was replaced by Hanks' medium and $7.4 \cdot 10^4$ Bq [^{14}C]valine were added instead of thymidine. The incorporation was stopped by an excess of non-labeled valine (500 μg/ml) and trichloroacetic acid was used as precipitating agent. The time of labeling was 30 min. Protein was determined by using the method of Lowry et al. [12] after dissolving the trichloroacetic acid pellet in 0.25 N NaOH. 5'-Nucleotidase activity was measured by using the method of Heppel and Hilmoe [16] on the basis of the release of P_i. P_i was determined by using the method of Chen et al. [17].

The homogeneity of the solubilized fractions was studied by gel filtration on Sepharose 6B (1 × 95 cm column) in Hanks' medium and by SDS-polyacrylamide gel electrophoresis. The affinity chromatography on Con A-Sepharose was performed according to the method of Allan et al. [18].

Neutral hexose content was determined by using anthrone reagent [19] and hexosamine content was measured according to the method of Dische and Borenfreund [20]. The method of Warren [21] was used for the determination of sialic acid.

Results

1. The optimal conditions for the solubilization of membranous proteins preserving the integrity of the cells were 37°C, 180 min and 0.24 mM deoxycholate (Table I). Although this temperature is not more favorable to DNA-synthesis of cells or to ^{51}Cr release than 25°C, it provides better protein solubilization. Longer incubation or higher deoxycholate concentration, in spite of their good solubilizing effect, cannot be used because these treatments result in marked damage to the cells. These conditions were applied for the large-scale separation (Fig. 1).

2. The elution pattern of solubilized protein fractions of the Sepharose 6B column can be seen in Fig. 2. The heterogeneity of the fractions was also supported by SDS-polyacrylamide gel electrophoresis. Affinity chromatography on Con A-Sepharose did not indicate the presence of any concanavalin A binding subfraction either in P_c or in P_d.

3. Carbohydrate analysis showed these fractions to contain only small amounts of hexose and hexosamine; sialic acid was found in the purified as well as in the crude P_d fraction, supporting the conclusion that P_d is derived from the plasma membrane (Table II). The plasma membrane origin of the solubilized glycoprotein fractions was further proved by the slightly enhanced 5'-nucleotidase activity.

4. The inhibitory effect of the solubilized fractions on the mitogen-activated DNA synthesis is shown in Fig. 3. The P_c fraction has only a mild effect but deoxycholate-solubilized protein (50 μg P_d) caused 50–60% inhibition. Both fractions also had an inhibitory action on DNA synthesis in the absence of

TABLE I

EFFECT OF DEOXYCHOLATE TREATMENT ON TONSILLAR LYMPHOCYTES

μg solubilized protein refers to 10^8 cells; ^{51}Cr release is expressed as cpm X 10^2 (±S.E.) and refers to 10^7 cells; [^{3}H]thymidine incorporation is expressed as cpm X 10^5 (±S.E.)/mg DNA.

