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Suppression of Stimulating Cell Activity by Microtubule-Disrupting Alkaloids

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Microtubule-disrupting alkaloids and protein fixatives were used to investigate the nature of an active process that must occur within stimulator cells in order for them to initiate a unidirectional mixed lymphocyte response (MLR). Brief treatment of the stimulator cells (SC) with glutaraldehyde (0.15%), formalin (0.6%), or lanthanum chloride (10^{-3} M) abolished their capacity to activate responder cells (RC). Pretreatment of SC with the microtubule-disrupting alkaloids, colchicine (c) (10^{-4} to 10^{-6} M) or colchicine + vincristine (c+v) (10^{-4} to 10^{-6} M) also abrogated their stimulating capacity. This capacity was not restored by the addition of supernates from untreated cultures, thereby excluding the possibility that the alkaloids acted by decreasing the release of soluble stimulatory factors from SC. The introduction of alkaloid-inactivated, mitomycin-treated RC as drug carriers did not affect the mitogenic response of untreated RC to concanavalin A. This excluded a significant leakage of alkaloids from the treated SC and uptake by RC during culture. Lumicolchicine produced no decrease in the stimulating capacity of SC. This suggested that the suppression induced by low concentrations of colchicine resulted from its specific disruption of microtubules. None of the above treatments quantitatively reduced the antigenicity of SC, as evaluated by humoral and cell-mediated lysis of the treated cells. Also, these treatments produced no significant changes in the specific binding of concanavalin A by SC. These results indicate there is a functional interaction of microtubular structures with cell surface antigens that appears to regulate either the capacity of SC to associate with RC, or the ability of SC to form and stabilize stimulatory antigenic configurations on the cell surface.

Key words: alkaloids, fixation, microtubules, mixed lymphocyte response

INTRODUCTION

The mixed lymphocyte response (MLR) is an in vitro reaction that results from co-culturing lymphocytes from two genetically different members of the same species (1). Interaction between the cells bearing disparate surface antigens results in a number of metabolic changes that culminate in enhanced DNA synthesis. Treatment of the lymphocytes from one donor with mitomycin C irreversibly inhibits their DNA synthesis and

allows them to act only as stimulator cells (SC) (2). This results in a unidirectional MLR in which only the untreated responder cells (RC) are stimulated to undergo increased DNA synthesis.

Initiation of the unidirectional MLR requires viable, metabolically intact cells. Cell-free alloantigens, in the form of membrane fragments (3) and soluble extracts (3–5), are poor stimulators of this response. Moreover, treatments that render whole stimulator cells nonviable, such as fixation (6) and freeze-thawing (7), completely abolish their stimulating capacity. In addition, the stimulating capacity is markedly reduced by such diverse agents as mild heat (50°C for 5 min) (3), ultraviolet light (8), iodoacetate (7) and ouabain (9). These agents can potentially alter components of the cell membrane and inhibit membrane-associated enzymes. On the other hand, not all metabolic inhibitors interfere with the stimulating capacity of SC. Mitomycin C (2) and puromycin (Ranney, unpublished observations) are two that are ineffective. These findings suggest that more is required of the stimulator cell than the simple presentation of alloantigens; a specific active process must also occur in these cells. Recently, functional interactions have been demonstrated to occur between the cytoskeletal structures and cell surface components of lymphocytes (10). The current study presents results indicating that such an interaction may be required for SC to initiate a mixed lymphocyte response.

