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Enumeration of Activated Thymus-Derived Lymphocytes by the Virus Plaque Assay

(cell-mediated immunity/mitogen stimulation/mixed lymphocyte culture)

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ABSTRACT Lymphocytes activated by antigens or mitogens acquire the capacity to replicate viruses, and the number of activated lymphocytes can be estimated by the virus plaque assay. Concanavalin A and pokeweed mitogen produced 33-fold and 17-fold increases in virus plaque-forming cells (V-PFC), respectively, above background, while lipopolysaccharide produced only a 2- to 3-fold increase. T (thymus-derived lymphocyte)-depleted lymphocyte populations, derived from anti-θ-treated or nude (athymic) mouse spleens, failed to produce V-PFC after culture with concanavalin A or pokeweed mitogen. The present studies thus demonstrate that the virus plaque assay measures activated T-lymphocytes.

A dissociation between the V-PFC response and cell proliferation was previously observed in antigen-stimulated cells cultured in the presence of mitotic inhibitors. In the present studies, while stimulation of CBA ($H2^k$) lymphocytes by DBA/2 ($H2^d$) cells produced high levels of thymidine incorporation, lymphocyte target-cell cytotoxicity, and V-PFC, stimulation of BALB/c ($H2^d$) lymphocytes against DBA/2 ($H2^d$) cells resulted in even higher levels of thymidine incorporation with a virtual absence of cytotoxic lymphocytes or V-PFC. These results indicate that proliferation is not a sufficient condition for permitting lymphocytes either to exert cytotoxicity on target cells or to replicate viruses, and suggest that there may be a correlation between the development of V-PFC and cytotoxic lymphocytes. They are consistent with the view that there are at least two functional subpopulations of T-lymphocytes.

Thymus-derived lymphocytes (T-cells) play a major role in cell-mediated immune responses such as delayed type hypersensitivity, allograft rejection, tumor immunity, and cellular resistance to infection (1). In addition they serve as helper cells in the production of antibodies to thymus-dependent antigens (2). The nature of T-cell function has been elucidated by *in vitro* studies of T-lymphocyte activation (3, 4), development of T-lymphocyte cytotoxicity (5, 6), and production of various products of activated lymphocytes or mediators (4, 7). However, the principal obstacle to a more complete understanding of the cell-mediated immune response has been the lack of a simple, general, and reproducible method for enumerating activated T-lymphocytes, analogous to the Jerne plaque assay for antibody-producing cells.

Abbreviations: V-PFC, virus plaque-forming cell; Con A, concanavalin A; PHA, phytohemagglutinin; PWM, pokeweed mitogen; LPS, lipopolysaccharide; VSV, vesicular stomatitis virus, T and B, thymus- and bone-marrow-derived (lymphocytes), respectively.

A virus plaque assay has been developed as a method for enumerating antigen- and mitogen-activated lymphoid cells (8, 9). The assay is based on the earlier observation that while unstimulated small lymphocytes do not support the replication of RNA viruses, antigen- or mitogen-stimulated lymphocytes rapidly acquired the capacity to do so (see ref. 8). The enhancement of virus production in culture is due to an increase in the number of lymphocytes replicating virus, and increased virus plaque-forming cells (V-PFC) have been observed in cultures of antigen-stimulated lymphocytes obtained from delayed hypersensitive human (10) and guinea pig donors (8) and in mixed lymphocyte cultures (11). The rate of increase in V-PFC in cultures stimulated with purified tuberculin (PPD) was linear over a 4-day culture period and unaffected by the mitotic inhibitor, vinblastine (10). This finding suggested the existence of a nondividing, antigen-sensitive cell, in contrast to the exponentially proliferating blasts cells in the same cultures.

The purpose of the present study is 2-fold: first, to clarify the nature of the virus plaque-forming cell, i.e., whether activated T- or B-lymphocytes or both are detected by the virus plaque assay; and second, to investigate the relationship of the V-PFC to the proliferating cells in mixed lymphocyte cultures and to the cytotoxic lymphocytes derived from them. The basic experimental design was to explore the enhancement of production of V-PFC in mouse spleens by mitogens that selectively stimulate T-lymphocytes (concanavalin A and phytohemagglutinin), B (bone-marrow-derived)-lymphocytes (lipopolysaccharide), and both T- and B-lymphocytes (pokeweed mitogen).

