Lymphocyte Mechanical Response Triggered by Cross-linking Surface Receptors

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ABSTRACT Using a recently developed method (Petersen, N. O., W. B. McConnaughey, and E. L. Elson, 1982, *Proc. Natl. Acad. Sci. USA.*, 79:5327–5331), we have measured changes in the deformability of lymphocytes triggered by cross-linking cell surface proteins. Our study was motivated by two previously demonstrated phenomena: the redistribution ("capping") of cross-linked surface immunoglobulin (sIg) on B lymphocytes and the inhibition of capping and lateral diffusion ("anchorage modulation") of sIg by the tetravalent lectin Concanavalin A (Con A). Both capping and anchorage modulation are initiated by cross-linking cell surface proteins and both require participation of the cytoskeleton.

We have shown that the resistance of lymphocytes to deformation strongly increased when sIg or Con A acceptors were cross-linked. We have measured changes in deformability in terms of an empirical "stiffness" parameter, defined as the rate at which the force of cellular compression increases with the extent of compression. For untreated cells the stiffness was ~0.15 mdyn/μm; for cells treated with antibodies against sIg or with Con A the stiffness increased to ~0.6 or 0.4 mdyn/μm, respectively. The stiffness decreased after completion of the capping of sIg. The increases in stiffness could be reversed to various extents by cytochalasin D and by colchicine. The need for cross-linking was demonstrated by the failure both of monovalent Fab' fragments of the antibodies against sIg and of succinylated Con A (a poor cross-linker) to cause an increase in stiffness. We conclude that capping and anchorage modulation involve changes in the lymphocyte cytoskeleton and possibly other cytoplasmic properties, which increase the cellular viscoelastic resistance to deformation. Similar increases in cell stiffness could be produced by exposing cells to hypertonic medium, azide ions, and to a calcium ionophore in the presence of calcium ions. These results shed new light on the capabilities of the lymphocyte cytoskeleton and its role in capping and anchorage modulation. They also demonstrate that measurements of cellular deformability can characterize changes in cytoskeletal functions initiated by signals originating at the cell surface.

Cross-linking lymphocyte surface proteins by multivalent ligands can initiate dramatic changes in the distribution and mobility of cell surface proteins. For example, cross-linking surface immunoglobulin (sIg)[1], the B lymphocyte antigen receptor, by bivalent anti-sIg antibodies first immobilizes (1) and then causes an active redistribution of the aggregated ("patched") sIg to one pole of the cell to form a "cap." Subsequently the cell shape changes by formation of a uropod at the site of the cap, and cell motility and chemotaxis can begin (2–4). Evidence from a variety of sources suggests that cytoskeletal contractility is involved in these events. They require cellular energy and can be inhibited by cytochalasins, which interfere with the assembly of actin microfilaments (2). Moreover, a morphological response similar to that which is observed after capping can be simulated by adding ATP in the presence of calcium and magnesium ions to glycerinated

[1] *Abbreviations used in this paper:* Anti-IgM, antibody specific for mouse immunoglobulin M; Con A, Concanavalin A; DME-HEPES, Dulbecco's modified Eagle's medium with 20 mM HEPES and 0.1% bovine serum albumin; FITC-Con A, Concanavalin A labeled with fluorescein isothiocyanate; IgM, immunoglobulin M; PBS, phosphate-buffered saline; s-Con A, succinylated Concanavalin A; sIg, lymphocyte surface immunoglobulin; 2xPBS, twofold concentrated phosphate-buffered saline.

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lymphocytes (5). Immunofluorescence studies have demonstrated co-localization with both patches and caps of many cytoskeletal components which might be involved in cellular contractility including actin (6, 7), myosin (8, 9), alpha-actinin (10, 11), calmodulin (12, 13), and spectrin (14, 15). More recently, evidence for the participation of myosin light chain kinase in these processes has suggested similarities between the regulation of capping and contraction of smooth muscle (16–18).

Other multivalent ligands such as the plant lectin Concanavalin A (Con A), which can bind to a wide range of cell surface glycoproteins, can elicit different but apparently related responses. On cells exposed to Con A at sufficiently high concentration the lectin does not cap, and it inhibits the capping of sIg-anti-sIg aggregates in a process called "anchorage modulation" (19, 20). Moreover, exposure to Con A paralyzes cell motility (21). Inhibition of capping by Con A probably results from retardation of the lateral mobility of the sIg molecules (22). Although Con A might bind directly to sIg, there is good evidence that its inhibitory effect on the mobility of sIg is exerted indirectly through the cytoskeleton. The inhibitory effect is reversed by colchicine and cytochalasin B (22, 23). Furthermore, experiments in which the binding of Con A was restricted to local regions on the lymphocyte surface demonstrate that patching and sIg mobility can be retarded at locations on the cell remote from the sites at which the Con A is bound (22, 24, 25). (Different responses to Con A can be observed under other conditions. On cells exposed to low concentrations of Con A [<5 μm/ml] or preincubated at low temperatures or with colchicine [which interferes with microtubule assembly] and then exposed to Con A, Con A-acceptor complexes can cap.)

In these responses there seems to be a reflexive pattern in the interactions from plasma membrane to cytoskeleton and then from cytoskeleton back to the plasma membrane. The changes in the cytoskeleton and in surface-cytoskeleton interactions which result from the initial cross-linking can, in turn, influence the mobility and distribution of both the cross-linked and other membrane proteins.

Although evidence for the involvement of cytoskeletal contractility in these processes comes from a variety of sources, the direction of cause and effect is not thoroughly established. Conflicting interpretations of the role of the cytoskeleton have been proposed (26, 27). In this study we questioned whether the proposed contractile response of the lymphocyte cytoskeleton to cross-linking surface proteins could be detected by direct mechanical measurements. We expected that the development of substantial contractile forces in the lymphocyte cytoskeletal cortex might exert a circumferential tension around the nucleus which would increase the resistance of the cell to deformation. To test this hypothesis, we have compared the resistance to compression of untreated cells with that of cells on which surface proteins have been cross-linked under conditions which lead to capping or anchorage modulation. This has been accomplished using a recently developed method for measuring the viscoelastic resistance of cells to deformation (28).

We have found that there was indeed a substantial cytoskeleton-dependent increase in the resistance of lymphocytes to deformation in cells exposed to surface cross-linking ligands. This elevated resistance decreased after capping or exposure to cytochalasin D or colchicine. Certain ligand-independent treatments, such as elevation of the intracellular calcium ion concentration or exposure to azide ion or hypertonic medium, also increased lymphocyte stiffness. These results demonstrate the use of direct measurements of cellular viscoelasticity as a probe of cellular and cytoskeletal function.

MATERIALS AND METHODS

Reagents

Con A labeled with fluorescein isothiocyanate (FITC-Con A) was purchased from Miles-Yeda, Rehovot, Israel; cytochalasin D, colchicine, A23187 (calcium ionophore), and sodium azide from Sigma Chemical Co., St. Louis, MO; and rhodamine-labeled succinylated-Con A (s-Con A) from Vector Laboratories, Inc., Burlingame, CA. Fluorescein-labeled, affinity-purified goat IgG antibody specific for mouse immunoglobulin M (anti-IgM) and fluorescein labeled $F(ab')_2$ fragments of this antibody were obtained from Cappel Laboratories, Cochranville, PA. Fluorescein-labeled monovalent Fab' fragments of the same antibody were prepared by reduction of the $F(ab')_2$ with 2-mercaptoethanol followed by alkylation with iodoacetamide and chromatography on Sephadex G-100 (29). SDS PAGE under nonreducing conditions detected no contamination by $F(ab')_2$ fragments. Covaspheres conjugated with FITC-Con A were prepared using MX beads purchased from Covalent Technology Corp., Ann Arbor, MI. The beads, which are polystyrene spheres 0.7 μm in diameter with carbonyl sites on their surfaces, were diluted 1:10 in distilled water and sonicated for 1 min in a bath sonicator (Bransonic 2) (Branson Sonic Power Co., Danbury, CT). They were then resuspended and reacted with 0.4 mg/ml FITC-Con A in phosphate-buffered saline (PBS) (pH 7.0) for 75 min at room temperature. The beads were then centrifuged at 8,000 rpm, washed and sonicated in PBS + 1% bovine serum albumin to saturate binding sites, and stored in PBS + 1% bovine serum albumin + 0.1% azide at 4°C.

