LECTIN-DRIVEN MATURATION OF CYTOTOXIC EFFECTOR CELLS: THE NATURE OF EFFECTOR MEMORY*

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In previous studies we (1, 2) and others (3–6) have demonstrated the activation of cytotoxic function in mouse lymphocytes by lectins, particularly concanavalin A (Con A). Cytotoxicity that is expressed through antigen-specific membrane receptors can be elicited by Con A in unprimed cells (1, 3, 4). Con A can also be used to regenerate cytotoxic function in cells that had previously been primed with alloantigen either in vitro (2, 6) or in vivo (5). In these reactions, Con A treatment of the so-called “memory” cells resulted in the recall of cytotoxicity directed specifically toward the original stimulating cell. The reinitiation of cytotoxicity by Con A in cells previously primed with alloantigen in vitro had at least some of the putative characteristics of a secondary reaction (2).

In the present study, we asked whether Con A could drive an unprimed population of T lymphocytes down the effector cell pathway, beyond the expression of cytotoxic function, to the development of effector memory cells. In order to answer this question, it became necessary at the same time to identify and characterize a series of reaction parameters by which memory could be defined. We propose that these parameters can be generally applied to an analysis of the state of differentiation of cytotoxic effector cells, regardless of the means used for generating the original cytotoxic function.

Materials and Methods

Animals. Inbred C57BL/6 and DBA/2 mice were obtained from Microbiological Associates, Bethesda, Md. C3H/HeJ mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. All mice used in the experiments described here were female and 6–9 wk old.

Cell Cultures. Cell suspensions from regional lymph nodes or spleens were prepared as previously described (1, 7). Where appropriate, erythrocytes were removed by treatment with 0.85% ammonium chloride at room temperature for 10 min. The cell suspensions were washed in phosphate-buffered saline (PBS) and resuspended in Dulbecco’s modified Eagle’s medium supplemented with fetal calf serum (FCS), penicillin-streptomycin, β-mercaptoethanol, and amino acids as previously described (7) (hereinafter referred to simply as Dulbecco’s medium).

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Abbreviations used in this paper: BUdR, 5’-bromodeoxyuridine; Con A, concanavalin A; FCS, fetal calf serum; [3H]TdR, tritiated thymidine; HU, hydroxyurea; MLC, mixed lymphocyte culture; PBS, phosphate-buffered saline.

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Primary stimulation with Con A was carried out in 250-ml Falcon plastic culture flasks (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). Lymphocytes were suspended at 3 × 10^6/ml in a total of 50 ml Dulbecco's medium containing 5 µg/ml Con A. After 48 h of incubation at 37°C in 7.5% CO₂ in air, the lymphocytes were collected, washed twice with PBS, and assayed for cytotoxicity as described below.

For preparation of Con A-primed lymphocytes, the 48-h cells were resuspended in medium at 3 × 10^6/ml and reincubated at 37°C for an additional 10 days in the absence of Con A. The medium was changed once at the end of 7 days total culture time. To test the reactivity of these cells, they were harvested, washed, and resuspended in Dulbecco's medium plus either Con A or allogeneic lymphocytes. For Con A restimulation, the Con A-primed cells were resuspended at 2 × 10^6/ml in Dulbecco's medium containing 2 µg/ml Con A, in either 17 × 100 mm Falcon plastic tubes or in 50-ml Falcon plastic culture flasks. As discussed in connection with Table I, Con A restimulation of primed cells was more effective in tubes than in flasks. For restimulation of Con A-primed cells with syngeneic or allogeneic lymphocytes, the primed cells plus irradiated (1,000 R) stimulator lymphocytes were cultured together for 48 h at a ratio of 1:1 at a final total cell concentration of 2 × 10^6/ml.

**Blastogenesis and DNA Synthesis.** At appropriate time intervals after initiation of primary or secondary cultures, sample tubes were removed from the incubator and the cell contents examined for evidence of cell transformation and DNA synthesis. Cell transformation was analyzed directly by hemacytometer counting in the presence of trypan blue. Cells with diameters greater than approximately 7 µm were scored as medium sized, and cells with diameters greater than approximately 10-11 µm were scored as large. The proportion of transformed or "blast" cells is determined from the sum of viable medium plus large lymphocytes divided by the total viable lymphocytes counted.

