necessary for the very survival of most tissue cultures, should be required for maximal motility. Other unreported studies show that motility is diminished in various media depleted (as in [6]) of serum factors, and that our results are not peculiar to factor-free medium. We can assert that BALB/c-3T3 requires a non-dialysable serum factor(s) not present in factor-free medium for maximal motility, but whether this factor(s) deserves the name "motility factor(s)" awaits serum fractionation studies to determine the specificity of the effect on motility.

Contrasting studies on an SV40-transformant of BALB/c-3T3 showed that these cells proliferated and moved well in factor-free medium. Moreover, no significant motility increase was observed following the addition of whole serum to such cells, demonstrating that this transformant is less dependent on serum factors for maximal motility than is the untransformed fibroblast.

We note finally that the methods used in this paper permit one to obtain a sparse population of BALB/c-3T3 cells synchronously arrested prior to the G2 phase of the cell growth cycle. This facility is useful for distinguishing the effects of cell–cell contact from those of proliferative arrest [7]. We have shown that such cells begin to synthesize DNA 23 h following serum addition and that they undergo mitoses approx. 10 h later. Since this time course closely resembles that found for 3T3 by Nilhausen & Green [4], it is probable that these sparse cells too are arrested in the G1 phase of the growth cycle.

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Biologically active Concanavalin A complexes suitable for light and electron microscopy

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Concanavalin A (ConA), a plant agglutinin which binds to simple sugars and polysaccharides containing terminal, non-reducing α-D-glucopyranosyl and mannopyranosyl residues, has been used to study carbohydrate receptors on the surface of normal as well as malignant cells [1, 2]. In addition, this agent has been shown to be a potent stimulator of DNA synthesis for murine thymus and thymus derived cells [3]. Thus morphologic examination of cell surfaces, using ConA as a marker, may elucidate surface membrane changes associated with cell activation and give some insight into the mechanics of cell stimulation. This report describes a technique for the covalent coupling of ConA to substances which are suitable for light and electron microscopic visualization of cell surfaces and carbohydrate receptors. These complexes have biologic functions similar to unmodified ConA.

ConA–ferritin and ConA–cytochrome peroxidase complexes were prepared using a modification of the glutaraldehyde coupling method of Avrameas [4]. It was initially

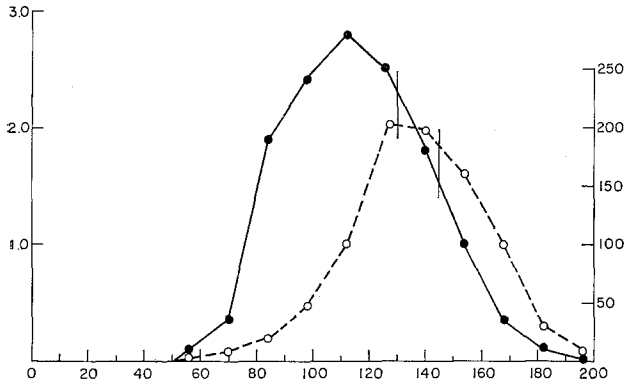

Fig. 1. Abscissa: elution vol (ml); *ordinate: (left)* A_{340}; *(right)* cpm $\times 10^3$. ●—●, OD; ○---○, radioactivity.

Elution profile of ConA–ConA and ConA–ferritin complexes on Sepharose 2B. Bed dimensions: 35 × 2.5 cm. Eluant: PBS, pH 7.4. Flow rate: 2 ml/min. The presence of ferritin was determined by optical density at 340 nm, while that of ConA was determined by the amount of ^{125}I ConA used as a tracer (cpm). The vertical lines indicate the samples pooled for mitogenic and electron microscopic studies.

observed that the addition of glutaraldehyde in concentrations as low as 0.025% to ConA in solution either alone or with another protein such as BSA, resulted in a significant loss of the mitogenic and binding activity of the ConA. However, prior saturation of the ConA binding sites with a carbohydrate ligand such as alpha-methyl-D-glucopyranoside (α-MG) protected the ConA so that the biologic activity was not lost during the glutaraldehyde cross-linking. Consequently, we prepared covalent complexes of ConA with ferritin and with cytochrome peroxidase utilizing ligand protection of the binding site.