MATERIALS AND METHODS

Animals. Female C57Bl/10, CBA, DBA/2, and BALB/c mice were obtained from Jackson Laboratories, Bar Harbor, Me., and were used at 6–12 weeks of age. Congenitally athymic (nude) mice (nu/nu) were used at 3.5 weeks of age.

Cell Suspensions and Cultures. Spleens were removed aseptically. Dissociated cells were washed twice, suspended in medium RPMI 1640 supplemented with 5% heat-inactivated fetal-calf serum (Reheis Chemical Co., Chicago, Ill., lot no. 72208), 2 mM L-glutamine, and 100 units of penicillin and 100 μg of streptomycin per ml, and adjusted to 15×10^6 viable cells per ml. The cells (1.5 ml) were cultured in large Leighton tubes (Bellco Glass Inc., Vineland, N.J.) at 37° in 5% CO_2

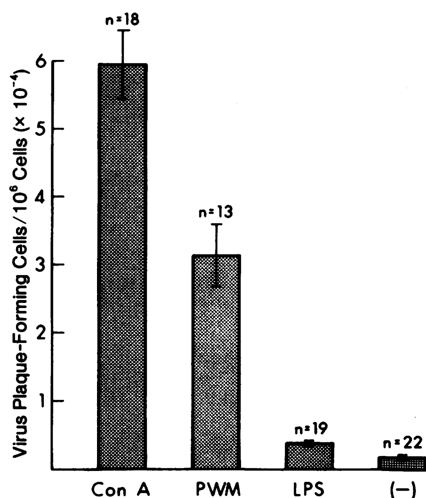

FIG. 1. Virus plaque-forming cells in mitogen-stimulated spleens. Normal C57B1/10 spleen cells were stimulated by Con A (2.5 μg/ml), PWM (1:10), or LPS (100 μg/ml) for 2 days. Results are expressed as V-PFC/10^6 viable cells plated (mean ± SE). n = number of experiments.

in air. Cell counts were made in 0.2% trypan blue; viable cells ranged from 80–95% after washing.

Mitogens. Concanavalin A (Con A) (Calbiochem, Los Angeles, Calif.) was purified on Sephadex (12). Pokeweed mitogen (PWM) (GIBCO, Grand Island, N.Y.) was reconstituted with distilled water. *Escherichia coli* 026:B6 lipopolysaccharide (LPS) (Difco Laboratories, Detroit, Mich.) was dissolved in phosphate-buffered saline (pH 7.2) at 1 mg/ml and boiled for 2 hr. The optimal doses for the virus plaque assay were Con A, 2.5 μg/ml, PWM, 1:10, and LPS, 100 μg/ml. Control cultures were prepared without mitogens in all experiments. Con A-stimulated cells recovered from cultures were treated twice with 0.1 M α-methyl-D-mannoside at 37° for 10 min to remove Con A.

Virus Plaque Assay (9). In brief, 1.5×10^6 viable cells were resuspended in 0.2 ml of 6% fetal-calf serum in Eagle's minimal essential medium (GIBCO Grand Island, N.Y.), infected with vesicular stomatitis virus (VSV) at a multiplicity of infection, for these experiments, of 100 for 2 hr at 37°. After infection cells were washed once and treated with 0.02 ml of guinea pig anti-VSV serum for 1 hr at 4° to neutralize free virus. After three washings, aliquots of suspensions of virus-infected cells were plated in a thin layer of agar on a monolayer of indicator L-cells in 60-mm plastic culture dishes. Three log dilutions of each sample were plated; each was done in duplicate. The plates were incubated for 2 days at 37° in 5% CO_2 in air, and the virus plaques were counted after they were stained with neutral red.

