Effects of concanavalin A and a succinylated derivative on lymphocyte proliferation and cyclic nucleotide levels

(lymphocyte activation/lectins/adenosine 3′:5′-cyclic monophosphate/guanosine 3′:5′-cyclic monophosphate)

JOHN W. HADDEN, ELBA M. HADDEN, JOHN R. SADLIK, AND RONALD G. COFFEY

Laboratory of Immunopharmacology, Memorial Hadden, John R. Sadlik, and Ronald G. Coffey, New York, N.Y. 10021

Communicated by Robert A. Good, February 23, 1976

ABSTRACT To better define cell surface-related changes involved in lymphocyte activation, we studied native concanavalin A (Con A) and succinylated concanavalin A (Suc-Con A) for their effects on proliferation and cyclic nucleotide levels of human peripheral blood lymphocytes. At optimal mitogenic concentrations, the two forms of Con A induce equivalent proliferation; however, the mitogenic activity of Con A progressively decreases above 50 µg/ml. In contrast, the mitogenic activity of Suc-Con A is not decreased even at 250 µg/ml. Lymphocytes stimulated by a range of concentrations of Suc-Con A (25–250 µg/ml) show progressive increases in levels of guanosine 3′:5′-cyclic monophosphate (cyclic GMP) during the first 10 min of incubation. During the same period, native Con A induces initial increases in cyclic GMP; however, above 25 µg/ml, lymphocytes treated with Con A show concentration-dependent declines in the elevated cyclic GMP levels. Although Suc-Con A has no significant effect on levels of adenine 3′:5′-cyclic monophosphate (cyclic AMP), Con A increases that are concentration- and time-dependent. The concentrations of Con A responsible for the early declines in cyclic GMP and the increases in cyclic AMP levels are those which in parallel studies induce less lymphocyte proliferation. The consistent increase in cyclic GMP levels caused by both Con A and Suc-Con A suggests that cyclic GMP is involved in the induction of the proliferative response. The increase in cyclic AMP levels caused by Con A, but not by its succinylated derivative, may be responsible for the decrease in mitogenic potential observed with high doses of the native mitogen.

The lymphocyte stimulated to undergo proliferation with plant lectins such as phytohemagglutinin and concanavalin A (Con A) has provided an important model for studying in vitro the early events of the initiation of proliferation in resting ("G0" or restricted "G1") cells. Because these mitogens need not enter the lymphocyte to initiate proliferation (1) and need be present on the lymphocyte surface only for a restricted time (2, 3), the most relevant initial events to focus upon are those related to the cell surface. After the lectin is bound, a number of early membrane events have been described. These include receptor aggregation and removal (termed patching and capping); increases in membrane transport of glucose, nucleosides, and potassium; and increases in phospholipid synthesis, calcium influx, and cellular levels of guanine 3′:5′-cyclic monophosphate (cyclic GMP) and adenine 3′:5′-cyclic monophosphate (cyclic AMP) (see refs. 4 and 5 for review).

The relationship of mitogen action to changes in intracellular levels of the cyclic nucleotides has been the subject of much attention in the analysis of these events. Although some investigators have observed mitogen-induced increases in lymphocyte cyclic AMP levels (6, 7), others have not (8–11). A number have observed increases in cyclic GMP levels (8, 12–14), whereas others have not (15). Although these differences may be ascribed to technical aspects of the experiments, it remains unclear whether the structure and charge of the mitogen may also be factors.

The observations of Gunther et al. (16) have provided a basis for approaching this question. These investigators have observed that Con A, when succinylated, exhibits mitogenic action equivalent to native Con A. Succinylated Con A (Suc-Con A) differs in two important ways: Suc-Con A fails to induce patch and cap formation and at high concentrations does not exhibit diminished mitogenic action. We have confirmed that Suc-Con A does not exhibit diminished mitogenic action and have shown further that both forms of Con A increase lymphocyte levels of cyclic GMP whereas only native Con A increases levels of cyclic AMP.

METHODS

Con A in highly purified, lyophilized form was obtained from Sigma Chemical Co. (St. Louis, Mo.). This Con A preparation was chromatographed on Bio-Gel P-100 according to Sawyer et al. (18), and the presence of both dimeric and tetrameric forms of Con A in this preparation was confirmed. This preparation was succinylated as described by Gunther et al. (16). Two successive derivatizations by this procedure were reported to yield nearly quantitative succinylation of native Con A. We obtained <3% detectable native Con A by polyacrylamide gel electrophoresis performed according to the methods described by Davis (17). Although negligible native Con A was detected after succinylation, succinylation of Con A in this manner yields an apparent spectrum of Con A charge species, presumably the result of variable degrees of succinylation. No further attempt was made to characterize the two major families of charge species observed on polyacrylamide gel electrophoresis. Suc-Con A was lyophilized and both Suc-Con A and Con A were stored at −20°C, under which condition their respective mitogenic activities remain stable for months. Fresh solutions were prepared prior to each experiment.

