

# IN VITRO SELECTION AND EXTENDED CULTURE OF ANTIGEN-SPECIFIC T LYMPHOCYTES

## I. Description of Selection Culture Procedure and Initial Characterization of Selected Cells

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The analysis of the functional properties of antigen-specific thymus-dependent (T) lymphocytes would be markedly aided if cell populations highly enriched in such lymphocytes could be prepared. Unfortunately, attempts to obtain antigen-specific depletion and/or enrichment of T lymphocytes by immunoadsorbents have been much less fruitful than has been true for bone marrow-derived (B) lymphocytes (1, 2). Although some success has been reported with conjugated Sepharose beads (3) and with derivatized nylon fibers (4), most investigators have been unable to reproducibly remove specific T lymphocytes with antigens conjugated to solid matrices. More promising results have been obtained in depleting alloantigen-specific cytotoxic T lymphocytes by allowing these cells to interact with monolayers of fibroblasts or tumor cells which bear the appropriate alloantigen (5, 6). This depletion is both temperature and time dependent (6), implying that some degree of cellular activity may be critical for specific adherence.

A possible general method to obtain populations of cells depleted of, or enriched in, T lymphocytes of any given specificity might be to associate the appropriate antigen with a cellular monolayer and to incubate T lymphocytes on such a monolayer under conditions which allow lymphocyte activation. In view of a series of recent studies emphasizing the importance of macrophage-associated antigen in the activation of DNA synthesis by specific T lymphocytes (7-9), monolayer of antigen-pulsed macrophage would seem ideal for this purpose. Indeed, Lipsky and Rosenthal (10) have recently shown that by 24 h after initiation of cell cultures, the number of lymphocytes from primed guinea pigs which adhere to macrophages which have been pulsed with an antigen to which the lymphocyte donor was immune is markedly greater than the number which adhere to nonpulsed macrophages or to macrophages pulsed with unrelated antigen.

In this communication, we report that an antigen-specific selection of T lymphocytes from peritoneal exudates of primed guinea pigs can be obtained by

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this approach. Purified peritoneal exudate lymphocytes (PELs),<sup>1</sup> a cell population highly enriched in T lymphocytes, were obtained from primed guinea pigs and were placed on culture dishes to which antigen-pulsed syngeneic macrophages had been allowed to adhere. 24 and 48 h later, nonadherent cells were removed by washing and the culture was maintained until day 7, at which time substantial numbers of cells could be harvested from the dish. These cells were principally T lymphocytes, contained few if any B lymphocytes, and were highly responsive to the antigen with which the macrophages adhering to the plate had been pulsed; the cells had little or no response to other antigens to which the lymphocyte donor had been responsive and to which the preculture population responded well. Moreover, these cells could be transferred to new antigen-pulsed monolayers and the activity of the cells was preserved for 2-5 wks. Our results suggest that populations of T cells can be selected *in vitro* for responses to specific antigens and that these selected cells can then be propagated *in vitro*. In some cases, cultures of cells maintained in this way begin to incorporate large amounts of [<sup>3</sup>H]thymidine in the absence of specific antigen. Cultures of such "antigen-independent" cells have been maintained for over 2 mo.

### Materials and Methods

**Animals and Immunization.** Inbred strain 2 and strain 13 guinea pigs were obtained from the Division of Research Services, National Institutes of Health, Bethesda, Md.; (2 × 13)F<sub>1</sub> guinea pigs were bred in our animal facility. Animals to be used as donors of lymphocytes were immunized in the four foot pads with complete Freund's adjuvant (CFA; Difco Laboratories, Detroit, Mich.) and with emulsions of 100 μg of one or more of the following antigens: ovalbumin (OVA; 5 times crystallized; Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.); 2,4-dinitrophenyl (DNP)-guinea pig albumin (GPA), containing 24 mol of DNP mol of GPA, prepared as previously described (11); a DNP derivative of the copolymer of L-glutamic acid and L-lysine (GL, mol wt 40,000; molar ratio of glutamic acid to lysine, 6:4, Pilot Chemicals, Inc., Watertown, Mass.) containing 8 mol of DNP mol of GL (12). Immunized animals were used as cell donors 2 wks to 2 mo after priming.

