

CELL-MEDIATED IMMUNE RESPONSES IN VITRO

I. SUPPRESSION OF THE GENERATION OF CYTOTOXIC LYMPHOCYTES BY CONCANAVALIN A AND CONCANAVALIN A-ACTIVATED SPLEEN CELLS*

BY DUANE L. PEAVY† AND CARL W. PIERCE§

*(From the Department of Pathology, Harvard Medical School,
Boston, Massachusetts 02115)*

(Received for publication 18 March 1974)

The recognition of two distinct pathways for differentiation of antigen-reactive T and B cells and the separate role that each of these cell types plays in cellular and humoral immune responses has greatly enhanced our understanding of the immune system. It is now well established that T cells have a critical role in the regulation of B-cell responses to antigen and may either enhance or suppress antibody responses depending on the experimental circumstances (1). In contrast, the required interactions among different cell types and the regulatory mechanisms involved in expression of cell-mediated immune responses have been less clearly defined. In one category of cellular immune responses, when the sensitizing antigen is a structural component of cell membranes, some T cells develop into cytotoxic lymphocytes (CL)¹ capable of destroying cells bearing that antigen. Thus, a manifestation of cell-mediated immune responses such as allograft responses (2), graft-vs.-host (GVH) reactions (3), and immune rejection of tumor cells is the destruction of target cells bearing antigens to which T lymphocytes have been sensitized (4). Data relating to cell interactions and regulatory mechanisms in these responses have been accumulating recently. Evidence for a lack of involvement of B lymphocytes in the generation of cytotoxic T lymphocytes has been obtained (5, 6). Most conclusive was the demonstration by Wagner et al. (7), that nearly pure populations of T cells, such as thymus cells or cortisone-resistant thymocytes, are able to generate strong CL responses in vitro. These studies are in complete agreement with the in vivo findings of Cerottini et al. (8) and

* This investigation was supported by U. S. Public Health Service Research Grant CA-14723 from the National Cancer Institute.

† Postdoctoral trainee of the National Institutes of Health, Training Grant AI-00426 from the National Institute of Allergy and Infectious Diseases.

§ Recipient of U. S. Public Health Service Research Career Development Award 5K4-AI-70173 from the National Institute of Allergy and Infectious Diseases.

¹ *Abbreviations used in this paper:* Con A, concanavalin A; CL, cytotoxic lymphocyte(s); GVH, graft-vs.-host reaction; HBSS, Hanks' balanced salt solution lacking sodium bicarbonate; MLC, mixed leukocyte cultures; PFC, plaque-forming cell(s).

demonstrate that the generation of CL occurs independently and without critical influence of B cells. Furthermore, a concept that functional heterogeneity exists and collaboration occurs among T-cell populations has been developing. Asofsky et al. (3, 9) have shown that in GVH responses cooperative cell interactions take place in which amplifier T cells augment the activity of the precursors of CL which inflict immunologic injury and target cell damage.

Rich and Pierce (10) reported that concanavalin A (Con A)-activated lymphoid cells suppress in vitro primary and secondary plaque-forming cell (PFC) responses by mouse spleen cells to heterologous erythrocytes. This lymphocyte population was thymus derived, resistant to irradiation, and characteristically similar to antigen-activated helper T cells. The present experiments were undertaken to determine if mitogen-activated T cells can also exert regulatory influences on the generation of cytotoxic T lymphocytes in vitro. The data clearly indicate that Con A-activated lymphocytes can suppress the development of CL generated in one-way mixed leukocyte cultures (MLC).

Materials and Methods

Mice.—Female C57BL/6 ($H-2^b$), Balb/c ($H-2^d$), and DBA/2 ($H-2^d$) mice, 2- to 4-mo of age (Jackson Laboratories, Bar Harbor, Maine), were maintained on acidified-chlorinated water and laboratory chow ad libitum.

Con A.—Twice recrystallized Con A (Nutritional Biochemicals Corporation, Cleveland, Ohio) was prepared for addition to cultures as described previously (11).

Cell Culture System.—Preparation of spleen cell suspensions and techniques of cell culture have been previously described in detail (11). One-way MLC were employed to generate CL activated toward $H-2^b$ or $H-2^d$ alloantigens (12). Cultures containing 25×10^6 responding spleen cells and an equal number of mitomycin C-treated allogeneic spleen cells were incubated in 5 ml of a completely supplemented Eagle's minimal essential medium containing 10% fetal bovine serum (lot E21806, Reheis Chemical Company, Kankakee, Ill.), in 60-mm plastic Petri dishes (no. 3002, Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) under modified Mishell-Dutton conditions (11) for the desired period of time.