Heparinized venous blood was obtained from healthy adult donors. Suspensions of lymphocytes were obtained by Ficolldiphenol diatrizoate gradient centrifugation (19). Cells were washed in Hanks' balanced salt solution and centrifuged at 150 X g to remove platelets. Final cell preparations were greater than 98% pure mononuclear cells (>85% lymphocytes by morphologic criteria); platelet contamination did not exceed 0.1% by weight.

Cells were cultured in Eagle's minimal essential medium, including 10% human AB serum, 2 mM L-glutamine, 100 units/ml of penicillin, and 100 µg/ml of streptomycin (Grand Island Biological Co., Grand Island, N.Y.), at a concentration of 1 X 10⁶ lymphocytes/0.2 ml in microculture plates (Falcon Plastics, Oxnard, Calif.). Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO₂ in air for 72 hr.

Abbreviations: cyclic AMP, adenosine 3′:5′-cyclic monophosphate; cyclic GMP, guanosine 3′:5′-cyclic monophosphate; Con A, concanavalin A; Suc-Con A, succinylated concanavalin A.
Kinetic experiments were incubated for 24, 48, 60, and 72 hr. Proliferation was assayed by the incorporation of 0.5 μCi of [3H]thymidine (specific activity 20 Ci/mmol, New England Nuclear, Boston, Mass.) measured during a terminal 4 hr pulse; cultures were terminated by a multiple automatic sample harvester (Otto Hiller Co., Madison, Wisc.), and thymidine incorporation into insoluble cellular constituents was assayed by liquid scintillation spectrometry. The data are calculated as the mean of [3H]thymidine incorporated per 10^5 lymphocytes for triplicate samples. Thymidine incorporation under these circumstances directly reflects lymphocyte DNA synthesis (20).

For cyclic nucleotide measurements, 1 x 10^5 lymphocytes were suspended in 1 ml of Hanks’ balanced salt solution in 12 x 75 mm plastic tubes (Falcon Plastics, Oxnard, Calif.). Lymphocytes were allowed to equilibrate for 1 hr at 37°. Solutions of Con A or Suc-Con A were added in 10 μl volumes for various times, followed by termination with 0.5 ml of cold 30% trichloroacetic acid. Samples were quickly frozen and stored at -20°. Samples were thawed, and 0.002 μCi of [3H]cyclic GMP (5 Ci/mmol, New England Nuclear) was added. The supernatant was removed after centrifugation and the protein in the pellet was determined by the method of Lowry et al. (21). Trichloroacetic acid in the supernatant was removed by five extractions with 2 volumes of ether. Cyclic nucleotides were separated on Dowex-1-formate columns according to Murad et al. (22); cyclic AMP was eluted with 10 ml of 1 M formic acid and cyclic GMP with 10 ml of 4 M formic acid. The eluates were evaporated to dryness on an Evapo-Mix (Buchler Co., Fort Lee, N.J.). Cyclic GMP was further purified by the QAE-Sephadex A-25 column method of Schultz et al. (23). Cyclic GMP was eluted with 0.1 M ammonium formate (pH 6.0) and the eluate was passed through a Dowex-50 H+ column (Bio-Rad Laboratories, Richmond, Calif.) to remove ammonium ion. The samples were then evaporated to dryness. The use of this extensive purification sequence has proven necessary to reliably detect cyclic GMP extracted from lymphocytes.

Both cyclic AMP and cyclic GMP samples were suspended in 300 μl of 10 mM Tris-HCl (pH 7.5) containing 2 mM magnesium chloride. Recovery of cyclic AMP was estimated by measuring the radioactivity of [3H]cyclic AMP (27 Ci/mmol, Schwartz/Mann, Orangeburg, N.Y.) standards containing 0.05 μCi/ml which were processed identically. Recovery of [3H]cyclic GMP was determined in each sample by measuring the radioactivity of a 50-μl aliquot. Aliquots treated with phosphodiesterase (Boehringer-Mannheim, New York, N.Y.) confirmed the purity (>95%) of all samples. Cyclic AMP was assayed by the method of Gilman (24) and cyclic GMP by the radioimmunoassay method of Steiner et al. (25) as modified by Goldberg et al. (26) with use of commercially available reagents (Collaborative Research, Inc., Waltham, Mass.). In the modified procedure the second antibody is omitted, and after incubation for 18-24 hr at 4° the samples are filtered on 0.45 μm HAWP filters (Millipore Corp., Bedford, Mass.). The final values are corrected for recovery and purity and are expressed as pmol/mg of protein.

