The demonstration that Concanavalin A (Con A), a polyclonal T-cell activator, mimics antigen in generating cytotoxic T lymphocytes (CTL) from normal lymphocytes (1-4) may help in defining not only the molecular nature of the stimulating antigenic determinants but also in identifying receptors on pre-killer cells involved in the activation process (5). If Con A and antigenic activation processes are mediated by similar pathways and by the same receptors, two predictions may be made: (a) It should be possible to use Con A and stimulating antigens interchangeably in the activation of secondary CTL and (b) antigenic stimulation should result in both specific activation and, to a lesser extent, nonspecific polyclonal activation.

This report tests the first prediction, i.e., whether lymphocytes primed to an allograft can be activated specifically into secondary cytotoxic T lymphocytes (2° CTL) by Con A. The secondary cytotoxic response was chosen to test this hypothesis because it characteristically appears early and is stronger than the primary response (6-8). The results reported here demonstrate that splenocytes primed to alloantigens can be activated in vitro into secondary CTL by both Con A. The cytotoxic activity is qualitatively indistinguishable from that obtained by antigenic stimulation.

Materials and Methods

Mice. C57BL/6 (H-2¹) and BALB/c (H-2²) mice, 8- to 12-wk old, were obtained from the Mammalian Genetics and Animal Production Section, Cancer Chemotherapy, National Service Center, National Cancer Institute.

Tumors. The following transplantable, ascitic tumors were maintained by weekly i.p. inoculation of 1 × 10⁷ tumor cells into the syngeneic host: EL-4 (a chemically induced lymphoma) in C57BL/6 mice and G-35 (a Gross virus-induced lymphoma) in BALB/c mice. D-6 (H-2¹) lymphoma was established in culture by Dr. E. Hays, U.C.L.A., from a spontaneous leukemia developed in C3H mice.

Immunizations. 10-20 million tumor cells were injected i.p. into the appropriate allogeneic
host: EL-4 (H-2b) into BALB/c (H-2d) mice, and G-35 (H-2~) into C57BL/6 (H-2b) mice. The immune mice were sacrificed 40-60 days after inoculation.

In Vitro Sensitization. Cultures were set in Falcon 50-ml flasks (cat. no. 3023), each containing a total of 25 × 10^6 responder cells and 5 × 10^6 stimulator cells (treated with 30 μg mitomycin C/10^7 cells) in 10 ml RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 5 × 10^-5 M 2-mercaptoethanol, 15 mM Hepes buffer, 2 mM glutamine, and 1% antimycotic-antibacterial mixture. The flasks were incubated in a horizontal position in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. Cells were harvested at 42-72 h, washed twice in phosphate-buffered saline, counted for viability, and brought to the desired cell concentration.

Con A Activation. This was set as above with the exception that Con A (Type IV, Sigma Chemical Co., St. Louis, Mo.) was added at the desired concentration. The preparation of blasts for use as target cell was done as described previously (9).

Assays for Cell-Mediated Cytotoxicity. The 51Cr release assay was used as described previously using 10^6 51Cr-target cells (9). Cytotoxic activity was occasionally expressed in lytic units per spleen as described by Cerottini et al., with the exception that one lytic unit was defined to represent the number of effector cells that kill 30% of the target (7). Lectin-dependent cellular cytotoxicity (LDCC) was performed as described previously (10).

Fractionation on Cell Monolayers. Adsorption of immune cells was carried out by the technique we have previously described (11).

[3H]Thymidine Incorporation. Proliferation was assessed by [3H]thymidine incorporation. A sample (0.3 ml) was taken from the culture flask, washed, resuspended in 0.6 ml fresh media, and dispensed in 0.2-ml aliquots in microtiter plates. To each well 1 μCi [3H]thymidine was added and the plates incubated for 6 h. The cells were harvested on a MASH multiharvester and counted for radioactivity. The results are expressed as mean count ± SD of triplicate samples with background subtracted. The background was usually less than 400 cpm.

Heterologous Anti-T Serum. Specific heterologous anti-mouse thymocyte serum was prepared as described (12).