Mitomycin C Treatment.—Spleen cells (100×10^6 cells/ml) were treated with mitomycin C (Sigma Chemical Co., St. Louis, Mo.) at a final concentration of $50 \mu\text{g/ml}$ for 30 min at 37°C . Cells were washed twice in Hanks' balanced salt solution lacking sodium bicarbonate (HBSS) and resuspended in culture medium at the desired density for addition to the MLC.

Tumor Cell Lines.—Tumors were maintained in ascitic form by serial transfer in syngeneic mice. P815 mastocytoma ($H-2^d$) and EL4 leukemia ($H-2^b$) cells were generously provided by Dr. Eric Martz of our department.

After harvest from the peritoneal cavity, $10\text{--}15 \times 10^6$ cells in 1-ml HBSS were mixed with 0.15 ml ^{51}Cr as Na_2CrO_4 (1 mCi/ml, $\approx 5 \mu\text{g Cr/ml}$, Radiochemical Centre, Amersham, England) in isotonic saline. After 30-min incubation at 37°C , target tumor cells were washed three times in HBSS and resuspended in culture medium at 5×10^5 cells/ml for use in the ^{51}Cr -release assay.

^{51}Cr -Release Assay.—Cells from triplicate MLC were harvested, pooled, and washed twice in HBSS. Cell number and viability by trypan blue exclusion were determined using a Cytograf 6300 A (Bio/Physics Systems Inc., Mahopac, N. Y.). Cytotoxic lymphocyte activity

generated in vitro was quantitatively titrated by the ^{51}Cr -release method of Cerottini et al. (8), modified as follows: Lymphocytes were adjusted to 10×10^6 viable cells/ml and serial twofold dilutions were prepared in culture medium. 0.1 ml of the respective cell dilutions and 0.1 ml of the ^{51}Cr -labeled target cells (5×10^5 cells/ml) were mixed in 10×75 mm glass test tubes in triplicate. The resulting viable spleen cell to tumor cell ratios were 20, 10, 5, 2.5, and 1.25. 0.5 ml of medium was added and the reaction mixtures were incubated without rocking at 37°C in a humidified atmosphere of 5% carbon dioxide and 95% air for 6 h. After centrifugation, radioactivity in the supernatant fluid was measured in a Packard autogamma scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.). Results are expressed as the percent of ^{51}Cr released as determined by the following formula:

$$\% \text{ } ^{51}\text{Cr} \text{ release} = \frac{100 \times ([^{51}\text{Cr} \text{ released by cytotoxic lymphocytes}] - [\text{spontaneous } ^{51}\text{Cr} \text{ released}])}{([^{51}\text{Cr} \text{ released by freeze-thawed target cells}] - [\text{spontaneous } ^{51}\text{Cr} \text{ released}])}$$

The standard error of the mean of released radioactivity in triplicate samples rarely exceeded 5%. Spontaneous release from P815 and EL4 tumor cells after 6 h was approximately 10–15% of that released in the freeze-thawed control. The maximum amount of releasable ^{51}Cr , determined by freezing and thawing target cells three times, was 90–95% of the total radioactivity incorporated into the cells. Chromium release was immunospecific and occurred only when in vitro sensitization was directed against antigens present on the respective target cells. Spleen cells cultured without alloantigens failed to cause a release of label greater than the spontaneous ^{51}Cr release when assayed with either syngeneic or allogeneic target cells.

Activation of Spleen Cells with Con A.— 50×10^6 spleen cells in 5-ml culture medium were incubated with or without $2 \mu\text{g/ml}$ Con A for 48 h. Cells from Con A-activated or control cultures were harvested, washed twice in HBSS, and resuspended in fresh culture medium at the desired cell density. Graded numbers of cells were added to the MLC in a vol of 0.1 ml.

Treatment of Spleen Cells with Anti- θ Serum and Complement.—Normal or Con A-activated spleen cells were treated with AKR anti- θ C3H serum and guinea pig serum as a complement source to specifically kill T cells; as a control, spleen cells were treated with brain-absorbed anti- θ serum as described in detail previously (13). After treatment, graded numbers of cells were added to the MLC in 0.1-ml vol.

Cell Irradiation.—Normal or Con A-activated spleen cells received 2,000 R of X irradiation from a General Electric Maximar 250 Type III X-ray therapy unit (General Electric, Medical Systems Div., General Electric Co., Milwaukee, Wis.) (10). After irradiation, cells were washed once with HBSS, resuspended in fresh culture medium at the desired cell density, and added to the MLC in 0.1-ml vol.