Cells

Mouse spleen lymphocytes were harvested from 8–16-wk-old BALB/c mice (Harlan Sprague Dawley, Inc., Indianapolis, IN) (30). A population of cells highly enriched in B lymphocytes (90% as judged by immunofluorescence detection of cells bearing sIg) was prepared from the spleens of athymic nude mice (obtained from Harlan Sprague Dawley, Inc.). The cells were suspended in Dulbecco's modified Eagle's medium with 20 mM HEPES (pH 7.3) and 0.1% bovine serum albumin (DME-HEPES, prepared by the Washington University Cancer Center). The cells were passed through nylon wool, centrifuged, and resuspended in Tris buffer + 0.75% NH_4Cl (pH 7.2) to lyse erythrocytes, then centrifuged immediately, and washed in DME-HEPES. Cell viability was 95% as judged by trypan blue exclusion. Cells were stored on ice or at room temperature in DME-HEPES.

Cell Treatments

A suspension of lymphocytes (0.2 ml, 2–5 × 10^6 cells/ml) was placed on a round glass coverslip (No. 2, 18-mm diam, Taylor Chemical Co., St Louis, MO), and the cells were allowed to settle and attach to the glass for 5–10 min at room temperature. The untreated cells adhered well to the glass; only a few detached when the coverslip was inverted and mounted in the cell poker chamber (28). The chamber was thermostatted and contained 10 ml of DME-HEPES. Lymphocyte deformability was measured at room temperature.

CON A: Lymphocytes were incubated with Con A at 30 μg/ml for 30 min at room temperature in DME-HEPES. The cells were then washed, allowed to adhere to glass coverslips for 5–10 min, and mounted in the cell poker as described in the legend to Fig. 2. Compounds to be tested for their ability to reverse the Con A-induced increase in cellular stiffness were incubated with the Con A-treated cells for 30–45 min before measurement of cellular deformability in the presence of the compound. The inhibitors included 1 μg/ml cytochalasin D (30 min), 10 μM colchicine (30 min), and 10 mM sodium azide (45 min). FITC-Con A-Covaspheres were added to cells preattached to glass coverslips and allowed to react with the cells for 10 min at room temperature. The cells were then washed and immersed in the cell poker chamber. Cells with beads on their surfaces were readily distinguished in the microscope. A fraction of the cells carried no beads. Hence control and modulated cells could be measured on the same coverslip.

ANTI-IGM: Lymphocytes were incubated on ice for 30 min with 100 μg/ml fluorescein-labeled anti-IgM antibody, or with 100 μg/ml $F(ab')_2$ or 200 μg/ml Fab'-labeled fragments of that antibody. The cells were washed at 4°C, allowed to attach to the glass coverslips for 5 min at room temperature, and then their deformability was measured. The binding of each of the antibody preparations to the cells was verified by fluorescence microscopy. Cells treated

in this way with divalent antibody or F(ab')$_2$ were in the midst of the capping process during the deformability measurement carried out at room temperature over ~20 min. Reversal by cytochalasin D or cholchicine of the modulation of cell stiffness by anti-IgM was carried out as described above. To prepare cells which had completed capping, we incubated lymphocytes exposed to anti-IgM as described above for an additional 30 min at 37°C before measurement of deformability at room temperature.

NONLIGAND TREATMENTS: Cells were exposed to several treatments which did not involve surface ligands (Con A or anti-IgM). Before exposure to hypertonic medium cells were allowed to settle on a coverslip for 10 min in a small volume of PBS. Twofold concentrated PBS (2xPBS) was then gradually added dropwise at room temperature for ~1 min. Cells were treated by sodium azide as described above. Cells were incubated with the ionophore A23187 at 1 μg/ml in the presence of 1.8 mM Ca^{2+} ions for 30 min at room temperature before measurement in the presence of the ionophore and Ca^{2+} ions. Reversal by cytochalasin D of the modulation of stiffness by nonligand treatments was carried out as described above. Prevention by cytochalasin D of the modulation of stiffness by azide ions was tested by incubating cells with cytochalasin D (1 μg/ml) and azide ion (10 mM) simultaneously for 45 min and measuring stiffness in the presence of both substances.

MEASUREMENTS OF CELLULAR DEFORMABILITY: The design and principles of operation of the Cell Poker have been described previously (28, 31). In our previous applications of this method, we studied spread cells with dimensions much larger than the diameter of the stylus tip. The lymphocytes studied in this work are much smaller cells. Hence rather than measuring the resistance to local indentation as before, we have in this work measured the resistance to small degrees of cellular compression. Cells with diameters of ~6 μm were compressed ~1μm using a stylus with tip diameter of ~4 μm as depicted in Fig. 1. The operation of the measurement is described schematically in Fig. 2. The cells, adherent to coverslips, are placed in the thermostatted chamber facing down toward the beam and stylus assembly, immersed in an appropriate medium or balanced salt solution. The entire assembly is mounted on a Leitz-McBain microscope fitted with a 32× Hoffman modulation contrast objective to permit observation of the cells and the positioning of the stylus with respect to the cell to be measured. In the experiments reported in this paper, the motor was programmed to execute a single triangular waveform (e.g., Fig. 2) with a velocity of 3.4 μm/s and total amplitude of 1.7 μm.

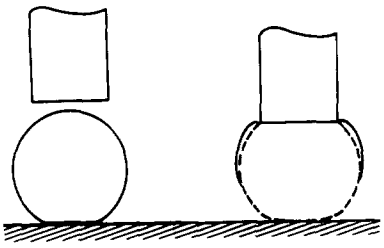

FIGURE 1 Schematic view of the deformation of a lymphocyte during compression by the glass probe tip of the Cell Poker.

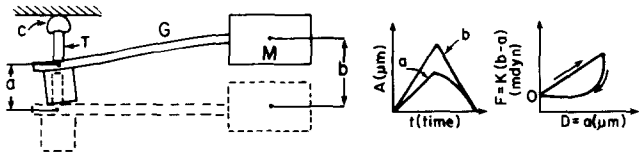

FIGURE 2 Schematic representation of a Cell Poker measurement (not drawn to scale). The bending of a horizontal glass beam (G) gauges the resistance to cellular deformation. A vertical glass stylus (*T*) with tip diameter 3–4 μm is mounted at the end of a 3-cm long horizontal vicor glass beam. The other end of the beam is mounted on a linear piezoelectric motor (*M*) which moves vertically according to a programmed waveform. Optical sensors monitor the vertical positions of the stylus and the motor. When the tip does not contact the cell (*C*) during its excursion, its displacement matches that of the motor (curve *b*). When the tip contacts the cell, the resistance of the cell to deformation retards the motion of the tip (curve a) and thereby bends the horizontal beam. The extent to which the beam is bent at each stage of the compression cycle is proportional to the force exerted on the tip by the cell and is determined by comparing the tip displacements in the presence and absence of cell contact (i.e., curves *a* vs. *b*). The force (*F*) exerted by the cell on the tip is then determined using the force constant of the beam (*k*), which has been obtained by a prior calibration. Then the plot of force versus tip displacement is obtained as shown. The degree of indentation (*D*) is measured from the point at which the force first deviates from zero.

Data Analysis

Ideally we should characterize the force versus displacement curves obtained in Fig. 2 in terms of a continuum mechanical analysis of the deformation of an object with the appropriate shape and viscoelastic properties. We could then quantitatively determine changes in the viscous and elastic contributions to cellular resistance to deformation. However, an appropriate analytical scheme has not yet been developed (32). Therefore, we used the slope of the first (ingoing) limb of the force versus deformation curve as an empirical measure of cellular viscoelasticity. This slope or "stiffness" in units of mdyn/μm increases whenever either the cellular elastic or viscous resistance to deformation increases. For many measurements, including all those of untreated cells, the ingoing limb of the force versus deformation plot was approximately linear (e.g., Fig. 4), thereby yielding an unambiguous determination of the stiffness. Frequently, however, we observed in treated cells a biphasic curve in which there was a small concave downward portion followed by a larger linear or concave upward phase (e.g., Fig. 3). In these instances, we fitted the first two-thirds of the plot to a linear form thereby eliminating the largest deviations from linearity which occur at the largest extents of compression. In plots that show a small concave downward initial phase this fitting procedure may underestimate the initial stiffness. This fitting procedure seems to provide a relatively low estimate of the changes in cell stiffness which we have observed. Furthermore, when cells stiffened due to the various treatments described in this work, a fraction of the cells fell off the coverslip and so could not be measured. It seems likely that these could be the stiffest cells. Hence we conclude that we have probably obtained a lower bound for the magnitude of the increases in cell stiffness which occurred in the cell population.

We considered the possibility that the measured changes in cell stiffness could have resulted from changes in cell shape. We tested this by comparing the vertical thicknesses of untreated cells with those of cells exposed to anti-IgM. The thickness of a cell was measured by raising the probe tip to the point at which it just came into contact with the cell as detected by microscopic observation. Then the probe was moved laterally away from the cell, and the distance of its tip from the substrate was measured using the calibrated sensor of the Cell Poker.

