CELL-MEDIATED MITOGENIC RESPONSE INDUCED BY
LEUKOAGGLUTININ AND
LENS CULINARIS LECTIN IN MOUSE LYMPHOCYTES

BY KEIKO OZATO, JOHN CEBRA, AND JAMES D. EBERT

(From the Carnegie Institution of Washington, Department of Embryology, Baltimore, Maryland 21210, and the Johns Hopkins University, Department of Biology, Baltimore, Maryland 21218)

This work was undertaken to characterize the response of mouse lymphocytes to a syngeneic stimulus after surface modification by lectins. As previously reported (1), cortisone-resistant thymocytes (CRT) manifest a high proliferative response to syngeneic mitomycin-C-treated spleen cells (Mito-Sp) 2-3 days after the initiation of culture when either responder or stimulator is briefly pretreated with a supermitogenic dose of concanavalin A (Con A). The reaction is distinct from mitogenic response to soluble Con A and reveals that syngeneic cells can lead to T-cell activation when membranes have been modified by lectin binding. In the present work we report that two additional lectins, namely leukoagglutinin and Lens culinaris lectin (LcH) are capable of generating closely similar responses in mouse T cells.

O'Brien et al. (2) and Beyer and Bowers (3) reported a comparable response in human and rat lymphocytes. They found that periodate-treated lymphocytes that had been inactivated with mitomycin-C stimulate a significant proliferative response in autologous untreated lymphocytes. Mills et al. (4) observed a similar lymphocyte activation generated by Con A pretreatment. Novogrodsky (5) reported that lymphocytes are stimulated by syngeneic cells that had been pretreated with neuraminidase and galactose oxidase. In these responses there was no evidence for a soluble mediator that could induce an equivalent response. Because of their common characteristics we speak of these reactions as cell-mediated mitogenic responses (CMMR).

The characteristics of CMMR were studied in two ways. First, various reagents were checked for their ability to produce CMMR. Results for 10 reagents including seven plant lectins are presented concerning (a) their ability to induce CMMR, (b) agglutinating, and (c) mitogenic properties. Second, we attempted to determine the cellular subpopulation that may act as responder and stimulator in CMMR. We asked whether specific cell combinations are necessary to generate the response. This question was studied with four different procedures.

1 Abbreviations used in this paper: CMMR, cell-mediated mitogenic response; Con A, concanavalin A; CRT, cortisone resistant thymocytes; FCS, fetal calf serum; LA, leukoagglutinin; LcH, Lens culinaris lectin; LPS, bacterial lipopolysaccharides; Mito-Sp, mitomycin-C-treated spleen cells; N-Con A, native Con A; Nu-Sp, spleen cells from nude mice; PNA, peanut agglutinin; SBA, soy bean agglutinin; WGA, wheat germ agglutinin.
Materials and Methods

Mice and Cells. Male mice from C57BL/10, B10.D2, and CBA/J (CBA) (4- to 8-wk old) were obtained from The Jackson Laboratory (Bar Harbor, Maine). Congenital athymic mice (Nu/Nu) were provided by Dr. W. H. Adler (Gerontology Research Center, National Institute of Child Health and Human Development, Public Health Service, Baltimore City Hospitals). Responder CRT were prepared from mice injected with 4 mg hydrocortisone sodium succinate (6). Spleen cells obtained from syngeneic normal mice were treated with mitomycin-C at 40 µg/ml for 30 min at 37°C, washed three times, and used as stimulator cells.

Lectins and Reagents. The following lectins were commercially obtained; Con A and leukoagglutinin (LA) (from Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.), wheat germ agglutinin (WGA), and soy bean agglutinin (SBA) (from Miles Laboratories Inc., Miles Research Products, Elkhart, Ind.). Lech was purified according to Howard et al. (7) with slight modifications (8), and peanut agglutinin (PNA) was prepared according to Novogrodsky et al. (9). Ca²⁺ ionophore A23187 was provided by The Eli Lilly and Company (Indianapolis, Ind.). Bacterial lipo polysaccharide (LPS) from Salmonella typhimurium was a gift from Dr. J. Shands (University of Florida). LPS from Escherichia coli was purchased from Difco Laboratories (Detroit, Mich.).

Pretreatment of Lymphocytes with Reagents and Subsequent Culture. To test the ability to induce CMMR stimulator Mito-Sp (0.5-1 × 10⁷/ml) were treated with various reagents in RPMI-1640 (Grand Island Biological Co., Grand Island, N. Y.) + 1% fetal calf serum (FCS) (Grand Island Biological Co.) for 30 min at 37°C. FCS was added to the incubation medium, because lectin treatment without serum resulted in firm adherence of lymphocytes to the plastic surface (10), which reduced cell recovery. After washing for three times 2-5 × 10⁶ Mito-Sp suspended in 0.5 ml RPMI-1640 + 10% FCS supplemented with gentamycin (50 µg/ml) were mixed with 2-5 × 10⁶ CRT and the cells were cultivated in plastic tubes (12 × 75 mm Falcon Plastics, Division of BioQuest, Oxnard, Calif.) for 72 h in humidified gas (10% CO₂, 7% O₂, and 83% N₂). To test for the generation of cytotoxic lymphocytes 1 × 10⁷ CRT were cultivated with 5 × 10⁶ Mito-Sp pretreated with lectins in Marbrook chambers for 4 days. To culture peritoneal macrophages, exudate cells were obtained by washing the peritoneal cavities of normal CBA mice (11) with RPMI-1640. They were incubated in plastic Petri plates (10 × 35 mm) at 1 × 10⁷ cells in 2 ml RPMI-1640 + 10% FCS. 60 min later the cells were discarded, and the culture was continued in the medium plus 20% FCS. 24 h later the cells that adhered to Petri plates were treated with mitomycin-C at 40 µg/ml for 30 min and then treated with 40 µg/ml Con A for 30 min at 37°C before mixing with 1 × 10⁶ CBA-CRT.