Results

Con A-Induced Activation of In Vivo Primed Splenocytes to a Tumor Allograft into Secondary Cytotoxic Lymphocytes. Primed splenocytes against a tumor allograft were cultured for 3-4 days with either the priming alloantigen or with Con A and tested for cell-mediated cytotoxicity by the 51Cr release assay (Table I). The stimulation in one-way mixed lymphocyte tumor reaction resulted in a very strong and specific cytotoxic activity against the stimulating target. In the absence of stimulating antigen, Con A also activated the primed splenocytes into cytotoxic lymphocytes with specificity directed against the priming alloantigen. The Con A-induced activation of CTL was shown in two different systems, namely, primed BALB/c anti-EL-4 and C57BL/6 anti-G-35. Cytotoxicity was not restricted to tumor target cells since 51Cr C57BL/6 blasts served as good as 51Cr EL-4 tumor (Exp. 4). Titrations of effector cells as a function of cytotoxic activity resulted in a linear relationship, and the slope of the curves was similar for both alloantigen and Con A activation. Phytohemagglutinin (PHA) was also able to activate 2° CTL in a manner similar to Con A. Lipopolysaccharide, a B-cell mitogen, however, was not effective (Exp. 5).

Normal splenocytes cultured with Con A were found to be not cytotoxic to syngeneic or allogeneic target cells. Failure to detect Con A-induced polyclonal activation was due to the assay conditions used in this study, namely, short cytotoxic assays (2-3 h) and low effector to target cell ratios (<20:1). Polyclonal activation was revealed when optimum conditions for demonstrating polyclonal activation were achieved, i.e., longer assay periods (18-36 h) and high effector target cell ratios (>200:1) (Exp. 1).
| Exp. no. | Responder splenocytes | Stimulator | Con A | Day tested | Percent specific \(^{51}Cr\) release (E:T 20:1) |
|---------|----------------------|------------|-------|------------|-----------------------------------------------|
|         |                      |            | \(\mu g/ml\) |            | EL-4 \((H-2^d)\) | G-35 \((H-2^d)\) |
| 1       | BALB/c anti-EL-4 (50d) | EL-4       | 0.25  | 4          | 32.2 ± 2.1 | 8.8 ± 0.6 |
| 1       | BALB/c anti-EL-4 (50d) |             | 1.0   | 4          | 50.3 ± 3.5 | 8.0 ± 0.3 |
| 1       | BALB/c anti-EL-4 (50d) |             | 2.0   | 4          | 51.6 ± 2.3 | 9.2 ± 0.4 |
| 1       | BALB/c anti-EL-4 (50d) |             | 4.0   | 4          | 49.3 ± 2.3 | 9.3 ± 0.2 |
| 1       | BALB/c anti-EL-4 (50d) |             |       | 3          | 49.1 ± 0.7 | 10.4 ± 0.3 |
| 1       | Normal BALB/c         | EL-4       | 2.0   | 4          | 3.2 ± 0.6  | 1.6 ± 0.5 |
| 1       | Normal BALB/c         |             | 4.0   | 4          | 4.5 ± 0.3  | 2.5 ± 0.7 |
| 1       | Normal BALB/c         |             | 1.0   | 3          | 8.2 ± 0.8  | 2.0 ± 0.4 |
| 2       | BALB/c anti-EL-4 (40d) | EL-4       | 1.0   | 2          | 63.2 ± 2.1 | 8.8 ± 0.6 |
| 2       | BALB/c anti-EL-4 (40d) |             | 2.0   | 2          | 55.3 ± 3.5 | 8.0 ± 0.3 |
| 2       | BALB/c anti-EL-4 (40d) |             | 4.0   | 2          | 51.6 ± 2.3 | 9.2 ± 0.4 |
| 2       | BALB/c anti-EL-4 (40d) |             |       | 2          | 49.3 ± 2.3 | 9.3 ± 0.2 |
| 2       | BALB/c anti-EL-4 (40d) |             |       | 3          | 49.1 ± 0.7 | 10.4 ± 0.3 |
| 2       | Normal BALB/c         | EL-4       | 2.0   | 2          | 3.2 ± 0.6  | 1.6 ± 0.5 |