TABLE 1. *Virus plaque-forming cells in T-depleted spleens*

Spleen cells	ΔV-PFC induced by mitogens		
	Con A	PWM	LPS
A. NMS + C treated	7,020	5,200	435
Anti-θ + C treated	45	48	544
B. nu/+	16,000	14,700	1,840
nu/nu	0	2,890	4,960

(A) C57B1/10 spleen cells were treated with anti-θ serum + complement on day 0. (cells 2.5×10^6/ml, anti-θ serum 1:32, absorbed guinea pig complement 1:12, final concentration, at 37° for 45 min). Surviving cells were cultured (5×10^6/ml) with mitogens for 2 days. The results are expressed as ΔV-PFC/10^6 cells treated. ΔV-PFC, V-PFC in mitogen-stimulated cultures; V-PFC in unstimulated cultures; NMS, normal mouse serum; C, complement. (B) Nude mouse spleen cells were cultured with mitogens for 2 days. The results are expressed as V-PFC/10^6 viable cells plated. nu/+, heterozygote littermate; nu/nu, homozygote nude mouse.

Isoantiserum and Complement. AKR anti-θC_3H serum was made by repeated thymocyte injection (13). Congenic anti-θ (θ/AKR^b anti-ASL1) serum and anti-PC.1 [(C57Bl/6 × DBA/2) F_1 anti-BALB/c MOPC 70A] serum was kindly given by Drs. E. A. Boyse and H. Sato. Rabbit complement was absorbed with EL-4 cells in the presence of EDTA (14).

Assays of Thymidine Incorporation and Lymphocyte Cytotoxicity. [^{3}H]Thymidine (2 μCi, specific activity 6.0 Ci/mmol) was added for a 24-hr period to assess DNA synthesis, and labeled cells were harvested at days 3 and 4. Lymphocyte cytotoxicity was assayed on day 4 by the method described by Cerottini and Brunner (15) at a lymphocyte: target cell ratio of 60:1. ^{51}Cr-release was assayed at 5 hr.

RESULTS

An increase in virus plaque-forming cells above the background (ΔV-PFC) in cultures stimulated by Con A, PWM, or LPS is clearly observed by the first day, reaching maximum usually on the second day of stimulation, and declining thereafter. In contrast, DNA synthesis in response to these mitogens, while high at 48 hr, generally increased slightly at 72 hr. The magnitude of the virus plaque-forming cell response to optimal doses of Con A, PWM, and LPS was compared on the second day of stimulation (Fig. 1). There was invariably a background of cells capable of replicating virus and producing infectious centers even in unstimulated cultures, on the order of 2000/10^6 viable spleen cells recovered from culture. Stimu-

TABLE 2. *Cell-surface antigens of virus plaque-forming cells*

Treatment at 2 day with	ΔV-PFC	
	Con A stimulation	LPS stimulation
A. NMS + C	105,000	
AKR anti-θC_3H + C	0	
Reduction	*100%*	
B. NMS + C	161,000	
Congenic anti-θ + C	4,700	
Reduction	*97%*	
C. NMS + C	174,000	7,670
Anti-PC.1 + C	133,000	3,530
Reduction	N.S.	*54%*

C57B1/10 (A, B) or BALB/c (C) spleen cells were stimulated by Con A (2.5 μg/ml) or LPS (100 μg/ml) for 2 days. Then cells (2.5×10^6/ml) were treated with AKR anti-θ C_3H serum (1:8), congenic anti-θ serum (1:40), or anti-PC.1 serum (1:320) + absorbed rabbit complement (1:16, final concentration). Results were expressed as ΔV-PFC/10^6 cells treated. N.S., not significant.