Antisera. Anti-Thy 1.2 (Anti-0) was prepared by 6 weekly injections of 10⁷ CBA/J thymocytes into AKR mice as described by Reif and Allen (12). The collected antiserum was heated at 56°C for 30 min before use.

Goat anti-mouse Fab was prepared by injecting 5-7 mg of mouse IgG that had been digested with papain and fractionated on CM cellulose together with Freund's complete adjuvant. The antiserum which reacted only with mouse immunoglobulins was precipitated with 37% ammonium sulfate and then absorbed onto Sepharose coupled with mouse IgG. The bound antibody was eluted with 0.1 M propionic acid, neutralized with Tris, and then dialyzed against phosphate-buffered saline. 85% of the recovered material bound to mouse IgG coupled to Sepharose on a second passage.

Goat anti-mouse IgG and rabbit anti-mouse IgG were purchased from Miles Laboratories Inc., Miles Research Products, Elkhart, Ind. and Cappel Laboratories, Inc., Downingtown, Pa., respectively. These antisera were diluted in RPMI-1640. Rabbit sera used as complement were previously absorbed with CBA spleen cells. Treatment of CBA spleen cells with anti-mouse Fab and anti-mouse IgG (1/40 dilution) in the presence of complement inhibited mitogenic response to LPS by more than 90% without affecting mitogenesis to Con A. To test the effect of the sera in CMMR, stimulator Mito-Sp (1 × 10⁷ cells in 0.5 ml) were treated with sera in the presence of complement (1/15 final dilution) for 45 min at 37°C followed by three washings before Con A pretreatment.

Agglutination. 5 × 10⁶ CBA spleen cells or thymocytes were incubated in 0.5 ml RPMI-1640 + 1% FCS containing various lectins or other reagents at different concentrations for 30 min at 37°C. Agglutination was classified roughly by microscopic observation of lymphocytes in a hemocytometer as follows; Strong (+ + +), when most of the cells were agglutinated in large clumps (more than 15 cells in a clump); medium (+ +), 5-10 cells were agglutinated in a clump; weak (+), less than 5 cells in a clump with some single cells; and none (-), most cells were dispersed.
Spleen Cell Fractionation on Nylon Wool Columns. A nylon wool column in a 10-ml syringe was prepared according to Julius et al. (13). Spleen cells were filtered through glass fiber and separated from erythrocytes by Ficoll-Hypaque (Lymphprep, Nyegarrd, Oslo) density gradient centrifugation. After mitomycin-C treatment, $2 \times 10^8$ cells were resuspended in 2 ml medium + 5% FCS, placed on the column equilibrated previously at 37°C, and allowed to stand for 45 min at 37°C. The effluent cells were obtained from the initial 25 ml of elution. The remaining cells were obtained by vigorous shaking of the recovered nylon wool with forceps in cold RPMI 1640.

Proliferative Response and Generation of Cytotoxic Lymphocytes. Lymphocytes cultured for 72 h were labeled with $[^3H]Tdr$ (1 μCi/ml) (sp act 14 Ci/mmol, Schwartz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.) for 12-20 h. DNA synthesis was assayed by measuring 5% trichloroacetic acid-insoluble material by scintillation counting. Blast cells were detected by staining the cells with Giemsa; enlarged cells (at least twice the diameter of small lymphocytes) with a distinct cytoplasmic space were counted as lymphoblasts. Generation of cytotoxic lymphocytes was assayed by $^{51}Cr$-release from target cells, mastocytoma P815-X2, in allogeneic combinations, and lymphoma EL-4 in C57BL/10 syngeneic combinations. Harvested lymphocytes were incubated with $[^5]Cr$-labeled target cells for 4 h at 37°C. Percent cytotoxicity per culture was obtained according to Cerottini et al. (14). Details of these assays are described elsewhere (1).

Results

Evaluation of Various Lectins for their Ability to Induce CMMR. In inducing CMMR a lectin may have one of the following roles. It may induce cellular contact by agglutination, or it may itself provide a stimulatory signal to the responder. If the former is the case, CMMR should be obtained by any agglutinating reagent which can form bridges between cells. In the latter case, we might find that only some lectins are capable of stimulating. Various lectins and reagents having different properties in agglutination and mitogenicity were tested for their ability to induce CMMR (Table I). Responders used were CBA-CRT, and stimulators were syngeneic Mito-Sp.