| 2       | Normal BALB/c         |             | 4.0   | 2          | 4.5 ± 0.3  | 2.5 ± 0.7 |
| 2       | Normal BALB/c         |             | 1.0   | 2          | 8.2 ± 0.8  | 2.0 ± 0.4 |
| 3       | BALB/c anti-EL-4 (60d) | EL-4       | 1.0   | 2          | 63.2 ± 2.1 | 8.8 ± 0.6 |
| 3       | BALB/c anti-EL-4 (60d) |             | 2.0   | 2          | 55.3 ± 3.5 | 8.0 ± 0.3 |
| 3       | BALB/c anti-EL-4 (60d) |             | 4.0   | 2          | 51.6 ± 2.3 | 9.2 ± 0.4 |
| 3       | BALB/c anti-EL-4 (60d) |             |       | 2          | 49.3 ± 2.3 | 9.3 ± 0.2 |
| 3       | BALB/c anti-EL-4 (60d) |             |       | 3          | 49.1 ± 0.7 | 10.4 ± 0.3 |
| 3       | Normal BALB/c         | EL-4       | 2.0   | 2          | 3.2 ± 0.6  | 1.6 ± 0.5 |
| 3       | Normal BALB/c         |             | 4.0   | 2          | 4.5 ± 0.3  | 2.5 ± 0.7 |
| 3       | Normal BALB/c         |             | 1.0   | 2          | 8.2 ± 0.8  | 2.0 ± 0.4 |
| 4       | BALB/c anti-EL-4 (60d) | EL-4       | 1.0   | 2          | 63.2 ± 2.1 | 8.8 ± 0.6 |
| 4       | BALB/c anti-EL-4 (60d) |             | 2.0   | 2          | 55.3 ± 3.5 | 8.0 ± 0.3 |
| 4       | BALB/c anti-EL-4 (60d) |             | 4.0   | 2          | 51.6 ± 2.3 | 9.2 ± 0.4 |
| 4       | BALB/c anti-EL-4 (60d) |             |       | 2          | 49.3 ± 2.3 | 9.3 ± 0.2 |
| 4       | BALB/c anti-EL-4 (60d) |             |       | 3          | 49.1 ± 0.7 | 10.4 ± 0.3 |
| 4       | Normal BALB/c         | EL-4       | 2.0   | 2          | 3.2 ± 0.6  | 1.6 ± 0.5 |
| 4       | Normal BALB/c         |             | 4.0   | 2          | 4.5 ± 0.3  | 2.5 ± 0.7 |
| 4       | Normal BALB/c         |             | 1.0   | 2          | 8.2 ± 0.8  | 2.0 ± 0.4 |
| 5       | BALB/c anti-EL-4 (50d) | EL-4       | 1.0   | 2          | 63.2 ± 2.1 | 8.8 ± 0.6 |
| 5       | BALB/c anti-EL-4 (50d) |             | 2.0   | 2          | 55.3 ± 3.5 | 8.0 ± 0.3 |
| 5       | BALB/c anti-EL-4 (50d) |             | 4.0   | 2          | 51.6 ± 2.3 | 9.2 ± 0.4 |
| 5       | BALB/c anti-EL-4 (50d) |             |       | 2          | 49.3 ± 2.3 | 9.3 ± 0.2 |
| 5       | BALB/c anti-EL-4 (50d) |             |       | 3          | 49.1 ± 0.7 | 10.4 ± 0.3 |

* Mice were primed in vivo to a tumor allograft and 40-60 days (d) later the spleens were removed and cultured in the presence of stimulating antigen or Con A.
† In this experiment the cytotexic assay was extended to 16 h in order to demonstrate polyclonal activation.
§ The numbers in parentheses represent the number of lytic units recovered in the culture.
| PHA was obtained from Wellcome Burroughs, England, and each vial was diluted to 5 ml. From this stock solution various amounts were used.
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Kinetics of the Proliferative and Cytotoxic Responses After Antigenic Stimulation and Con A Activation. With Con A concentrations of 0.5 and 1.0 μg/ml, both radioactive incorporation and CTL activity reached a maximum at day 3 and then declined at day 4 (Fig. 1). With <0.5 μg/ml Con A, weak CTL activity and relatively weak incorporation of radioactivity was obtained. Antigenic stimulation resulted in maximum CTL and radioactive incorporation at day 4. These experiments demonstrated that Con A activation of CTL follows the same early kinetics observed after secondary antigenic stimulation, and the proliferative response appears to parallel the cytotoxic response.