DNA synthesis was measured by the rate of incorporation of [³H]thymidine into DNA. Cell cultures in which proliferation was to be measured were centrifuged, resuspended in 1.0 ml of culture medium, and triplicate 250-µl aliquots were removed to 12 × 75 mm plastic culture tubes. 2 µCi of tritiated thymidine ([³H]Tdr) in 25 µl was added to each tube for 1 h. The cultures were flushed and washed with cold PBS, and precipitated with cold 10% TCA in the presence of a small amount of protein carrier. The TCA precipitates were dissolved in NCS (Amerham/Searle Corp., Arlington Heights, Ill.) and counted in Omnifluor toluene (New England Nuclear, Boston, Mass.).

**Hydroxyurea (HU) and 5'-Bromodeoxyuridine (BUdR) Treatment.** The use of HU and BUdR in experiments involving generation of cytotoxic function in mouse lymphocytes has been described in detail elsewhere (7), and the procedures used herein have not been changed.

**Cytotoxicity Assay.** Cytotoxicity was determined using a ⁵¹Cr-release assay. Cells harvested from primary or secondary cultures were washed and resuspended at various concentrations in Dulbecco's medium. Target cells were prepared by incubating 10⁵ P815 mastocytoma cells (maintained by serial passage in DBA/2 mice) in 0.25 ml of Na₂Cr₂O₄ (0.5-1.0 mCi/ml) plus 0.05 ml FCS for 60-90 min. Assays were carried out in triplicate in 12 × 75 mm plastic tubes containing 1.0 ml of effector cell suspension and 0.25 ml of a suspension (10⁴/ml) of ⁵¹Cr-labeled target cells. The lengths of the assay periods and the ratio of effector:target cells used was variable and is defined under each table or figure.

**Results**

**Generation of Cytotoxic Function in Lymphocytes Previously Activated by Con A.** The cytotoxic response of lymphocytes that had been primed 12 days previously with 5 µg/ml Con A, to various alloantigenic cells and to 2 µg/ml Con A, is shown in Table I. (Cells primed 6-8 days previously showed no response to either stimulus.) In experiments 1-4, Con A-primed C57BL/6 cells responded within 24 h to both types of stimuli by developing into blast cells and becoming cytotoxic. [It should be emphasized that in all of these experiments we are examining cytotoxicity mediated via a cell surface antigen receptor, as opposed to lectin-dependent cell-mediated cytotoxicity. The definition and characteristics of this response have been described by us and by others (1, 3, 4).] The degree of blastogenesis was always greater in the case of Con A restimulation than for
Regeneration of Cytotoxic Function in Con A-Primed Lymphocytes by Alloantigen and by Con A

| Exp. | Reacting strain | Percent ^51Cr release from P815 when restimulated with: |
|------|-----------------|----------------------------------------------------------|
|      |                 | C3H | C57BL/6 | DBA/2 | Con A |
| 1    | C57BL/6         | 1.6(6.3) | 45.2 (34.0) | 41.5 (90.0) |
| 2    | C57BL/6         | -4.9(5.0) | 42.7 (40.3) | 53.8 (80.9) |
| 3    | C57BL/6         | 9.3(18.8) | 47.7 (28.6) | 4.8 (90.7) |
| 4    | C57BL/6         | -0.5(3.7) | 39.5 (48.1) | 17.1 (96.1) |
| 5    | C3H             | 3.7 (1.3) | 12.8(25.0) | 29.9 (31.1) | 32.5 (55.2) |
| 6    | C3H             | 1.8 (13.4) | 27.8(15.2) | 49.6 (25.1) | 1.9 (75.9) |

Lymph node plus spleen cells from the indicated reacting strain were incubated with 5 μg/ml Con A for 48 h, after which they were washed, resuspended in medium without Con A, and returned to the incubator for an additional 10 days (medium changed on days 5-6). On day 12 of total culture, the cells were harvested, washed, and restimulated for 24 h with either Con A (2 μg/ml), or an equal number of the indicated strain of irradiated mouse lymphocytes. After 24 h of secondary culture, the cells were harvested, washed, counted, and incubated with ^51Cr-labeled P815 (H-2^d) target cells for 12 h at a ratio of 100:1. The values indicated are the averages of triplicate samples, corrected for spontaneous release (values in parentheses are percent transformed cells in the cultures at the time of cytotoxicity assay). In assays of Con A-restimulated cells, α-methyl mannoside (0.1 M) was included in the assay tubes.