MATERIALS AND METHODS

Mixed Lymphocyte Cultures

Stimulator cells (SC) and responder cells (RC) were prepared from the peripheral blood of unrelated human donors by centrifugation on a ficol-hypaque gradient (11). This cell preparation consisted of lymphocytes and mononuclear cells. Mixed lymphocyte cultures were performed in microtiter plates using 6×10^5 SC and 2×10^5 RC per well, as described by Hartzman et al. (2). The cells were cultured in 0.2 cc of RPMI 1640 containing 10% fetal calf serum (FCS) (Associated Biomedic Systems) for 120 hr at 37°C in 5% CO_2. ^{3}H-thymidine (1 μCi/culture, 6 Ci/mmole) was added during the last 8 hr of incubation. The cultures were harvested on a multiple automated sample harvester (Microbiological Associates) and solubilized in NCS (Amersham-Searle) for scintillation counting. In all experiments except those involving fixation with glutaraldehyde, formalin, and lanthanum chloride, the SC were treated with mitomycin C (25 μg/ml in serum-free RPMI 1640 for 30 min at 37°C) (2), to irreversibly inhibit their DNA synthesis and induce a unidirectional response by RC. These results are expressed as the stimulation index, which is calculated as follows:

$$\frac{\text{CPM in stimulated cultures}}{\text{CPM in unstimulated controls}}$$

Pretreatment of Stimulating Cells

For experiments involving the fixation of SC under mild conditions in glutaraldehyde or formalin, the cells were suspended in 1 cc of serum-free RPMI 1640, and the respective fixative added in 1 cc of the same medium to produce a final concentration of 0.15% (w/v) for glutaraldehyde and 0.6% (w/v) for formalin. Brief fixation was carried out for 15 sec, after which 13 cc of medium was added to rapidly decrease the concentration of fixative. The cells were then centrifuged at 600 g for 10 min, washed twice with 10 cc of serum-free medium, and suspended in RPMI 1640 containing 10% FCS for culture. Brief

fixation in 10^{-3}M lanthanum chloride ($LaCl_3$) was performed similarly except that the fixation was carried out in tris-NaCl (12).

For experiments involving the pretreatment of SC with alkaloids, the cells were incubated with colchicine (c) (10^{-4} to 10^{-6} M) (Sigma), lumicolchicine (a photoinactivated derivative of colchicine) (10^{-4} to 10^{-6}M), vincristine (v) (10^{-4} to 10^{-6}M) (Lilly) or c + v in equimolar concentrations (10^{-4} to 10^{-6}M) in serum-free RPMI 1640 for 2 hr at 37°C. During the last 30 min, mitomycin C was added as described above, and the cells were washed twice prior to culturing. Control SC were incubated for 90 min in serum-free medium without alkaloids prior to treatment with mitomycin C.

Preparation of Lumicolchicine

Lumicolchicine was prepared by exposing a 100 μg/ml solution of colchicine (Sigma) to ultraviolet light (λ 3654 Å) as described by Mizel and Wilson (13).

Mitogen Stimulation

To test for the possible leakage of microtubule-disrupting alkaloids from the pretreated SC during culture, alkaloid-inactivated, mitomycin-treated RC that could not incorporate ^{3}H-thymidine or act as stimulator cells, but could function only as drug carriers were cultured together with untreated RC. Concanavalin A was then added in optimal mitogenic concentrations (5 μg/ml) to the cell mixture. Incorporation of ^{3}H-thymidine by the untreated population of RC in the cell mixture was determined after 72 hr of culture. This result was compared with the mitogen-stimulated incorporation of radiolabel by untreated RC alone, and the results were expressed as a percentage of the latter value.

Specific Binding of Concanavalin A

Concanavalin A (con A) was purified from jack bean meal as described by Agrawal and Goldstein (14) and was radiolabeled with ^{3}H-acetic anhydride as described by Rittenhouse et al. (15). The specific saturation binding of lectin to untreated and alkaloid-treated SC was compared by adding ^{3}H-con A at a final concentration of 100 μg/ml to 1×10^7 SC in each category and determining the counts bound in the presence and absence of 0.1 M α-methyl-D-mannopyranoside (15).