Occasionally we have used Student's *t* test to determine the statistical significance of the difference between the means of two measured distributions of stiffness values. We should note, however, that the application of this test to our measurements is of uncertain validity. The distributions of stiffness, especially of modulated cells, may not be normal, nor can we assert that the variances of the compared distributions are the same. Nevertheless, we have used Student's *t* test for the sake of simplicity, despite these possible violations of the preconditions for its application, to provide a rough estimate of statistical significance in the few instances in which the presence or absence of a significant difference might be questioned.

RESULTS

As seen in Figs. 3*B* and 4, *A–F*, measurements on untreated and on some of the treated primary spleen lymphocytes yielded an approximately linear dependence of force on extent of compression for compressions of ~1 μm. Therefore, it is appropriate to use the slope of this plot as an empirical "stiffness" coefficient with units mdyn/μm to characterize quantitatively the resistance of the lymphocyte to compression. The control population of untreated mixed B and T spleen lymphocytes had an average stiffness of 0.15 ± 0.08 mdyn/μm. Furthermore, at comparable extents of deformation the force exerted on the stylus tip by the cell was smaller as the deformation decreased (tip force decreasing) than it was as the deformation increased (tip force increasing). This hysteresis corresponds to a dissipation of energy and in studies of fibroblast deformability was attributed at least in part to cytoplasmic viscosity (28, 31). The hysteresis seen in Fig. 3*B* and that generally observed in the measurements on untreated

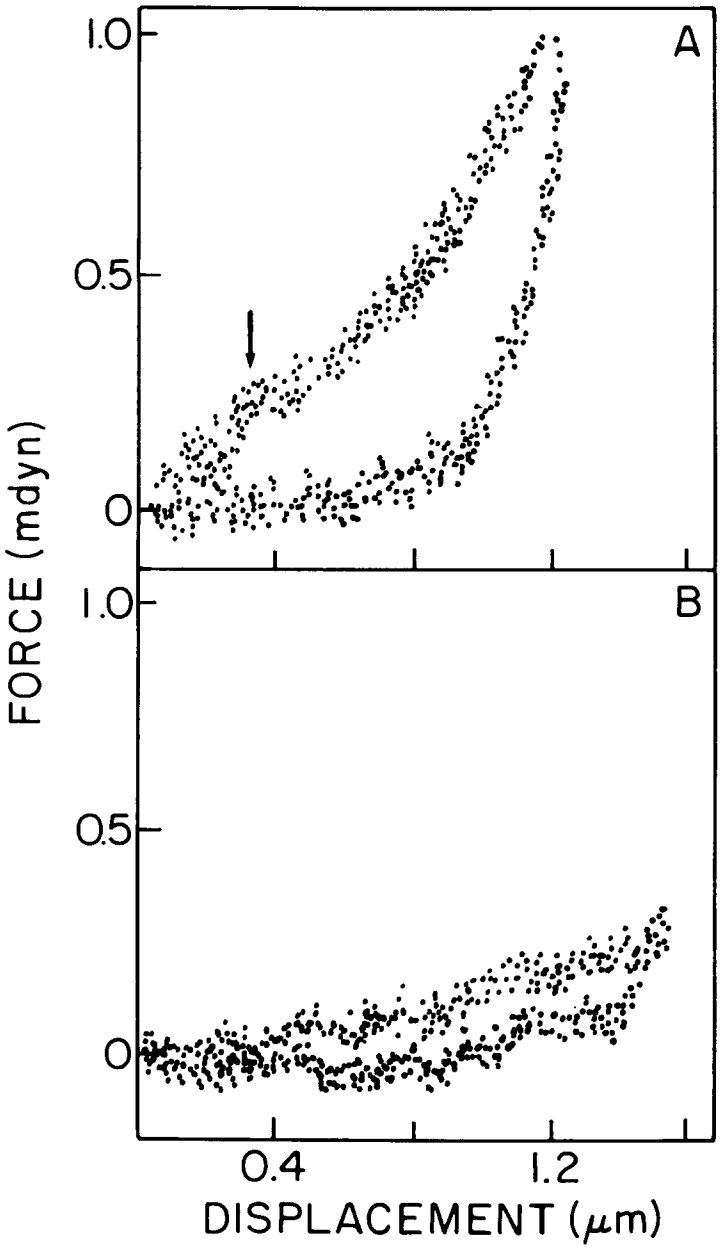

FIGURE 3 Examples of force versus displacement data obtained on lymphocytes using the Cell Poker. (*A*) B lymphocyte with sIg cross-linked by anti-IgM antibody. The curve shows a small region of negative curvature just after the probe tip contacts the cell surface. This is followed by a much larger phase with positive curvature. This bimodal appearance is observed in ~50% of the measurements made on cells treated with anti-IgM which show an increased stiffness. Although the existence of the initial phase with negative curvature is not convincingly established by a single curve, its appearance in a substantial fraction of the measurements made on cells treated in this way suggests that it represents an authentic mechanical property. As discussed in the text, the initial phase might represent resistance to bending; the later phase, resistance to stretching. The value of the stiffness obtained by fitting the first two-thirds of this curve is 0.64 mdyn/μm. (*B*) Control measurement on an untreated lymphocyte. The stiffness value obtained from this measurement is 0.18 mdyn/μm.

lymphocytes was typically much smaller than seen in adherent fibroblasts.

Cross-linking sIg using an anti-IgM antibody caused a marked change in the observed force-deformation curve as shown in Fig. 3*A*. The rate of increase of force with compression (i.e., the stiffness) and the hysteresis area were both substantially increased. Modulation of the cellular viscoelasticity by the various treatments used in this work not only increased the cellular resistance to deformation but also produced changes in the detailed shapes of the force-compression curves. Linear plots were often obtained. Frequently, however, the plots deviated from linearity. Often they were biphasic with a small early concave-down component followed by a much larger linear or concave-up component. Fig. 3*A* provides an example. As an approximate estimate of stiffness from these measurements we chose the average slope of the first two-thirds of the in-going branch of the force-deformation plots as described in Materials and Methods.

In principle, the observed change in stiffness might result from a change in cell shape. For example, cross-linking sIg might have caused the cells to spread more and thereby reduce their thickness normal to the substrate. This reduction in cell thickness could account for an increase in stiffness (32). We tested the effect of cross-linking sIg on cell thickness as described in Materials and Methods. We observed that the average thickness ± SD of 20 untreated cells was 4.76 ± 0.33 μm. For 20 cells in the midst of capping after treatment with anti-IgM (100 μg/ml), the average thickness was 4.77 ± 0.32 μm. Hence we cannot attribute the observed increases in stiffness to decreases in cell thickness. The diameter of the cells as observed through the microscope was similar to their height so that even these lymphocytes which adhere to a solid substrate preserve an approximately spherical shape.

Our quantitative measurements revealed a consistent pattern: lymphocytes became stiffer in response to various treatments such as cross-linking of sIg or Con A acceptors or exposure to azide ion, to a calcium-specific ionophore and extracellular calcium ions, or to hypertonic medium. More-

over, we observed that the stiffer modulated cells adhered less well to glass coverslips and had a rougher appearing surface compared with untreated cells.

Con A

When a mixed population of mouse spleen lymphocytes was treated with FITC-Con A at a concentration of 30 μg/ml, the cells displayed a bright ring of diffuse fluorescence. No caps or patches of aggregated Con A were detected over a 2-h period at room temperature. This observation verifies that the mobility of the Con A acceptor complexes had been retarded and that the cells were under anchorage modulation. This modulation was likely to preserve the cells in a constant cytoskeletal state during the measurement of deformability (~30 min). Fig. 4, *a* and *d* characterizes the effect of Con A on the deformability of a population of mixed spleen lymphocytes. Upon exposure to Con A, there was a large increase in the average resistance to deformation; the stiffness values increased almost threefold to 0.40 ± 0.20 mdyn/μm. The distribution of values was broad with an SD of 50% of the mean. A considerable fraction of the treated cells continued to have stiffness values in the same range as the untreated control cells. Note that the standard deviation of stiffness values relative to the mean was also large (30–50%) for control cells. This heterogeneity in deformability is possibly related to the heterogeneity of immunological functions in this mixed population of lymphocytes (see below). As seen in Fig. 4*a*, the increase in stiffness caused by Con A was correlated with a large increase in the hysteresis area of the force-compression plots; this suggests that viscous forces contribute to the increased resistance to deformation.