RESULTS

Effects of Soluble Con A on the Generation of Cytotoxic Lymphocytes In Vitro.—The effects of Con A on the generation of CL was examined by adding varying concentrations of soluble Con A to the MLC at initiation. Spleen cells from C57BL/6 mice were incubated 5 days with mitomycin C-treated DBA/2 spleen cells, and the CL response was measured by lysis of ^{51}Cr -labeled P815 mastocytoma cells. At submitogenic concentrations (0.05–0.1 $\mu\text{g/ml}$) of Con A, neither enhancement nor suppression of CL responses was observed (Fig. 1). Cells from these cultures had approximately the same cytotoxic activity as cells from control cultures; 50% lysis was observed at lymphocyte/target cell ratios of 5:1–7:1. In contrast, marked suppression of cytotoxic activity was observed with mitogenic concentrations (1–5 $\mu\text{g/ml}$) of Con A. With submito-

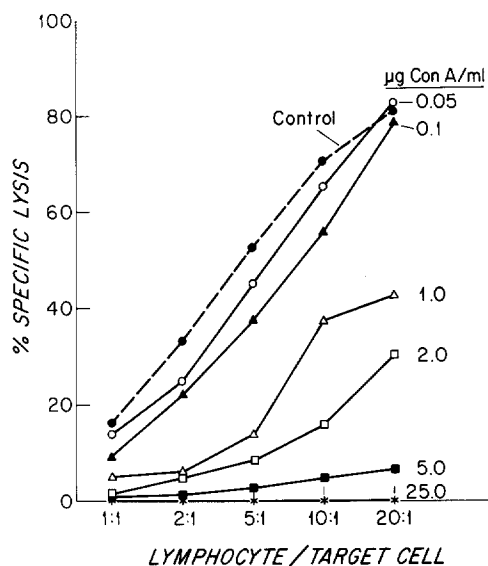


FIG. 1. Effects of soluble Con A on the generation of cytotoxic lymphocytes in vitro. MLC of C57BL/6 and mitomycin C-treated DBA/2 spleen cells were incubated with the indicated concentrations of Con A. Percent specific lysis of ^{51}Cr -labeled P815 mastocytoma target cells was determined on day 5.

genic concentrations of Con A, total cell recovery and viability were comparable in control and Con A-treated cultures. Mitogenic concentrations of Con A, however, substantially increased the numbers of total and viable cells per culture. Higher Con A concentrations (25 $\mu\text{g}/\text{ml}$), which led to complete suppression of CL responses, decreased viable cell recovery to such an extent that firm interpretation of the data was prevented.

The suppressive effects of Con A were not unique to spleen cells from C57BL/6 mice which are relatively poor responders to Con A as determined by [^3H]thymidine incorporation (14). In experiments employing the same strain combinations in reversed roles, DBA/2 spleen cells were incubated with mitomycin C-treated C57BL/6 spleen cells and assayed on day 5 with EL4 leukemia target cells. Analogous results were obtained over the range of concentrations of Con A tested.

Effects of Con A Added after Culture Initiation on the Generation of Cytotoxic Lymphocytes.—Enhancement or suppression of PFC responses mediated by Con A is dependent on the time Con A was added to the culture (11). Although profoundly inhibitory for PFC responses when added at culture initiation, mitogenic concentrations of Con A enhanced PFC responses fivefold when added to cultures at 48 h. We have performed analogous experiments. 2 μg Con A/ml were added at intervals after initiation of the MLC and the CL

response was assayed on day 5. Soluble Con A was clearly inhibitory when added at culture initiation (Fig. 2). Whereas significant suppression of CL responses was still obtained by adding Con A 24 h after culture initiation, the ability of Con A to inhibit 5-day responses was lost after 48 h. In contrast to the enhancement of PFC responses by mitogenic concentrations of Con A added at 48 h, no increase in the CL response was observed under these conditions.

Effects of Con A-activated Spleen Cells on the Generation of Cytotoxic Lymphocytes In Vitro.—The suppression of CL responses in MLC by mitogenic concentrations of soluble Con A could be induced either by: (a) the activation of a suppressor cell population; or (b) the diversion of alloantigen-sensitive precursor cells into blastogenesis and mitoses which does not result in the generation of CL. Experiments were designed to investigate whether Con A activates a population of spleen cells which could suppress generation of CL responses in vitro. C57BL/6 spleen cells were incubated with or without 2 μg Con A/ml for 48 h and graded numbers of viable control or Con A-activated cells were added at initiation to MLC of C57BL/6 and mitomycin C-treated DBA spleen cells. CL responses were measured 5 days later. When as few as 10^6 Con A-activated spleen cells were added to the MLC containing 25×10^6 responding C57BL/6 spleen cells, CL responses were significantly diminished (Fig. 3). Moreover, the addition of increasing numbers of Con A-activated cells (up to 2.5×10^6 cells) resulted in increased inhibition of the CL responses. When