RESULTS

Succinylated Con A and native Con A both induce marked proliferation of human peripheral blood lymphocytes, as measured by thymidine incorporation at 3 days of culture. Fig. 1 compares the effects of the two over a broad concentration range on lymphocyte proliferation, as measured by [3H]thymidine incorporation between 68 and 72 hr of culture. Con A shows maximal mitogenic action between 25 and 50 μg/ml, and above these concentrations progressively less proliferation is observed. Suc-Con A shows comparable mitogenic action to Con A at about 50 μg/ml; however, at higher concentrations, no diminution of the proliferative response is observed. Kinetic experiments (data not shown) indicate that both Con A and Suc-Con A, over a range of concentrations produce progressive increases in [3H]thymidine incorporation between 24 and 72 hr of culture. The effects on thymidine incorporation of Suc-Con A occur later than those of Con A; thus, at 48 hr the effects of Con A (25-150 μg/ml) are greater than those of Suc-Con A, as observed by Wang et al. (27).

The effects of various concentrations of the two forms of Con A on lymphocyte cyclic GMP levels are depicted in Fig. 2. Suc-Con A at all concentrations tested (Fig. 2A) produces progressive increases in cyclic GMP levels over the first 10 min of incubation; however, Con A (Fig. 2B), although producing similar increases in cyclic GMP levels at optimal mitogenic concentrations, shows a progressive decline in cyclic GMP levels at concentrations at or above 50 μg/ml after 5 min of incubation. These higher concentrations of Con A are those inducing less proliferation (Fig. 1). As occurs with proliferation, lymphocytes from individual subjects vary considerably in the cyclic GMP responses to Con A stimulation. Experiments with five different subjects with Con A showed increases in cyclic GMP levels ranging from 0.14 to 1.03 and with Suc-Con A ranging from 0.08 to 1.16 pmol/mg of protein above control. Maximal increases observed with 25-50 μg/ml of Con A and Suc-Con A over the first 15 min of incubation averaged 5.2- and 4.5-fold control levels, respectively. Over the range of concentrations studied, Con A generally showed early peaks (5 min or less) with a rate of increase of 0.19 ± 0.03 pmol/mg of protein per min, whereas Suc-Con A showed progressive increases over the first 10 min with a slower rate of increase, 0.07 ± 0.03 pmol/mg of protein per min.

The effects of the two forms of Con A on lymphocyte cyclic AMP levels are depicted in Fig. 3. Suc-Con A (Fig. 3A), at all concentrations tested, produced no significant increases in cyclic AMP levels over the first 10 min of incubation; however, Con A (Fig. 3B) produced progressive, concentration-dependent increases in cyclic AMP levels over this period. The cyclic AMP increases observed with higher concentrations of Con A can be
thymus-derived lymphocytes question of the specificity of induction effects to lymphocytes, does not provide, in contrast to those with Con A as a function of concentration and time.

correlated with the decline of proliferation (Fig. 1) and the early decline of cyclic GMP levels (Fig. 2B).

Table 1 compares the effects of Suc-Con A and Con A on lymphocyte cyclic nucleotide levels at 15 min of incubation. Evident at this time are the significant increases in cyclic AMP levels with Con A but not Suc-Con A. Cyclic GMP levels at this time are less than those at 10 min; nevertheless, with the mitogen concentrations used, the averaged levels with Suc-Con A are significantly greater than those with Con A.

**DISCUSSION**

In contrast to the more generally used mitogenic lectin, phytohemagglutinin, Con A is commercially available in highly purified form and is easily modified to yield a succinylated derivative (16). The biological activity of the Suc-Con A differs from the native Con A in that it is 10-fold less agglutinating for lymphocytes, does not induce patch and cap formation, and does not inhibit anti-Ig-induced capping (28). These two forms of Con A provide, then, useful reagents with differing biological effects to probe cell surface-related changes involved in the induction of proliferation in relation to those involved in agglutination and patch and cap formation. In addition, these reagents make it possible to examine the mechanisms by which high concentrations of lectin mitogens fail to induce proliferation.