Cytoskeletal inhibitors weakened the increase in lymphocyte stiffness caused by Con A. Fig. 4 depicts examples of

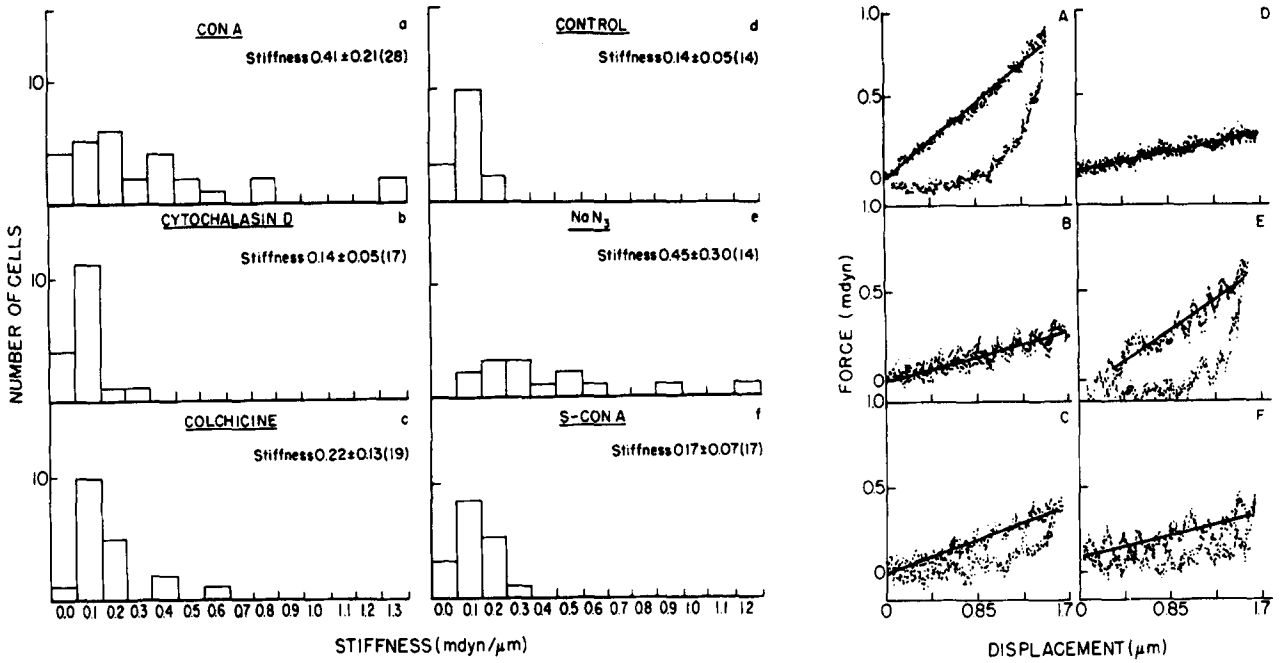

FIGURE 4 Effects of various inhibitors on the increased stiffness of lymphocytes treated with Con A (at 30 μg/ml). (*a–e*) Distributions of stiffness values obtained on lymphocytes treated first with Con A and then with the listed inhibitors as described under Materials and Methods. (*f*) Distribution of stiffness values obtained on lymphocytes treated with s-Con A (at 30 μg/ml). (*A–F*) Representative force versus displacement curves from each of the populations shown in *a–f*. The linear fit to the ingoing limb of the plot which determines the stiffness value is superimposed. The stiffness values obtained, which are close to the means for the respective populations, are (*A*) 0.54, (*B*) 0.16, (*C*) 0.24, (*D*) 0.13, (*E*) 0.43, and (*F*) 0.15 mdyn/μm.

TABLE I

Modulation of Deformability of Mouse Spleen Lymphocytes by Con A

Ligand	Stiffness ± SD		Treatment after Con A	Stiffness + SD	
	mdyn/μm	*N*		*mdyn/μm*	*N*
–	0.14 ± 0.06	(23)			
–	0.14 ± 0.05	(14)	Cytochalasin D	0.13 ± 0.04	(15)
Con A	0.41 ± 0.21	(28)	Cytochalasin D	0.14 ± 0.05	(17)
Con A	0.41 ± 0.21	(28)	Colchicine	0.22 ± 0.13	(19)
Con A	0.41 ± 0.22	(19)	Cytochalasin D + 0.5 μM Colchicine	0.17 ± 0.06	(18)
Con A	0.49 ± 0.27	(12)	NaN_3	0.45 ± 0.30	(14)
S-Con A	0.17 ± 0.07	(17)			
Covaspheres-Con A	0.39 ± 0.23	(17)			

Cells were incubated with Con A or s-Con A (30 μg/ml) for 30 min at room temperature, then washed and incubated with inhibitors for 30 min at room temperature. We measured the deformability with inhibitors present. Cytochalasin D (1 μg/ml), colchicine (10 μM), NaN_3 (10 mM). *N*, Number of cells measured.

typical force-deformation plots for cells subjected to various treatments along with histograms which characterize the responses of the several populations of treated cells. The results are summarized in Table I. The increase in stiffness provoked by Con A was entirely reversed by cytochalasin D (Fig. 4, *b* and *B*). In contrast, cytochalasin D had no effect on the deformability of the already soft control cells (Table I). Colchicine partially reversed the stiffening effect of Con A. Sodium azide, which poisons oxidative energy production and depletes the concentration of intracellular ATP, prevents capping but not the inhibition of the lateral mobility of sIg by Con A (2, 22). Surprisingly, azide ion alone caused an increase in lymphocyte stiffness comparable with that produced by Con A (Table III) and did not affect the stiffness of cells already treated with Con A. A dimeric fragment of Con A, s-Con A, which is a less effective cross-linker of surface proteins than the tetrameric parent molecule, did not significantly increase lymphocyte stiffness.

Although the involvement of the cytoskeleton was demonstrated by the effects of colchicine and cytochalasin D, it might be supposed that the generation of a uniform layer of cross-linked Con A acceptor complexes over the cell surface could contribute directly to the observed increase in stiffness. To test this possibility, we have treated a mixed population of spleen lymphocytes with 0.7-μm spheres (covaspheres) to which FITC-Con A was covalently attached. We measured the deformability of cells which had 10 or more beads on their surfaces, a percentage of surface coverage shown previously with Con A platelets to be at or above the threshold for anchorage modulation of sIg mobility (22). These cells had a discontinuous surface distribution of Con A including ~25% of the surface area which was in contact with the glass coverslip before addition of the beads and which was therefore free of the beads. The cells treated in this way showed an increase in resistance to compression similar to that caused by soluble Con A (Table I). Hence continuity of the surface layer of cross-linked Con A acceptor complexes was not necessary for the observed increase in stiffness. Cells on the same coverslip which had no beads attached to their surfaces had the same stiffness values as untreated control cells.

Surface Immunoglobulin

We verified that cells observed as soon as possible after incubation with anti-IgM at room temperature were in the midst of the capping process. Using fluorescence-labeled antibody, about half of the cells were seen to have patches of aggregated antibody-sIg complexes. Approximately 50% of these labeled B cells showed caps in various stages of development at the end of the 25-min measurement period. A typical example of the increases in resistance to compression and in the hysteresis area of the force-compression curve caused by cross-linking sIg is shown in Fig. 3. On the average, there was a three- to fourfold increase in stiffness in a population of unfractionated mouse spleen lymphocytes. As is shown in Fig. 5, *a* and *b*, there was a wide distribution of stiffness values in the treated cells, with some cells in the treated population having values in the same range as cells in the untreated control population. That this modulation of cellular viscoelasticity depended on cross-linking the sIg molecules is shown in Fig. 5, *c–f*. Fab′ fragments of the anti-mouse IgM antibody elicited only a slight increase in stiffness which is, however, statistically significant to $p < 0.005$ according to Student's *t* test. (This small increase in stiffness might have been caused by a small contamination of divalent antibody fragments, although none was detected by gel electrophoresis, or it could have been a genuine response to the binding of monovalent ligands.) When cells to which the Fab′ fragments of the goat anti-mouse IgM were bound were treated with divalent rabbit antibody directed against goat IgG, the resulting cross-linking of the Fab′-sIg complexes caused an increase in the resistance to compression which was comparable with that provoked by the intact anti-IgM antibody or the divalent $F(ab')_2$ fragment. Fig. 5, *b*, *d* and *f* show that stiffness values were distributed over a wide range in modulated cells comparable to that seen in cells treated with Con A. This could have resulted from heterogeneity in the cell population which included lymphocytes (T cells among others) which do not have immunoglobulin on their surfaces, from heterogeneity in the response among cells bearing sIg, and from the cell-to-cell variation in the extent