alloantigen stimulation; the degree of cytotoxicity developed toward the P815 (H-2^d) target cells was, however, about the same in both cases (except for experiments 3, 4, and 6 which were cultured in flasks instead of tubes; see below). It is particularly interesting to note the substantial levels of reactivity to allogeneic cells at 24 h; in normal, unprimed lymphocytes, cytotoxicity would be completely undetectable before about 48 h (7). In experiments 5 and 6, the abilities of the target-specific strain and a third-party strain to generate cytotoxicity toward the P815 target cells are compared. As in the case of alloantigen-primed memory cells (2), we observed substantial cross-reactivity between strains in the regeneration of cytotoxicity in Con A-primed lymphocytes.

The effect of the geometry of the culture vessel on the response of primed cells to Con A and alloantigen can be seen by comparing experiments 3, 4, and 6 with the other experiments. In experiments 3, 4, and 6, restimulation was carried out in plastic culture flasks (Falcon no. 3012) rather than 17 × 125 mm plastic tubes (Falcon 2054). While no difference in cytotoxicity or blastogenesis was detected in the two situations when alloantigen was used for restimulation, Con A restimulation of cytotoxicity in flasks was very poor compared with tubes. Con A-induced blastogenesis, on the other hand, was identical in flasks and tubes. Experiments in which restimulation with Con A in flasks vs. tubes was compared directly within the same experiment (not shown) confirmed that flask restimulation of cytotoxicity was only about 1/3 as efficient as tubes, whereas blastogenesis was unaffected.

Kinetics of the Response of Normal and Con A-Primed Lymphocytes to Con A. Our data on the time of exposure to Con A required for maximal generation of cytotoxicity in fresh normal and in Con A-primed cells is shown in Fig. 1. We define maximal cytotoxicity as that developed when Con A is left in the cultures until the time of assay (24 or 48 h). Unprimed cells developed minimal cytotoxic-
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FIG. 1. Time of exposure to Con A required for maximal development of cytotoxic function. Fresh, normal C57BL/6 lymph node cells, or Con A-primed C57BL/6 lymphocytes (prepared as described in the Materials and Methods section) were incubated with 5 μg/ml or 2 μg/ml of Con A, respectively. At various time points, the cells were collected, washed, and resuspended in medium containing 0.1 M α-methyl mannoside, and returned to the incubator. Unprimed cells (△—△) were harvested and assayed for cytotoxicity after 48 h total culture; primed cells (●—●) were harvested for assay after 24 h of total culture. The values shown are the averages of three experiments, and are plotted as the average percent of maximal activity developed in the cultures.

Fig. 1. Time of exposure to Con A required for maximal development of cytotoxic function. Fresh, normal C57BL/6 lymph node cells, or Con A-primed C57BL/6 lymphocytes (prepared as described in the Materials and Methods section) were incubated with 5 μg/ml or 2 μg/ml of Con A, respectively. At various time points, the cells were collected, washed, and resuspended in medium containing 0.1 M α-methyl mannoside, and returned to the incubator. Unprimed cells (△—△) were harvested and assayed for cytotoxicity after 48 h total culture; primed cells (●—●) were harvested for assay after 24 h of total culture. The values shown are the averages of three experiments, and are plotted as the average percent of maximal activity developed in the cultures.

ity if the lectin was removed before 6 h, but essentially maximal reactivity after 8 h of exposure. Con A-primed cells, on the other hand, developed a maximal response to Con A after only 3 h of exposure to Con A.

The time of onset of blastogenesis caused by Con A in normal and Con A-primed cells is shown in Fig. 2. In normal cells, the first discernable medium-sized lymphocytes (7–10 μm) appear at 15 h, and the overall proportion of medium and large cells rises to an average of about 50% at 24 h. At this latter time there are about equal numbers of medium and large cells in the cultures. Significant numbers of medium-sized lymphocytes are always discernable in Con A-treated cultures of Con A-primed cells starting between 6 and 9 h, and these cultures reach maximal transformation by 12–15 h, at which time almost all of the transformed cells are of the large size class (≥10 μm).