Detection of SC Antigens by Antibody-Mediated-Complement Dependent Cytolysis

The effects of fixation and alkaloid treatment on serologically detectible histocompatibility (HLA) antigens of SC were determined by the microcytotoxicity assay of Pincus and Gordon (16), using HLA-2 positive SC, monospecific anti-HLA-2 antiserum (kindly provided by Dr. Kamal Mittal) and rabbit complement (Pel-Freeze, Inc., Rogers, Ark.). The reciprocal titer of antiserum required to lyse 50% of the treated SC (SC_t) was expressed as a percentage of the titer required to lyse 50% of the untreated SC (SC_u), as follows:

$$\% \text{ of control titer} = 100 \times \left[\frac{\log_2 \text{ reciprocal titer } SC_t}{\log_2 \text{ reciprocal titer } SC_u} \right]$$

Detection of SC Antigens by Cell-Mediated Lympholysis

Killer cells, capable of destroying specific lymphoid targets, were generated in vitro against the surface determinants of untreated SC according to the method of Lightbody and Bach (17), by culturing RC (2×10^6/cc) together with mitomycin-treated SC ($6 \times$

10^6/cc) that had not been subjected to fixation or treatment with alkaloids and harvesting the specific killer cells on the 6th day of culture. The effects of fixation and alkaloid treatment on the surface determinants of SC were then determined by pulse-labeling the fixative or alkaloid-treated SC with $Na_2\ ^{51}Cr_2O_7$, using these cells as targets for the specific killer cells, and determining their extent of lysis in a 12 hr chromium release assay (17). The results were expressed as a percentage of the lysis of untreated SC as follows:

$$\% \text{ of control} = 100 \times \left[\frac{\left(\frac{E-S}{T-S}\right)_{\text{for } SC_t}}{\left(\frac{E-S}{T-S}\right)_{\text{for } SC_u}} \right]$$

E = release of ^{51}Cr in experimental tubes (sensitized killer cells + targets)
S = spontaneous release of ^{51}Cr in control tubes (unsensitized cells + targets)
T = total release of ^{51}Cr by radiolabelled targets after freezing and thawing (3X)

RESULTS

The mechanism of fixation-induced changes in stimulating capacity was initially investigated by treating the stimulator cells for 15 sec with low concentrations of protein fixatives that had been shown by Bubbers and Henney (18) to produce minimal alterations of cell-surface antigens. The effects of such fixation on the stimulatory capacity of SC are shown in Fig. 1. Glutaraldehyde, formalin, and lanthanum chloride all reduced the stimulation index to less than 0.5% of the untreated value. To determine if these treatments quantitatively altered the surface antigens of SC, the presence of these antigens on untreated and fixed cells was compared by 1) antibody-mediated-complement dependent lysis (AML), 2) cell-mediated lysis (CML), and 3) specific binding of ^{3}H-con A (con A) (Fig. 2). Antibody-mediated lysis and con A binding were essentially unchanged after all treatments, whereas cell-mediated lysis was slightly enhanced by these treatments. Similar results (data not shown) were obtained for cells fixed with lanthanum chloride and glutaraldehyde. By these criteria, abolishment of the stimulating capacity produced by brief fixation did not appear to result from the alteration of cell surface antigens.

To further elucidate the active process required for generation of stimulating activity, SC were pretreated with pharmacologic agents that had been demonstrated to disrupt microtubules and increase the mobility of certain cell-surface receptors (10). Colchicine, at concentrations of 10^{-6} to 10^{-4}M, caused a progressive decrease in the stimulating capacity of SC (Fig. 3). In contrast, similar concentrations of the photoinactivated derivative, lumicolchicine, produced no significant reduction in their stimulating capacity. The possibility that significant quantities of colchicine had leaked from the treated stimulator cells and inhibited the transport of ^{3}H-thymidine into responder cells during culture (13) was investigated as follows. Untreated RC were cultured together with genetically identical cells that had been treated with colchicine and mitomycin C, such that they could function only as drug carriers, but not as stimulator or responder cells. Con A was then added to the cell mixture and the incorporation of ^{3}H-thymidine by the untreated population of RC was determined as described above. The mitogenic response of untreated RC was not significantly altered by the presence of colchicine-carrying cells (Fig. 3). This excluded the leakage of colchicine from treated SC, in quantities sufficient to inhibit the transport and incorporation of radiolabel by RC.