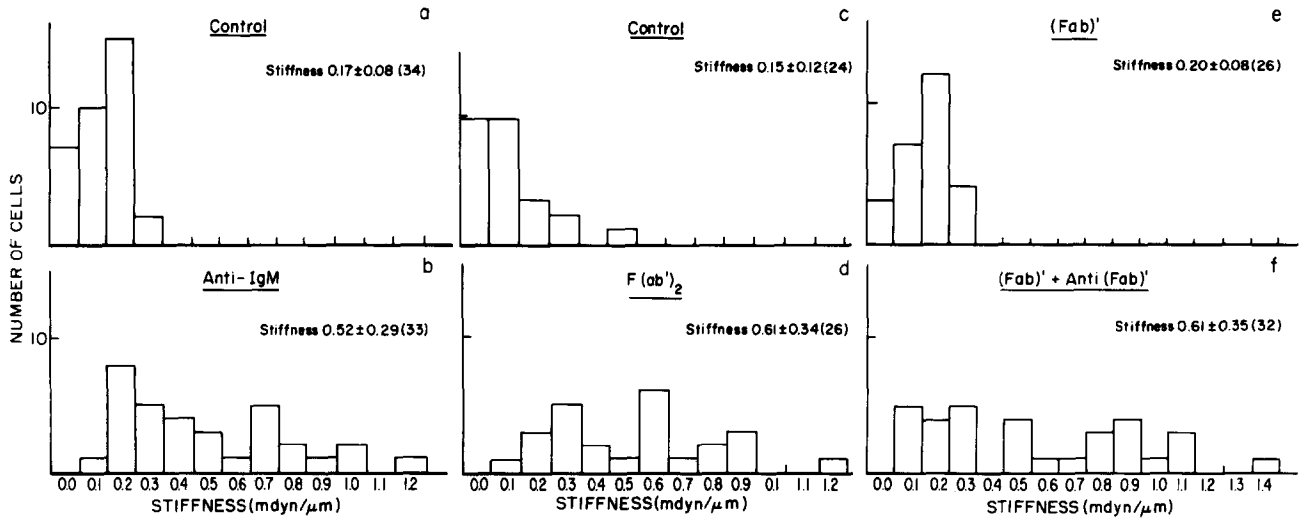

FIGURE 5 Requirement for sIg cross-linking to produce an increase in cell stiffness. (*a* and *b*) Control (untreated) cells and cells treated with anti-IgM. (*c* and *d*) Control and cells treated with $F(ab')_2$ fragments of anti-IgM. (*e* and *f*) Cells treated with monovalent Fab′ fragments of anti-IgM and cells treated with Fab′ fragments and bivalent anti-Fab′ antibody.

TABLE II

Modulation of Deformability of Mouse Spleen Lymphocytes by Anti-IgM Antibodies

Ligand	Condition	Stiffness + SD	
		mdyn/μm	*N*
Effect of Capping			
–	Control	0.16 ± 0.09	(34)
Anti-IgM	During capping*	0.56 ± 0.30	(42)
Anti-IgM	After capping‡	0.27 ± 0.10	(55)
Effects of inhibitors			
–	Control	0.16 ± 0.08	(46)
Anti-IgM	During capping*	0.47 ± 0.27	(70)
Anti-IgM	Cytochalasin D§	0.30 ± 0.11	(18)
Anti-IgM	Colchicine§	0.23 ± 0.12	(27)
Anti-IgM	Cytochalasin D + Colchicine	0.19 ± 0.10	(45)
Requirement for cross-linking‖			
–	Control	0.15 ± 0.06	(56)
Fab′ (anti-IgM)		0.20 ± 0.09	(81)
Fab′ + anti-Fab′	During capping*	0.61 ± 0.35	(32)
$F(ab')_2$	During capping*	0.61 ± 0.34	(26)

All mechanical deformability measurements were done at room temperature. *N*, Number of cells measured.

* Incubated with anti-IgM (100 μg/ml) for 30 min at 0°C. Washed at 4°C and allowed to settle on substrate for 10 min at room temperature. Measured during subsequent 20-min interval.

‡ After incubation with anti-IgM and adherence to substrate as above, incubated for an additional 30 min at 37°C on substrate.

§ Cells prepared as described (*) were subsequently incubated with cytochalasin D (1 μg/ml), colchicine (10 μM), or both for 30 min at room temperature.

‖ Fab′ fragments (200 μg/ml), anti-Fab′ (100 μg/ml), $F(ab')_2$ (100 μg/ml).

of capping in the population of measured cells.

Cytochalasin D, in contrast to its complete reversal of the response to Con A, only partly reversed the increased stiffness caused by anti-IgM (Table II). Colchicine was comparable to cytochalasin D in its partial reversal of the increased stiffness and had similar effects on cells treated either with Con A or anti-IgM. Colchicine and cytochalasin D acting together were more effective than either was separately and appeared to be able to reverse the response to anti-IgM almost completely.

Completion of the capping process also partially reversed the increase in stiffness induced by anti-IgM antibodies (Table II) (Fig. 6). Lymphocytes which were treated with anti-IgM as described above were incubated at 37°C for an additional 30 min to produce the cells which had completed capping. With the use of fluorescence-labeled anti-IgM, we observed that 80% of labeled cells had formed caps after 10 min under these conditions. Previous work suggests that dissipation of the cap by internalization or shedding has already begun at this time (33, 34). By 30 min at 37°C capping was over. Of the cells incubated for this period, some had elongated shapes and a few had a spot of fluorescence. A fluorescent rim was detected on none of the cells, but most cells showed a diffuse weak fluorescence which might have resulted from internalized surface complexes. Fluorescent particles, possibly shed from the cell surface, were seen in the medium. The deformability measurements were carried out at room temperature. As seen in Fig. 6, the average stiffness of cells after capping was 0.27 ± 0.10 mdyn/μm, about half the value obtained from cells in the midst of capping (0.56 ± 0.30 mdyn/μm). In cells which had completed capping as in untreated control cells the distribution of stiffness values was narrower, the cell surface apparently smoother, and the cells were more firmly attached to the glass coverslip. Finally, we observed that incubation of cells at 37°C for 15 min which have not been exposed to surface cross-linking agents caused a small increase in the stiffness from 0.16 ± 0.07 to 0.20 ± 0.08 mdyn/μm. This difference was of marginal statistical significance ($0.05 < p < 0.1$).

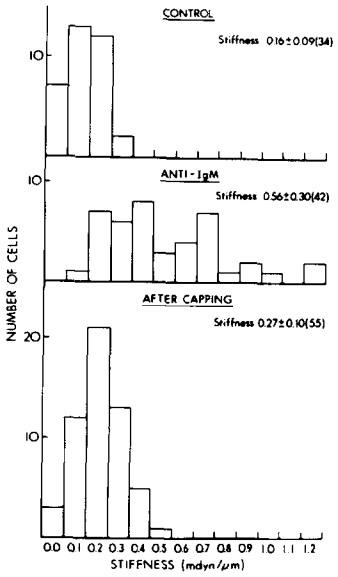

FIGURE 6 Change of stiffness with time during and after the capping of sIg. (*Top panel*) Control. (*Middle panel*) Cells treated with anti-IgM on ice for 25 min were then measured during the capping process at room temperature. During the 20-min measurement interval the percentage of capping cells increased from 10% to 50%. (*Bottom panel*) Cells treated as in previous panel and then incubated for 30 min at 37°C before measurement at room temperature. Most of the B cells in this sample have eliminated their fluorescent caps. Hence stiffness diminishes at this late stage of the process.

Table II summarizes the modulation of deformability of mouse spleen lymphocytes by anti-IgM. Cross-linking sIg induced a larger increase in stiffness than did Con A under comparable conditions despite the fact that Con A should bind to all the lymphocytes whereas less than half of the cells bear sIg. (According to Student's *t* test, the mean stiffness of cells treated by Con A was significantly different from that of cells treated by $F[ab']_2$ fragments of anti-IgM and from that of cells treated by Fab′ fragments cross-linked by a second antibody at $p < 0.005$ and from cells treated by anti-IgM at $p < 0.025$.)

