POKEWEED MITOGEN-, CONCANAVALIN A-, AND PHYTOHEMAGGLUTININ-INDUCED DEVELOPMENT OF CYTOTOXIC EFFECTOR LYMPHOCYTES

An Evaluation of the Mechanisms of T Cell-Mediated Cytotoxicity

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Cytotoxic effector lymphocytes (CEL)[1] play a major role in various forms of tumor immunity, autoimmunity, and allograft rejection and a number of experimental systems have been developed to study lymphocyte-mediated cytotoxicity in vitro. Thus, lymphocytes from sensitized animals destroy target cells in tissue culture which carry the specific antigens against which the lymphocyte donor was sensitized. Lymphocytes from nonimmunized donors may also become cytotoxic to target cells in the presence of phytohemagglutinin (PHA) or antibody (for review see reference 1). While PHA-induced cytotoxicity has been extensively studied, lymphocyte cytotoxicity induced by other mitogens such as concanavalin A (Con A) and pokeweed mitogen (PWM) (2–5) has received less attention.

In the present studies Con A and PWM were as effective as PHA in inducing the development of CEL in cultures of chicken lymphocytes. However, it was demonstrated that the killer cells induced by PWM and Con A were capable of destroying only certain selected target cells. This "target cell specificity" was also demonstrated in mixtures of target cells appropriate and inappropriate for each mitogen. Our data suggest that mitogen-induced target cell lysis requires cross-linking between target and aggressor cell, that nonspecific additional recruitment of CEL does not occur, and that soluble lymphotoxins are not involved in this type of cytotoxic reaction.

Materials and Methods

Preparation of Leucocytes.—8-12-wk old inbred line 96 chickens (Hyline, Johnstown, Iowa) served as donors of various lymphoid organs. Leucocyte suspensions were prepared from thymus, bursa of Fabricius, spleen, bone marrow, and peripheral blood by previously described methods (9). Briefly, spleen, bursa, and thymus were teased apart with forceps and sus-

[1] *Abbreviations used in this paper:* ag, agammaglobulinemic; anti-Ig, anti-immunoglobulin serum; ATS, antithymocyte sera; B, bursal derived; BRC, burro red cells; BSS, balanced salt solution; CEL, cytotoxic effector lymphocytes; CRC, chicken red cells; Con A, concanavalin A; FCS, fetal calf serum; HRC, human red cells; PHA, phytohemagglutinin P; PWM, pokeweed mitogen; T, thymic-derived; TdRH3, [^{3}H]thymidine.

pended in serum-free RPMI 1640 (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 50 U penicillin/ml, 50 μg streptomycin/ml, and 2 mM glutamine. This media was subsequently used for all washing procedures and for culturing of the cells. Undispersed clumps of tissue were allowed to settle by gravity, the supernatant single cell suspensions removed, and the cells washed once in culture media. Bone marrow cells were obtained from femura which were opened at both ends and flushed out with medium, using a syringe and a 19 gauge needle. The cells were suspended in RPMI 1640 by repeated aspiration through a pipet and washed twice. Peripheral blood was obtained by heart puncture and centrifuged for 10 min at $80 \times g$. The leucocyte-rich supernatant was then withdrawn and the cells washed once in medium. All cell suspensions were finally resuspended at a cell density of 5×10^6 per milliliter.

Purification of Lymphocytes.—Spleen and bone marrow suspensions were freed from phagocytic cells by incubation on cotton wool columns according to the method of Rosenstreich et al. (6). Further purification was achieved by incubation of the column effluent cells in 100 $\times$ 15 mm plastic petri dishes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) for 3 h, after which the nonadherent cell population was carefully decanted. Lymphocyte populations obtained by these combined techniques contained less than 0.1% of phagocytic cells as tested by the uptake of latex particles. In some experiments bone marrow cells were also purified by Ficoll-Hypaque gradients, using the method of Böyum (7).