LA and LcH CAN GENERATE CMMR. Among the reagents tested LA, a purified form of phytohemagglutinin (15, 16) and LcH, a lectin from *Lens culinaris* (7, 8, 17) both T-cell mitogens' induced CMMR (Figs. 1 and 2). Significant DNA synthesis on day 3 occurred when untreated CRT were cultured with syngeneic Mito-Sp that had been briefly treated with either lectin. Optimal concentrations are 25 and 100 μg/ml for LA and LcH, respectively. The optimal ratio of responder to stimulator was about 1:1 to 1:2 in these culture conditions. The blast cells generated per culture were $2.1 \times 10^5$ cells in LcH system and $0.9 \times 10^5$ cells in LA system. A significant level of CMMR occurred even when the cell ratio was 1:5 (with LA) and 1:8 (with LcH). Under optimal conditions the level of $[^3H]Tdr$ incorporation was almost as high as the standard mitogenic response of CRT to the soluble forms of these lectins. In both cases Mito-Sp treated with lectins did not show significant proliferation when cultured alone. Evidently, the response was produced by CRT, since no response was found when CRT were pretreated with mitomycin-C. Syngeneic thymocytes were much less stimulatory as compared to Mito-Sp. In the LA system, thymocytes showed practically no ability to generate the response, and with LcH the response to thymocytes was about 1/8 of Mito-Sp. This result is consistent with the findings in Con A-induced CMMR (1).

K. Ozato, J. Somerville, and J. D. Ebert. 1977. *Lens culinaris* lectin, a T cell mitogen and concanavalin A have common receptors. Manuscript submitted for publication.
Table I

Lectins Capable of Inducing CMMR

| Lectin or reagent | Agglutination | Property of lectins* | Induction of CMMR |
|-------------------|---------------|----------------------|-------------------|
| Native-Con A      | ++            | + (T cell)           | +                 |
| Succinyl-Con A    | +             | + (T cell)           | +                 |
| LA                | ++            | + (T cell)           | +                 |
| LcH               | ++            | + (T cell)           | +                 |
| WGA               | ++            | -                    | -                 |
| PNA               | ++            | -                    | -                 |
| SBA               | -             | -                    | -                 |
| LPS from E. coli  | -             | + (B cell)           | -                 |
| from Salmonella   | -             | + (B cell)           | -                 |
| Ca²⁺ Ionophore (A23187) | -     | ?                    | -                 |

* Induction of CMMR was tested in CBA-CRT stimulated by syngeneic Mito-Sp pretreated with the reagents at 10, 25, 50, 100, 200, and 300-500 μg/ml, except the Ca²⁺ ionophore which was tested at 10⁻⁴, 10⁻⁵, 10⁻⁶, 10⁻⁷, and 10⁻⁸ M. Agglutination was studied at 50 and 250 μg/ml (at 10⁻⁸ and 10⁻⁷ M in Ca²⁺ ionophore).

Fig. 1. LA-induced CMMR. CBA-CRT (2.5 x 10⁶) were mixed with the same number of syngeneic Mito-Sp (●) pretreated with LA or mitomycin-C-treated thymocytes (○) pretreated with LA. CRT were pretreated with mitomycin-C before mixing with syngeneic Mito-Sp (△). Mito-Sp LA pretreated were cultured alone (△). The value indicates [³H]Tdr incorporation on day 3 (mean of duplicate).

We tested next whether a soluble mediator could be responsible for the reaction. Supernates were obtained from cultures of Mito-Sp alone, 24 and 48 h after the initiation of culture after pretreatment with 25 μg/ml LA or 100 μg/ml LcH. Both undiluted samples and samples diluted 1:1 with fresh medium, were
tested within 24 h of collection for their ability to stimulate syngeneic CRT. DNA synthesis on day 3 remained at background level, and no detectable lymphoblasts were generated in the culture.

CMMR induced by LcH and LA is similar to the phenomenon described previously with Con A but with certain differences. In Con A-dependent CMMR either responder or stimulator cells may be pretreated to obtain the effect (1). This was possible since a 30-min Con A treatment is not mitogenic in CRT by itself. In contrast, LA and LcH triggered mitogenesis in CRT by a 30-min pulse treatment (details are reported elsewhere).3 Because of this phenomenon, we did not pursue further the effects of pretreatment of responder cells by these lectins, although the addition of Mito-Sp to CRT pretreatment with the lectins enhanced their proliferation, implying that CMMR did take place.