Concentration of deoxycholate	Time of treatment (min)	Solubilized protein (μg)			^{51}Cr release			[^{3}H]Thymidine incorporation		
		0°C	25°C	37°C	0°C	25°C	37°C	0°C	25°C	37°C
Control	90	7.3 ± 0.7	9.8 ± 0.9	10.0 ± 0.8	7.0 ± 0.7	7.2 ± 0.4	7.3 ± 0.7	2.1 ± 0.1	2.0 ± 0.1	1.7 ± 0.0
	180	8.2 ± 0.9	17.7 ± 1.6	50.0 ± 2.0	7.4 ± 0.4	7.7 ± 0.6	7.9 ± 0.6	2.0 ± 0.3	1.5 ± 0.2	1.5 ± 0.2
	24 h	51.0 ± 6.8	187.0 ± 7.8	210.0 ± 14.0	16.3 ± 0.7	40.0 ± 1.7	51.0 ± 0.9	1.4 ± 0.1	1.2 ± 0.1	1.2 ± 0.1
0.024 mM	90	18.6 ± 1.7	29.0 ± 3.1	30.0 ± 4.0	7.3 ± 0.3	7.7 ± 0.7	7.6 ± 0.1	1.9 ± 0.2	1.9 ± 0.2	1.9 ± 0.1
	180	20.0 ± 2.2	32.0 ± 2.4	60.0 ± 1.3	7.9 ± 0.6	7.8 ± 1.2	8.0 ± 0.5	1.7 ± 0.1	1.7 ± 0.1	1.5 ± 0.0
	24 h	56.0 ± 6.2	171.0 ± 10.0	225.0 ± 6.7	17.6 ± 1.1	39.0 ± 3.9	59.0 ± 0.5	1.3 ± 0.1	0.9 ± 0.1	0.6 ± 0.1
0.12 mM	90	27.0 ± 2.7	30.0 ± 1.6	4.40 ± 0.5	7.9 ± 0.6	7.6 ± 0.8	8.2 ± 0.1	2.1 ± 0.1	1.8 ± 0.1	2.0 ± 0.0
	180	40.0 ± 4.6	.56.0 ± 6.3	72.0 ± 1.6	7.5 ± 0.9	7.7 ± 0.8	8.4 ± 0.5	2.0 ± 0.2	1.6 ± 0.1	1.6 ± 0.1
	24 h	74.0 ± 5.2	169.0 ± 9.2	250.0 ± 19.7	21.0 ± 0.6	40.0 ± 2.4	59.0 ± 0.8	1.0 ± 0.1	0.5 ± 0.1	0.6 ± 0.1
0.24 mM	90	60.0 ± 14.0	50.0 ± 6.2	66.0 ± 1.3	7.2 ± 0.8	7.9 ± 0.4	8.4 ± 0.4	1.8 ± 0.1	1.7 ± 0.1	2.1 ± 0.0
	180	80.0 ± 8.4	58.0 ± 5.1	80.0 ± 8.1	8.2 ± 0.6	8.0 ± 0.9	8.4 ± 0.5	1.6 ± 0.2	1.3 ± 0.1	1.7 ± 0.2
	24 h	159.0 ± 12.0	202.0 ± 21.0	aggregated	43.0 ± 0.8	50.0 ± 3.1	78.0 ± 2.2	0.8 ± 0.1	0.9 ± 0.1	0.7 ± 0.0
0.48 mM	90	62.0 ± 8.7	69.0 ± 7.9	110.0 ± 3.4	10.9 ± 0.9	11.8 ± 1.0	18.0 ± 0.2	1.3 ± 0.1	1.4 ± 0.2	1.6 ± 0.1
	180	87.0 ± 9.3	102.0 ± 14.3	130.0 ± 16.0	10.8 ± 0.7	17.0 ± 1.1	22.0 ± 0.1	1.2 ± 0.1	0.9 ± 0.1	1.0 ± 0.1
	24 h	202.0 ± 16.0	269.0 ± 26.0	aggregated	72.0 ± 3.7	71.0 ± 9.1	85.0 ± 1.2	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
Averages of 0 min treatment		7.6 ± 1.3	8.3 ± 1.0	6.7 ± 0.8	1.2 ± 0.1	1.6 ± 0.5	1.5 ± 0.6	2.2 ± 1.2	2.3 ± 0.1	2.4 ± 0.1

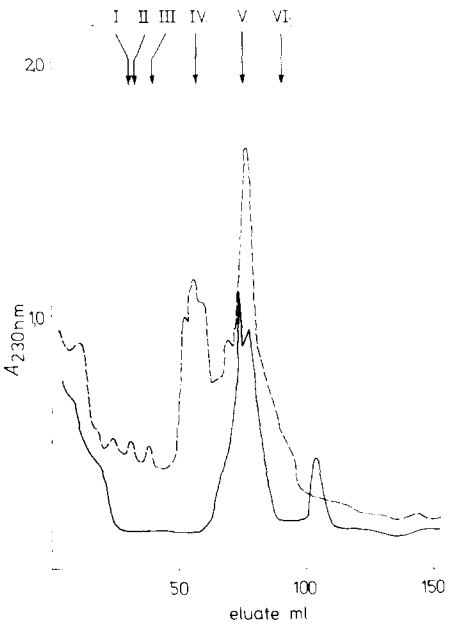

Fig. 2. The elution profile of solubilized proteins on Sepharose 6B column. — — — —, P_c fraction; ———, P_d fraction. Standard proteins: I, = blue dextran; II, IgG (M_r $1.55 \cdot 10^5$); III, bovine serum albumin dimer (M_r $1.36 \cdot 10^6$); IV, bovine serum albumin monomer (M_r $6.8 \cdot 10^4$); V, ribonuclease (M_r $1.4 \cdot 10^4$); VI, phenol red dye.

mitogens (control samples). Inhibitions caused by the solubilized membrane components were about the same when P_c and P_d fractions were added to the cell cultures only at 24 h of the mitogen activation (not demonstrated).