One milliliter containing 42 mg of ConA (2× crystallized; Nutritional Biochemical Corp., Cleveland, Ohio) in physiologically buffered saline, pH 7.4, (PBS) was added to 1 ml of 1 M α-MG (Pfahnstiehl Laboratories, Waheegan, Ill.) in PBS and allowed to mix for 30 min at room temperature. Ten μg of ^{125}I-ConA, radioiodinated using the chloramine T method [5], had been added to this mixture for purposes of following the fate of ConA during the preparation. Two ml of horse spleen ferritin, (130 mg; Miles Laboratories, Kankakee, Ill.), was then added, and the reaction volume stirred for 5 min at room temperature. At this time, 0.1 ml of 1% glutaraldehyde, final concentration of 0.025%, was added and the mixture gently stirred for 60 min at room temperature to allow coupling of the α-MG-ConA to the ferritin. The reaction mixture was then dialysed against several changes of PBS containing glycine (2 mg/ml) to stop the coupling reaction. The association constant for the binding of α-MG to ConA is such that most of the sugar was removed from the Con A during dialysis [6]. The dialysed mixture which now contained ConA–ferritin as well as ConA–ConA and ferritin–ferritin complexes was mixed with 30 ml of Sephadex G-200 (Pharmacia, Uppsala) suspended in PBS. The ConA–ferritin and the ConA–ConA complexes which contained free binding sites, were bound to the dextran molecules present on the outer surface of the Sephadex

Table 1. *DNA synthetic response of murine thymocytes to ConA and ConA–ferritin*

Medium	Native ConA 1 μg/ml	ConA–Ferritin 5 μg/ml	2 μg/ml	1 μg/ml
cpm 485	35 381	7 464	38 187	26 699

1.5×10^6 murine thymocytes were cultured for 72 h in 5% fetal calf serum to which was added medium, native ConA, or ConA–ferritin from which the concentration of ConA was calculated by determining the percentage of ^{125}I ConA in the final preparation (see text). DNA synthesis was determined by the addition of ^{3}H-TdR during the last 12 h of culture, and is expressed as cpm.