The use of this mitogen preparation with heterogeneous populations of peripheral blood mononuclear cells raises the question of the specificity of its action on target cells. Like phytohemagglutinin, Con A is preferentially mitogenic for thymus-derived lymphocytes (T-lymphocytes) in human

**Table 1. Lymphocyte cyclic nucleotide levels 15 min after Con A stimulation**

<table>
<thead>
<tr>
<th>Lectin and concentration (µg/ml)</th>
<th>Cyclic AMP pmol/mg of protein ± SEM (n = 3)*</th>
<th>Cyclic GMP pmol/mg of protein ± SEM (n = 3)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>117 ± 6</td>
<td>0.386 ± 0.08</td>
</tr>
<tr>
<td>100</td>
<td>203 ± 18</td>
<td>0.313 ± 0.02</td>
</tr>
<tr>
<td>250</td>
<td>344 ± 42</td>
<td>0.233 ± 0.02</td>
</tr>
<tr>
<td>Suc-Con A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>79 ± 9</td>
<td>0.637 ± 0.17</td>
</tr>
<tr>
<td>100</td>
<td>76 ± 8</td>
<td>0.403 ± 0.08</td>
</tr>
<tr>
<td>250</td>
<td>91 ± 6</td>
<td>0.510 ± 0.01</td>
</tr>
<tr>
<td>Control</td>
<td>78 ± 5</td>
<td>0.170 ± 0.05</td>
</tr>
</tbody>
</table>

*Data are pooled from three experiments with different individuals, which accounts for the large SEM. The increases in cyclic AMP observed with Con A but not Suc-Con A are progressive and significant (P < 0.01). The averaged increases in cyclic GMP observed at 15 min with Con A are significantly above controls (P < 0.01) and significantly less than with Suc-Con A (P < 0.01).

**DISCUSSION**

in peripheral blood (29). Both phytohemagglutinin and Con A induce a degree of proliferation in lymphocytes bearing immunoglobulin on their surface (B-lymphocytes); however, this action appears to result from a delayed, recruitment phenomenon dependent upon the presence of activated T-lymphocytes (29-31). In addition to T- and B-lymphocytes, human peripheral blood mononuclear preparations contain monocytes whose contribution to the proliferative response of lymphocytes to mitogens has not been fully clarified. Recent studies of Drs. Norman Berlinger and Robert A. Good (personal communication) indicate that the proliferative response of human peripheral blood lymphocytes induced by Con A is virtually unaffected by the removal of the monocyte population. In addition, monocyte-free human peripheral blood lymphocytes show

**FIG. 2. Effects of Con A and Suc-Con A on lymphocyte cyclic GMP levels.** The effects of Suc-Con A (A) and Con A (B) over a concentration ranging from 25 to 250 µg/ml on cyclic GMP levels of lymphocytes from a single individual are depicted to demonstrate the difference in profile of the response with the two forms of Con A as a function of concentration and time.

**FIG. 3. Effects of Con A and Suc-Con A on lymphocyte cyclic AMP levels.** The effects of Suc-Con A (A) and Con A (B) on cyclic AMP levels of lymphocytes from five individuals were examined. The effects of Con A at concentrations of 50 µg/ml and higher in increasing cyclic AMP levels at 10 min of incubation are significant (P < 0.01).
an unaltered dose-response curve for native Con A. This latter point is relevant because it has been suggested, on the basis of studies with mouse spleen cells, that the lack of effect on proliferation of high lectin concentrations may result from the activation of a suppressor population that is glass-adherent, presumably macrophages (32). The available evidence indicates that the early events observed after Con A stimulation involve principally, if not exclusively, the T-lymphocyte.

The results of the present experiments demonstrate that both Con A and Suc-Con A induce human lymphocyte proliferation of comparable magnitude; however, Suc-Con A at concentrations above 50 μg/ml does not show the progressive decline in the proliferative response exhibited by Con A. These results confirm those obtained with murine (16) and human lymphocytes (27).

In the present study, it was also observed that both Suc-Con A and Con A produce significant early increases in cyclic GMP in lymphocytes. The present results corroborate those which indicate that cyclic GMP is involved in the induction of proliferation of lymphocytes (8).* The increases in cyclic GMP observed are comparable to those obtained with other mitogenic agents in lymphocytes (8, 12–14), fibroblasts (33, 34), epidermal cells (35), salivary gland (36), and uterus (37). The cyclic GMP increases induced by mitogenic agents have been further related in the lymphocyte to calcium influx (5) and to actions of cyclic GMP and calcium to initiate RNA synthesis in the isolated nucleus, in parallel to events which occur in the nucleus of the intact lymphocyte when stimulated by mitogenic agents (5, 33, 38). Cyclic GMP-mediated phosphorylation of nonhistone acidic nuclear proteins in lymphocytes (39) and nuclear localization of cyclic GMP (40) and of cyclic GMP binding proteins (41) further link cyclic GMP to nuclear and particularly nucleolar ribosomal RNA synthesis. These observations have provided the basis for the hypothesis that cyclic GMP and calcium represent components of a membrane-to-nucleus signal sequence mediating the cell surface action of mitogenic agents that induces nuclear activation (5, 38).