5. The effect of P_c and P_d on the incorporation of labeled macromolecular precursors in short-time-incubated lymphocytes is illustrated in Fig. 4. P_d has stronger inhibitory capacity on the spontaneous DNA synthesis than P_c (35% compared to 10%, 100 μg), but inhibition with both fractions was negligible in the first 30 min of the incubation. The amino acid incorporation

TABLE II

CHEMICAL AND ENZYMATIC CHARACTERIZATION OF SOLUBILIZED FRACTIONS

n.d., not determined. Crude preparations were stopped before freeze-drying while the purified proteins were prepared according to the scheme depicted in Fig. 1. Results for sialic acid, hexosamine and hexose are expressed as μg/mg protein.

Fraction	5'-Nucleotidase (nmol P_i/mg protein per min)	Sialic acid	Hexosamine	Hexose
Intact cells	29.7 ± 1.3	6.0 ± 0.7	n.d.	n.d.
Crude P_c	39.2 ± 3.0	14.1 ± 1.1	n.d.	n.d.
Crude P_d	45.4 ± 3.2	40.5 ± 2.2	n.d.	n.d.
Residual cells	28.8 ± 3.3	5.1 ± 1.6	n.d.	n.d.
Purified P_c	n.d.	19.1 ± 1.6	13.4 ± 2.2	8.4 ± 0.9
Purified P_d	n.d.	33.3 ± 2.7	16.4 ± 3.0	5.0 ± 1.1

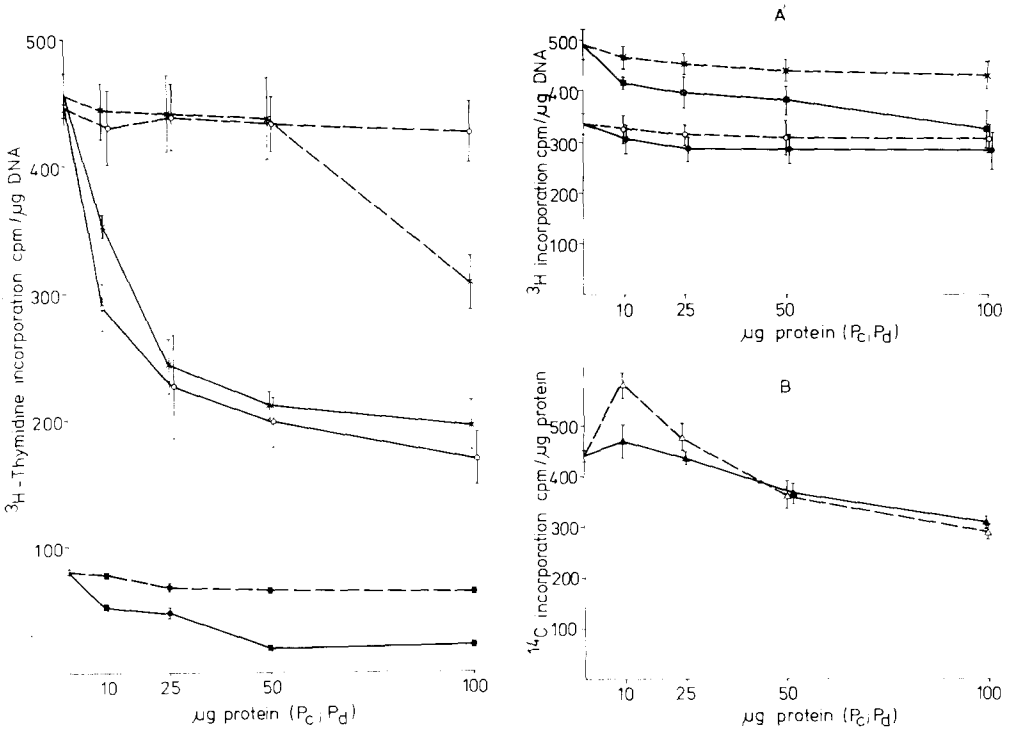

Fig. 3. The effect of membranous proteins (P_c and P_d) on the mitogen-activated DNA synthesis of human tonsillar lymphocytes. S.E. values were calculated from six experiments. — — — —, P_c fraction; ———, P_d fraction; ●, non-activated; ○, concanavalin A-activated; X, phytohemagglutinin-activated.