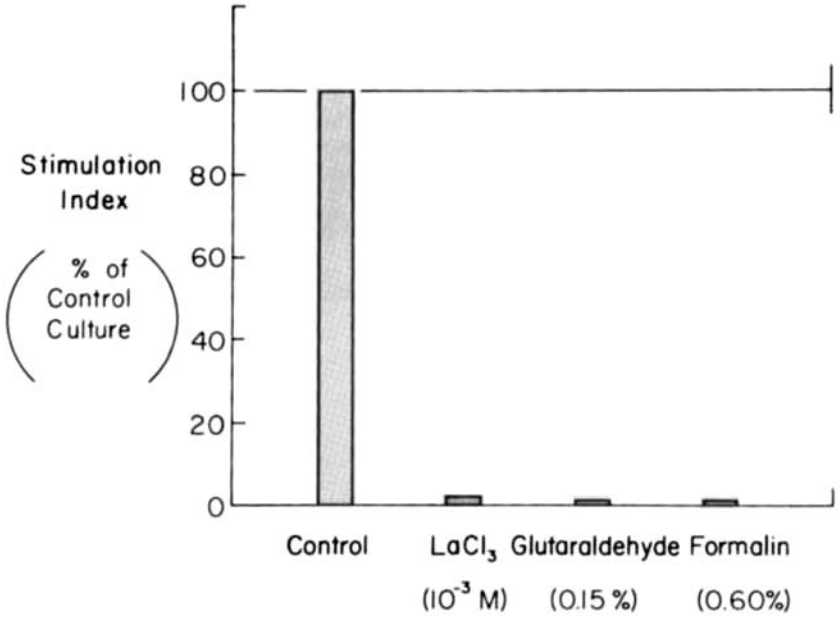

Fig. 1. Effects of protein fixatives on SC stimulating capacity. Results are expressed as a percentage of the maximal stimulation index achieved in normally activated control cultures using mitomycin-treated SC. The maximal stimulation index = (CPM_{A+B_m}/CPM_{A+A_m}) = (55,805 ± 1,278 CPM/ 683 ± 102 CPM) = 81.7 ± 1.9. CPM = the incorporation of ^{3}H-thymidine into the DNA of RC 120 hr after initiation of culture, A = responding cells, B = stimulating cells, and the subscript m designates the treatment of SC with mitomycin C. In the experimental cultures, SC were pretreated with $LaCl_3$ (10^{-3}M), glutaraldehyde (0.15%), and formalin (0.6%), as shown above.

The effects of combined pretreatment of SC with vincristine and colchicine were also investigated. Vincristine acted synergistically with colchicine, such that a nearly maximal (71%) decrease in stimulating capacity was produced by 10^{-6}M colchicine + 10^{-6}M vincristine, 2 log concentrations lower than that required for colchicine alone (10^{-4}M) (Fig. 3). Again, none of these pretreatments quantitatively altered the surface antigens of SC as determined by antibody-mediated lysis and ^{3}H-con A binding; all treatments except lumicolchicine slightly enhanced cell-mediated lysis (Fig. 2).

The effects of 10^{-6}M c + v on the release of soluble stimulatory products from SC was investigated by transferring day 1 through day 4 supernates from normally activated mixed lymphocyte cultures to the test cultures (containing RC + pretreated SC) (Table I). None of these supernates reversed the alkaloid-induced decrease in the stimulating capacity of SC. Thus, the alkaloid effect did not appear to result from a suppressed release of soluble stimulatory factors by SC during initiation of the mixed lymphocyte response.

DISCUSSION

The disruption of stimulating capacity by brief exposure to low concentrations of fixatives that do not appear to significantly alter SC surface determinants indicates that initiation of the mixed lymphocyte response depends on more than a simple presentation of SC antigens to the responding cells. Moreover, the significant reduction of stimulating