TABLE 3. *In vitro cytotoxicity, DNA synthesis, and V-PFC in mixed lymphocyte cultures*

Combinations	DNA synthesis		*In vitro* cytotoxicity (%)		Virus plaque assay	
	cpm	S.I.	DBA-M	DBA-L	V-PFC/10^6	ΔV-PFC/10^6
CBA x DBA/2_m	24,300 ± 2,410	3.87	78.0 ± 11.6	60.0 ± 11.3	65,700 ± 3,600	59,100
CBA	6,280 ± 2,470				6,610 ± 1,380	
BALB/c x DBA/2_m	32,000 ± 8,250	7.36	2.2 ± 1.4	−0.5 ± 2.2	5,790 ± 1,900	3,180
BALB/c	4,340 ± 807				2,610 ± 1,390	

CBA or BALB/c spleen cells (1×10^6) were cultured with mitomycin C (25 μg/ml)-treated DBA/2 spleen cells (DBA/2_m) (1×10^6) in 1 ml of medium RPMI 1640 + 5% fetal-calf serum in 12 × 75 mm plastic culture tubes for 4 days at 37°. Aliquots of cells recovered from cultures were assayed both for cytotoxicity (% ^{51}Cr release) against P815 mastocytoma target cells and for virus plaque-forming cells (V-PFC/10^6 plated). Parallel cultures were labeled with [^{3}H]thymidine for 72–96 hr. S.I., stimulation index. In separate experiments, lymphocyte cytotoxicity was tested against DBA/2 lymphoblasts (DBA-L), obtained by stimulating normal DBA/2 spleen cells with 2.0 μg/ml Con A for 3 days. Lymphocyte: target cell ratio was 60:1.

lation by Con A produced a 33-fold increase in virus plaque-forming cells above background, PWM produced a 17-fold increase, and LPS produced a slight though statistically significant increase, about 2–3 times background. The ΔV-PFC response to the B-cell mitogen LPS represented only 3.6% of that produced by Con A or 7.0% of that produced by PWM in the same spleen cultures.

In order to assess the contribution of T- and B-lymphocytes to the production of V-PFC, two types of T-depleted lymphocyte populations were studied. First, anti-θ serum (AKR anti-θC_3H) and complement-treated spleen cells were stimulated by Con A, PWM, and LPS and compared with spleen cells treated with normal-mouse serum plus complement. A 99% diminution in the specific virus plaque-forming cells inducible by Con A or PWM was observed in three experiments in the anti-θ-treated spleens (Table 1). The conditions for anti-θ treatment were selected such that 90% of C57Bl/10 thymocytes were killed but no diminution in Jerne PFC to sheep erythrocytes was observed. Second, culturing congenitally athymic (nude) mouse spleens with Con A in two experiments failed to stimulate an increase in V-PFC, whereas 16,000 V-PFC/10^6 cells were induced in control spleens (Table 1). PWM induced as many V-PFCs as Con A in control spleens, but only a marginal increase in V-PFC in nude mouse spleen. LPS produced small responses of comparable magnitude in both nude and control spleens.

It is thus clear that the virus plaque assay is thymus-dependent, but it is not yet formally demonstrated that V-PFCs are indeed T-cells. However, stimulation of thymocytes, a population containing fewer than 1% B-cells, with Con A produced about 3% V-PFC on day 2. Additionally, Con A-stimulated spleen cells were treated with anti-θ serum and guinea pig complement on day 1 of stimulation, and, while 20–30% of the cells were killed, a 70–80% diminution in V-PFC was obtained. However, the same anti-θ treatment failed to diminish significantly the Con A-stimulated V-PFC on day 2 and killed only 2% of the cells, consistent with the finding that activated lymphocytes are more resistant to immune cytolysis by anti-θ (16). When a 4-fold increased concentration of anti-θ serum was used with absorbed rabbit complement, 65% of the Con A-stimulated cells could be killed on day 2; V-PFC were reduced in this case by more than 99% (Table 2*A*). To rule out nonspecific effects with high doses of the AKR anti-θ serum, these results were confirmed by use of a sample of congenic (θ/AKR^b → ASL_1) anti-θ serum (Table 2*B*). Together, these experiments demonstrate that virtually all V-PFC after Con A stimulation are, in fact, θ-positive T-lymphocytes.