A close cell contact is not sufficient to generate CMMR. The capacity of generating CMMR in nonmitogenic lectins is summarized in Table I, together with the agglutinating properties of the lectins. The results were obtained from tests over a wide range of lectin concentrations. WGA (18) and PNA (5) were capable of agglutination lymphocytes to an extent comparable to the T-cell mitogenic lectins but were deficient in the induction of CMMR. SBA (19) which did not agglutinate lymphocytes did not produce CMMR. LPS, a B-cell mitogen (20), and Ca2+ ionophore, a mitogen for other mammalian cells (21), were not

---

3 Ozato, K., and J. D. Ebert. 1977. Transfer of concanavalin A between responding lymphocytes and syngeneic stimulating cells in cell mediated mitogenic response. Manuscript submitted for publication.
CELL-MEDIATED MITOGENIC RESPONSE

Table II

| Responder strain | Stimulator strain | C57BL/10 Pretreatment | B10 D2 Pretreatment | CBA Pretreatment |
|------------------|-------------------|-----------------------|---------------------|------------------|
|                  | LA    | LcH | Untreated | LA    | LcH | Untreated | LA    | LcH | Untreated |
| C57BL/10         | 19.3 ± 0.6 | 32.0 ± 0.6 | 0.3 ± 0.01 | 24.0 ± 0.6 | 36.2 ± 0.5 | 5.5 ± 0.1 | 26.4 ± 1.3 | 38.5 ± 1.6 | 7.8 ± 0.3 |
| B10.D2           | 25.5 ± 0.8 | 34.1 ± 0.7 | 6.3 ± 0.2 | 21.3 ± 0.7 | 26.8 ± 0.3 | 0.4 ± 0.1 | 23.1 ± 1.3 | 37.3 ± 1.0 | 4.5 ± 0.2 |

* Mito-Sp obtained from three strains were pretreated with LA (50 μg/ml) or LcH (100 μg/ml) or medium (untreated), washed, then mixed with the same number of CRT (2 × 10^6). The value represents mean of triplicates ± SD.

active in the generation of CMMR. In summary, the response is not correlated with the agglutinating property, and only lectins with T-cell mitogenic properties generate CMMR.

CMMR IN ALLOGENEIC COMBINATIONS. We tested the response in two other strains of mice with different H-2 haplotypes, both in syngeneic and allogeneic combinations. Untreated CRT from H-2^b (C57BL/10) and H-2^d (B10.D2) mice exhibited significant proliferative responses in the presence of syngeneic Mito-Sp that had been pretreated with LA or LcH (Table II). In allogeneic combinations, when Mito-Sp stimulators were pretreated with the lectins, DNA synthesis exceeded normal MLR by more than fourfold. The enhanced DNA synthesis was found in all the combinations tested, i.e., H-2^b vs. H-2^d, H-2^b vs. H-2^k, H-2^d vs. H-2^b and H-2^d vs. H-2^k. Soluble supernate alone obtained from lectin-pretreated allogeneic Mito-Sp cultures did not produce noticeable CRT proliferation. Further, the response in allogeneic combinations is consistently higher than in syngeneic combinations.

Suppression of the Generation of Allogeneic Cytotoxic Lymphocytes as a Consequence of CMMR. In this series of experiments two questions were asked: (a) Is the generation of cytotoxic T lymphocytes in allogeneic combinations affected when the response is combined with CMMR? (b) Does CMMR in syngeneic combination lead to the generation of cytotoxic lymphocytes towards syngeneic target cells?

In allogeneic CMMR, C57BL/10 CRT were stimulated with B10.D2 Mito-Sp after pretreatment either with LA or LcH. The cytotoxic activities on day 4 were measured as ^31Cr-release from the target cells P815, and are shown in Fig. 3. Treatment of stimulator Mito-Sp with either LA or LcH resulted in a drastic suppression of cytotoxic activity. The suppression was significant at concentrations as low as 25 μg/ml of LA, 50 μg/ml of LcH. At higher concentrations (100-200 μg/ml) at which more extensive cell division had occurred (See Figs. 1 and 2), the cytolytic activities were almost completely suppressed. Washing of harvested lymphocytes from LcH-treated cultures with α-methyl-d-mannopyranoside (0.2 M in RPMI-1640 for 15 min at 37°C) before executing the cytotoxicity test did not restore cytotoxic activity.

Cytolytic activity toward lectin-coated target cells was also studied, since CRT could have responded to lectin-modified components of stimulators. C57BL/10 CRT cultures stimulated with B10.D2 Mito-Sp pretreated with LA or LcH at 100 μg/ml were used as effector lymphocytes. Pretreatment of target cells with
either lectin at 25 \( \mu g/ml \) for 30 min followed by washing did not change the cytolytic activity.

Cytotoxic activity is a syngeneic combination examined in C57BL/10 is shown in Fig. 3. Little cytotoxicity was observed in CMMR culture with lectin when tested with normal target cells. Pretreatment of target cells with either lectin (25 \( \mu g/ml \) for 30 min) showed only a marginal elevation of cytotoxicity: 8.1\% in LA-pretreated target vs. 6.2\% in untreated and 6.8\% in LcH-pretreated target vs. 5.0\% untreated tested in CMMR induced by corresponding lectin. This level of cytotoxicity is too low to allow comparison to cytotoxicities toward syngeneic target cells reported in other systems (22-24). Similar suppression of allogeneic cytolysis and the absence of syngeneic cytolysis were found previously in Con A-induced CMMR (1).

**Determination of the Lymphocyte Subpopulation Responsible for CMMR.** All experiments on subpopulation analysis were carried out with Con A-induced CMMR, that is CRT (2-5 \( \times 10^6 \) cells) were mixed with an equal number of syngeneic Mito-Sp in 0.5 ml medium and were cultured for 72 h, after either responder or stimulator was pretreated with Con A for 30 min at 37\(^\circ\)C at 50 \( \mu g/ml \) (1).