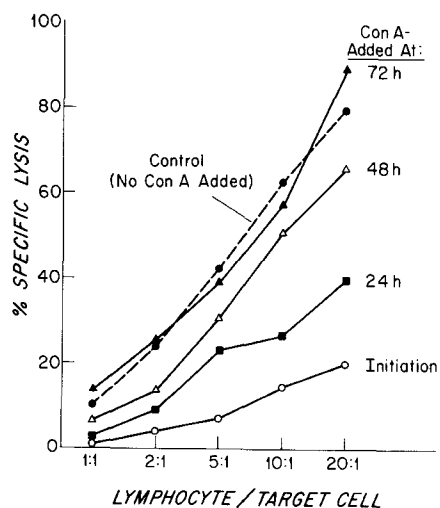


FIG. 2. Effects of adding soluble Con A at intervals after culture initiation on cytotoxic responses in vitro. 2 μg Con A/ml were added to the MLC of C57BL/6 and mitomycin C-treated DBA/2 spleen cells at initiation or 24, 48, and 72 h thereafter. ^{51}Cr -release assays were performed on day 5 using P815 mastocytoma as target cells.

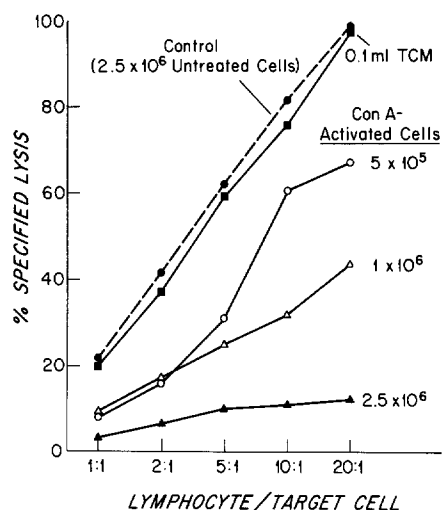


FIG. 3. Suppression of cytotoxic responses in MLC by Con A-activated spleen cells. Graded numbers of viable control or Con A-activated cells or an equal volume of culture medium were added to MLC of C57BL/6 and mitomycin C-treated DBA/2 spleen cells at initiation. Cytotoxic responses were assayed 5 days later on ⁵¹Cr-labeled P815 mastocytoma target cells.

normal control cells were added, however, the cytotoxic activity generated in the MLC was unaffected. Percent viability and total cell recovery in all cultures were comparable. Analogous results were obtained in MLC employing Balb/c suppressor and responding cells and mitomycin C-treated C57BL/6 spleen cells where cytotoxic activity was assayed on EL4 target cells.

The suppressive effect of Con A-activated spleen cells on CL responses assayed after 5 days incubation was most pronounced when suppressor cells were added at culture initiation. Addition after 24 h resulted in approximately 50% less inhibition, while no reproducibly significant suppression was observed when suppressor cells were added at 48 h or later. No enhancement of CL responses was noted under any of the conditions employed. However, since all cultures were assayed after 5 days incubation, suppressor cells added at 48 h or later were able to influence the MLC for only up to 72 h, which may have been too brief a period to achieve suppression by 5 days. These experiments were expanded and CL responses were measured on days 5 and 7, times when suppressor cells had been present for at least 5 days. The addition of 2.5×10^6 Con A-activated C57BL/6 spleen cells to MLC of C57BL/6 and mitomycin C-treated Balb/c spleen cells at initiation inhibited both 5 and 7 day CL responses (Figures 4 a and 4 b). However, when the suppressor cells were added 48 h after initiation of the MLC, neither 5 nor 7 day CL responses were suppressed. The addition of 2.5×10^6 control spleen cells to the MLC at initia-

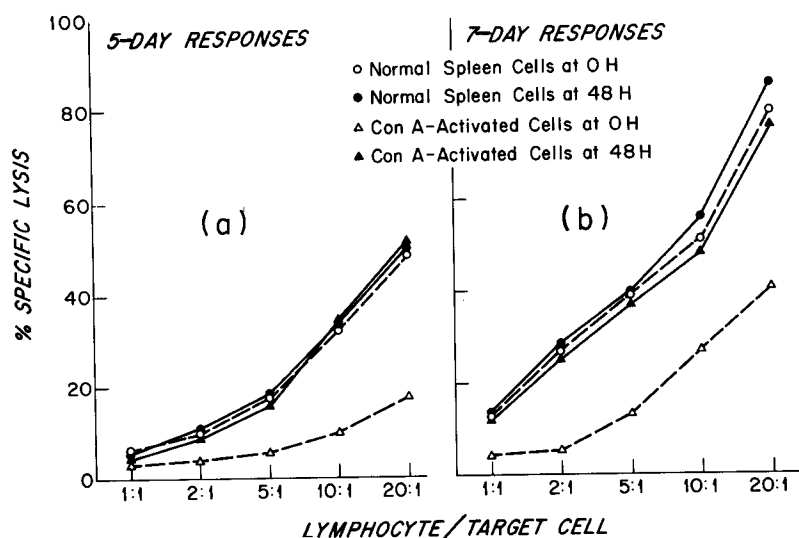


FIG. 4. Effects of Con A-activated spleen cells on cytotoxic lymphocyte responses assayed 5 and 7 days after culture initiation. 2.5×10^6 viable control or Con A-activated C57BL/6 spleen cells were added to MLC of C57BL/6 and mitomycin C-treated Balb/c spleen cells at initiation or 48 h later. Responses were measured 5 days (Fig. 4 a) and 7 days (Fig. 4 b) after culture initiation on ^{51}Cr -labeled P815 mastocytoma target cells.