Preparation of ^{51}Cr-Labeled Target Cells.—Human, burro, or chicken blood was mixed with equal amounts of Alsever's solution and then stored at 4°C for 2–14 days. Before use red cells were washed twice with balanced salt solution (BSS) and 10^7 erythrocytes incubated with 200 μCi of sodium^{51}chromate (Amersham/Searle Corp., Arlington Heights, Ill.) for 30 min at 37°C in the presence of 5% fetal calf serum (FCS). After labeling the cells were washed again three times in BSS and finally resuspended in BSS containing 5% FCS at a cell density of 5×10^5/ml. To exclude a lytic effect of the mitogens themselves on the tested erythrocytes, 5×10^6 unlabeled erythrocytes were incubated with 5×10^4 ^{51}Cr-labeled target cells and various dilutions of mitogen. No lysis was observed in any of these experiments.

Mitogens and Absorptions.—In preliminary experiments a range of concentrations of each mitogen was tested. These were 1–100 μl/ml of PHA (Difco Laboratories, Detroit, Mich.), 1–100 μl/ml of PWM (Grand Island Biological Co.), and 1–100 μg/ml of Con A (Calbiochem, San Diego, Calif.). Optimal doses to induce CEL were 10 μl/ml of PHA, 10 μl/ml of PWM, and 10 μg/ml of Con A. Appropriately diluted mitogens were absorbed twice with packed suspensions of washed red cells at a ratio of 3:1 for 30 min at 4°C.

Cytotoxicity Tests.—Cytotoxicity tests were performed as previously described (8). All leucocyte suspensions were cultured in 16 $\times$ 125 mm plastic culture tubes (Falcon Plastics). Unless otherwise indicated, the incubation period was 48 h. $\frac{1}{10}$ ml of mitogen solution and 5×10^4 ^{51}Cr-labeled erythrocytes were added simultaneously to the leucocyte suspension at the beginning of the experiments. After incubation, the culture tubes containing a total volume of 1.2 ml were centrifuged for 10 min at $1{,}300 \times g$ and 0.6 ml of the supernatant removed. Both supernatant and the remaining 0.6 ml (referred to as pellet in the formula) of each culture were counted separately in an Auto-gamma counter (Packard Instrument Co., Downers Grove, Ill.).

The results were calculated by the following formula:

$$\%\ ^{51}\text{Cr release} = \frac{\text{supernatant cpm} \times 2}{\text{supernatant cpm} + \text{pellet cpm}} \times 100.$$

Every determination was done in triplicate and results expressed as mean percent ^{51}Cr release $\pm$ 1 SE.

Lymphocyte Transformation Studies.—Our methodology for culturing chicken leucocytes and assessing lymphocyte transformation by the uptake of [^{3}H]thymidine (TdRH3) has been

described in detail (9). Briefly, 5×10^6 spleen or bone marrow cells were cultured in 1 ml of serum-free RPMI 1640 for 72 h with various doses of PHA, Con A, and PWM, and the uptake of TdRH3 in the TCA-insoluble material was determined. All cultures were done in triplicate and results are given as mean counts per minute per culture (cpm) $\pm$ SE.

Induction of Agammaglobulinemia.—To induce agammaglobulinemia in chickens, cyclophosphamide—(Cytoxan; Mead Johnson, Evansville, Ind.) treatment of newborn chickens was performed as previously described (9). All surviving chickens were tested for the presence of detectable serum immunoglobulins by double immunodiffusion in agar (10) at the age of 6 wk. Chickens having less than 0.1% of the normal adult immunoglobulin level by this method were defined as agammaglobulinemic (ag) and subsequently used. At various occasions leucocyte suspensions from the spleen and peripheral blood of normal and ag chickens were also tested for the presence of cells with surface bound immunoglobulins by a modification of the method of Kincade et al. (11). Briefly, cell suspensions containing 5×10^6 mononuclear cells were incubated with 0.8 μm latex beads at 37°C for 45 min to label phagocytic cells. The washed cell suspension was then incubated with either a polyspecific rabbit antichicken immunoglobulin antiserum or with a specific antichicken L chain antiserum. After 30 min of incubation at 0°C the cells suspensions were washed with cold BSS containing 1% human albumin, and then incubated with FITC-conjugated sheep antirabbit IgG. Control cell suspensions, incubated with FITC conjugated antirabbit IgG alone were always included. After three additional washes, the number of lymphocytes with surface-bound immunoglobulins were enumerated under oil immersion using a combination of incident light fluorescence and phase contrast microscopy.