Fig. 4. The effect of membranous proteins (P_c and P_d) on the short-time incorporation of [^{3}H]thymidine (A) and [^{14}C]valine (B) into the macromolecules. S.E. values were calculated from three experiments. For details see Materials and Methods. (A) ○- - - - -○, P_c; ●———●, P_d (30 min incubation); X- - - - -X, P_c; ⊠———⊠, P_d (60 min incubation) (B) △- - - - -△, P_c; ▲———▲, P_d (30 min incubation).

into proteins was enhanced in the presence of 10—20 µg P_c protein while larger amounts resulted in an inhibition. No characteristic difference was observed between P_c and P_d fractions when they were present in greater amounts. The uptake of thymidine and valine within short periods was not influenced by P_c and P_d fractions (Table III).

Discussion

Deoxycholate is generally used for the solubilization of membranous proteins from various cells [8—10]. The optimal conditions of the deoxycholate treatment of human tonsillar lymphocytes were based on our experiments in which the concentration of the detergent, incubation time and temperature were varied. Our purpose was to achieve maximal protein solubilization without considerable cell disruption. 0.24 mM deoxycholate was found to solubilize a large amount of proteins from the cells without any significant cell damage compared to the control (Table I). This mild treatment is very likely to promote only the solubilization of the loosely integrated membrane

TABLE III

THE UPTAKE OF LABELED PRECURSORS BY THE CELLS

Protein added (μg)		[^{3}H]Thymidine (cpm/10^7 cells)			[^{14}C]Valine (cpm/10^7 cells)	
		0 min	30 min	60 min	0 min	30 min
Control		60 ± 5	2880 ± 155	1350 ± 97	360 ± 24	16 800 ± 256
P_c	10	72 ± 7	2868 ± 198	1211 ± 86	341 ± 17	17 050 ± 103
	25	55 ± 6	2824 ± 145	1234 ± 79	344 ± 26	16 661 ± 121
	50	70 ± 6	2794 ± 144	1170 ± 78	380 ± 21	16 998 ± 113
	100	70 ± 7	2825 ± 111	1174 ± 59	362 ± 19	16 007 ± 134
Pd	10	42 ± 5	2791 ± 156	1328 ± 67	394 ± 31	16 624 ± 157
	25	68 ± 5	2782 ± 178	1268 ± 54	374 ± 20	16 006 ± 104
	50	31 ± 4	2850 ± 132	1270 ± 46	380 ± 30	16 200 ± 115
	100	31 ± 5	2784 ± 144	1282 ± 59	388 ± 26	16 509 ± 125

proteins. The P_c fraction may contain the peripheral proteins soluble in buffered saline. The P_d fraction, solubilized by 0.24 mM deoxycholate, may consist mainly of the loosely integrated membranous proteins because it was obtained from the cells after previous treatment with Hanks' medium (Fig. 1). We suppose that the P_c and P_d fractions were mostly derived from the membrane of the lymphocytes, for ^{51}Cr release from chromium-labeled cells did not change after 3 h of treatment with 0.24 mM deoxycholate, demonstrating the intactness of the cells. The membranous origin of the P_c and P_d fractions was also supported by their content of sialic acid and 5'-nucleotidase.