Normal and Con A-primed lymphocytes are not nearly so different in their response to Con A in terms of the time of onset of DNA synthesis (Fig. 3). The rate of DNA synthesis in cultures of normal lymph node cells plus Con A is first distinguishable from that in cultures without Con A beginning at 18 h, whereas DNA synthesis in cultures of Con A-primed cells plus Con A is first distinguishable from controls beginning at about 15 h. The rate of increase in DNA synthesis, and maximal DNA synthetic values per unit number of viable cells, are also not very different in the two types of cultures.

The Effect of Inhibitors of DNA Synthesis and Gene Expression on the Regeneration of Cytotoxicity in Con A-Primed Cells. A key question is whether the cells responding to Con A in the cultures of Con A-primed lympho-
cytes are responding in a qualitatively or simply a quantitatively different way from normal, unprimed lymphocytes. For example, the data on initiation of DNA synthesis (Fig. 3) suggest that the slightly enhanced values seen with primed cells could easily be accounted for on the basis of selective enrichment of Con A-sensitive cells. The data on the length of time required for exposure to Con A to develop a maximal cytotoxic response (Fig. 1) and on time of onset of blastogenesis (Fig. 2), on the other hand, suggest there may be qualitative differences in the two populations. This possibility is further strengthened by the observation that strong cytotoxicity can be generated in Con A-primed cells by alloantigen in 24 h; a primary cytotoxic response in vitro requires 44-48 h to develop (7). Nevertheless, it is not inconceivable that these differences could be accounted for by selective enrichment of potentially reactive cells, and could thus reflect quantitative rather than qualitative differences in the two cell populations. In a previous study of the differences in reactivity of alloantigen-primed and unprimed cells, to alloantigen in the primary and secondary mixed lymphocyte culture (MLC) reaction (7), we found that unprimed cells required both DNA synthesis and new gene expression in order to develop cytotoxic function, whereas alloantigen-primed lymphocytes require neither. These parameters thus provided evidence for a qualitative difference in the primary and secondary cell-mediated immune reactions to alloantigens. Wagner and Röllinghoff (8) and MacDonald et al. (9) also reported a requirement for DNA
FIG. 3. Onset of Con A-induced DNA synthesis in unprimed and in Con A-primed cells. Fresh, normal C57BL/6 lymph node cells, or Con A-primed C57BL/6 lymphocytes (prepared as described in the Materials and Methods section) were incubated in 12 x 75 mm tubes with 5 μg/ml or 2 μg/ml Con A, respectively. At 3-h intervals, sample tubes were removed and assayed for capacity to incorporate [3H]TdR as described in the Materials and Methods section. The points shown are the average of triplicate samples, standard deviation was in all cases less than 10%. (△—△), Unprimed cells without Con A; (○—○), unprimed cells plus 5 μg/ml Con A; (O—O), Con A-primed cells plus 2 μg/ml Con A.

synthesis in order to generate cytotoxicity in the primary, but not in the secondary, MLC. We have now examined the primary and secondary generation of cytotoxicity in lymphocytes using Con A, with respect to these two parameters, and the data are presented in Tables II and III.

In the experiments shown in Table II, HU was used to block DNA synthesis in unprimed and in Con A-primed cells during exposure to Con A. Because of possible nonspecific cytotoxic effects of HU on cells exposed to the drug for more than 24 h (10), in the case of unprimed cells HU was present only from just before the initiation of DNA synthesis (16 h) to the time of assay for cytotoxicity (44-48 h). We found that, just as for the primary and secondary MLC reactions, unprimed lymphocytes required DNA synthesis in order to develop cytotoxicity with Con A, whereas Con A-primed lymphocytes could develop full cytotoxicity in the absence of DNA synthesis. Also, as previously reported for the primary and secondary MLC (7), blocking DNA synthesis has essentially no effect on initial blastogenesis in primary or secondary lectin-generated reactions. The modest reductions in the proportion of blasts seen in the primary reaction to Con A in the presence of HU (Table II) is almost certainly due to the fact that the blast cells in the cultures without HU continue to divide and expand within the culture during the second 24-h period, whereas the blasts in the cultures with HU represent primary blasts that cannot divide and expand. When examined at
The Effect of HU on Con A-Driven Generation of Cytotoxic Functions in Unprimed and in Con A-Primed Lymphocytes

| Exp. | Unprimed cells | Con A-primed cells |
|------|----------------|--------------------|
|      | -HU (Percent reduction) | +HU (Percent reduction) | -HU (Percent reduction) | +HU (Percent reduction) |
| 1    | 21.0 (93.0) | 117.1 | 10.6 (97.4) | 10.7 (99.0) |
| 2    | 13.0 | 3.5  | 73.1 | 33.1 (92.5) | 34.1 (94.4) |
| 3    | 31.7 (87.3) | 17.7 (72.2)* | 44.2 | 7.8 (94.8) | 7.2 (93.3) |
| 4    | 11.7 (93.1) | 3.4 (87.3) | 71.0 | — | — |
| 5    | 27.6 (78.0) | -4.6 (56.6) | 116.6 | 23.9 (93.9) | 22.7 (96.4) |