Antisera.—Antichicken thymocyte sera (ATS) were raised in burros by injecting 10^9 thymocytes from chickens less than 3 wk old, emulsified in complete Freund's adjuvant, followed by a booster injection of the same cell dose in incomplete Freund's adjuvant. Antichicken immunoglobulin serum (anti-Ig) was raised in sheep according to methods previously described (10). This antiserum reacted only with immunoglobulin as determined by immunoelectrophoresis against normal and agammaglobulinemic chicken serum. Chicken spleen and bone marrow cells were incubated for 30 min at 37°C in the presence of a 1/5 dilution of the different antisera and their corresponding control sera which had been heated at 56°C for 30 min. After incubation the cells were washed three times in RPMI 1640, readjusted to a cell density of 5×10^6/ml and used in the cytotoxicity assay as described.

RESULTS

Effect of Different Mitogens on the Development of CEL in Chicken Spleen Cell Cultures.—Various combinations of three different target cells, HRC, BRC, and CRC, and three different mitogens PHA, Con A, and PWM, were investigated. PHA-induced lysis of all three types of target cells (Table I). However, in the presence of PWM only HRC were lysed, while BRC and CRC were unaffected. Similarly, Con A-treated spleen cells lysed only BRC, but not HRC and CRC. PHA-stimulated cultures released 30–50% ^{51}Cr during 48 h of culture, while the amount of cytotoxicity, induced by PWM and Con A was usually somewhat higher in the range of 45 to 70%. Control spleen cells cultured without mitogen released about 5 to 15% ^{51}Cr from the various targets during 48 h of culture. The three mitogens themselves had no direct lytic effects on the tested erythrocyte target cells. "Target cell specificity" was observed not only with spleen cells but also when leucocytes from other lymphoid organs were investigated. Therefore, in subsequent exper-

TABLE I

Lysis of Different Target Erythrocytes by Chicken Spleen Cells in the Presence of PHA, PWM, and Con A

	Chicken red cells	Burro red cells	Human red cells
None	10.0 ± 0.6*	6.0 ± 0.6	8.8 ± 0.8
PHA	52.7 ± 2.0	27.4 ± 1.0	31.5 ± 2.0
PWM	13.8 ± 0.5	6.5 ± 0.7	60.0 ± 1.0
Con A	14.2 ± 1.2	50.6 ± 1.3	6.3 ± 1.2

* Mean percentage of ^{51}Cr release ± SE.

iments only three mitogen target cell combinations were used, consisting of PWM + HRC, Con A + BRC, and PHA + CRC.

Effect of Preabsorption on the Activity of PWM and Con A.—The observed "target cell specificity" of two mitogens which are both known to stimulate DNA synthesis in chicken lymphocyte cultures (9) was further investigated. PWM and Con A were absorbed with erythrocytes from human, burro, and chicken origin. These experiments demonstrated that the ability of Con A to induce cytotoxicity could be removed only by absorption with BRC (Table II). Preabsorption with HRC and CRC had no effect under the conditions tested. Similarly the activity of PWM was removed by absorption with HRC, but not with BRC or CRC. In the same experiment the ability of the absorbed mitogens to induce lymphocyte transformation was also determined by the uptake of TdRH3. Absorption with CRC did not affect the mitogenic activity of either mitogen, while preabsorption with the appropriate target cells removed the mitogenic activity of both PWM and Con A. It might be inferred from these experiments that only BRC have receptors for Con A and only HRC have receptors for PWM.

Effect of Mixing of Different Target Erythrocytes.—In order to determine whether lymphocytes, when stimulated by one mitogen and the appropriate target, will also lyse inappropriate targets, the following experiment was designed. Chicken spleen cells were incubated simultaneously with PWM, unlabeled HRC, and ^{51}Cr-labeled BRC. Conversely, Con A was added together with unlabeled BRC and labeled HRC. The results of this experiment are summarized in Table III. They clearly show that nonspecific lysis of inappropriate target cells did not occur at a time when there was significant lysis of the appropriate targets.