tion or 48 h thereafter had no effect on 5 or 7 day CL responses. Thus, the failure of suppressor cells to inhibit CL responses when added to the MLC at 48 h was not due to insufficient time for these cells to mediate their effects. Instead, suppression of CL responses requires that suppressor cells be added during the critical early induction phase of the CL response.

Kinetics of CL Responses in MLC Containing Con A-Activated Suppressor Cells.—The suppression of cytotoxic responses in MLC by Con A-activated suppressor cells could be due to: (a) a failure to initiate development of CL; (b) partial diminution of CL development due to limited clonal expansion or terminal differentiation of activated precursors of CL; (c) the abrogation of a CL response which initially developed normally; or (d) a delay in development of CL which would eventually reach normal levels. Kinetic studies were undertaken to determine which of these alternatives were valid. Control or Con A-activated Balb/c spleen cells were added at initiation to MLC of Balb/c and mitomycin C-treated C57BL/6 spleen cells; cytotoxic responses against EL4 target cells were measured on days 3, 4, 5, and 6. By day 3 weak but detectable CL responses had developed; no measurable differences were found between control cultures and cultures to which Con A-activated suppressor cells had been added (Fig. 5 a). On days 4, 5, and 6, CL responses in control MLC increased progressively (Fig. 5 b-d). However, during this time, a definite and continued inhibition of CL responses occurred in cultures containing

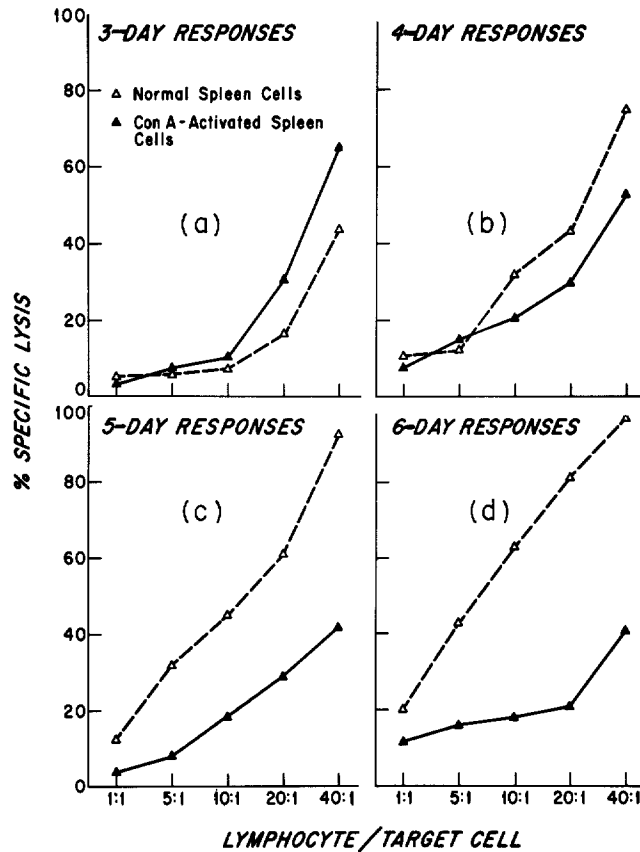


FIG. 5. Kinetics of cytotoxic lymphocyte responses in MLC containing Con A-activated spleen cells. 2.5×10^6 viable control or Con A-activated Balb/c spleen cells were added to MLC of Balb/c and mitomycin C-treated C57BL/6 spleen cells at initiation and cytotoxic responses were measured 3 (Fig. 5 a), 4 (Fig. 5 b), 5 (Fig. 5 c), and 6 (Fig. 5 d) days later on ^{51}Cr -labeled EL4 leukemia target cells.

suppressor cells. Viable cell recoveries from control and experimental cultures declined at comparable rates. These data indicate that the suppression is the result of an abrogative effect of suppressor cells on CL responses which initially develop normally during the first 3-4 days of culture, but subsequently abruptly abort and decrease precipitously from day 4 to 6.