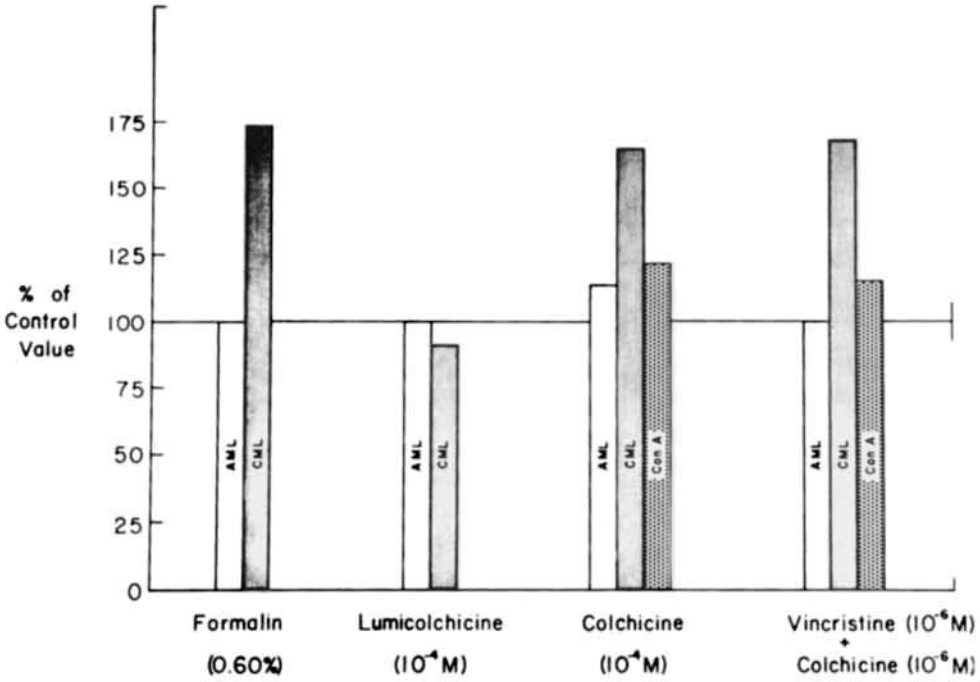

Fig. 2. Effects of fixatives and alkaloids on SC surface determinants. Results are expressed as percentages of the control values (see Materials and Methods). AML (antibody-mediated lysis) = reciprocal titer of anti-HLA-2 antiserum producing a 50% lysis of variously treated HLA-2 positive SC in the presence of rabbit complement (reciprocal titer for untreated SC = 128); CML (cell-mediated lysis) = ^{51}Cr release from variously treated, radiolabeled SC targets in the presence of killer cells directed against the surface antigens of control SC (see text) (specific release from untreated SC targets = 57%); Con A = specific binding of ^{3}H-concanavalin A (100 μg/ml) by variously treated SC (specific binding by untreated SC = 1384 CPM/10^7 cells). Treatments of SC included formalin (0.6%), lumicolchicine (10^{-4}M), colchicine (10^{-4}M), and c + v (10^{-6}M).

TABLE I. Capacity of Supernates from Normally Activated Lymphocyte Cultures to Reconstitute the Stimulating Capacity of Alkaloid-Treated SC

Treatment of stimulating cells[a]	Supernate[c]	None	Day[d] 1	Day 2	Day 3	Day 4
		Stimulation index[b]				
1. –	–	38.8 ± 1.4	–	–	–	–
2. c + v	–	12.5 ± 0.5	–	–	–	–
3. c + v	$A + A_m$	–	12.0 ± 0.4	10.8 ± 0.2	10.5 ± 0.2	11.7 ± 0.4
4. c + v	$A + B_m$	–	10.4 ± 0.2	10.3 ± 0.2	11.3 ± 0.4	10.6 ± 0.3

[a]All stimulating cells were also treated with mitomycin C; c + v = colchicine (10^{-6}M) + vincristine (10^{-6}M).

[b]Of test cultures, containing SC treated as indicated; stimulation index as defined in the text.

[c]Transferred to the test cultures on day 0, from normally activated baseline ($A + A_m$) and stimulated ($A + B_m$) supernatant production cultures, 1–4 days after their initiation. Final dilution in test cultures = 1:3.

[d]Days after initiation of supernatant production cultures.