Kinetics of Phytomitogen-Induced Cytotoxicity.—Kinetic studies were performed using chicken spleen cells and the three appropriate mitogen-target cell combinations. In these experiments a very similar pattern of reactivity was found with all three mitogens (Table IV). Significant cytotoxicity usually could be detected within 4–8 h. The percentage of ^{51}Cr release then gradually increased and the reactions were completed within 24–48 h. Control cultures of

TABLE II

Comparison of Con A and PWM-Induced Cytotoxicity and Lymphocyte Transformation after Absorption of the Mitogens with Different Erythrocytes

Mitogen	Absorbed with	^{51}Cr release*	Lymphocyte transformation‡
		%	
None	—	15.3 ± 1.4	710 ± 63
Con A	—	64.3 ± 3.4	25,372 ± 1,640
Con A	HRC	87.1 ± 3.4	26,848 ± 1,280
Con A	BRC	22.1 ± 1.9	570 ± 42
Con A	CRC	60.6 ± 1.6	27,348 ± 1,420
None	—	16.8 ± 1.6	—
PWM	—	73.3 ± 3.3	14,512 ± 740
PWM	HRC	10.9 ± 0.2	748 ± 45
PWM	BRC	61.7 ± 1.3	7,680 ± 753
PWM	CRC	70.6 ± 2.9	15,116 ± 821

* Con A was tested with ^{51}Cr-labeled burro erythrocytes (BRC) as targets and PWM with ^{51}Cr-labeled human erythrocytes (HRC).

‡ TdRH3 uptake was measured at 72 h, results are given as mean cpm of triplicate cultures ± 1 SE.

TABLE III

Effect of Mixing Different Target Erythrocytes on PWM- and Con A-Induced Development of Cytotoxic Effector Cells in Chicken Spleen Cultures

Mitogen	Target cells	
	[^{51}Cr]HRC + unlabeled BRC	[^{51}Cr]BRC + unlabeled HRC
None	5.1 ± 0.5	15.3 ± 0.3
PWM	36.6 ± 4.7	14.1 ± 1.6
Con A	7.6 ± 1.6	42.2 ± 1.4

TABLE IV

Kinetics of Mitogen-Induced Cytotoxicity by Chicken Spleen Cells When Different Mitogen Target Cell Combinations Are Used

Hours	PWM-HRC*	Con A-BRC	PHA-CRC
4	23.7 ± 0.6‡	19.9 ± 0.9	17.3 ± 1.3
8	34.5 ± 2.0	28.4 ± 1.1	20.2 ± 0.6
12	37.5 ± 2.0	30.0 ± 1.3	23.6 ± 1.7
24	53.5 ± 2.4	53.7 ± 2.6	30.4 ± 3.7
48	67.3 ± 1.8	69.6 ± 1.6	38.9 ± 0.7

* Pokeweed mitogen–human red cells; concanavalin A–burro red cells; phytohemagglutinin–chicken red cells.

‡ Mean percent ^{51}Cr release of triplicate samples ± SE, all mitogen-free controls of this experiment were $<11\%$.

spleen cells and targets without mitogen released from 6 to 13% chromium during this incubation period.

Preincubation of Lymphocytes With Mitogen.—Since significant target cell killing could be demonstrated within a few hours after the addition of the mitogens, we sought to determine if the lymphocytes were actually activated to become cytotoxic during this period of incubation, or whether these early cytotoxic effects could be due to a pre-existing population of effector cells which only required a link to the target for expression of their cytotoxic potential. Spleen cells were preincubated for 8 h in the presence of Con A, PWM, or saline as a control. Labeled target cells were then added and the cultures incubated for another 8 h with or without additional mitogen. Although 8 h of incubation is ordinarily sufficient for very significant target cell killing (Table IV), it was found that lymphocytes preincubated with mitogen for 8 h were subsequently no more cytotoxic than those preincubated with saline (Table V). This suggests that new killer cells are not generated during this period but rather that pre-existing effector cells become cytotoxic only after linkage to the target cell by the mitogen. Longer periods of preincubation of lymphocytes with mitogen have been reported to induce the development of blast cells with high cytotoxic activity (2). Our data is not necessarily in conflict with these observations since our experiments were principally concerned with the period of time during which no morphologic change in the lymphocytes could be detected.