Although the stimulation by LPS was invariably less than 10% of that produced by Con A or PWM, it was of interest to explore the nature of the V-PFC in this case. Because the degree of stimulation of LPS for V-PFC was much less than for thymidine incorporation, only a subpopulation of activated B-lymphocytes could be involved. One such subpopulation, the plasma cells, have been distinguished by the PC.1 alloantigen on their surface (17). When LPS-stimulated cultures of BALB/c spleens were treated with anti-PC.1 serum plus complement in three experiments, the number of V-PFC was diminished between 40 and 70% (Table 2*C*). The same conditions of anti-PC.1 treatment killed 85–90% of PC.1 (+) BALB/c myeloma MPC-11 and fewer than 5% of BALB/c thymocytes. This result suggests that the slight increase in V-PFC induced by LPS is largely attributable to plasma cells that are differentiated sufficiently to contain the PC.1 antigen and to replicate viruses.

In previous work (10), evidence was presented that indicates a dissociation of cell proliferation and the V-PFC response in antigen-induced activation of lymphocytes from delayed hypersensitive donors and in mitogen-induced activation of lymphocytes. Recently, a striking dissociation between the proliferative response and development of cytotoxic lymphocytes in the mixed lymphocyte culture system has been observed (36). BALB/c ($H2^d$) lymphocytes can be stimulated in mixed lymphocyte cultures to incorporate thymidine by mitomycin-treated DBA/2 ($H2^d$) cells that are identical at the major histocompatibility locus (18, 19). Such activated BALB/c lymphocytes are, however, incapable of lysing ^{51}Cr-labeled DBA/2 mastocytoma target cells or Con A-stimulated DBA/2 spleen cells *in vitro*. In contrast, CBA lymphocytes ($H2^k$) stimulated by DBA/2 ($H2^d$) cells both proliferate and differentiate into cytotoxic lymphocytes. When the virus plaque assay was performed on these two combinations of mixed lymphocyte cultures, a marked increase in V-PFC was observed in only one combination (Table 3). CBA lymphocytes stimulated by DBA/2 cells exhibited proliferation, target cell killing, and virus plaque-forming cells, while the BALB/c against DBA/2 combination, which incorporated equal or greater amounts of thymidine, failed to lyse DBA/2 target cells or produce virus. It should be noted that the mixed lymphocyte culture stimulation in both combinations

is abolished by treatment with anti-θ serum (36). Although only two combinations of mixed lymphocyte cultures have been studied here, these experiments demonstrate that proliferation is not a sufficient condition for permitting lymphocytes either to exert direct cytotoxicity on target cells or to replicate viruses, and suggest that there may be a correlation between the development of V-PFC and cytotoxic lymphocytes.

DISCUSSION

While the Jerne plaque assay, which permits enumeration of antibody-producing cells to certain antigens, has permitted a clearer understanding of the development of antibody-producing cells, the inability to enumerate antigen-sensitive T-lymphocytes has remained a major handicap to the understanding of cellular immunology.

A virus plaque assay was introduced in an attempt to deal with this problem (8, 9). It is based on the observation that small lymphocytes are incapable of replicating various viruses, but when stimulated with mitogens or antigens, they become capable of doing so. By estimating the number of lymphocytes activated to produce virus by means of an infectious centers assay, the enhancement of virus replication was shown to be due to an increase in the number of virus-producing cells. Previous studies with this assay had indicated (*i*) increases in V-PFC were observed when cells obtained from delayed-hypersensitive donors were stimulated with specific antigens (8), (*ii*) at the electron microscopic level the virus-producing cell was an enlarged small lymphocyte (20), (*iii*) the absolute efficiency of the virus plaque assay for murine θ (+) lymphomas after a 2-hr infection with VSV was about 60%, and (*iv*) the kinetics of activation of antigen-sensitive cells by antigen was linear over a 4-day period and not affected by mitotic inhibitors (10). It was proposed that the virus plaque assay detects nondividing antigen-sensitive cells, characteristic of differentiated effector cells in cell-mediated immunity (4), similar to cells producing the migration inhibitory factor (21, 22) and target-cell destruction (23).