Characterization of Con A-Activated Suppressor Cells.—The effect of X irradiation and treatment with anti- θ serum and complement to kill T cells on the generation and functional activity of Con-A activated suppressor cells was investigated. Portions of Balb/c spleen cells were treated before or after activation with $2 \mu\text{g}$ Con A/ml for 48 h as follows: (a) anti- θ serum and comple-

ment; (b) brain-absorbed anti- θ serum and complement; (c) 2,000 R X irradiation; and (d) untreated. Con A-activated cells (2.5×10^6) recovered after these treatments were added to the MLC of Balb/c and mitomycin C-treated C57BL/6 spleen cells at initiation. Cytotoxic activity against EL4 target cells was measured 5 days later. Untreated Con A-activated spleen cells markedly suppressed CL responses (Table I). Treatment of spleen cells with anti- θ serum and complement to kill T cells before or after activation with Con A abolished the suppressive capacity of the cells. The control of spleen cells treated with brain-absorbed anti- θ serum and complement showed that these manipulations did not diminish the suppressive capacity of Con A-activated suppressor cells. These results clearly demonstrate that suppressor cells are T cells. In addition, suppressor T-cell activity as abolished by X irradiation of spleen cells before activation with Con A. However, when spleen cells were X irradiated after Con A activation, suppression of CL responses was comparable to that achieved with unirradiated Con A-activated cells.

DISCUSSION

The results of these experiments clearly indicate that Con A can substantially inhibit the generation of cytotoxic lymphocytes in vitro. Suppression of CL responses was achieved by the addition of mitogenic concentrations of soluble Con A or spleen cells activated by mitogenic concentrations of Con A. The suppression was critically dependent upon the concentration and time of addition of Con A or Con A-activated spleen cells. Suppression of CL responses under these experimental conditions was equally apparent using spleen cells from C57BL/6, DBA/2, and Balb/c mice as the responder cells in the MLC or as Con A-activated cells. In part, these results are similar to those of Perl-

TABLE I
*Effect of X Irradiation and Anti- θ Serum and Complement
on Con A-Activated Suppressor Cell Activity*

Treatment	Time of treatment relative to Con A- activation	% Specific lysis 20:1 Lymphocyte/Target Cell*
Normal spleen cell control	—	85
Con A-activated spleen cell control	—	37
Anti- θ and C	Before	82
	After	80
Brain-absorbed anti- θ and C	Before	45
	After	38
X irradiation, 2,000 R	Before	80
	After	37

* 2.5×10^6 control or Con A-activated Balb/c spleen cells were added to MLC of 25×10^6 Balb/c and 25×10^6 mitomycin C-treated C57BL/6 spleen cells at initiation. ^{51}Cr -release assays were performed on day 5 using EL4 leukemia as target cells.

mann et al. (15), who observed that treatment of phytohemagglutinin-stimulated human lymphocyte cultures with Con A stimulated incorporation of tritiated thymidine, increased viable cell recovery, but abrogated cytotoxic responses to chicken erythrocytes.

Under appropriate conditions, lymphocytes previously incubated with Con A nonspecifically lyse target cells (16, 17). Since cytotoxicity is a function of viable cells (8), it is possible to argue that the inhibitory effects described here may be mediated by direct cytotoxicity of Con A-activated cells upon the responding effector cell population(s). Suppression of CL responses was not due to direct cytotoxicity or the elaboration of toxic substances for several reasons: (a) in a parallel study, Con A, itself, in the concentrations used in these experiments failed to confer cytotoxic activity to spleen cells for either syngeneic or allogeneic tumor cells;² (b) addition of mitogenic concentrations of soluble Con A, which suppressed CL responses, increased the viable cell recovery; (c) the 75–90% suppression of CL responses caused by Con A-activated spleen cells was not associated with a corresponding reduction in viable cell recovery; (d) suppression of CL responses was not due to interference with the lytic process in the assay since addition of Con A or Con A-activated cells to the ⁵¹Cr-release assay did not alter the result;² and (e) addition of either Con A or Con A-activated spleen cells to MLC 48 h or more after initiation had no effect on the generation of CL.

The population in Con A-activated spleen cells which mediates suppression of CL responses in MLC was characterized. The data clearly indicate that suppressive effects were mediated by T cells, since suppressor cell activity was abrogated by treatment of spleen cells with anti- θ serum and complement before or after activation with Con A. Furthermore, generation of suppressor T-cell activity was sensitive to irradiation before Con A activation, whereas after Con A activation, suppressor T-cell function was radioresistant. In these regards, suppressor T cells are similar to helper T cells known to regulate B-cell responses to complex multideterminant antigens (1).