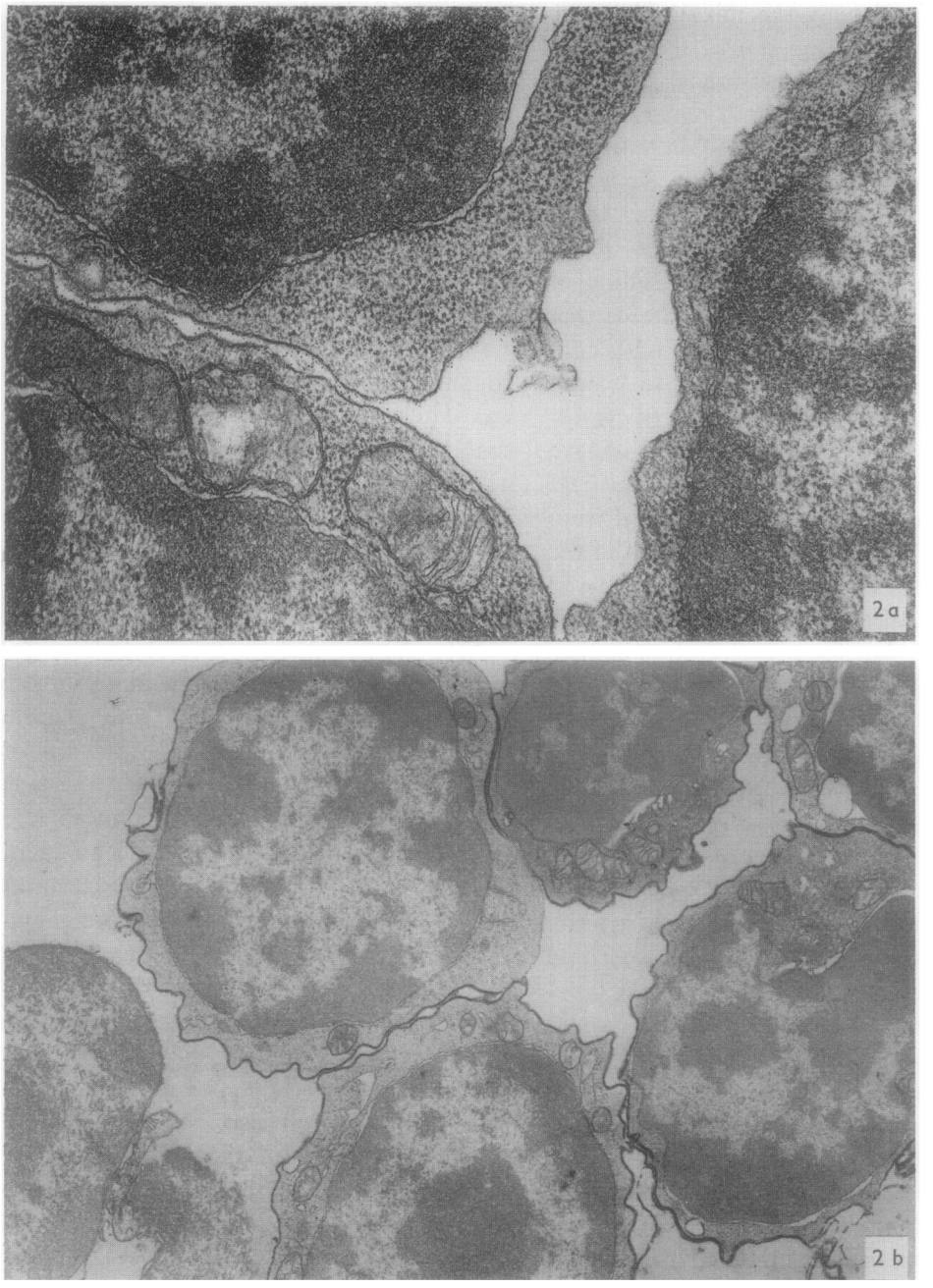

Fig. 2. (*a*) Electron micrograph of ConA-ferritin bound to murine thymocytes. Note the patchy distribution of the ConA–ferritin label. × 53 000; (*b*) Electron micrograph of ConA-cytochrome peroxidase bound to murine thymocytes. The distribution of this label is similar to that noted for ConA–ferritin, with areas of the cell surface negative for the reaction product. × 13 600.

beads. After allowing the mixture to react for 1 h at room temperature, the gel was washed on a sintered glass funnel with PBS to remove ferritin–ferritin complexes. The Con A–Con A as well as the Con A–ferritin complexes were eluted from the Sephadex with 1 M α-MG, and the eluted material passed over Sepharose 2B (Pharmacia) which had been equilibrated with PBS. Elution of the material with PBS revealed the pattern demonstrated in fig. 1. Arbitrarily, tubes containing balanced ratios of Con A to ferritin were pooled as indicated, and dialysed against several changes of PBS at 4°C to remove any α-MG still bound to the Con A. The final concentration of Con A was calculated from the percentage of radioactivity present in the sample and, as shown in table 1, the material was as biologically active as native Con A when tested for its capacity to stimulate murine thymocytes to incorporate ^{3}H-thymidine into DNA.

The localization of Con A binding sites on thymocyte surfaces was studied by adding Con A–ferritin complexes to murine thymocytes for ½ h at 4°C. These cells were then washed three times over 5% bovine serum albumin suspended in medium to remove unbound Con A–ferritin, and then prepared for electron microscopy by glutaraldehyde-osmic acid fixation. As can be seen (fig. 2*a*) a patchy localization of the Con A–ferritin on the surface membrane was noted.