**Characterization of the Stimulating Cells. Effect of Anti-Mouse Ig or Anti-Thy 1.2.** To eliminate B cells or T cells from stimulator Mito-Sp, goat anti-mouse Fab and goat (or rabbit) anti-mouse IgG or anti-Thy 1.2 were used. The effects were studied in three strains of different \( H-2 \) haplotypes, either when stimulator or responder cells were pretreated with Con A. As seen in Table III, the response was abrogated in all cases when stimulator cells were...
TABLE III

Effect of Anti-Mouse Ig or Anti-Thy 1.2 on Stimulator Cells in Con A-Induced CMMR*

| Treatment of stimulators          | Exp. I. Con A pretreatment: stimulators | Exp. II. Con A pretreatment: responders |
|----------------------------------|----------------------------------------|----------------------------------------|
|                                  | B10.D2 | CBA/J | C57BL/10 | CBA/J |
| Goat anti-mouse Fab (1/40)       | 1.0 ± 0.1 | 0.9 ± 0.1 | 1.4 ± 0.1 | 1.8 ± 0.2 |
| Goat anti-mouse Fab (1/80)       | 0.8 ± 0.1 | 2.0 ± 0.2 | 3.2 ± 0.1 | 3.7 ± 0.2 |
| Goat anti-mouse IgG (1/40)       | 1.3 ± 0.1 | 3.1 ± 0.1 | 1.0 ± 0.1 | 4.5 ± 0.3 |
| Rabbit anti-mouse IgG (1/40)     | 1.1 ± 0.1 | 1.8 ± 0.1 | 0.9 ± 0.1 | 3.0 ± 0.2 |
| Normal goat serum (1/40)         | 28.0 ± 1.3 | 18.3 ± 1.3 | 18.0 ± 1.2 | 13.3 ± 0.8 |
| Normal rabbit serum (1/40)       | 26.4 ± 1.7 | 17.6 ± 1.1 | 15.3 ± 1.0 | 12.5 ± 0.7 |
| Anti-thy 1.2 (1/40)              | 25.3 ± 3.0 | 19.0 ± 1.7 | 21.1 ± 1.8 | 20.2 ± 1.3 |
| Normal mouse AKR serum (1/40)    | 29.3 ± 3.0 | 13.4 ± 1.2 | 23.8 ± 2.1 | 19.9 ± 1.1 |

* Mito-Sp were treated with the antisera and complement and washed. The dilution of antiserum is shown in parenthesis. In Exp. I, Mito-Sp were further pretreated with Con A before mixing with syngeneic CRT. In Exp. II, Mito-Sp were mixed with CRT pretreated with Con A. The values represent means of triplicates ± SD.

exposed to, anti-mouse Ig. Anti-mouse Fab was as effective as anti-mouse IgG. Only slight variations among different strains were noted. On the other hand, anti-Thy 1.2 treatment of stimulator Mito-Sp had no significant effect.

EFFECT OF NYLON WOOL FRACTIONATION OF STIMULATOR CELLS. The capacity of spleen cells to stimulate CMMR was tested with subpopulations fractionated with a nylon wool column. CBA-Mito-Sp filtered through glass fiber were fractionated into a T-cell-enriched effluent population and a non-T-cell-enriched fraction that had been retained in the column. As shown in Table IV, the effluent population was much less stimulatory than the unfractionated population, whereas the retained cells exhibited higher stimulatory activity. Thymocytes were totally inactive in inducing a response. These experiments indicate that stimulatory activity depends on the presence of Ig-positive cells and/or Fc-receptor-positive cells, and thymocytes or T cells are much less capable of stimulating.

Since macrophages have been reported to induce the activation of syngeneic T cells in various responses (4, 5, 11), we explored the possible role of macrophages as stimulators in CMMR in two ways: (a) elimination of adherent cells from the spleen population by passage through plastic Petri plates; and (b) testing peritoneal exudate cells for their ability to induce CMMR. First, spleen cells were recovered after two consecutive incubations on plastic Petri plates, each for 45 min at 37°C in RPMI-1640 + 10% FCS. 85% of the stimulatory activity was retained in the nonadherent population. Cocultivation of CBA-CRT with syngeneic peritoneal exudate cells which had been pretreated with Con A neither generated a significant DNA synthesis nor lymphoblasts.

Characterization of Responder: Lack of Con A-Induced CMMR in Spleen Cells from Nude Mice. To determine the nature of the responder subpopulation CMMR was studied in congenital athymic nude mice. Either responder
TABLE IV
The Effect of Spleen Cell Fractionation on the Stimulatory Activity in CMMR

| Experimental condition | Stimulator population | Effluent | Remaining | Unfractionated | Thymocytes |
|------------------------|-----------------------|----------|-----------|----------------|------------|
| Con A pretreatment: responders* | 2.54 ± 0.2 | 7.93 ± 0.55 | 5.0 ± 0.3 | 0.2 ± 0.01 |
| Con A pretreatment: stimulators† | 2.59 ± 0.1 | 6.32 ± 0.4 | 4.7 ± 0.2 | 0.3 ± 0.01 |

* CBA-CRT (2 × 10⁶) pretreated with N-Con A were mixed with 1 × 10⁶ nylon wool-fractionated syngeneic Mito-Sp cells.
† Fractionated CBA-Mito-Sp were further treated with Con A before mixing with syngeneic CRT. The value represents mean of triplicates ± SD.