Ligand-independent Treatments

Cross-linkage of surface proteins by multivalent ligands is not a necessary condition for their capping. This is well-demonstrated by the induction of capping of several proteins on lymphocyte membranes by hypertonic medium (e.g., 2xPBS) in the absence of cross-linking ligands (35, 36). Exposure of lymphocytes to 2xPBS increased cellular stiffness to an extent comparable to that produced by Con A (Table III). Morever, the breadth of the distribution of stiffness values obtained in hypertonic medium was also comparable with that produced by cross-linking ligands. Hence the broad distribution of values generated by cross-linking can not result only from differences among cells in the numbers or affinities

TABLE III

Modulation of Deformability of Mouse Spleen Lymphocytes by Ligand-Independent Treatments

	Control		Treated		Reversal by cytochalasin D	
	Stiffness ± SD		Stiffness ± SD		Stiffness + SD	
	mdyn/μm	*N*	*mdyn/μm*	*N*	*mdyn/μm*	*N*
NaN_3	0.17 ± 0.07	(43)	0.47 ± 0.22	(43)	0.44 ± 0.25	(26)
NaN_3 + cytochalasin D	0.15 ± 0.08	(18)	0.14 ± 0.07	(17)	-	
A23187 + Ca^{2+}	0.19 ± 0.11	(36)	0.41 ± 0.17	(41)	0.43 ± 0.22	(16)
2xPBS	0.18 ± 0.10	(23)	0.49 ± 0.28	(25)	0.34 ± 0.27	(22)
Cytochalasin D	0.14 ± 0.05	(14)	0.13 ± 0.04	(15)	-	

Cells treatments and measurement as described in Materials and Methods. *N*, Number of cells measured.

of binding sites. The increase in stiffness produced by 2xPBS was partly reversed by cytochalasin D. Incubation of cells in DME-HEPES plus 0.3 M mannitol caused an increase in stiffness to an average value of 0.52 ± 0.15 mdyn/μm, comparable with that caused by 2xPBS. Incubation in DME-HEPES plus 0.2 M mannitol caused a substantially smaller increase in stiffness to an average value of 0.27 ± 0.15 mdynes/μm.

Treatment of lymphocytes with a metabolic inhibitor or increase of their intracellular calcium ion concentration also induced an increase in stiffness without cross-linking cell surface proteins (Table III). Incubation of lymphocytes with 10 mM sodium azide for 30–45 min at room temperature caused a marked increase in stiffness and induced a spikey appearance of the cell surfaces. This increase in resistance was not reversed by subsequent exposure of the treated cells to cytochalasin D. In contrast, cytochalasin D did prevent an increase in stiffness when the cells were incubated with it and azide simultaneously. Incubation of lymphocytes with the ionophore A23187 at a concentration of 1 μg/ml in the presence of calcium (1.8 mM) at room temperature for 30–45 min also increased cell stiffness. The increase in stiffness caused by the ionophore was not reversed by subsequent addition of cytochalasin D.

B Lymphocytes

Unfractionated spleen lymphocytes consist of ~42% B cells as detected by immunofluorescence staining for sIg. The current version of the Cell Poker does not permit detection of fluorescence from the cells on which the deformability measurements are being carried out. Therefore we could not selectively measure the changes in stiffness of B cells in an unfractionated population of spleen lymphocytes. Hence our measurements included randomly B, T, and null cells. It would, however, be useful to observe specifically changes in B cell stiffness to be able to relate them more directly to earlier studies of the redistribution and mobility of surface proteins and changes in cytoskeletal organization which have been carried out on B cells. Therefore, we have repeated the measurements described above on lymphocytes prepared from the spleens of nude athymic mice which, according to our immunofluorescence observations, consisted of >90% B cells. The results, shown in Table IV, obtained after treatment with anti-IgM antibody, the ionophore A23187, or sodium azide were very similar to those obtained previously with the mixed population of cells. The response to anti-IgM seems paradoxical because less than half of the cells in the mixed population carried sIg. Con A increased the stiffness of B cells less than that of the mixed cells, suggesting that Con A may cause a larger increase in the stiffness of T- than of B-cells.

DISCUSSION

We have shown by direct mechanical measurements that cross-linking Con A acceptors or sIg on lymphocyte surfaces caused a large (three- to fourfold) increase in the cellular resistance to compression. Not only the stiffness (i.e., the slope) but also the hysteresis of the measured plots of force versus compression was increased by surface cross-linking. This suggests that both elastic and viscous resistance to deformation were increased. To determine the relative contributions of increases in elastic and viscous resistance will, however, require further experimental and theoretical work (32).

An important general conclusion from the results summarized in Fig. 7 is that measurements of deformability can reveal and monitor changes in physiological state via changes in cellular mechanical characteristics. We look forward to investigating analogous processes in other cell types. Lymphocytes, however, offer certain specific advantages for mechanical studies. The fact that they are initially approximately spherical and that they remain so, apart from relatively small deviations of shape such as formation of a uropod, facilitates the development of an interpretive mechanical model. Furthermore, because the layer of cytoplasm surrounding the nucleus in lymphocytes is relatively thin and apparently homogeneous, we expect that its mechanical properties may be dominated by the cytoskeletal cortex which lies immediately beneath the plasma membrane. Hence mechanical responses of the cortical cytoskeleton may be more easily detected in lymphocytes than in fibroblasts, which have a larger cytoplasmic volume occupied by a cytoskeleton of a variegated and apparently more complex organization. In addition these observations of mechanical changes should increase our understanding of the mechanisms of capping of cross-linked sIg together with the ensuing morphological changes and dynamic processes (2) and of anchorage modulation of capping and of the lateral mobility of membrane proteins by Con A (19, 20, 22, 25). The responses to cross-linking of sIg are thought to be related to normal cellular activities including directional polarization and locomotion (2) whereas the physiological significance of the responses to Con A (e.g., in mitogenesis of T cells) is unknown.

The Increased Cell Stiffness Induced by Con A Parallels Anchorage Modulation of Lateral Mobility

Exposure of lymphocytes to Con A at a concentration of 30 μg/ml caused an approximately threefold increase in stiffness (Table I). There are several similarities between this modulation of deformability and the previously observed modulation of lateral mobility (22, 25) (Table I) (Figs. 5 and

TABLE IV

Modulation of Deformability of B Spleen Lymphocytes

Treatment	Stiffness ± SD	N	Control-Stiffness ± SD	N
Con A	0.25 ± 0.08	(17)	0.14 ± 0.09	(19)
Anti-IgM	0.50 ± 0.19	(19)	0.13 ± 0.06	(36)
NaN_3	0.42 ± 0.18	(18)	0.20 ± 0.06	(18)
A23187 + Ca^{+2}	0.41 ± 0.16	(17)	0.17 ± 0.10	(18)

B lymphocytes were prepared from the spleens of nude athymic mice. Cells were treated as described in Materials and Methods identically to the mixed populations of lymphocytes. *N*, Number of cells measured.

7). (*a*) Both phenomena can be triggered by locally bound Con A. Cells bearing Con A beads have substantial surface areas free of Con A. Hence the increase in stiffness in these cells does not result simply from the creation of a cross-linked surface matrix around the cell. The increase, therefore, in stiffness and the inhibition of lateral mobility are both reflections of global cellular responses to locally applied signals. (*b*) Both phenomena require cross-linking of Con A receptors; binding of s-Con A, an ineffective cross-linker, is insufficient. (*c*) Both phenomena are reversed by cytoskeletal inhibitors. The Con A–induced increase in stiffness is reversed entirely by cytochalasin D and partially by colchicine. The modulation of lateral mobility is reversed partially by colchicine or cytochalasin B separately and entirely by the two inhibitors acting together. Whether this is a significant difference or is due to the greater potency of cytochalasin D relative to cytochalasin B as an inhibitor of actin assembly remains to be tested. (*d*) Neither anchorage modulation of diffusion nor the increase in stiffness is reversed by azide ion.

These similarities between the modulation of lateral mobility and cell stiffness by Con A suggest that the two phenomena may have a causal connection. It has been suggested that anchorage modulation of mobility could result from an effect of Con A on the state of the cytoskeleton or on the linkage of cell surface proteins to the cytoskeleton (22). Our results demonstrate the occurrence of the former although not its relationship to inhibition of mobility. The effect of Con A on the linkage of sIg to the lymphocyte cytoskeleton remains to be tested.

There is a significant overlap of the deformability distributions in control and in Con A-treated cells (Fig. 5, *a* and *d*). This is unlikely to be due to unresponsiveness of a defined fraction of the mixed lymphocyte population (e.g., either B or T cells). Both B and T lymphocytes bind and respond to Con A. On a mixed population of cells preincubated at 0°C, Con A will cap at 37°C even when applied at high concentration (37). Most of the capped cells are T cells. As seen in Table IV, B cells from nude mice do respond significantly although less on the average than the mixed population from normal mice. If B cells from nude and normal mice are comparable in their response to Con A, then one must conclude that T cells display a stronger mechanical response to Con A than do B cells.