Properties of Cytotoxic Effector Cells Activated by Con A. The specific Con A inhibitor, α-methyl-D-mannose pyranoside (α-mm) failed to inhibit (cell-mediated cytotoxicity (CMC) of effector cells generated by Con A demonstrating that CMC is not mediated by contaminant Con A present in the cultures (Table II, Exp. 1).

Other properties of Con A-induced CTL are summarized in Table III. Treatment with specific anti-T serum and complement abrogated cytotoxicity demonstrating that the effector cells are thymus dependent. The cytotoxic activity was
TABLE II

Specific CMC and Nonspecific LDCC

| Exp. no. | Effector lymphocytes | Inhibitor α-mm | CMC* | LDCC‡ |
|----------|----------------------|---------------|------|-------|
| 1        | BALB/c anti-EL-4§     |               | 38.8 ± 0.2 | 20.8 ± 0.3 |
|          | BALB/c anti-EL-4§     | 2.5 x 10⁻¹    | 38.8 ± 0.9 (0) | 3.8 ± 0.5 (82) |
|          | BALB/c anti-EL-4§     | 5 x 10⁻¹      | 38.8 ± 1.6 (0) | 12.4 ± 0.6 (40) |
|          | BALB/c anti-EL-4§     | 10⁻²          | 39.2 ± 0.3 (0) | 20.6 ± 0.1 (0) |
| 2        | BALB/c anti-EL-4§     |               | 33.8 ± 0.8 | 22.4 ± 1.3 |
|          | BALB/c anti-EL-4‡     | 41.6 ± 2.9    | 12.5 ± 1.5 |
|          | C57BL/6 anti-G-35§    | 36.3 ± 1.4    | 10.0 ± 1.4 |
|          | C57BL/6 anti-G-35¶    | 61.3 ± 1.1    | 41.3 ± 3.0 |

* CMC was assessed against ⁵¹Cr EL-4 or ⁵¹Cr G-35 at an effector-target ratio of 20:1.
‡ LDCC was assessed against ⁵¹Cr D6 (H-2b) target cells in the presence of 1.0 μg Con A (Exp. 1) and against syngeneic ⁵¹Cr EL-4 or ⁵¹Cr G-35 target cells in the presence of 10 μl PHA (Exp. 2).
§ Activated by Con A (1.0 μg/ml).
¶ Activated by antigen.

| TABLE III

Properties of Cytotoxic Effector Cells Activated by Concanavalin A and by Alloantigens

| Effector cells | Activation | Treatment | CMC to ⁵¹Cr EL-4 | Percent inhibition |
|----------------|------------|-----------|------------------|--------------------|
| BALB/c anti-EL-4 (60d) | Con A (1 μg/ml) | None | 34.6 ± 1.0 | 95 |
| BALB/c anti-EL-4 (60d) | Con A (1 μg/ml) | Absorbed on BALB/c monolayer* | 38.0 ± 1.1 | 95 |
| BALB/c anti-EL-4 (60d) | Con A (1 μg/ml) | Absorbed on C57BL/6 monolayer | 0.7 ± 0.6 | 96 |
| BALB/c anti-EL-4 (60d) | Con A (1 μg/ml) | Anti-T serum + C' | 3.6 ± 1.9 | 90 |
| BALB/c anti-EL-4 (60d) | Con A (1 μg/ml) | Adherence to plastic* | 30.2 ± 0.3 | 95 |
| BALB/c anti-EL-4 (60d) | EL-4a | None | 41.3 ± 0.7 | 95 |
| BALB/c anti-EL-4 (60d) | EL-4a | Absorbed on BALB/c monolayer | 39.5 ± 0.3 | 90 |
| BALB/c anti-EL-4 (60d) | EL-4a | Absorbed on C57BL/6 monolayer | 6.4 ± 0.7 | 85 |
| BALB/c anti-EL-4 (60d) | EL-4a | Anti-T serum + C | 4.8 ± 1.0 | 88 |
| BALB/c anti-EL-4 (60d) | EL-4a | Adherence to plastic | 43.3 ± 0.5 | 90 |