The completeness of deoxycholate removal is always a crucial problem in the study of the deoxycholate-solubilized proteins. In our case, the possibility that the effects attributed to the P_d fraction were caused by traces of the detergent could be excluded. Deoxycholate is dissolved optimally in ethanol (solubility 200 g/l). Washing three times with absolute ethanol (three aliquots of 50 ml) after dialysis should guarantee the total removal of the detergent (there were approx. 5 mg deoxycholate in a freeze-dried preparation). Nevertheless, it is conceivable that traces of deoxycholate are bound to the P_d fraction but these small amounts of detergent are unlikely to cause an inhibition of the thymidine incorporation (Figs. 3 and 4), because when lymphocytes were incubated for 3 h at 37°C in the presence of 0.24 mM deoxycholate (0.1 mg/ml), the rate of DNA synthesis in the cells was about the same as that in control cells incubated without any detergent (Table I). Moreover, the P_c fraction which might not contain deoxycholate in higher amounts, also had an inhibitory action on the DNA, as well as on the protein synthesis. Therefore, we conclude that the inhibitory effect of the P_d fraction on the synthesis of macromolecules in lymphocyte targets is not based upon its contamination with deoxycholate.

Purified P_c and P_d preparations do not represent homogeneous protein populations according to their elution profiles from Sepharose 6B (Fig. 2), but P_d is less heterogeneous. Both fractions inhibited the macromolecular synthesis in lymphocytes, however, P_d must have higher amounts of inhibitory components.

Membranous proteins serve as receptors for various lectins [1,2,4,7,15]:

the inhibition of lectin-binding prevents the mitogenic effect. The inhibition of the mitogen-activated DNA synthesis in our experiments cannot be explained by postulating the presence of lectin receptors in the solubilized fractions for the following reasons: (1) P_d affected not only the mitogen-activated but also the spontaneous (non-activated) DNA synthesis (Figs. 3 and 4); (2) the solubilized fractions did not contain any protein bound to Con A-Sepharose; (3) the neutral hexose and hexosamine content (glucose and mannose are haptens for concanavalin A, *N*-acetylgalactosamine for phytohemagglutinin) is quite low in both fractions; (4) an inhibitory effect could also be observed when the P_d fraction was added to the cultures at 24 h of the stimulation; the binding of the lectins and the triggering effect must have taken place during 1 day.

P_d probably does not affect the binding of lectins to the cell surface, but it may influence other processes during DNA synthesis. Since both inhibitory proteins do not block the uptake of thymidine in short-time-incubated cells, their site of action must be either at the nucleotide phosphorylation or in nucleotide triphosphate polymerization. Inhibition of DNA polymerase by an inhibitor of DNA synthesis was reported by Lee and Lucas [22]. Further experiments would be necessary in connection with the effect of our fractions on DNA polymerase and on enzymes of the salvage mechanism of thymidine.

On the basis of our results, we assume that membranous glycoprotein fractions (P_d, P_c) are lymphokine-like materials. This possibility is supported by our earlier experiments [23—25] according to which lymphokine-like proteins can be isolated by ammonium sulfate from the medium of 4-h-incubated human lymphocytes without in vitro activation. Lymphokines are produced by activated lymphocytes in vitro; they are the putative mediators of cellular immunity. It has been described that one of them (lymphotoxin) is associated with the membrane of mitogen-activated human lymphocytes [26]. We suppose that synthesis of soluble protein mediators is a normal function of lymphocytes and the activation of cells is not necessarily a requirement for their production. In our case, the lymphokine-like factor was obtained from the membrane of unactivated lymphocytes, it apparently has been synthesized in vivo by the cells.

The question arises as to whether the factor described here might be related to the known inhibitory lymphokines, e.g., to inhibitor of DNA synthesis [27—30], to soluble inhibitory factor [31] or to lymphocyte chalons [32—34] derived from lymphocytes and affecting the DNA synthesis in their target cells. Similar to inhibitor of DNA synthesis, soluble ihibitory factor and lymphocyte chalon, our fraction can inhibit the stimulated DNA synthesis of lymphocytes, but it also decreases the non-activated DNA synthesis; this is not a typical attribute of these factors. At the same time, our factor differs from inhibitor of DNA synthesis and lymphocyte chalon in its origin and in its molecular weight. The soluble inhibitory factor described by Wolf et al. [31] is also derived from human, unactivated tonsillar lymphocytes, except that it was isolated from the medium of 7-day-cultured cells.

According to our results and on the basis of certain similarities to factors mentioned above, we suppose that lymphokine-like proteins (glycoproteins?) are continuously produced in human lymphocytes and they are able to incor-

porate into the membrane of the cells. It is assumed that they may play some role in the cell-cell interactions, and in the normal homeostatic regulation of lymphocytes.

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