Average: 84.4

Lymph node plus spleen cells from C57BL/6 mice were used either directly (unprimed cells) or after 12 days of culture involving exposure to Con A as described in Table I (Con A-primed cells). Unprimed cells were incubated with 5 µg/ml Con A. After 14-16 h of culture, HU (0.5 mM) was added to half the culture tubes. All primary cultures were harvested at 44-48 h, washed, counted, and incubated with 51Cr-labeled P815 target cells in 0.1 M α-methyl mannoside for 14 h. Values shown are percent 51Cr released, and are averages of triplicate samples (values in parentheses are percent transformed cells at time of assay). Con A-primed cells were washed, counted, and incubated with 2 µg/ml Con A for 24 h, in the presence or absence of 0.5 mM HU. At the end of this treatment, the cells were washed and incubated with 51Cr-labeled P815 target cells in 0.1 M α-methyl mannoside for 6 h.

Effect of BUdR on Con A-Driven Generation of Cytotoxic Function in Unprimed and in Con A-Primed Lymphocytes

| Exp. | Unprimed lymphocytes | Con A-primed lymphocytes |
|------|----------------------|--------------------------|
|      | -BUdR (Percent reduction) | 10 µg/ml BUdR | 50 µg/ml BUdR | Percent reduction | -BUdR (Percent reduction) | 10 µg/ml BUdR | 50 µg/ml BUdR | Percent reduction |
| 1    | 33.4 | 19.5 | 41.8 | 14.7 | 56.2 | 32.7 | 26.2 | 19.9 | 29.3 | 10.4 |
| 2    | 57.5 | 29.9 | 48.0 | 21.6 | 62.4 | 15.4 | 11.9 | 22.6 | 15.6 | -1.3 |
| 3    | 22.5 | 17.5 | 22.7 | 13.5 | 41.2 | 28.2 | 26.5 | 6.1 | 36.4 | 6.4 |

Average: 37.5

Unprimed and Con A-primed C57BL/6 cells were prepared as outlined in Tables I and II. Unprimed cells were incubated with 5 µg/ml Con A, either in the absence of, or in the presence of 10 µg/ml or 50 µg/ml of BUdR. The cultures were protected from light during the entire procedure. After 44-48 h, the cells were harvested, washed, and incubated with 51Cr-labeled P815 target cells for 14-16 h. Con A-primed cells were handled similarly, except that they were incubated with 2 µg/ml Con A, were harvested after 24 h, and were assayed for 6-8 h. Values shown are percent 51Cr released, average of triplicate samples, corrected for background release.

24 or 30 h (data not shown) cultures of normal cells plus Con A display the same degree of blastogenesis whether or not HU is present.

The drug BUdR has been shown, at appropriate doses, to inhibit the expression of new genes in differentiating eukaryotic systems, without affecting either DNA synthesis or the expression of those genes already functioning within the cell (11). We previously used this drug to demonstrate that the primary MLC reaction requires new gene expression, while the secondary reaction does not (7). The effect of BUdR on the lectin-driven generation of cytotoxicity in unprimed and in Con A-primed lymphocytes is shown in Table III. As can be seen from the data, BUdR had an inhibitory effect on the lectin-driven generation of cytotoxicity in unprimed cells, but only a slight effect in Con A-primed cells. The
significance of the incomplete reduction of cytotoxicity in unprimed cells at 48 h of culture will be discussed below.