d, days.
* Experiments with monolayer absorptions and adherence to plastic Petri dishes were done by testing the nonadherent fractions for cytotoxic activity without readjustment of effector-target cell ratio.

recovered in the nonadherent cell fraction, demonstrating that cells adherent to plastic are not the effector lymphocytes. Absorption of effector cells on monolayers prepared with the priming alloantigen specifically removed the cytotoxic effector cells, demonstrating antigenic specificity of the effector cells.

Complementarity of Con A and Antigen in Activation of 2° CTL. If activation of 2° CTL by Con A and alloantigen is mediated by the same mechanisms, it would be expected that mixtures of suboptimal concentration of Con A and antigen should be additive and stimulatory. Indeed, the addition of Con A to the cultures resulted in augmentation of CTL activity as measured by ⁵¹Cr release and by total lytic activity recovered. This augmentation of cytolytic activity was also noticed with submitogenic concentrations of Con A (0.1-0.25 μg/ml) and with nonstimulatory responder:target ratio (10,000:1). The augmentation of CTL activity was synergistic in all cases (Fig. 2).
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FIG. 2. Primed BALB/c spleen cells to EL-4 were cultured in vitro with Con A, EL-4, or a mixture of Con A and EL-4. 3 days later, the cells were tested for cytotoxic activity against \(^{31}Cr\) EL-4 cells. Three responder to stimulating ratios (R/S) were used to correspond to R/S 100:1, R/S 1,000:1, and R/S 10,000:1, using constant number of responder splenocytes. Three effector:target cell ratios were used (20:1, 5:1, and 1.25:1), and an estimate of the number of lytic units for each culture was calculated and represented in parentheses. The results in the figure represent cytotoxicity at E:T of 5:1.

Discussion

Evidence is presented which demonstrates that primed splenocytes to an allograft can be activated by Con A into secondary CTL with specificity for the priming alloantigens. The cytotoxic activity induced by Con A is independent of the presence of Con A in the cytotoxic assay, thus clearly not LDCC. The data is consistent in that identical populations of primed lymphocytes respond to either Con A or antigen and suggest that Con A binds to the antigen-binding receptors or to functionally similar receptors present on the membrane. This idea is best illustrated from several independent lines of evidence indicating the absence of obvious qualitative differences in the CTL responses generated by Con A and by antigen, namely, Con A-induced activation resembles antigenic stimulation in the kinetics of appearance of CTL, strength of CTL activity, and specificity of killing. Furthermore, mixture of suboptimal concentrations of Con A and stimulating antigen resulted in augmentation of CTL activity.

The generation of CTL has been shown to be mediated by cooperation of at least two T-cell subclasses (5, 13). Our present studies cannot distinguish whether Con A activation of \(^{2}\) CTL directly triggers the prekiller cells (SD reactive cells), the proliferating helper cell, or both cells. The availability of specific antisera directed against subclasses of T cells should be helpful in answering this question (14).