Mitogen-Induced Cytotoxicity By Leucocytes from Various Lymphoid Organs of the Chicken.—The cytotoxic potential of mitogen-stimulated leucocyte cultures from chicken thymus, bursa of Fabricius, bone marrow, and peripheral blood was compared. A vigorous response was found in blood and bone marrow cells which equalled the reaction of spleen cells (compare Tables I and VI). Cultures of thymocytes were only weakly reactive when stimulated with PHA, but showed significant cytotoxic reactions when PWM and Con A and their

TABLE V

Effect of Preincubation with Mitogen on the Subsequent Lysis of Target Cells by Chicken Spleen Cells

8 h preincubation with	8 h 2nd incubation with [^{51}Cr]HRC	
	+ PWM	− PWM
Saline	25.8 ± 0.8	5.3 ± 0.2
PWM	28.6 ± 1.1	27.3 ± 0.4
	8 h 2nd incubation with [^{51}Cr]BRC	
	+ Con A	− Con A
Saline	27.1 ± 0.9	8.9 ± 1.1
Con A	28.2 ± 1.3	26.3 ± 2.1

appropriate target cells were used. Bursal cells were either not cytotoxic at all or more frequently very low in activity (as in the experiment shown in Table VI).

Cytotoxic Reactivity of Leucocytes from Agammaglobulinemic Chickens.—To determine the role of bursal-derived B cells in the mitogen-induced development of CEL, spleen cells from normal chickens were compared with those from ag chickens which lacked B cells detectable by immunofluorescence studies (Table VII). The comparison between these two cell populations is shown in Table VIII. No differences were found between normal and ag chicken spleen cells when various mitogen-target cell combinations were tested. These findings clearly indicate that mitogen-induced cytotoxicity does not require B cells and suggest that it is a function of thymic derived (T) cells.

Investigation of "Purified" Spleen Cells.—Spleen cells prepared in the usual manner contained 10–15% phagocytic cells as determined by the uptake of latex particles. After passage over cotton wool columns and subsequent incubation in plastic petri dishes, populations were obtained which contained less than 0.1% phagocytic cells. These populations were as active in performing PHA-, PWM-, and Con A-induced cytotoxicity as normal unpurified cell

TABLE VI

Development of Cytotoxic Effector Lymphocytes in Leucocyte Cultures from Various Lymphoid Organs of the Chicken

Mitogen/target	Thymus	Bone marrow	Blood	Bursa
PHA-CRC	18.8 ± 0.6*	34.5 ± 1.8	36.5 ± 1.5	21.6 ± 0.8
None-CRC	15.9 ± 0.9	10.7 ± 0.3	10.9 ± 1.6	15.5 ± 1.0
PWM-HRC	26.4 ± 4.7	67.6 ± 2.2	36.7 ± 1.0	14.2 ± 2.1
None-HRC	9.5 ± 1.5	11.2 ± 0.2	5.1 ± 0.9	10.9 ± 0.4
Con A-BRC	24.6 ± 0.7	68.4 ± 1.2	49.5 ± 1.7	13.2 ± 0.3
None-BRC	7.1 ± 1.1	15.1 ± 0.8	9.8 ± 0.7	10.2 ± 0.9

* Mean percent ^{51}Cr release of triplicate samples ± 1 SE.

TABLE VII

Percentage B Cells in Various Lymphoid Organs from Normal- and Cyclophosphamide-Induced Agammaglobulinemic Chickens*

	Normal	Agamma*
	%	%
Spleen	24.2 ± 6 (7)‡	0.0 (8)
Thymus	8.6 ± 4 (7)	0.0 (8)
Blood	18.8 ± 5 3)	0.0 (4)
Bursa	89.7 ± 5 (8)	—

* At least 1,000 cells were scanned in every preparation for the presence of cells with surface-bound immunoglobulins.

‡ Number of birds examined are given in parenthesis.

TABLE VIII

Comparison of the Development of Cytotoxic Effector Cells in Spleen and Bone Marrow Cultures from Normal and Agammaglobulinemic Chickens

Mitogen/target	Normal chicken		Agammaglobulinemic chicken	
	Spleen	Bone marrow	Spleen	Bone marrow
PHA-CRC	40.9 ± 0.3	50.1 ± 0.9	42.7 ± 3.6	52.1 ± 1.2
None-CRC	10.0 ± 1.2	8.3 ± 0.6	10.1 ± 0.6	9.2 ± 1.0
PWM-HRC	60.0 ± 1.0	62.3 ± 1.6	75.2 ± 2.9	73.2 ± 2.6
None-HRC	8.8 ± 0.8	7.7 ± 0.4	8.1 ± 0.3	7.9 ± 0.8
Con A-BRC	53.9 ± 2.1	58.6 ± 2.0	57.3 ± 1.9	60.2 ± 1.6
None-BRC	15.2 ± 0.5	13.2 ± 0.9	15.6 ± 2.3	14.1 ± 1.0