TABLE V
Lack of Con A-Induced CMMR in the Spleen Cells from Nu/Nu Mice

| Experimental condition | Con A pretreatment: responders | Con A pretreatment: stimulators | |
|------------------------|--------------------------------|---------------------------------|---|
| Responders vs. stimulators | Con A pretreatment | cpm × 10⁻³ | SI | Con A pretreatment | cpm × 10⁻³ | SI |
| Nu-Sp | Nu-Mito-Sp | + | 5.6 ± 0.4 | 1.81 | 5.2 ± 0.1 | 1.86 |
| Nu-Sp | CBA-Mito-Sp | + | 11.3 ± 0.7 | 1.55 | 18.3 ± 0.8 | 1.8 |
| CBA-CRT | CBA-Mito-Sp | + | 42.7 ± 2.1 | 105.8 | 97.8 ± 5.6 | 88.9 |
| | | - | 0.4 ± 0.01 | 1.1 ± 0.1 |

* Responders (2.5 × 10⁶) were mixed with 2 × 10⁶ stimulators.
cpm × 10⁻³ indicates [³H]TdR incorporation on day 3 (mean of triplicates ± SD).
SI (stimulation index) is obtained by Con A-pretreated culture/untreated culture.

spleen cells from nude mice (Nu-Sp) or mitomycin-C-treated stimulator (Nu-Mito-Sp) were pretreated with N-Con A and mixed with untreated partner Nu-Sp, two spleens being pooled in an experiment. The results are shown in Table V. Only slight increases (2–2.5 times control) in proliferation were found upon pretreatment with Con A of either Nu-Sp or Nu-Mito-Sp. As compared to CBA-CRT vs. CBA-Mito-Sp cultures, stimulation indices (DNA synthesis in CMMR/control DNA synthesis) in cells from nude mice were 50 times less. In an allogeneic combination (Nu-Sp vs. CBA-Mito-Sp) Nu-Sp showed little sign of CMMR.

The nature of responder cells was studied further with anti-Thy 1.2-treated spleen cells. Treatment of CBA spleen cells with the antibody (diluted 1/40) in the presence of complement before mixing with syngeneic Mito-Sp diminished CMMR by 85–92%, regardless which partner cells were pretreated with Con A.

Discussion

The Nature of CMMR. In this communication, we deal with a T-cell proliferative reaction to syngeneic lymphocytes after their surface alteration with LA or
LcH. The responses are equivalent to the Con A-induced reaction reported previously (1). Others reported similar responses exerted by different reagents (2, 3, 5). In these responses, soluble mediators have not been detected. Although soluble factors that induce proliferation of lymphocytes have been found in the supernate of activated lymphocytes (25), in CMMR, stimulators were inactivated before culture. Because of the obligatory involvement of stimulator cells we have called the reaction CMMR. The response is characteristic in that the presence of stimulator cells pretreated with an appropriate lectin (or other reagents) is required. Examination of various lectins led to the following conclusion: (a) Close cell contact that can be obtained by agglutination is not sufficient for the generation of CMMR. (b) Lectins that lead to CMMR possess the property of T-cell-specific mitogens. Other reagents like periodate or treatment with neuraminidase followed by galactose oxidase which induce equivalent mitogenesis fall into the same category; they are mitogenic selectively in T cells (26).

The uniqueness of CMMR compared to other types of immune responses can be summarized as follows.

**CMMR is distinct from the mitogenic response to soluble mitogens.** Standard mitogenic responses to soluble mitogens are generated in thymocyte populations or "pure" T-cell populations (19), whereas in CMMR-specific stimulator cells are required; thymocytes cannot act as stimulator cells.

**CMMR is not limited to histocompatible combinations.** There is no apparent restriction for H-2 gene products in the generation of CMMR. All three lectins capable of generating CMMR (Con A, LA, and LcH) exhibited augmented DNA synthesis in every allogeneic combination tested. In contrast, T-B cooperation in antibody formation is reported to require compatibility in H-2 gene products, and allogeneic combinations do not produce a cooperative effect (27), although the opposite result has been reported as well (28).

**CMMR is distinct from the response to altered self antigens.** It is known that modification of syngeneic stimulators by virus infections or by trinitrophenol leads to the generation of cytotoxic T lymphocytes specific for syngeneic target cells (22-24). In these systems it is postulated that the responses are against altered self antigens. Although CMMR is similar to these systems in that a syngeneic response is produced, the response is limited to proliferation. We failed to detect a significant level of cytotoxic activity toward syngeneic cells, whether they were treated with lectins or not. Moreover, in allogeneic combinations the generation of cytotoxic lymphocytes is severely impaired after CMMR.