**Preparation of "Monolayers" of Adherent Peritoneal Exudate Cells.** Nonimmunized inbred guinea pigs received intraperitoneal injections of 25 ml of sterile mineral oil (Marcol 52, Humble Oil & Refining Co., Houston, Texas) and the resulting peritoneal exudate was harvested 3-4 days later as described by Rosenstreich et al. (13). These cells were washed in Hanks' balanced salt solution (BSS) and then incubated with antigen (100 μg/ml) for 30 min at 37°C in a culture medium comprised of RPMI 1640 with 25 mM HEPES buffer (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% fetal calf serum (Microbiological Associates, Inc., Bethesda, Md.), penicillin (100 μg/ml), and streptomycin (100 μg/ml). This medium will subsequently be referred to as complete medium. 10-15 × 10<sup>6</sup> cells were added to sterile 6-cm tissue culture dishes (Falcon Plastics, Div. of Bio Quest Los Angeles, Calif.) and cultured for 2 h at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>-95% air. In some experiments, larger or smaller culture dishes were used and the number of peritoneal exudate cells (PECs) plated adjusted accordingly. After 2 h, nonadherent cells were aspirated from the dishes. The adherent cells were washed four times by the addition of small volumes of Hanks' BSS, followed by shaking and swirling of the dishes and aspiration. Complete medium (2.5 ml) was added to the dishes at the conclusion of washing.

Morphologic evaluation of cells remaining on the plate revealed that 5-7% were lymphocytes (or could not be distinguished from lymphocytes). In individual experiments, PECs were incubated with

<sup>1</sup> *Abbreviations used in this paper:* BSS, balanced salt solution; CFA, complete Freund's adjuvant; EAC, rabbit antish sheep erythrocyte antibodies and mouse complement; GL, copolymer of L-glutamic acid and L-lysine; GPA, guinea pig albumin; [<sup>3</sup>H]TdR, [*methyl*-<sup>3</sup>H]thymidine; OVA, ovalbumin; PECs, peritoneal exudate cells; PELs, peritoneal exudate lymphocytes; PPD, purified protein derivative of tuberculin.

latex particles before plating. In such cases 70–80% of cells which were adherent after 2 h contained latex particles.

*Preparation of PELs and Culture on Antigen-Pulsed Adherent PECs.* PELs were purified from oil-induced peritoneal exudates by passage over rayon wool columns as described by Rosenstreich et al. (13). Cell populations purified in this manner contain variable numbers of macrophages (10–30%); the remaining cells are largely lymphocytes of which less than 5% (often less than 1%) have surface immunoglobulin or the C3 receptor as judged by the ability to form rosettes with sheep erythrocytes coated with IgM antibody and complement (C). Thus, PELs are a population highly enriched in T lymphocytes. PELs ( $10\text{--}30 \times 10^6$ ) in 2.5 ml of complete medium were added to dishes to which antigen-pulsed macrophages had been previously allowed to adhere. The dishes were incubated for 24 h at 37°C, the nonadherent cells aspirated, and the adherent cells washed four times. 5 ml of fresh complete medium were added to the dishes which were cultured for an additional 24 h and the aspiration and washing procedure repeated. The cultures were “fed” by the addition of 5 ml of fresh complete medium 4 days after the initiation of the cultures. After 1 wk, nonadherent cells were harvested and a portion were transferred to new monolayers of antigen-pulsed adherent PECs. The remainder were evaluated for in vitro DNA synthetic responses to a variety of antigens. Weekly transfers were continued as long as the cultured cells appeared to expand or maintain their numbers on the adherent PEC monolayer.

*Assay of Antigen-Stimulated DNA Synthetic Response.* Lymphocytes harvested from culture dishes or purified directly from peritoneal exudates were cultured in complete medium in microtiter U plates (Cooke Laboratory Products Div., Dynatech Laboratories Inc., Alexandria, Va.). To each well was added a total vol of 200  $\mu$ l containing 1 or  $2 \times 10^5$  lymphocytes,  $1 \times 10^5$  PECs from nonimmune guinea pigs, and either BSS or antigen, at a final concentration of 100  $\mu$ g/ml. Fresh PECs from nonimmune syngeneic donors, were added in all cases because of the possibility that cells harvested from dishes 1 wk after initiation of culture might be deficient in macrophages. The cell mixture was cultured for 72 h and 1  $\mu$ Ci of [methyl- $^3$ H]thymidine ( $^3$ H]TdR; 6.7 Ci/mmol; New England Nuclear, Boston, Mass.) added for the last 16 h of culture. Cells were processed on a Harrison microharvester (14) and incorporation of  $^3$ H]TdR measured by liquid scintillation counting. In some experiments, the PECs obtained from nonimmune animals were incubated with mitomycin C (50  $\mu$ g/ml) for 40 min at 37°C and washed four times before being added to the wells of the microtiter culture plate.