An alternative approach to the cell surface localization of Con A binding sites involved the use of Con A–peroxidase complexes. Con A was coupled to cytochrome peroxidase (C-Pox) which had been isolated from baker's yeast (a generous gift of Dr Takashi Yonetani). This enzyme is well suited for linkage with Con A as it contains no detectable carbohydrate which may be bound by Con A. Horseradish peroxidase on the other hand, has a high mannose content, and is bound noncovalently by the Con A [7, 8]. This leads to the precipitation of Con A–Horseradish peroxidase complexes during the coupling procedure. Forty-two milligrams of Con A in PBS were added to 1 ml of α-MG in PBS. The α-MG was allowed to bind to the Con A for 60 min at room temperature. Ten milligrams of cytochrome peroxidase (0.5 ml) was then added and allowed to mix for 5 min after which 1% glutaraldehyde was added to a final concentration of 0.025%. After the coupling had proceeded for 1 h, the mixture was dialysed against several changes of PBS containing glycine (2 mg/ml), and then mixed with Sephadex G-200 as described previously. The C-Pox–C-Pox complexes were then eluted by washing with PBS, and the C-Pox–Con A as well as the Con A–Con A complexes removed by elution with α-MG.

After further dialysis against PBS to remove α-MG, the complexes, without further purification, were incubated with murine thymocytes for morphologic evaluation of Con A binding sites. After washing, the cells were fixed in suspension with an equal volume of 2% glutaraldehyde in Tyrode solution, washed with PBS, and the cytochemical reaction product developed with 3,3-diamino benzedine for 10–30 min at room temperature according to the method of Graham & Karnovsky [9]. The cells were then processed for both electron and light microscopy. Light microscopy showed gross patchy localization of dark reaction product, while the electron micrographs (fig. 2*b*) showed a pattern of binding identical to that noted for Con A-ferritin. Areas of the membrane devoid of reaction product are seen on both exposed membrane surfaces as well as at sites of cell membrane apposition. This implies that the localization is not due to simple trapping of reaction product.

Con A–ferritin and Con A–horseradish peroxidase complexes suitable for electron micro-

scopy have been described previously [10, 11]. However, in the case of the Con A–ferritin the material was not shown to be biologically active and in the case of the Con A–horseradish peroxidase, the linkage was not covalent. The techniques described here provide a simple general method for the preparation of biologically active Con A covalently linked to markers suitable for electron and light microscopic evaluation of cell surfaces. Utilizing these techniques, biologically active compounds useful for morphologic studies of cell surfaces may be prepared with other plant agglutinins. These compounds should be helpful in studies concerning surface membrane changes occurring during cell activation, as well as studies concerning the surface membrane architecture of normal and malignantly transformed cells.

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Inhibition of RNA, DNA and Protein Synthesis by Antisera in Mouse Leukemic Cells L5178YR

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Summary

An in vitro system containing a rabbit serum medium-adapted subline of L5178Y cells and rabbit antiserum was used to study the effects of antibodies, in the absence of complement, on cells in culture. Heat-inactivated antiserum inhibits cell growth and metabolism. These actions occur slowly. RNA synthesis is inhibited after 4 h, but the effects on the rates of protein and DNA synthesis become demonstrable only after 6 to 8, and 8 h, respectively. Maximal inhibition can be obtained after 24 to 48 h. These effects together with the apparent involvement of energy generating systems suggest that some indirect mechanism, triggered as a result of primary antigen-antibody reactions on the cell surface, is responsible.

We have reported that heat-inactivated rabbit antisera, in the absence of complement, inhibited cell growth, colony formation in soft agar, both uptake and incorporation of amino acid into cell proteins, and killed mouse leukemia L5178YR cells in culture [11, 12]. The inhibitory action of antisera was a slow process and became demonstrable only after 6 h and reached a maximum after 24 to 48 h. Energy sources such as glucose and succinate, but not malate, were found to alleviate, at least partially, the harmful effects of antisera. In order to delineate the possible site of initial action of antiserum we studied the time course effects of antisera on the rates of RNA, DNA and protein synthesis.

Materials and Methods

L5178YR cells: These have been described previously [11].

Antisera: The sera were prepared as described before [11], collected aseptically and stored at −70°C. They were heat-inactivated (56°C, 30 min) just before use. Heat-inactivated preimmune sera and other normal sera were used as controls.

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