**The Lymphocyte Subpopulation Involved in CMMR.** The population analysis in Con A-induced CMMR indicated first that stimulation depends largely on the presence of Ig-bearing lymphocytes (and perhaps Fc receptor-bearing cells). T cells and adherent cells do not seem to be major stimulators in our experimental system. Second, the population that can respond is also limited. T cells are capable of responding to a much higher degree than non-T cells.

Therefore, it seems that in our experimental conditions responder T cells are stimulated by Ig-positive lymphocytes in the predominant pathway of CMMR. However, the possibility that macrophages are partially participating in the stimulation (4, 5) is not entirely excluded since it is difficult to achieve absolute
elimination of macrophages. Furthermore, the anti-mouse Ig that we used to suppress stimulatory activity could have reacted with Fc receptors of macrophages. With respect to cell combination, CMMR shows a similarity to MLR in which B cells are potent stimulators and T cells are primary responders (29). We show in a separate paper that CMMR requires viable stimulator cells, which is also similar to MLR. Dyminski and Smith (30) reported that the helper-like function of syngeneic spleen cells that is found in T-cell proliferative response to allogeneic thymocytes depends on the presence of Ig-bearing cells. The reaction they found is analogous to our finding because the response is dependent on syngeneic combinations.

The opposite reaction to the one studied here, the activation of B cells induced by thymocytes treated with Con A, has been reported (31). This response is weak but in its dependence on T-B-cell interaction and on lectin is comparable to our observations. We regard the response as a minor pathway of CMMR. The slight stimulation that we found in the experiment with Nu-Sp may be due to this type of CMMR.

Recently, Kuntz et al. (32) reported that human T-cell proliferation is produced by autologous non-T cells, that is Ig-positive cells, and K cells. In contrast to our system no lectin is involved. Conceivably, human lymphocytes can form the necessary cell interactions without surface modification. Alternately, human serum might already contain substances resembling lectins which facilitate the response. Therefore, we suggest that CMMR is not restricted to the specific conditions used in this study, but reflects a type of internal regulatory mechanisms. Thus, CMMR may provide a useful experimental model system to study cellular interaction and communication.

Summary

The proliferative response of mouse lymphocytes to syngeneic cellular stimulation upon membrane modification with lectins was studied. Brief pretreatment of stimulator cells (mitomycin-C-treated spleen cells) followed by mixed culture with syngeneic cortisone-resistant thymocytes resulted in a significant proliferative response in the thymocytes. This effect was not due to a soluble mediator and was similar to the mitogenic response after Con A-induced membrane modification reported previously. Because of its general characteristics, we refer to this response as cell-mediated mitogenic response (CMMR).

Cell contact between stimulator and responder cells was necessary but not sufficient for the induction of the response. The lectins that generated CMMR were T-cell mitogens. CMMR was generated in all the syngeneic combinations tested and even in allogeneic combinations. No detectable cytotoxic activity towards syngeneic target cells was produced after CMMR. Moreover, CMMR in allogeneic combinations led to the suppression of the generation of specific cytotoxic lymphocytes. Population analysis with antibodies against T or B cells, nylon wool fractionation of stimulator cells, and tests with peritoneal macrophages and with spleen cells from athymic mice revealed that CMMR depends predominantly on the interaction between responder T cells and stimulator Ig-positive lymphocytes.

We wish to thank Doctors I. B. Dawid and M. Plaut for their critical reading of the manuscript. The capable technical assistance of Ms. D. Somerville and Ms. B. Smith is greatly acknowledged.
Received for publication 12 May 1977.

