Kinetics of the Reaction of Kidney-Bean Leucoagglutinin with Human Lymphocytes

The leuco- and erythroagglutinating properties of extracts from kidney beans (*Phaseolus vulgaris*) have been known for over half a century. In 1960 NOWELL[1] discovered that the bean extracts were capable of stimulating lymphocytes to undergo mitotic division in vitro. It has been shown that these biological activities are exerted by glycoproteins, which can be divided into one purely leucoagglutinating and probably several erythro- and leucoagglutinating glycoproteins. The kidney-bean leucoagglutinin has been extensively purified and described[2]. It has been shown that the process of lymphocyte stimulation in vitro is initiated by the attachement of the leucoagglutinin to the cell membrane[3]. The kinetics of this reaction have not previously been investigated in detail.

Materials and methods. Blood was collected from healthy volunteers of both sexes (age 20–35 years) by venous puncture and the lymphocytes were isolated[4]. The resulting cell suspension contained 99–100% pure, small- and medium-sized lymphocytes. Homogeneous kidney-bean leucoagglutinin was isolated and crystallized as described elsewhere[5]. Leucoagglutinin was labelled with tritium by acetylation with H^3-acetic acid anhydride, as previously described[6].

All experiments were performed in 16×110 mm polycarbonate tubes (Daniel Oy, Finland), which showed negligible unspecific absorption of leucoagglutinin (below 1.0% of added leucoagglutinin). The basal incubation medium consisted of Eagle's minimum essential medium, supplemented with 0.2% methylcellulose (Methocel cP, Fluka AG, Switzerland). After incubation the cells were washed twice with ice-cold phosphate-buffered saline, pH 7.4, transferred to glass tubes and solubilized with Soluene (Packard Inc., USA). The cell-bound radioactivity was determined by counting in an automatic liquid scintillator (Wallac Oy, Finland) in Bray's solution with 4 g/l Omnifluor (New England Nuclear Corporation Inc., USA). Quenching was corrected for, using external standards[7].

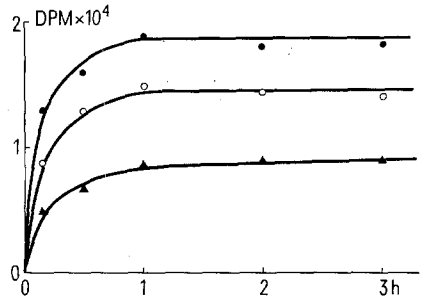

Fig. 1. Binding of leucoagglutinin to lymphocytes in the absence and presence of serum, –●–, 0; –○–, 15; and –△–, 50% (1.5 µg/ml leucoagglutinin and 10^6 lymphocytes, 37 °C).

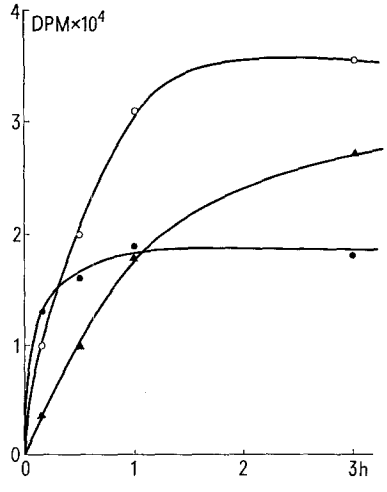

Fig. 2. Effect of different temperatures on the binding of leucoagglutinin to lymphocytes. –△–, 4 °C; –○–, 20 °C; and –●–, 37 °C. Other conditions as in Figure 1.

Results. The results of the studies of different serum concentrations and temperatures on the binding of the leucoagglutinin to lymphocytes are shown in Figures 1 and 2. Increasing the temperature from 4 to 37 °C makes the binding to cells more rapid. However, the largest amount of leucoagglutinin was bound at 20 °C. The cells bound about twice as much leucoagglutinin at 20 °C as at 37 °C. At 37 °C the binding is completed within 1 h. The effect of serum was studies only at 37 °C. As can be seen from the Figure, serum competes with the cells for the agglutinin. As the kidney-bean leucoagglutinin is a metalloprotein containing Ca and Mn[5], the effect of the chelating agent EDTA on the binding was investigated. 0.01 *M* EDTA greatly inhibits the attachment of the leucoagglutinin to the cells and in addition is also capable of detaching already bound leucoagglutinin.