TABLE IX

Comparison between Mitogen-Induced Cytotoxicity of Column-Purified and Unpurified Spleen Cell Populations

Mitogen/target	Column-purified	Unpurified
HRC-None	4.8 ± 0.6	5.5 ± 1.8
HRC-PWM	54.3 ± 1.6	61.4 ± 2.5
BRC-None	5.1 ± 0.6	10.5 ± 1.0
BRC-Con A	59.6 ± 2.4	64.6 ± 2.5
CRC-None	4.7 ± 0.3	7.4 ± 0.5
CRC-PHA	50.3 ± 2.3	45.7 ± 3.3

suspensions (Table IX). These results indicate that mitogen-induced target cell lysis is not dependent on the presence of a phagocytic cell population.

Effect of ATS and Anti-Ig on Mitogen-Induced Cytotoxicity.—It has been shown that PHA-induced cytotoxicity can be inhibited by preincubation of the aggressor cells with antilymphocyte serum (12). In our studies preincubation of chicken spleen cells with burro antichicken thymocyte serum in the absence of complement led to a complete blocking of the PHA-, PWM-, and Con A-induced cytotoxicity (Table X). Normal burro serum which was tested in parallel cultures had no effect. In addition, a sheep antichicken Ig serum was tested under the same conditions. This antiserum had no inhibitory effect on the development of CEL. No differences were seen between the anti-Ig serum and the normal sheep serum.

Comparison of Lymphocyte Transformation of Spleen and Bone Marrow Cells.—Since bone marrow cells have been shown to be equally cytotoxic as spleen cells in our studies (Table VIII) in vitro lymphocyte transformation of cells from these two organs was also compared. Spleen cells of chicken responded vigorously to all three mitogens, as measured by the uptake of TdRH3. However, bone marrow cells were unreactive over a wide range of mitogen doses (Table XI).

TABLE X

Effect of Antithymocyte Serum and Antiimmunoglobulin Serum on the Development of Cytotoxic Effector Lymphocytes in Cultures of Chicken Spleen Cells*

	Antithymocyte serum (burro)	Normal burro serum	Antiimmunoglobulin (sheep)	Normal sheep serum
HRC-PWM	7.3 ± 1.5	40.7 ± 0.9	59.3 ± 5.7	58.7 ± 4.2
HRC-None	6.7 ± 0.6	10.9 ± 1.5	7.8 ± 1.1	9.3 ± 1.2
BRC-Con A	22.5 ± 1.0	52.3 ± 4.8	62.1 ± 3.4	64.2 ± 5.1
BRC-None	16.1 ± 1.0	15.3 ± 0.2	14.2 ± 1.1	13.7 ± 1.4
CRC-PHA	16.4 ± 1.4	25.7 ± 1.5	33.2 ± 1.6	36.0 ± 1.8
CRC-None	14.5 ± 1.6	11.3 ± 0.2	10.0 ± 0.9	11.2 ± 0.4

* Chicken spleen cells were incubated in 1/5 dilutions of the antisera and their corresponding control sera without complement for 45 min and then washed three times before culturing.

TABLE XI

Comparison of Lymphocyte Transformation in Culture of Chicken Spleen and Bone Marrow Cells

	Spleen	Bone marrow
Control	683 ± 38*	1,840 ± 211
PHA	73,112 ± 3,120 (5 μl/ml)	2,130 ± 188 (5 μl/ml)
PWM	44,817 ± 1,070 (5 μl/ml)	1,931 ± 180 (10 μl/ml)
Con A	1,103,012 ± 6,540 (5 μg/ml)	2,240 ± 212 (10 μg/ml)

* Lymphocyte transformation was determined by the uptake of TdRH3 at 72 h. Results are given as mean counts per minute of triplicate samples ± 1 SE.

‡ Various concentrations of the three mitogens were tested with both cell types. Only the maximal responses are listed.