The observed increases in virus plaque-forming cells in delayed type hypersensitivity and mixed lymphocyte cultures suggested that the virus plaque assay selectively detects activated T-lymphocytes. The present work was designed to examine critically that possibility. It has been established that Con A and PHA in mice selectively activate T-lymphocytes (24–26), while LPS activates only B-lymphocytes (26, 27), and PWM probably activates both T- and B-lymphocytes (25). In the present studies, when normal mouse spleen cells containing both T- and B-lymphocytes were stimulated by these mitogens, a marked increase in V-PFC was obtained with Con A (6% of the cells) and PWM (3% of the cells), but only a marginal increase with LPS (0.2%) (Fig. 1). That the virus plaque-forming cells were indeed activated T-lymphocytes was demonstrated by several experiments. When T-lymphocyte-deprived populations, i.e., nude or normal mouse spleen cells treated with anti-θ serum and complement were stimulated with the T-cell mitogens, Con A and PWM, little or no increase in V-PFC was obtained (Table 1). This observation established that T-lymphocytes were required for the production of V-PFC. Second, mouse thymocytes, thought to contain fewer than 1% B-lymphocytes, could be stimulated by Con A to produce as many as 3% virus plaque-forming cells. Third, it could be demonstrated that V-PFC obtained even 24 or 48 hr after stimulation by Con A could be eliminated by treatment with anti-θ serum. However, higher concentrations of anti-θ serum together with absorbed rabbit complement were required for complete abolition of V-PFC by Con A-treated cells after 2 days of stimulation, but the specificity of this effect was confirmed by use of a congenic anti-θ (θ/AKR^b anti ASL1) serum (Table 2). These experiments demonstrate unambiguously that the V-PFC in Con A-stimulated mouse spleens are in fact activated T-lymphocytes.

While never greater than 10% of the response to Con A, the small V-PFC response in LPS-stimulated cultures requires some comment. LPS and PWM produced a similar degree of thymidine incorporation, and yet the V-PFC responses differed widely. The equal thymidine incorporation suggests that poor survival of B-lymphocytes in culture cannot explain this disparity. The small increase in V-PFC in activated B-lymphocytes could be due either to a very low efficiency of virus replication in activated B-lymphocytes or to replication of virus in a small subpopulation of activated B-lymphocytes. The latter possibility was suggested by two previous observations: an IgM-producing murine plasmacytoma, MOPC-104, was found to replicate VSV, and, at the electromicroscopic level, the few LPS-stimulated lymphocytes associated with virus had dilated rough-surfaced endoplasmic reticulum characteristic of plasma cells (20). Use of a specific isoantiserum to a cell membrane-associated differentiation antigen, PC.1, plus complement in the present experiments markedly diminished the LPS-induced V-PFC (Table 2), thus demonstrating that many of these V-PFC are differentiated, PC.1-positive plasma cells. In addition, macrophages represent a major portion of the background V-PFC in unstimulated cultures and may also be activated by LPS to produce virus. In the practical sense, the contribution of the non-T-cell V-PFC represented less than 10% that of activated T-lymphocytes in spleen, and would be even further diminished by use of cell sources containing fewer B-lymphocytes and macrophages, e.g., peripheral blood or lymph nodes, or treated with immunoadsorbants.

Mixed lymphocyte culture reactions generally occur when T-lymphocytes are stimulated by cells that differ across a major histocompatibility antigen (28). Non-H2 different congenic combinations generally do not result in stimulation (29). However, an increasing number of exceptions to this generalization have been recognized, and specific loci responsible for mixed lymphocyte cultures have been postulated (30, 31). One of the exceptions is found in the stimulation of BALB/c (H2^d) lymphocytes by DBA/2 (H2^d) cells (18, 19). An M-locus that is not linked to H2 is responsible for this stimulation (31). In this system, BALB/c spleen or thymus cells proliferate when stimulated by mitomycin C-treated DBA/2 spleen cells, but do not develop lymphocyte cytotoxicity against ^{51}Cr-labeled P815 mastocytoma cells (derived from DBA/2 mice). These DBA/2-stimulated BALB/c lymphocytes similarly failed to lyse DBA/2 primary lymphoblasts obtained by stimulating normal DBA/2 spleen cells with Con A, thus excluding the possibility that the failure of activated BALB/c lymphocytes to lyse mastocytoma target cells reflected a lack of normal DBA/2 cell-surface antigens in the mastocytoma cells. In contrast, CBA (H2^k) spleen cells, when stimulated by DBA/2 (H2^d) cells, proliferate and also are capable of exerting cytotoxicity on DBA/2 target