The reactions between lectins and blood cells have previously been assumed to conform to the laws of mass action. Then the binding of the leucoagglutinin (L) to lymphocytes (C) can be considered as a reversible bimolecular reaction:

$$L + C \underset{k_2}{\overset{k_1}{\leftrightarrow}} LC, \qquad (1)$$

where C denotes the cellular binding site and k_1 and k_2 are the rate constants for the reactions. The association constant K is then

$$K = \frac{k_1}{k_2} = \frac{(LC)}{(L)\,(C)}. \qquad (2)$$

This formula refers to the primary association of leucoagglutinin with lymphocytes but does not refer to the process of cell agglutination. The reversibility of the reaction was ascertained according to WURMSER and FILITTI-WURMSER[8]. As can be seen from Figure 3, the reaction is completely reversible. The equilibrium values for different concentrations of leucoagglutinin were plotted, as suggested by SCATCHARD[9], according to the equation

$$\frac{(LC)}{(L)} = K\,(n - LC), \qquad (3)$$

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where LC is the number of molecules of leucoagglutinin bound to the lymphocytes, L is the concentration of free leucoagglutinin in moles per litre and n is the number of binding sites per lymphocyte.

Plotting LC/L against LC will yield a straight line, if the reaction is homogeneous with respect to K. As can be seen from Figure 4, this is not the case. The intercept on the LC axis is n and the intercept on the LC/L axis is Kn. The intercepts were obtained by extrapolation. Values of $1.6–2.4 \times 10^6$ binding sites per lymphocyte were obtained for the lymphocytes from 5 persons examined. The classical first-association constant K_o is $7–8 \times 10^7$ M^{-1}. The average association constant K_{av} decreased with increasing concentration of leucoagglutinin and the lower limit is $0.5–2 \times 10^7$ M^{-1}.

Discussion. Previous studies on the temperature-dependence of the reactions between lectins and cells are confirmed[10]. The largest amount of leucoagglutinin is bound at 20 °C, but increasing the temperature to 37 °C speeds up the binding. Serum interferences greatly with the binding to the cells and this may, at least partly, be the reason for the inhibitory action of high concentrations of serum on in vitro lymphocyte stimulation. The nature of the substance in serum which interferes with the cellular attachment of the leucoagglutinin is not known for the moment, but most probably it is a glycoprotein[11].

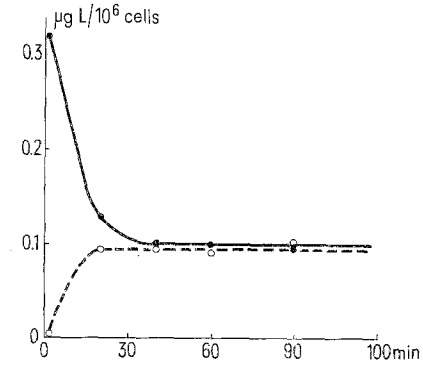

Fig. 3. Reversibility of the reaction between lymphocytes and kidney-bean leucoagglutinin. The filled circles denote the binding of leucoagglutinin to lymphocytes saturated at 40 µg/ml and then diluted to 0.8 µg/ml. The open circles show the absorption to lymphocytes at 0.8 µg/ml.

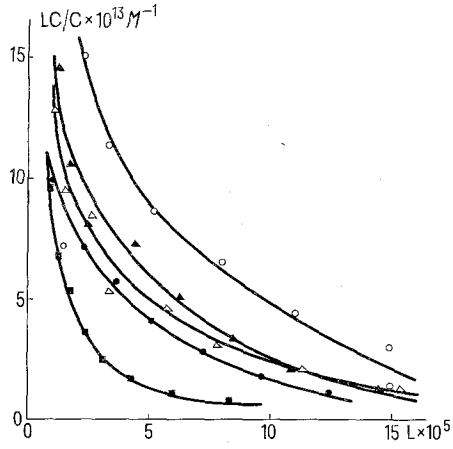

Fig. 4. SCATCHARD plots of the reaction between lymphocytes and leucoagglutinin. Curves from 5 individuals.