Rich and Pierce (10), as well as Dutton (18), have described inhibition of PFC responses by Con A-activated suppressor T cells. Although the suppressor cells which influence the humoral and cell-mediated immune responses are sensitive to treatment with anti- θ serum and complement and resistant to irradiation after activation, they may represent different populations of regulatory T cells. Differences between the activities of suppressor T cells in the two types of immune responses are evident. First, PFC responses were inhibited by suppressor T cells added after 48-h incubation (10), whereas CL responses were inhibited only when suppressor cells were added at MLC initiation. Second, the degree of suppression induced by Con A-activated cells was consistently greater on PFC responses. These differences may be due to dis-

² Peavy, D. L., and C. W. Pierce. Unpublished observations.

parate sensitivity of the assays and differences in the number of initially responding cells, i.e., it has been estimated that approximately $1/10^6$ spleen cells are antigenically sensitive to SRBC determinants (19) while more than $1/10^2$ cells are believed to participate in allograft responses (20). The resolution of the question of whether the same or different subpopulations of suppressor T cells regulate PFC and CL responses must await further experimentation.

Suppression of CL responses required that suppressor cells be present in the MLC during the first 24 h of incubation. If addition of suppressor cells was delayed 48 h, no inhibition of 5 or 7 day CL responses resulted. Thus, precursors of CL must be exposed to suppressor cells during the early, yet undefined, interactions among alloantigens, and T cells participating in CL responses. However, kinetic studies of CL responses in MLC indicated that suppression occurred late in the response. CL responses in MLC containing suppressor T cells after 3 and 4 days incubation were similar to control responses. Actual suppression of these cultures manifested later as CL responses fell precipitously on day 5 and 6. Thus, suppression of CL responses by Con A-activated T cells cannot be regarded as a failure to initiate an immune response, but represents an abortive attempt. It is possible that activated T cells may inhibit the clonal expansion and terminal differentiation of alloantigen-sensitive T cells into cytotoxic killer cells. In this regard, suppressor cells may behave as a homeostatic mechanism limiting the generation of inordinately large numbers of cells sensitive to major and minor histocompatibility antigens. It is of great interest that the activities of suppressor T cells in both PFC and CL responses are similar. That is, in both types of responses suppressor T cells: (a) must be present during the early induction phase of the response; (b) do not block initiation of the response; but, (c) act to abort full expression of the response during the later phase of exponential expansion of the effector cells.

Although both Con A and Con A-activated cells can suppress CL responses, it is possible that suppression is mediated thru different mechanisms. Since suppression by Con A-activated cells requires that these cells be present during the first 24 h of the MLC, it seems unlikely that there is sufficient time for Con A to activate suppressor cells which then suppress the CL response. Con A may stimulate the alloantigen-reactive T cells into blastogenesis which does not result in the generation of CL, thus appearing to suppress this response. Investigations of the mechanisms by which Con A and Con A-activated cells suppress CL responses are currently in progress.

The development of in vitro techniques has enabled investigators to determine that the generation of CL responses is a complex process involving different cell types (21). While ample evidence has been offered that T cells are necessary for both afferent and efferent phases of cytotoxic responses, the possibility that a cooperative interaction occurs among subsets of T cells in the development of allograft responses has not yet been thoroughly examined.

Recent data from several laboratories (22, 23) have shown that synergy results from interactions among thymocytes and peripheral T cells in cellular immunity to transplantation antigens. Our observations are consistent with this emerging concept of T-cell-T-cell interaction in cellular immune responses in the sense that they demonstrate that T lymphocytes may also exert a regulatory influence and suppress CL responses.

SUMMARY

The effects of soluble concanavalin A (Con A) or Con A-activated spleen cells on the generation of cytotoxic lymphocytes (CL) in mixed leukocyte cultures (MLC) were examined. Mitogenic concentrations of soluble Con A or small numbers of Con A-activated spleen cells substantially inhibited CL responses. The suppression was partial rather than absolute and was critically dependent upon the concentration and time of addition of soluble Con A or Con A-activated spleen cells to the MLC.

Suppressive effects of Con-A activated spleen cells were mediated by T cells since suppressor cell activity was abrogated by treatment of spleen cells with anti- θ serum and complement before or after Con A activation. X irradiation of spleen cells before Con A treatment also abrogated generation of suppressor cell activity. After activation by Con A, however, the function of suppressor cells was radioresistant.

Although the precise mechanism(s) of suppression is, as yet, unknown, the precursors of CL must be exposed to Con A-activated cells during the early phases of the immune response for suppression to occur. Kinetic studies revealed that suppression of CL responses was not due to a failure to initiate an immune response, but represented a response which developed initially, but subsequently aborted. The relevance of these observations to the concepts of T-cell-T-cell interaction and regulatory control of immune responses by T cells is discussed.

It is a pleasure to thank Professor Baruj Benacerraf for his advice and assistance during the progress of these experiments and in the preparation of the manuscript.