In the present study it was observed that Con A, but not Suc-Con A, induces increases in cyclic AMP levels in the lymphocytes. This effect of Con A is concentration-dependent in a way that relates to the effect of Con A at a concentration of 50 μg/ml or higher to induce progressively less proliferation. The effect of Con A in increasing cyclic AMP levels is progressive with time during the initial 15 min of incubation. Because increases in cyclic AMP induced by Con A are correlated in time and concentrations of Con A with the early declines in elevated cyclic GMP levels, we suggest that the increases in cyclic AMP may be responsible for "blunting" the cyclic GMP response. These results, demonstrating cyclic AMP increases with Con A, confirm those of Parker and coworkers (7, 15).

It is of interest that other agents that increase cyclic AMP levels in lymphocytes are inhibitory to the proliferative response induced by mitogens (6, 42, 43). In addition, it has been observed that high concentrations of phytohemagglutinin increase cyclic AMP levels (6, 44) and that agents which lower the elevated cyclic AMP levels reverse the depressed proliferative response (5, 44). These observations, taken in conjunction with those of the present study, indicate that cyclic AMP is not an essential component of the early events of lymphocyte activation, as has been suggested by others (45, 46), and suggest that the effect of high concentrations of mitogens to induce less proliferation involves an inhibitory influence which is mediated by cyclic AMP.

The different biological effects on Con A and Suc-Con A has been attributed to the conversion of Con A from the tetrameric to the dimeric form as a result of succinylation (16). The inference from this interpretation is that as a result of the reduced valence of Suc-Con A less surface crosslinking of receptors results; therefore, aggregation, patch and cap formation, and reduced mitogenicity of high concentrations do not occur to the same extent. Sawyer et al. (18) have observed that commercial Con A is a mixture of tetrameric and dimeric forms of Con A, an observation confirmed in the present study. These workers noted that the dimer and tetramer species of Con A have similar mitogenic activity without differences at high concentrations. We, therefore, interpret our results to indicate that succinylation modifies the dose-response curve of the effect of Con A on proliferation not because of a conversion of Con A from a tetramer to a dimer, but rather because of the alteration in charge of the Con A induced by succinylation.

That Suc-Con A does not significantly induce agglutination and modify the mobility of cell surface receptors indicates that such effects are unrelated to the mitogenic aspects of lectin action. The question of the inter-relationship of the observed cyclic AMP increases, the inhibition of mitogenic action and various receptor mobility phenomena remains open. Experiments (46) in which fluorescent antibodies against cyclic AMP were used to localize the site at which lectin mitogens increase cyclic AMP in lymphocytes demonstrate microaggregates of cyclic AMP at the cell surface. These aggregates may represent micropatch formation or intermembranous particle aggregation occurring under these conditions. Patch formation may therefore be directly related to adenylate cyclase activation and local cyclic AMP accumulation. It has been suggested that cyclic AMP is directly related to microtubular aggregation in lymphocytes (cf. ref. 47). Microtubular protein is a known substrate for phosphorylation by an endogenous cyclic AMP-dependent protein kinase (48), and cyclic AMP-dependent phosphorylation of non-nuclear proteins has been observed in lymphocytes (49). It has been suggested that components of an organized submembranous assembly involving microtubules are responsible for both the modulation of surface receptor mobility and the regulation of inhibition of mitogenic stimulation (27, 28). Such a hypothesized mechanism of high-dose mitogen inhibition of proliferation offers one explanation for how cyclic AMP may exert its antiproliferative effects in lymphocytes.

We acknowledge Drs. C. Ching and C. Lopez for technical assistance and Drs. G. Litman and C. Stackpole for constructive suggestions. This study was supported by grants from the National Institutes of Health (NIH-07548 and CA-16988) and the American Heart Association (72-643). J. W. H. is the recipient of an Established Investigatorship of the American Heart Association.


---

* The levels of cyclic GMP previously reported by us (8) were incorrectly labeled as pmol/10⁵ cells; they should be corrected to read pmol/10⁶ cells.
Immunology: Hadden et al.