It has previously been shown that DETA is highly inhibitory of lymphocyte stimulation with kidney-bean extracts. KAY[12] postulated that this was due to either impaired cellular binding or binding in a form not compatible with stimulation. LINDAHL-KIESSLING[4] arrived at the conclusion that EDTA in 0.01 M concentrations prevents the cellular attachment of kidney-bean phytohaemagglutinin. The results of the present investigation confirm that EDTA prevents the binding of leucoagglutinin to lymphocytes and in addition is also capable of detaching already bound leucoagglutinin. As the leucoagglutinin molecule has been shown to contain considerable amounts of Ca and Mn, an interaction between EDTA and these metals seems highly probable.

The cellular receptors for phytohaemagglutinins have been shown to be glycopeptides or polysaccharides[11], but the structure of the leucoagglutinin receptor is not known. The figure of 2.0×10^6 binding sites per lymphocyte for the leucoagglutinin may be compared with the values of 6.6×10^6 for a lentil lectin[13] and 2.7×10^6 for kidney-bean erythroagglutinin[14].

Previous investigators who studied the kinetics of the reactions between lectins and cells have generally assumed that these reactions are completely reversible[10]. However, this fact has not been unequivocally proved before, with the exception of the reactions of the lentil lectin, which can be washed off cells with D-mannose solutions[13]. The method used in the present investigations shows that the reaction between human lymphocytes and leucoagglutinin is completely reversible under cell-culture conditions and that a dynamic equilibrium between bound and unbound leucoagglutinin exists, an equilibrium which conforms to the laws of mass action and thermodynamics.

The association constant and number of binding sites for the leucoagglutinin were determined as suggested by SCATCHARD[9]. The curvilinear plots in Figure 4 indicate that the reaction between the leucoagglutinin and the lymphocytes is not homogeneous with respect to K. However, this does not prevent the calculation of K_o and the number of receptor sites on the lymphocytes from the intercepts of the curves. The reason for the inhomogeneity may be either heterogeneity in the receptors of the lymphocytes or in the binding sites of the leucoagglutinin. Of these explanations, the first-mentioned is most probable, as the leucoagglutinin used has been shown to be homogeneous by very rigorous criteria, including isoelectric focusing, disc electrophoresis, ion-exchange chromatography, and crystal structure, and in addition is composed of 4 identical subunits[2]. The results of this study would be explained if it were assumed that the lymphocytes contain two kinds of receptors for the leucoagglutinin, i.e. high- and low-affinity receptors. The occurrence of several kinds of receptors for kidney-bean agglutinins on lymphocytes has not been demonstrated before. The high-affinity receptor may consist of two low-affinity receptors combining with the same leucoagglutinin molecule. When leucoagglutinin is added in concentrations causing lymphocyte stimulation, it will interact almost exclusively with the high-affinity receptors. Therefore, it seems reasonable to infer that the high-

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affinity receptors are connected with the process of lymphocyte activation. If, as suggested, the high-affinity receptors consist of two low-affinity receptors, it means that lymphocytes, in order to become activated, require the bivalent attachment of the stimulating molecule to the cell membrane. Leucoagglutinin in higher concentrations react predominantly with the low-affinity receptors or monovalently and does not stimulate lymphocytes as shown previously[2].

It has been shown that leucoagglutinin does not have to be internalized in the lymphocytes during stimulation[3]. Thus the stimulatory process is initiated by the establishment of a dynamic equilibrium between lymphocyte receptors of high-affinity type and the mitogen molecules.

Zusammenfassung. Es wurde die Kinetik der Reaktion zwischen Lymphozyten und Leukoagglutinin aus *Phaseolus vulgaris* untersucht.