**Discussion**

We (1) and others (3, 4) have shown previously that Con A can drive mouse T cells to the expression of antigen receptor-mediated cytotoxic function which is indistinguishable from that developed by reaction to alloantigen, except of course that the former is polyclonal while the latter is clonally restricted. We (2) and others (5, 6) have also used Con A to elicit the re-expression of cytotoxic function in mouse effector memory cells generated by reaction to alloantigen. In the present investigation this line of thought was pursued further to determine whether or not Con A could also be used to induce a state of “memory” in lymphocytes not previously exposed to alloantigen. In the course of carrying out these studies, we found it necessary to develop a clear set of parameters by which memory could be defined, since the precise nature of memory in cytotoxic cells is not at present well established. The simple fact that development of proliferation and cytotoxicity in primed cells seems to be “quicker and stronger” than in unprimed cells is certainly less than satisfactory because it says nothing at all about the actual state of differentiation of the two cell populations. We have thus tried to determine whether the apparent differences in reactivity between primed and unprimed cells are due to qualitative or quantitative differences in the two populations, or a combination of both. The information presented here using Con A integrates well with information we and others have previously derived regarding primary and secondary reactions generated with specific alloantigen.

In this study we have examined the following criteria in comparing the generation of cytotoxic function in unprimed and in Con A-primed mouse lymphocytes: length of time required for exposure to Con A in order to develop a maximal cytotoxic response; time of onset and rate of DNA synthesis; time of onset and degree of blastogenesis; sensitivity to an inhibitor of DNA synthesis; and sensitivity to an inhibitor of new gene expression.

The length of time required for exposure to Con A in order to develop a maximal cytotoxic response in unprimed mouse lymph node cells is between 6 and 8 h, whereas the length of time of exposure to Con A required by primed cells to develop a maximal response is 3–4 h. In our opinion, it would be difficult to account for this difference on a basis of enrichment of Con A-sensitive cells alone. In our hands, the fraction of unprimed mouse lymph node cells responding to Con A in a primary reaction is about 35–40% (unpublished observations). Thus even if 100% of the primed cells react to Con A in the secondary reaction, increased reactivity due to enrichment of responding cells would not exceed a factor of two or three, and this in itself would be insufficient to account for the differences seen in Fig. 1. It could be argued that primed cells are simply “parked” synchronously in a different stage of the cell cycle from unprimed cells, and thus as a population need a less prolonged stimulus to be activated. This would be compatible with our observations on the speed and apparent synchrony of blastogenesis (Fig. 2). However, in view of our observations on the length of time required for initiation of DNA synthesis in the two populations (see below) this also seems an unlikely explanation. We thus conclude that the length of
time of exposure to Con A required for commitment to a maximal cytotoxic response indicates a genuine qualitative difference in the state of the two cell populations.

We conclude from the information presented in Fig. 3 that the time of onset and kinetics of DNA synthesis is not a valid criterion for distinguishing primed from unprimed cells. While superficially DNA synthesis in primed cells does appear to be "quicker and stronger" than in unprimed cells, a more careful analysis suggests that the difference between the two populations is in fact rather minor, both in terms of time required to initiate DNA synthesis after receiving a stimulus, and in the rate and magnitude of synthesis. We believe the difference observed could easily be accounted for by enrichment as described above, and thus probably represents a quantitative rather than a qualitative difference in the two cell populations.

The differences between primed and unprimed cells in terms of the blastogenic response to Con A would seem to reflect the opposite case. In unprimed cells, there are no medium- or large-sized lymphocytes observable in Con A-treated vs. nontreated cultures before 15 h, after which a slow but steady increase is observed up to 24 h. At 18 h, almost 100% of the nonsmall lymphocytes scored are medium in size, and at 24 h only about half of the lymphocytes scored as blasts are large (≥10 μm). In primed cells, on the other hand, some medium-sized lymphocytes are already visible by 6 h. The most dramatic increase is between 9 and 12 h (Fig. 2), and by 15 h virtually all of the lymphocytes scored as transformed are of the large class size. Primed cells thus differ from unprimed cells both in time of onset of blastogenesis and in the average time required to go from small to large lymphocytes. It is difficult to imagine an explanation for these differences based on quantitative considerations. The very rapid transition from small through medium to large-sized lymphocytes suggests the possibility that the primed cells are more closely synchronized than are unprimed cells. However, the time elapsed before the first appearance of the first medium-sized lymphocytes is substantially different in primed vs. unprimed cells. In addition, the length of time required to begin DNA synthesis is, as pointed out above, almost the same in both populations.