Exposure of human erythrocytes to wheat germ agglutinin increases their viscous (38) and elastic (39) resistance to deformation by micropipette aspiration. The viscous resistance was attributed to increased viscous drag of the aqueous medium on the wheat germ agglutinin molecules bound to the cell surface (38). It could not be certainly decided whether

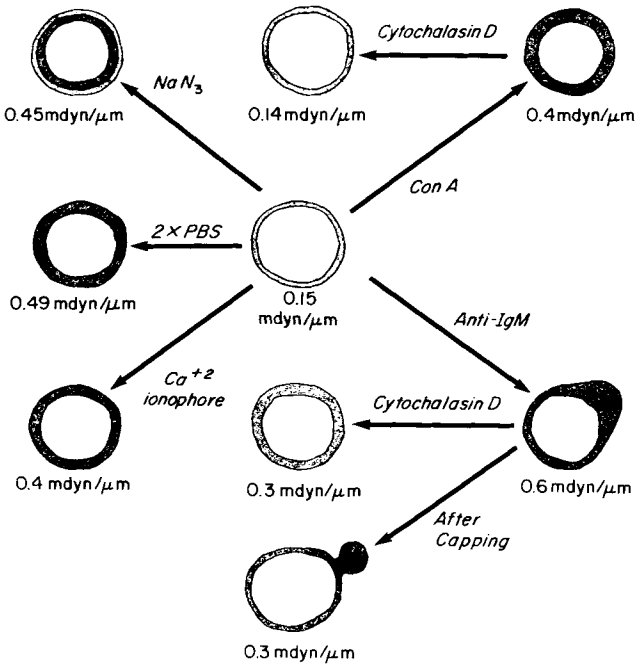

FIGURE 7 Schematic model of experimental results. The increases in stiffness elicited by surface cross-linking and various ligand independent treatments are shown as resulting from the development of a mechanically resistant cytoskeletal shell around the nucleus. This may decrease cellular deformability both by contracting around the nucleus and thereby generating resistance to stretching the tensed cytoskeletal matrix (symbolized in the figure by the increased density of the shell) and by resisting bending (symbolized by an increased thickness of the shell) as discussed in the text. The cytoskeletal shell of increased density is shown extending to the cell surface in most instances to account for the corresponding retardation of the lateral mobility of cell surface proteins which is observed in capping and anchorage modulation. The response to NaN_3 is exceptional; there is no effect either on the baseline lateral mobility of cell surface proteins nor on anchorage modulation. The effects of 2xPBS and of the ionophore A23187 plus calcium ions on the lateral mobility of sIg have not yet been determined. The former treatment does, however, induce capping of several cell surface proteins (35, 36), and the latter generates sIg patches in some cells (34). The effect of cytochalasin D in completely or partially reversing the increase in stiffness caused by various treatments is also shown as is the partial reversal that occurs after completion of capping. Not shown in this scheme are the following observations: cytochalasin D partly reverses the increase in lymphocyte stiffness caused by 2xPBS but not that caused by azide ion or A23187 plus calcium ion; simultaneous incubation of cells with both cytochalasin D and azide ion prevents the increase in stiffness otherwise caused by the latter (Table III); colchicine partly reverses to a similar extent the increases in stiffness caused by anti-IgM and Con A (Table II).

the increased elastic resistance resulted from the formation of an external cross-linked matrix or from an effect of wheat germ agglutinin on the erythrocyte cytoskeletal cortex as had previously been suggested (39, 40). The effectiveness of Con A-beads in increasing stiffness and of cytochalasin D in reversing the increase suggests that the observed effects of Con A on lymphocyte deformability depend little, if at all, on either surface viscosity or a cross-linked surface matrix; rather, the increases in stiffness are mediated through the cytoskeleton.

Cross-linking sIg Increases Lymphocyte Stiffness and Initiates Capping

The sequence of dynamic functions initiated by cross-linking sIg, including capping, formation of a uropod, and induction of cellular motility, involves the active movement of one part of the cell relative to others and therefore the performance of mechanical work. As pointed out above, cytoskeletal contractility seems to be involved in these processes. Our studies of this system were motivated by the supposition that mechanical tensions generated in the cell during capping might cause a detectable increase in the overall resistance of the cell to compression. We did, indeed, observe a substantial increase in cellular stiffness due to cross-linking sIg but we cannot yet confidently interpret this as the result of a cytoskeletal contraction.

Changes in cellular stiffness occurred over the same time period as the redistribution of surface molecules and cytoskeletal components during capping (Table II) (Fig. 6). Whereas the average stiffness of cells in the midst of capping was 0.56 mdyn/μm, cells measured at a later stage in the process and an average stiffness of 0.27 mdyn/μm. Hence the stiffness diminished when the capping of the sIg had completed. This reinforces the suggestion that the same cytoskeletal processes are involved in capping and in the increase of stiffness. Moreover, the distribution of stiffness values was narrower after capping (Fig. 6). This suggests that the cells with the highest initial stiffness values relaxed first or that all modulated cells converged to a modestly elevated stiffness value after capping. Measurements of the kinetics of the increase and relaxation of stiffness relative to the formation and dissipation of the cap should help to clarify the relationship between these two phenomena. The relationship between ligand-dependent capping and ligand-dependent increases in stiffness is further reinforced by the fact that both functions require cross-linking of the ligand-receptor complexes on the cell surface (Table II).

The effect of cytoskeletal inhibitors on capping are complex and differ from the effects on anchorage modulation (2, 19). Similarly, the effects of these inhibitors on the increases in stiffness caused by anti-IgM and Con A differ. Cytochalasin D only partially reversed the increase in stiffness caused by cross-linking sIg (Table II) in contrast to its complete reversal of the Con A effect (Table I). This difference in sensitivity provides a further indication that the two cross-linking ligands provoke significantly different cytoskeletal changes. It has previously been shown that cytochalasin B is only partially effective in reversing sIg caps but reverses Con A caps completely (2). Colchicine does not inhibit and may even enhance capping (2), and it partly reversed the increase in stiffness caused by anti-IgM. Disruption of microtubules could reverse stiffening either by enhancing the rate of capping (19) or by a direct effect on the mechanical properties of the cell. Colchicine and cytochalasin D together were more effective than either was separately (Table II).

In a mixed population of spleen lymphocytes, less than half should be sIg-bearing B cells. A population enriched in B cells (to the 90% level) was obtained from the spleens of nude athymic mice. Treatment by anti-IgM antibodies elicited a very similar effect on stiffness in the nude and wild type populations of spleen lymphocytes (Table IV). This suggests (*a*) that the stiffness of T cells was increased in the presence of modulated B cells even without ligand binding to the T cells, or (*b*) that the absence of T cells in the population obtained from nude mice modulated the increase in B cell stiffness caused by anti-sIg. B cells from nude mice have been shown to cap inefficiently (41).

Sodium Azide Increases Cellular Stiffness without Affecting the Mobility or Distribution of Plasma Membrane Proteins

The threefold increase in stiffness provoked by sodium azide is comparable to that caused by Con A. In contrast to Con A, however, sodium azide has no effect on the lateral mobility of sIg (1, 22). It interferes neither with the formation of patches of sIg cross-linked by antibody (2) nor with anchorage modulation of sIg mobility by Con A (22). Moreover, azide does not impair the adherence of cross-linked sIg to detergent-insoluble cytoskeletons (42) or to detergent-extracted actin (43). These results suggest that azide modulates the state of the cytoskeleton in these cells without disturbing its interaction with cell surface components.

Earlier work has demonstrated effects of azide on the morphology of lymphocytes including increased expression of microvilli (44) and the formation of a constriction ring on cells bearing caps of cross-linked sIg (33). We have observed that azide-treated lymphocytes seem to have rougher surfaces under modulation contrast optics and to adhere less well than untreated cells to glass coverslips. These morphological effects and the increase in stiffness which we have observed might be attributed to an irreversible contraction of actomyosin analogous to a rigor state of muscle (34). This is consistent with our observation that the azide-induced increase in stiffness was not reversible by cytochalasin D but could be prevented by incubating cells with cytochalasin D simultaneously with azide (Table III). In the latter case we suppose that the cytochalasin disassembled actin filaments before the ATP content of the poisoned cells was sufficiently depleted to cause rigor contraction. (See reference 45 for other examples of the ineffectiveness of cytochalasins in the presence of azide ion.) Nevertheless, it does not appear that cellular actin and myosin are entirely immobilized in azide-treated cells; both proteins co-patch with aggregated sIg in the presence of azide (7). Furthermore, azide has been reported to enhance the polymerization of actin in thymocytes and to render the actin resistant to depolymerization by DNase I (46). These results were also interpreted in terms of an azide-induced rigor state.