cells (36). In the present experiments an increase in virus plaque-forming cells was obtained only in CBA spleens stimulated by DBA/2 cells (Table 3). It should be mentioned, however, that BALB/c spleen cells are able to produce V-PFC when stimulated by Con A or major histocompatibility antigens. These experiments demonstrate that lymphocyte proliferation in mixed lymphocyte cultures is not a sufficient condition for generating either cytotoxic lymphocytes or V-PFC. They further suggest a parallelism between the cytotoxic effector cells and the virus plaque-forming cells, although more detailed experiments will be required to ascertain whether the V-PFC and cytotoxic lymphocytes are identical.

The dissociation of virus production and cytotoxic activities from the proliferative response clearly reflects a heterogeneity within T-lymphocyte populations seen in many experimental situations (24, 32-34). At least two hypotheses can be invoked to explain these findings. One would hold that there are two or more specialized subpopulations of T-lymphocytes. Perhaps one proliferates and cooperates with a second, which then becomes capable of effector function. An alternative view, in analogy to the generation of fully differentiated, terminal plasma cells from precursor cells (35), would be that there is only one population of T-lymphocytes, but at various stages in their differentiation from precursors, the cells perform different biological functions. For example, in early stages they may be capable of antigen recognition and clonal expansion but not effector function; in their most differentiated state they may carry out cytotoxicity and mediator production but be incapable of further proliferation. At the present time it is not possible to discriminate between these alternatives. The virus plaque assay should provide a useful quantitative technique for approaching these problems.

NOTE ADDED IN PROOF

Since this paper was submitted for publication, several reports have been published demonstrating a dissociation between cell proliferation and lymphocyte cytotoxicity in mixed lymphocyte cultures in human [Eijsvoogel, V. P., du Bois, R., Melief, C. J. M., Zeylemaker, W. P., Raat-Koning, L. & de Groot-Kooy, L. (1973) *Transplant, Proc.* **5**, 415-420; Bach, F. H., Segall, M., Zier, K. S., Sondel, P. M., Alter, B. J. & Bach, M. L. (1973) *Science* **180**, 403-406] and in mouse preparations [Alter, B. J., Schendel, D. J., Bach, M. L., Bach, F. H., Klein, J. & Stimpfling, J. H. (1973) *J. Exp. Med.* **137**, 1303-1309]. Differences at the MLC locus alone generated lymphocyte proliferation but not cytotoxic lymphocytes; it was necessary that both MLC locus and major histocompatibility differences be present and that the target cell differ from the lymphocytes at a major histocompatibility locus in order to be killed. Study of the virus plaque assay, in the present paper, is consistent with these results and further suggests that the functional differences between proliferating lymphocytes and cytotoxic lymphocytes are intrinsic to the lymphocytes themselves and independent of target cells.