The Con A activation of \(^{2}\) CTL is reminiscent of the recently reported studies by Alter et al. (15) who demonstrated that lymphocyte-defined (LD) antigens stimulate \(^{2}\) CTL with specificity to the primary stimulating cellular antigens. These results and the work presented here suggest that Con A and LD antigens, or functionally similar antigens, may trigger prekiller cells by similar mechanisms via a single pathway. Accordingly, it may be expected that the triggering events induced by these two activating agents would be indistinguishable. Several shared properties have been demonstrated here and in other studies: (a) Con A and LD antigens are mitogenic as demonstrated by \(^{3}H\) thymidine incorporation; (b) Con A and LD antigens both activate specific \(^{2}\) CTL from antitigen-
cally primed cells under similar assay conditions (i.e., short-term assays with low E:T ratios). The cytotoxic activity generated is qualitatively indistinguishable and in many cases quantitatively alike. (c) Since Con A activation is thought to be nonspecific, LD activation ought to be also nonspecific, i.e., no requirement for antigenic identity of the priming LD with the secondary LD stimulus. Indeed, studies of Alter et al. (15) and ours (unpublished) have demonstrated that activation of 2° CTL by LD antigen does not require homology with the primary LD. Targets with different LD determinants were able to trigger 2° CTL with specificity to the primary antigens. The role of SD antigens in 2° CTL activation, however, cannot be ruled out completely since SD antigenic contaminants may still be present with the primed responder cells. (d) Con A and LD antigens are expected to be complementary in the activation process. Evidence is presented here showing that mixing of suboptimal nonmitogenic concentrations of Con A and antigen with primed responding lymphocyte in cultures resulted in augmentation of 2° CTL activity. The amplification was synergistic in several responder:target ratios tested. Synergism was also obtained with optimal mitogenic concentration of Con A. The synergistic activity obtained may be the result of specific triggering of primed lymphocytes and/or expansion of responding cells by mitogenic factors. The augmentation of 2° CTL activity by mitogenic Con A concentrations as shown here is in contrast to Con A-induced suppression of 1° CTL generation in one-way mixed leukocyte response (16). It may be that primed lymphocytes are refractory to Con A-induced suppression. Further studies are being investigated to test this possibility, and (e) Con A has been shown to be a polyclonal T-cell activator of unprimed lymphocytes (1-4), whereas LD antigens have not been examined for polyclonality. In the 1° response, LD antigens have been shown to cooperate with SD antigens to generate strong and specific CTL, and in the 2° CTL response LD antigens bypass the SD antigenic requirement. The functional role of LD antigens in the 1° response vs. the 2° response may still be the same, and the differences may be reconciled by assuming that LD antigens have low affinity for uncommitted prekiller cells and high affinity for primed cells. The activation of 1° CTL by LD antigens is helped by the SD antigens then serving as focusing device. By this mechanism, specificity of CTL, rather than polyclonality, is also achieved by virtue of SD antigens binding to specific antigen-binding receptors present on corresponding clones of lymphocytes.

In drawing a comparison between Con A and LD antigen function, another question that remains to be investigated is whether LD antigens also play a role in the cytotoxic step of CMC. Con A has been shown to mediate nonspecific CMC by specifically immunized effector cells (10, 17). The mechanism of the LDCC reaction may be explained on the basis of contact between effector and target cells brought about by lectin agglutination. After the contact, Con A triggers the effector cell to induce cytotoxicity. It will be of interest of determine whether LD antigens can effectively mediate nonspecific cytotoxicity by similar mechanisms.

Our results indicate that there are functional similarities between Con A and LD antigens or functionally similar antigens in ability to stimulate CTL, and we propose that they may, in fact, be stimulating prekiller cells by the same mechanism.
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Summary

A secondary specific cytotoxic response is obtained when lymphocytes primed in vivo to a tumor allograft are exposed to Con A in culture. The secondary cytotoxic cells generated are specific to target cells bearing antigens of the primary sensitizing cells and are qualitatively indistinguishable from the response obtained upon secondary antigenic stimulation. The cell-mediated cytotoxicity is independent of concanavalin A (Con A) and is not affected by the Con A-specific inhibitor, α-methyl-d-mannose pyranoside. Furthermore, cultures containing a mixture of submitogenic concentrations of Con A and stimulating antigens showed synergy and augmentation of cytotoxic activity. It is suggested that activation of prekiller cells by Con A into CTL may be mediated via the same or similar receptors normally triggered by the stimulating antigens. Functional similarities between Con A and the lymphocyte-defined antigens of the major histocompatibility complex region are discussed.

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Note Added in Proof. Independent studies in agreement with our findings have been recently reported (Heininger, D., M. Touton, A. Chakrabarty, and W. J. Clark. J. Immunol. In press. Received for publication 20 September 1976.

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