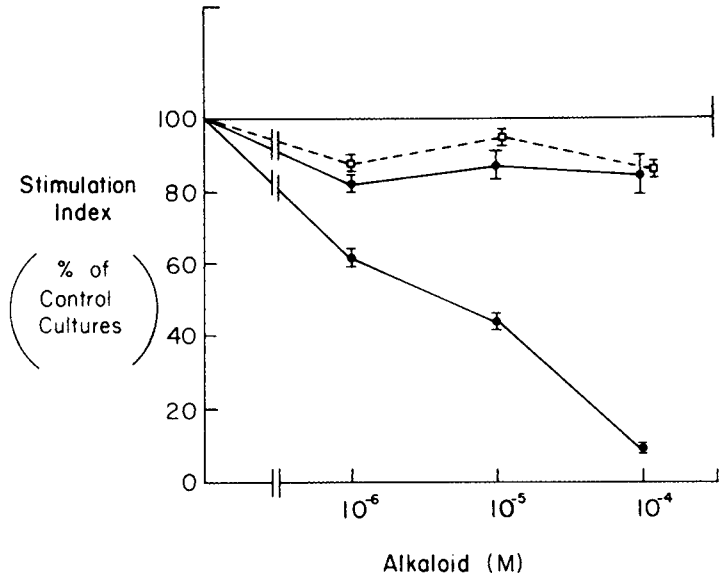

Fig. 3. Effects of colchicine and lumicolchicine on SC stimulating capacity. Results are expressed as a percentage of the maximal stimulation index in control cultures (= 81.7 ± 1.9), as in Fig. 1. SC pretreated with colchicine (10^{-6} to 10^{-4}M) + mitomycin C (25 μg/ml) (lower •——•); SC pretreated with lumicolchicine (10^{-6} to 10^{-4}M) + mitomycin C (25 μg/ml) (upper •——•); mitogenic response of RC to con A (5 μg/ml) in the presence of genetically identical, colchicine-inactivated, mitomycin-treated cells (□– – □) (see text).

capacity achieved with low (10^{-6}M) concentrations of colchicine suggests that the active process required for stimulation involves the interaction of microtubules with specific cell-surface antigens of SC that initiate the MLR. The specificity of colchicine as an inhibitor of this cellular process is supported by the inability of lumicolchicine to produce similar effects at any of the concentrations tested. Lumicolchicine does not bind tubulin nor disrupt microtubules but does produce the other alterations in cellular functions that are characteristic of colchicine at equivalent concentrations (13). Furthermore, the synergistic effects of vincristine and colchicine at low concentrations further support the specificity of colchicine for this cellular process, since 10^{-6}M vincristine markedly stabilizes the binding of colchicine to tubulin (19).

The functional interaction of colchicine-sensitive (microtubular) structures with cell-surface antigens has been well documented for both lymphoid (10) and nonlymphoid cells (12). In lymphoid systems, colchicine has been shown to alter the lateral mobility of certain cell-surface receptors (10). This suggests that the colchicine-induced disruption of microtubules in SC might function by interfering with an interaction between microtubules and cell-surface antigens that appears to be necessary for formation and stabilization of stimulatory configurations on the SC surface. Alternatively, colchicine could produce this effect by reducing the capacity of stimulating cells to associate with responding cells. This latter possibility is less likely because of the present data indicating that colchicine treatment of SC increases rather than decreases their lysis by specific killer cells (Fig. 2). Since this type of lysis depends on the initial association between targets and specific killer cells (20), the enhanced lysis of treated SC when employed as targets constitutes evidence that

these cells retain the capacity to associate with other cells of the lymphoid system. Therefore, it appears likely that microtubular structures regulate the stimulating capacity of allogeneic cells by influencing either the lateral mobility or the configuration of their surface receptors. These findings potentially explain the inability of soluble alloantigens (4, 5) and soluble tumor antigens (21) to stimulate vigorous immune responses in vitro. Further studies of this interaction between cytoskeletal elements and cell-surface determinants should provide valuable information concerning the regulation of lymphocyte responses to cell-surface antigens and serve as good models for the investigation of cell-to-cell interactions in general.

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