REFERENCES

1. Katz, D. H., and B. Benacerraf. 1972. The regulatory influence of activated T cells on B cell responses to antigen. *Adv. Immunol.* **15**:1.
2. Sprent, J., and J. F. A. P. Miller. 1972. Interaction of thymus lymphocytes with with histoincompatible cells. III. Immunological characteristics of recirculating lymphocytes derived from activated thymus cells. *Cell. Immunol.* **3**:213.
3. Cantor, H., and R. Asofsky. 1972. Synergy among lymphoid cells mediating the graft-versus-host response. III. Evidence for interaction between two types of thymus-derived cells. *J. Exp. Med.* **135**:764.
4. Wagner, H., and M. Rollinghoff. 1973. In vitro induction of tumor-specific immunity. I. Parameters of activation and cytotoxic reactivity of mouse lymphoid

- cells immunized in vitro against syngeneic and allogeneic plasma cell tumors. *J. Exp. Med.* **138**:1.
5. Wigzell, H. 1971. Cellular immunoabsorbents. *Prog. Immunol.* **1**:1105.
 6. Hayry, P., L. C. Andersson, S. Nordling, and M. Virolainen. 1972. Allograft response in vitro. *Transplant. Rev.* **12**:91.
 7. Wagner, H., A. W. Harris, and M. Feldmann. 1972. Cell-mediated response in vitro. II. The role of thymus and thymus-derived lymphocytes. *Cell Immunol.* **4**:39.
 8. Cerottini, J. C., A. A. Nordin, and K. T. Brunner. 1971. Cellular and humoral response to transplantation antigens. I. Development of alloantibody-forming cells and cytotoxic lymphocytes in the graft-versus-host reaction. *J. Exp. Med.* **134**:553.
 9. Asofsky, R., H. Cantor, and R. E. Tiglaar. 1971. Cell interactions in the graft-versus-host response. *Prog. Immunol.* **1**:369.
 10. Rich, R. R., and C. W. Pierce. 1973. Biological expressions of lymphocyte activation. II. Generation of a population of thymus-derived suppressor lymphocytes. *J. Exp. Med.* **137**:649.
 11. Rich, R. R., and C. W. Pierce. 1973. Biological expressions of lymphocyte activation. I. Effects of phytoimitogens on antibody synthesis in vitro. *J. Exp. Med.* **137**:205.
 12. Wagner, H., and M. Feldmann. 1972. Cell-mediated immune responses in vitro. I. A new in vitro system for the generation of cell-mediated cytotoxic activity. *Cell. Immunol.* **3**:405.
 13. Mosier, D. E., and C. W. Pierce. 1972. Functional maturation of thymic lymphocyte populations in vitro. *J. Exp. Med.* **136**:1484.
 14. Williams, R. M., and B. Benacerraf. 1972. Genetic control of thymus-derived cell function. I. In vitro DNA synthetic response of normal mouse spleen cells stimulated by the mitogens Concanavalin A and phytohemagglutinin. *J. Exp. Med.* **135**:1279.
 15. Perlmann, P., H. Nilsson, and M. A. Leon. 1970. Inhibition of cytotoxicity by Concanavalin A in vitro. *Science (Wash. D. C.)*. **168**:1112.
 16. Schwartz, H. J., and F. Wilson. 1971. Target cell destruction in vitro by Concanavalin A-stimulated lymphoid cells. *Am. J. Pathol.* **64**:295.
 17. Stavy, L., A. J. Treves, and M. Feldman. 1972. Capacity of thymic cells to effect target cell lysis following treatment with Concanavalin A. *Cell. Immunol.* **3**:623.
 18. Dutton, R. W. 1972. Inhibitory and stimulatory effects of Concanavalin A on the response of mouse spleen cell suspensions to antigen. I. Characterization of the inhibitory cell activity. *J. Exp. Med.* **136**:1445.
 19. Shearer, G. M., G. Cudkowicz, M. Connell, and R. L. Priore. 1968. Cellular differentiation of the immune system in mice. I. Separate splenic antigen-sensitive units for different types of anti-sheep antibody-forming cells. *J. Exp. Med.* **128**:437.
 20. Simonsen, M. 1970. On the nature and measurement of antigenic strength. *Transplant. Rev.* **3**:22.
 21. Wagner, H., M. Feldmann, W. Boyle, and J. W. Schrader. 1972. Cell-mediated immune responses in vitro. III. The requirement for macrophages in cytotoxic reactions against cell-bound and subcellular alloantigens. *J. Exp. Med.* **136**:331.

22. Wagner, H. 1973. Synergy during in vitro cytotoxic allograft responses. I. Evidence for cell interaction between thymocytes and peripheral T cells. *J. Exp. Med.* **138**:1379.
23. Cohen, L., and M. L. Howe. 1973. Synergism between subpopulations of thymus-derived cells mediating the proliferative and effector phases of the mixed lymphocyte reaction. *Proc. Natl. Acad. Sci. U. S. A.* **70**:2707.