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1. Katz, D. H. & Benacerraf, B. (1972) *Advan. Immunol.* **15**, 1-94.
2. Miller, J. F. A. P. & Mitchell, G. F. (1969) *Transplant. Rev.* **1**, 3-42.
3. Hirschhorn, K. (1969) in *Mediators of Cellular Immunity*, eds. Lawrence, H. S. & Landy, M. (Academic Press, New York & London), pp. 3-12.
4. Bloom, B. R. (1971) *Advan. Immunol.* **13**, 101-208.
5. Perlmann, P. & Holm, G. (1969) *Advan. Immunol.* **11**, 117-193.
6. Cerottini, J. C. & Brunner, K. T. (1973) *Advan. Immunol.*, in press.
7. David, J. R. & David, R. R. (1972) *Progr. Allergy* **16**, 300-449.
8. Bloom, B. R., Jimenez, L. & Marcus, P. D. (1970) *J. Exp. Med.* **132**, 16-30.
9. Jimenez, L. & Bloom, B. R. (1971) in *In Vitro Methods in Cell-Mediated Immunity*, eds. Bloom, B. R. & Glade, P. R. (Academic Press, New York & London), pp. 553-559.
10. Jimenez, L., Bloom, B. R., Blume, M. R. & Oettgen, H. F. (1971) *J. Exp. Med.* **133**, 740-751.
11. Jimenez, L. & Bloom, B. R. (1970) in *Proceedings of the Fifth Leukocyte Culture Conference*, ed. Harris, J. E. (Academic Press, New York & London), pp. 467-472.
12. Agrawal, B. B. L. & Goldstein, I. J. (1965) *Biochem. J.* **96**, 23c-25c.
13. Cerottini, J. C., Nordin, A. A. & Brunner, K. T. (1970) *Nature* **228**, 1308-1309.
14. Boyse, E. A., Hubbard, L. & Stockert, E. (1970) *Transplantation* **10**, 446-449.
15. Cerottini, J. C. & Brunner, K. T. (1971) in *In Vitro Methods in Cell-Mediated Immunity*, eds. Bloom, B. R. & Glade, P. R. (Academic Press, New York & London), pp. 369-373.
16. Lohmann-Matthes, M. L. & Fischer, H. (1972) *Eur. J. Immunol.* **2**, 290-292.
17. Takahashi, T., Old, L. J. & Boyse, E. A. (1970) *J. Exp. Med.* **131**, 1325-1341.
18. Dutton, R. W. (1965) *J. Exp. Med.* **122**, 759-770.
19. Festenstein, J. (1966) *Ann. N.Y. Acad. Sci.* **129**, 567-572.
20. Nowakowski, M., Feldman, J. D., Kano, S. & Bloom, B. R. (1973) *J. Exp. Med.* **137**, 1042-1059.
21. Bloom, B. R., Gaffney, J. & Jimenez, L. (1972) *J. Immunol.* **109**, 1395-1398.
22. Rocklin, R. E. (1973) *J. Immunol.* **110**, 674-678.
23. Mauel, J., Rudolf, B., Chapuis, B. & Brunner, K. T. (1970) *Immunology* **18**, 517-535.
24. Stobo, J. D. (1972) *Transplant. Rev.* **11**, 60-86.
25. Greaves, M. & Janossy, G. (1972) *Transplant. Rev.* **11**, 87-130.
26. Andersson, J., Sjoberg, O. & Möller, G. (1972) *Transplant. Rev.* **11**, 131-177.
27. Gery, I., Kruger, J. & Spiesel, S. Z. (1972) *J. Immunol.* **108**, 1088-1091.
28. Wilson, D. B., Howard, J. C. & Nowell, P. C. (1972) *Transplant. Rev.* **12**, 3-29.
29. Dutton, R. W. (1966) *J. Exp. Med.* **123**, 665-671.
30. Bach, F. H., Bach, M. L., Sondel, P. M. & Sundharadas, G. (1972) *Transplant. Rev.* **12**, 30-56.
31. Festenstein, H., Abbasi, K., Sachs, J. A. & Oliver, R. T. D. (1972) *Transplant. Proc.* **4**, 219-222.
32. Asofsky, R., Cantor, H. & Tigelaar, R. E. (1971) in *Progress in Immunology*, ed. Amos, B. (Academic Press, New York & London), pp. 369-381.
33. Sprent, J. & Miller, J. F. A. P. (1971) *Nature, New Biol.* **234**, 195-198.
34. Wagner, H. (1972) *J. Immunol.* **109**, 630-637.
35. Williamson, A. R. & Askonas, B. A. (1972) *Nature* **238**, 337-339.
36. Howe, M. L. & Berman, L. (1973) *J. Immunol.*, in press.