Characterization of the CEL in the Bone Marrow.—A number of experimental approaches were employed to further characterize the CEL of the bone marrow. It became readily apparent that bone marrow cells from B cell free ag chickens did not differ in mitogen-induced cytotoxicity from matched normal controls (Table VIII). Passage of bone marrow cells over cotton wool columns and subsequent incubation in plastic petri dishes did not remove their cytotoxic potential (Table XII). These findings suggest that the cell of the bone marrow which performs mitogen-induced killing of target cells is neither a B cell nor a mature phagocytic cell. Additionally, fractionation of bone marrow cells on Ficoll-Hypaque gradients was performed. This technique resulted in a cell population which was enriched in lymphocytes, but still contained a significant number of cells from the hemopoietic series. This cell population was also fully active in performing PHA-, PWM-, or Con A-induced cytotoxicity. These observations suggest that the cytotoxic effector cell of the bone marrow is also a T lymphocyte. However, participation of other cell populations of the bone marrow cannot be completely ruled out.

TABLE XII

Effect of Various Treatment on the Mitogen-Induced Cytotoxicity of Chicken Bone Marrow Cells

Mitogen/target	Untreated	Column purification	Ficoll-Hypaque
PWM-HRC	67.3 ± 2.8	68.6 ± 5.3	82.1 ± 9.1
None-HRC	7.3 ± 0.8	6.2 ± 0.7	7.2 ± 1.4
Con A-BRC	58.7 ± 2.5	63.7 ± 2.4	68.1 ± 7.0
None-BRC	9.3 ± 1.8	10.1 ± 1.1	7.6 ± 0.6
PHA-CRC	48.1 ± 3.1	53.2 ± 6.1	55.1 ± 2.4
None-CRC	7.8 ± 1.8	6.5 ± 0.4	5.8 ± 0.6

DISCUSSION

In the present investigation it was demonstrated that PWM and Con A are as effective as PHA in inducing the development of CEL. PWM and Con A also have been shown to be active in certain other in vitro cytotoxic systems (2, 3), but Perlman et al. found Con A to be inactive under conditions where PHA induced lymphocyte-mediated target cell lysis (4). We have clearly demonstrated that characteristics of the target cell are crucial in this kind of in vitro cytotoxic reaction. Thus, PWM-treated chicken spleen cells lysed only HRC, but not CRC and BRC. Similarly Con A-induced cytotoxicity was directed only against BRC, but not against the two other types of targets. Preabsorption experiments also strengthened this observation since absorption only with the appropriate target cell removed subsequent activity in the cytotoxicity assay.

This "target cell specificity" was also used as an experimental tool to shed some light on the mechanisms of mitogen-induced in vitro lymphocyte cytotoxicity. In cultures containing mixtures of target cells appropriate and unappropriate for a given mitogen, only the appropriate target cells were lysed in the presence of this mitogen. This provides strong evidence against a significant role of soluble lymphotoxins in this type of lymphocyte-mediated target cell lysis. Further, it suggests that target cell killing can only be initiated when cross-linking between the targets and the lymphocytes has been accomplished, and that the mitogen provides this link. This cytotoxic process has been previously shown to be dependent only on energy requiring events and to be independent of the later events of mitogen induced lymphocyte transformation such as protein, RNA, and DNA synthesis (13). Our studies demonstrate that significant target cell lysis can be detected at an early time in culture and that lymphocytes preincubated with mitogen do not have enhanced cytotoxic capacity. This suggests that mitogen does not induce differentiation of a new effector cell population and that many of the CEL detected by this assay may be a pre-existing class of cells requiring only a link to the target for expression of their cytotoxic potential.

Another concern of this study was to more precisely characterize the ag-

gressor cell in mitogen-induced cytotoxicity, since it has been previously shown that not all lymphoid cells can be stimulated by mitogens to become cytotoxic. For example, peripheral leukocytes from patients with chronic lymphocytic leukemia were found to be ineffective (14). We have found that spleen and peripheral blood leukocytes are highly active, while thymocytes show a lower but significant capacity to lyse target cells. Bursal cells either showed no or a very low activity in our experiments. Since the bursa of Fabricius in chickens is populated predominantly by B cells (11) this observation suggests that B cells are not the effector cells in mitogen-induced cytotoxicity.