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Thermal Injury: Release of a Cytotoxic Factor

Recent studies from these laboratories have suggested the presence of a specific inhibitor of muscular contraction in the serum of acute thermally injured patients[1]. A toxic glycoprotein isolated from in vitro scalded human skin inhibited the formation of adenosine 5'-triphosphate (ATP)-induced tension by glycerol extracted muscle fibers[2,3]. Earlier studies have shown that the 'toxic glycoprotein', when administered i.v., proved lethal to mice, and, when incorporated into the growth medium, inhibited the growth of Hela and HEP_2 cells[4].

Sensitized lymphocytes are known to mediate allograft and tumor immunity as well as delayed hypersensitivity reactions. Lymphocytes-mediated-cytotoxicity against various target cells can be induced by several methods which include the addition of lymphocytes specifically sensitized against the corresponding target cells, or the addition of antigen unrelated to the target cells, to a mixture of target lymphocytes specifically sensitized against the added antigen. Investigation of the mechanisms of cell-mediated immune reactions and induction of lymphocyte-mediated cytotoxicity against various target cells in various types of trauma, viral and neoplastic is valuable for the understanding of the cell-mediated reactions[5–8] and of the immunological state of the victim.

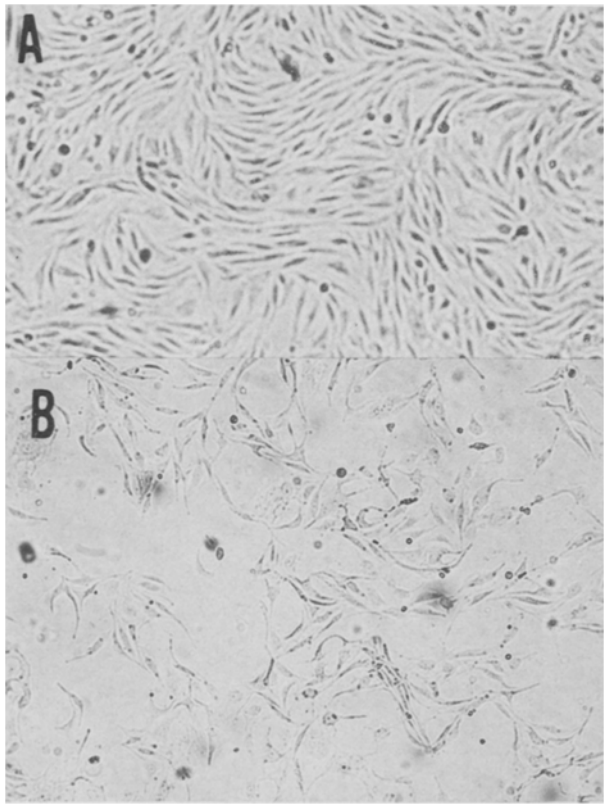

Fig. 1. Cytotoxic effect of the 'acute burn serum inhibitor' on rabbit heart fibroblasts: 48 h growth of rabbit heart fibroblasts in basal Eagles medium in presence of 0.10 ml of extract from normal serum (A), and from serum of thermally injured patient with 60% total body injury.

The present study was performed to investigate the cytotoxic effects of an 'acute burn serum inhibitor' isolated from the serum of acute thermally injured patients, on fibroblast of rabbit heart culture, and on the growth and migration of lymphocytes from normal and thermally injured subjects.

Methods. Blood samples were obtained from Sumner L. Koch Burn Ward at Cook Country Hospital. The blood was allowed to clot at room temperature, and the serum was separated at $1000 \times g$ for 15 min. The serum (10 ml) was diluted with an equal volume of physiological saline, then mixed with one-fifth volume of glacial acetic acid and an equal volume of butanol. The aqueous phase was separated after centrifugation for 60 min at $1000 \times g$, and processed as described for the 'toxic glycoprotein'[4,9]. The 'acute burn serum inhibitor' was finally dialyzed against physiological saline and made up to a final volume of 10 ml.

Fibroblasts of primary heart cultures were obtained from albino rabbits, 3–4 lbs. The hearts were removed, rinsed free of blood and cut into small pieces in 10 ml of Earle's salt solution. The solution then decanted, and the

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