References
1. Ozato, K., and J. D. Ebert. 1976. Concanavalin A potentiates syngeneic response in murine lymphocytes. J. Exp. Med. 143: 1.
2. O'Brien, R. L., J. W. Parker, P. Paolilli, and J. Steiner. 1974. Periodate-induced lymphocyte transformation. IV. Mitogenic effect of NaIO₄ treated lymphocytes upon autologous lymphocytes. J. Immunol. 112: 1884.
3. Beyer, C. F., and W. E. Bowers. 1975. Periodate and concanavalin A induce blast transformation of rat lymphocytes by an indirect mechanism. Proc. Natl. Acad. Sci. U.S.A. 72: 3890.
4. Mills, G., V. Montecone, and V. Paetkau. 1976. The role of macrophages in thymocyte mitogenesis. J. Immunol. 117: 1325.
5. Novogrodsky, A. 1976. A chemical approach for the study of lymphocyte activation. In Mitogens in Immunobiology. J. J. Oppenheim and D. L. Rosenstreich, editors. Academic Press, Inc. New York. 43.
6. Blomgren, H., and B. Andersson. 1969. Evidence for a small pool of immunocompetent cells in the mouse thymus. Exp. Cell Res. 57: 185.
7. Howard, I. K., H. J. Sage, M. D. Stein, N. M. Young, M. A. Leon, and D. F. Dyckes. 1971. Studies on a phytohemagglutinin from the lentil. II. Multiple forms of Lens culinaris hemagglutinin. J. Biol. Chem. 246: 1590.
8. Ticha, M., G. Entlicher, J. V. Koštíř, and J. Kocovrek. 1970. Studies on phytohemagglutinins. IV. Isolation and characterization of a hemagglutinin from the lentil Lens esculenta, Moench. Biochim. Biophys. Acta. 221: 292.
9. Novogrodsky, A., R. Lotan, A. Ravid, and N. Sharon. 1975. Peanut agglutinin, a new mitogen that binds to galactosyl sites exposed after neuraminidase treatment. J. Immunol. 115: 1243.
10. Wong, S. Y., B. M. Longenecker, F. Pazderka, and R. F. Ruth. 1975. Immobilization of lymphocytes at surfaces by lectins. Exp. Cell Res. 92: 428.
11. Rosenthal, A. S., and E. M. Shevach. 1973. Function of macrophages in antigen recognition by guinea pig T lymphocytes. I. Requirement for histocompatible macrophages and lymphocytes. J. Exp. Med. 138: 1194.
12. Reif, A. E., and J. M. Allen. 1964. The AKR antigen and its distribution in leukemias and nervous tissues. J. Exp. Med. 120: 413.
13. Julius, M. H., E. Simpson, and L. A. Herzenberg. 1973. A rapid method for the isolation of functional thymus-derived murine lymphocytes. Eur. J. Immunol. 3: 645.
14. 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.
15. Weber, T. 1969. Isolation and characterization of lymphocyte-stimulating leucoagglutinin from red kidney beans (Phaseolus vulgaris). Scand. J. Clin. Lab. Invest. Suppl. 111: 33.
16. Skoog, V. T., T. H. Weber, and W. Richter. 1974. Studies on the interaction between mitogens and human lymphocytes in vitro. Exp. Cell Res. 85: 339.
17. Young, N. M., M. A. Leon, T. Takahashi, I. K. Howard, and H. J. Sage. 1971. Studies on a phytohemagglutinin from the lentil. III. Reaction of Lens culinaris hemagglutinin with polysaccharides glycoproteins and lymphocytes. J. Biol. Chem. 246: 1596.
18. Burger, M. M., and A. R. Goldberg. 1967. Identification of a tumor specific determinant on neoplastic cell surfaces. Proc. Natl. Acad. Sci. U.S.A. 57: 359.
19. Novogrodsky, A., and E. Katchalski. 1973. Transformation of neuraminidase-treated lymphocytes by soy bean agglutinin. Proc. Natl. Acad. Sci. U.S.A. 70: 2515.
20. Andersson, J., G. Möller, and O. Sjöberg. 1972. Selective induction of DNA synthesis in T and B lymphocytes. *Cell Immunol.* 4: 381.
21. Maino, V. C., N. M. Green, and J. J. Crumpton. 1974. The role of calcium ions in initiating transformation of lymphocytes. *Nature (Lond.)*. 251: 324.
22. Doherty, P. C., and R. M. Zinkernagel. 1974. T cell-mediated immunopathology in viral infections. *Transplant. Rev.* 19: 89.
23. Koszinowski, U., and H. Ertl. 1975. Lysis mediated by T cells and restricted by H-2 antigens of target cells infected with vaccinia virus. *Nature (Lond.)*. 255: 552.
24. Shearer, G. M. 1974. Cell-mediated cytotoxicity to trinitrophenyl-modified syngeneic lymphocytes. *Eur. J. Immunol.* 4: 527.
25. Jacobsson, H., and H. Blomgren. 1975. Characterization of mouse cells releasing or responding to mitogenic factor induced by phytohemagglutinin *in vitro*. *J. Immunol.* 114: 1631.
26. Novogrodsky, A. 1974. Selective activation of mouse T and B lymphocytes by periodate, galactose oxidase and soy bean agglutinin. *Eur. J. Immunol.* 4: 646.
27. Katz, D. H., T. Hamaoka, and B. Benacerraf. 1973. Cell interactions between histoincompatible T and B lymphocytes. II. Failure of physiologic cooperative interactions between T and B lymphocytes from allogeneic donor strains in humoral response to hapten-protein conjugates. *J. Exp. Med.* 137: 1405.
28. Heber-Katz, E., and D. B. Wilson. 1976. Collaboration of allogeneic T and B lymphocytes in the primary antibody response to sheep erythrocytes *in vitro*. In *Mitogens in Immunobiology*. J. J. Oppenheim and D. L. Rosentreich, editors. Academic Press, Inc. New York. 375.
29. Cheers, D., and J. Sprent. 1973. B lymphocytes as stimulators of a mixed lymphocyte reaction. *Transplantation (Baltimore).* 15: 336.
30. Dyminski, J. S., and R. T. Smith. 1975. Evidence for a B-cell-like helper function in mixed lymphocyte culture between immunocompetent thymus cells. *J. Exp. Med.* 141: 360.
31. Elfenbein, G. J., and M. C. Gelfand. 1975. Proliferation of mouse bone marrow-derived lymphocytes *in vitro*: One mechanism of response to concanavalin A and phytohemagglutinin. *Cell Immunol.* 17: 463.
32. Kuntz, M. M., J. B. Innes, and M. E. Weksler. 1976. Lymphocyte transformation induced by autologous cells. IV. Human T-lymphocyte proliferation induced by autologous or allogeneic non-T lymphocytes. *J. Exp. Med.* 143: 1042.