We thus suggest that the differences in onset and rate of blastogenesis reflect a qualitative difference in the differentiative state of the two cell types, which cannot be accounted for by quantitative differences or by cell synchrony.

The rationale for, and the interpretation of, the experiments involving inhibitors of DNA synthesis and gene expression was presented in detail in the Results section, and has been discussed elsewhere (2, 7). By both of these criteria, Con A-primed cells are clearly qualitatively different, in their response to Con A, from unprimed cells. The failure to inhibit the generation of cytotoxicity completely with BUDR does, however, warrant comment. In previous experiments using BUDR to inhibit generation of cytotoxicity in primary MLC, cytotoxic function in treated and control cultures was measured on days 3 or 4, and was found to be essentially completely suppressed at a dose of 50 μg/ml (7). In the present experiments, cytotoxic function in treated and control cultures was assayed at 48 h, and was reduced only about 50%. We have also noted (unpublished observations) that cytotoxicity generated at 48 h in a primary MLC is only partly inhibited by BUDR. Although we have no direct evidence, we interpret
this as being due to the fact that at 48 h, only one or two cell generations have
probably been completed since the onset of DNA synthesis, and thus BUdR is
only incorporated into some fraction of the reacting cell population. Presumably,
if we allowed the Con A-generated primary cytotoxic response to mature fur-
ther, we would see greater suppression of cytotoxicity in BUdR-treated cultures
as compared with controls, since a higher proportion of the cells would have
incorporated BUdR. Unfortunately, in most cases the cytotoxic response begins
to degenerate in our primary Con A cultures by 72 h, and so the effect of BUdR
at times beyond 48 h becomes difficult to assess.

A final difference between unprimed and Con A-primed cells that we would
note is the response to alloantigen. As shown in Table I, Con A-primed cells
develop a strong cytotoxic response to alloantigen which they have not previ-
ously seen within 24 h. In a previous publication (7), we showed that cytotoxicity
in unprimed cells in response to alloantigen is undetectable before 44–48 h,
using assay conditions considerably more sensitive than those used here. Since
as far as we can determine the primary response to Con A in mouse lymphocytes
is polyclonal, then a quantitative enrichment of a particular alloantigen-sensi-
tive clone seems most unlikely. Therefore, we feel that the enhanced rate of
development of cytotoxic function in Con A-primed cells in response to alloanti-
gen represents yet another qualitative difference in the two cell populations.

At present we do not have any insight into how Con A is acting at the cell
membrane to activate cytotoxic function. The T-cell receptor for alloantigen may
well have carbohydrate moieties for which Con A is specific, and the entire
chain of events relating to generation and expression of cytotoxic function may
be the result of activation of the cell via nonspecific interaction with the antigen
receptor. Alternatively, a mitogenic signal could be delivered through some
other cell surface structure, which leads ultimately to expression of cytotoxic
function. Whatever the mechanism by which Con A triggers cytotoxic immune
function in lymphocytes, in all of our studies to date we have been unable to
detect any difference in the process of either generation or regeneration of
cytotoxicity in lymphocytes, whether triggered polyclonally by lectin or in a
restricted fashion by alloantigen. By the criteria defined in the present study we
conclude that the end result of Con A stimulation of unprimed mouse lympho-
cytes, under appropriate conditions, is the development of a bona fide memory
state. The use of Con A activation of mouse lymphocytes as a model for the study
of development of cytotoxic responses, therefore, seems justified, and in view of
the greater proportion of cells responding, may have several advantages over
antigen activation.

Summary

In an attempt to define further the activation of effector T-cell function with
concanavalin A (Con A), we examined the ability of Con A to generate effector
memory in mouse lymph node cells in vitro. In the course of these studies, it
became necessary to define parameters by which memory could be defined.
These parameters include length of time of exposure to signal required to
generate full cytotoxic function; time of onset, and kinetics, of blast cell forma-
tion; requirement for DNA synthesis; sensitivity to the drug 5'-bromodeoxyuri-
dine; and kinetics of the cytotoxic response to alloantigen. By these criteria,
mouse lymph node cells exposed 12 days previously to Con A behave qualitatively differently from unprimed mouse lymphocytes. We found that the time of onset and kinetics of DNA synthesis could not be used to distinguish primary and secondary cytotoxic responses. We propose that the parameters defined in these studies can be applied generally in determining whether a given cytotoxic response involves primed (memory) or unprimed cells.

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