There have been conflicting data reported about the role B and T lymphocytes play in various cytotoxic reactions. Specific target cell killing by lymphocytes from sensitized donors has been demonstrated to be a T cell function (15). By contrast, good evidence has been presented that antibody-mediated lymphocyte-dependent cytotoxicity is not performed by T lymphocytes (16, 17). The chicken provides a unique model to determine the contribution of B cells to mitogen-induced cytotoxic reactions since complete elimination of B cells and agammaglobulinemia can be readily induced with cyclophosphamide treatment during the first days of extraembryonic life. We have found that the development of CEL in spleen and bone marrow cultures from ag chickens, stimulated by PHA, PWM, or Con A is completely normal. This indicates that B cells are not needed in these cytotoxic reactions and suggests that these reactions are a function of T cells. This concept is further supported by the observation that anti-Ig sera did not block this reaction, while antithymocyte sera completely inhibited target cell killing. Very recently it was found that mitogen-induced lymphocyte cytotoxicity in mice is also a T cell function (18).

To exclude the possibility that phagocytic cells were responsible for the target cell lysis observed, chicken spleen cells were freed of adherent cells by a combined technique of incubation-filtration through cotton wool columns and adherence to plastic petri dishes. The resulting cell population which contained less than 0.1% cells, capable of phagocytosis of latex particles was as effective in killing of target cells as an unpurified spleen cell population containing a 100-fold greater concentration of phagocytes. Thus, these data effectively eliminate a requirement for either phagocytic cells or B cells in mitogen-induced target cell lysis and again strongly suggests that these reactions are a function of T lymphocytes.

An interesting dissociation in mitogen-induced responses of some cell populations has become apparent during these studies. We have previously reported that the in vitro proliferative responses of chicken lymphocytes to PHA, PWM, and Con A are also a function of T lymphocytes (9). Spleen, peripheral blood, and thymus cells are all capable of proliferative responses as well as cytotoxic reactions with mitogen stimulation. Bone marrow cells, however, do not proliferate after stimulation with any of the three phytomitogens, while the same cells are as active as spleen cells in performing vigorous cytotoxic reactions. The bone marrow of the chicken contains a significant number of T cells

as evidenced by the ability of chicken bone marrow cells to elicit a graft-vs.-host reaction (19). Our studies suggest, but do not definitively prove that the effector cells in the bone marrow are also T lymphocytes. It is intriguing to hypothesize that the dissociation in mitogen-induced cell reactivities may be detecting functional differences between two different types of thymic-dependent lymphocytes.

This dissociation in mitogen-induced lymphocyte reactions also may explain why high lymphocyte:target cell ratios are required in the cytotoxicity test even though these phytomitogens are capable of inducing proliferation in a majority of peripheral T lymphocytes. Thus, the subpopulation of T cells responsible for mitogen-induced target cell lysis may only represent a small population of the total peripheral T cell population.

SUMMARY

Cultures of chicken lymphoid tissues were tested for their capacity to lyse ^{51}Cr-labeled chicken, burro (BRC), and human red blood cells (HRC) in the presence of phytomitogens. PHA-stimulated cultures lysed all three types of targets, while PWM and Con A showed a "target cell specificity" for HRC and BRC, respectively. In mixtures of target cells only the appropriate targets were lysed by lymphocytes activated by either Con A or PWM indicating that soluble lymphotoxins do not play a major role in these reactions. Preincubation experiments suggested that there may be a population of pre-existing aggressor cells which only require linking to the targets by the mitogens for activation of their cytotoxic potential.

Strong cytotoxic reactions were found with spleen cells, peripheral blood leucocytes, and bone marrow cells. Thymocytes were less active but could be stimulated for significant cytotoxicity, while bursal cells were generally unreactive. Spleen cells from agammaglobulinemic chickens totally lacking serum immunoglobulins and B cells with surface-bound immunoglobulins were as active as cells from normal chickens. The activity of spleen cells, from which phagocytic cells were removed was also unimpaired. These results indicate that the development of cytotoxic effector lymphocytes in mitogen-treated leucocyte cultures is a property of T lymphocytes. Although bone marrow cells fail to proliferate in response to these phytomitogens, they do have strong cytotoxic reactivity suggesting that different subsets of thymic-derived lymphocytes are responsible for mitogen-induced transformation and mitogen-induced cytotoxicity.

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