These observations as well as our own suggest alternative interpretations of the inhibition of capping by azide. It is usually supposed that azide prevents capping by interfering with an energy-requiring process which is needed to move the cross-linked sIg molecules to one pole of the cell. Perhaps, however, depletion of the G actin pool by azide inhibits a specific actin polymerization process necessary for capping (46). Alternatively, the stiffening responsible for the decreased deformability of azide-treated lymphocytes might also prevent movement of patched sIg to the cap by immobilizing components which participate in the process.

Exposure to Hypertonic Medium or to a Calcium Ionophore Plus Extracellular Calcium Increases Lymphocyte Stiffness without Need for Surface Cross-linking

Exposure of lymphocytes to hypertonic medium, either 2xPBS or PBS containing high concentrations of mannitol,

stimulates capping of several uncross-linked proteins (35, 36) and increases lymphocyte stiffness (Table III). Assuming that sIg does not aggregate in the absence of a multivalent ligand, the capping of surface proteins which have not been cross-linked argues against hypotheses which interpret capping in terms of a membrane flow (27). The capping and stiffening responses could both be interpreted in terms of a contraction of the cortical cytoskeleton induced by exposure to hypertonic medium. We cannot, however, rule out that other factors (e.g., a thickening of the cytoskeleton consistency) could cause or at least contribute to the stiffening effect. These ligand independent responses do, however, demonstrate that surface cross-linking is an exclusive requirement neither for the generation and transmission of an activating signal for capping nor for the development of the putative interactions from cytoskeleton to cell surface which draw the sIg molecules into the cap. Exposure to hypertonic medium initiates both processes.

It has been suggested that an increase in the concentration of cytoplasmic calcium ions plays a role in capping (2, 47) although evidence to the contrary also exists (48). In the absence of cross-linking ligands, an increase in the concentration of cytoplasmic calcium ions causes a redistribution of myosin and even a low frequency of patching of sIg (49). The increase in lymphocyte stiffness which we have observed in the presence of A23187 and calcium ions is consistent with a possible role for calcium ions in cytoskeletal activation. The fact that increasing cytoplasmic calcium ion concentration by A23187 does not cause capping but rather inhibits or even reverses capping indicates that additional factors must regulate the potentially antagonistic effects of calcium ions on the cytoskeleton (50–53).

Our experimental results also verify that the mechanisms by which the calcium ionophore and cytochalasin D inhibit and reverse caps are different. The former caused an increased cellular stiffness which is consistent with a contractile response of the cytoskeleton. The latter caused a reduced cell stiffness which is consistent with the expectation that cytochalasins interfere with the assembly of microfilaments which are principal determinants of the increased stiffness caused by cross-linking ligands.

The Observed Increase in Cell Stiffness Might Result from a Contraction of the Cortical Cytoskeleton and/or an Increase in Its Resistance to Bending

A mechanical interpretation of the observed increases in cellular viscoelasticity requires that we consider the overall structure of lymphocytes. Morphometric studies have shown that human lymphocytes are small, approximately spherical cells. Almost three-fourths of the diameter of the cell is occupied by the nucleus which is surrounded by a spherical shell of cytoplasm which is ~1 μm thick (54). We shall suppose that mouse and human lymphocytes are sufficiently similar that we need not distinguish between them for present purposes. Our measurements show that the deformability of untreated cells was insensitive to cytochalasin D (Tables I and IV). Previous studies have shown that cytochalasin B causes a marked reduction in the stiffness of the perinuclear regions of adherent fibroblasts but has little, if any, detectable effect on the resistance of the nucleus to indentation (28). Assuming that the results obtained on fibroblasts are pertinent to our lymphocyte measurements, we conclude that the resistance to compression of untreated lymphocytes was dominated by the nucleus, expected from the earlier work to be insensitive to cytochalasins, or that the cytoplasmic consistency of these lymphocytes did influence our measurements, but, unlike fibroblasts, was unaffected by cytochalasins. We consider the former to be the more likely alternative. Then by the same logic, the ability of cytochalasin D to reverse the increased stiffness triggered by anti-IgM and Con A suggests these cellular mechanical responses take place in the cytoplasmic shell.

Earlier studies have suggested that cross-linking sIg initiates an actomyosin contraction which drives capping and related dynamic cellular responses (2, 7, 55) and which may be regulated by a mechanism similar to that which controls contraction of smooth muscle (12, 13, 16–18). More recent electron microscopic studies have shown that the amount of actin filaments bound to the cytoplasmic surface of the plasma membrane is substantially increased in cells with cross-linked sIg or Con A acceptors (56). Furthermore, the filaments were frequently arranged in a ringlike pattern which was especially prevalent in cells prepared under conditions which promote capping. Therefore the simplest model for the observed increase in cellular stiffness might also be based on a contraction of the cytoskeletal cortex around the nucleus. An isometric tension could develop due either to a uniform circumferential or radial contraction of the cortex. Then, in contrast to untreated cells in which the relatively weak resistance to compression is expected to result mainly from shearing the nucleus, the treated cells could be substantially compressed only by stretching the tensed cortex. The stronger the cortical contraction, the greater the increase in resistance to cellular compression.

Detailed examination of the dependence of force on the extent of compression of modulated cells suggests, however, that additional factors may be involved. Frequently there was a biphasic dependence of resisting force on extent of compression. Especially in cells treated with anti-IgM or A23187 plus calcium ions there was often a small initial component in which force increased rapidly but with negative curvature (i.e., concave down). This first phase was then followed by a much longer phase during which the rate of increase of force either remained constant or increased with increasing compression to produce linear or concave-upward plots (see Fig. 3). This change in the sign of the curvature of the force versus compression curve from negative to positive is not seen in the simple elastic stretching of osmotically swollen human erythrocytes, which should be a good model of spherical cells surrounded by tensed membranes (57). Rather, the biphasic characteristics of the force-compression curves suggest an additional component in the resisting force. Theoretical and experimental studies of macroscopic model systems (58, 59) have suggested that the additional component might be a bending resistance which develops from a mechanical thickening of the cytoskeletal shell around the nucleus. More theoretical and experimental work will be required to test this hypothesis (32).

Conclusions

Our experimental results are summarized in Fig. 7. Based on these results we conclude the following: (*a*) Cross-linking

sIg on Con A acceptors on primary mouse spleen lymphocytes caused an increase in the resistance of the cells to deformation. Simple binding of monovalent Fab fragments of antibody against sIg or of s-Con A to its acceptors (without cross-linking) was insufficient to elicit an increase in cellular stiffness. (*b*) The observed increases in cellular stiffness depended on the integrity of the cytoskeleton; they could be reversed to varying extents by such cytoskeletal inhibitors as colchicine and cytochalasin D. Differences in the effectiveness of the inhibitors in reversing the Con A– and anti-IgM-induced increases in stiffness suggest differences in the cytoskeletal processes involved in these functions. Furthermore, Con A beads applied locally to the cell surface (leaving other regions of the surface devoid of Con A) caused an increase in stiffness comparable to that observed by soluble Con A. Hence the observed increase in stiffness cannot be attributed simply to the development of a cross-linked matrix over the entire cell surface. Rather, it must involve a change in the state of the cytoskeleton. (*c*) There are many correlations between the observed increases in cell stiffness and capping or anchorage modulation. These include the effects of cytoskeletal inhibitors and the evolution of stiffness over the same time period as the development and dissipation of caps. Hence it is likely that the changes in the distribution and mobility of cell surface proteins are related to the changes in the mechanical properties of the cytoskeleton. The mechanism and structural basis of this relationship remain to be determined. (*d*) Various ligand-independent treatments also cause an increase in cell stiffness. Hence changes in deformability can be generated in the absence of surface cross-linking. ([*i*] Exposure of lymphocytes to hypertonic medium, which had previously been shown to cause capping of several membrane proteins, also produced an increase in cell stiffness. This demonstrates that capping is still correlated with an increase in stiffness even in the absence of surface cross-linking. [*ii*] Exposure of lymphocytes to azide ion prevents capping (2) and caused an increase in cell stiffness without influencing the mobility and distribution of surface proteins. This change in deformability could be a kind of rigor effect resulting from ATP depletion. [*iii*] Increasing intracellular calcium ion concentration by an ionophore caused an increase in stiffness although it prevents capping.) (*e*) Cell poking measurements of cellular deformability can provide a sensitive assay of changes in the mechanical and functional state of the cytoskeleton and of the complex formed by the plasma membrane and the cytoskeletal cortex.

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