*Detection of Surface Markers of Lymphoid Cells and Phagocytosis of Latex Particles.* Lymphoid cells bearing surface immunoglobulin were detected as previously described (15), by incubation with a fluoresceinated rabbit antiguinea pig immunoglobulin antibody and examination with a fluorescence microscope equipped with an incident light ultraviolet illuminator.

Lymphoid cells bearing receptors for the third component of C (C3) were detected by their ability to form rosettes with sheep erythrocytes coated with the IgM fraction of rabbit antisheep erythrocyte antibodies and mouse C (IgM EAC). Lymphoid cells bearing receptors for IgG were similarly detected by rosette formation with sheep erythrocytes coated with the IgG fraction of rabbit antisheep erythrocyte antibodies (IgG EA). These methods have been described in detail (16). Under the conditions used in these studies the IgG EA reagent only binds to the monocyte receptor for cytophilic antibody. Lymphoid cells capable of forming rosettes with rabbit erythrocytes were detected by the procedure of Stadercker et al. (17). These cells have been previously shown to be T lymphocytes. The ability of the cells to phagocytose was judged by exposing the cell populations to 1- $\mu$ m latex particles in medium containing 10% unheated normal guinea pig serum for 30 min at 37°C. The cells were then smeared and stained with Giemsa. The fraction of cells containing latex particles was then determined by counting 200 cells.

## Results

*Specific “Selection” of PELs Mediating Antigen-Stimulated DNA Synthetic Responses.* PELs obtained from immunized guinea pigs were placed on culture dishes to which antigen-pulsed nonimmune syngeneic PECs had previously been allowed to adhere. As described in the Materials and Methods, nonadherent cells were discarded at 24 and 48 h and cells were harvested from the culture fluid 7 days after initiation of the culture. These cells were tested for specific

responsiveness to antigen by culturing them in microtiter plates with no antigen, with the antigen with which the adherent PECs on the culture dish had been pulsed, or with another antigen to which the lymphocytes donor had been immunized but to which the adherent PECs on the culture dish had not been exposed.

This selection procedure is illustrated in Fig. 1. Results of representative experiments are presented in Fig. 2 and in Table I. In the experiment summarized in Fig. 2, PELs were obtained from strain 2 guinea pigs which had been immunized with OVA in CFA. A sample of these cells were directly tested for [ $^3\text{H}$ ]TdR incorporation in response to antigen. These cells responded both to OVA and to purified protein derivative of tuberculin (PPD). Their responsive-

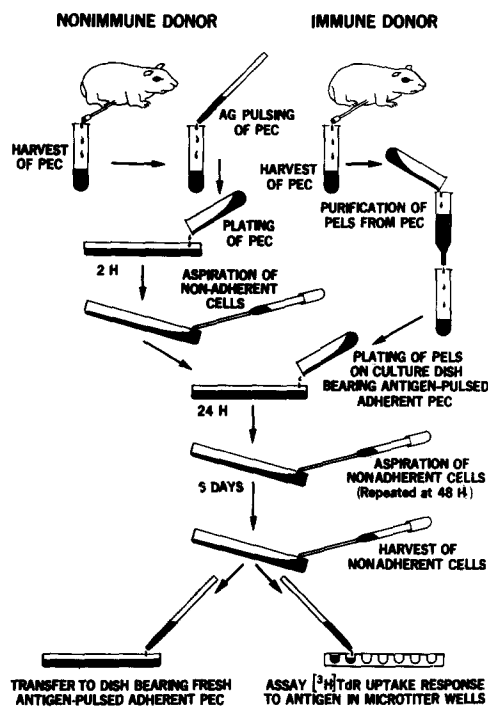


FIG. 1. Summary of the selection culture procedure.

ness to PPD can be attributed to immunization with CFA containing killed *Mycobacterium tuberculosis* organisms. A second portion of the PELs from this donor were placed in a culture dish bearing adherent PECs pulsed with OVA and a third portion was cultured on a dish bearing adherent PECs pulsed with PPD. Cells were harvested after a 7 day selection culture, as outlined above. Those obtained from the dish bearing OVA-pulsed adherent PECs displayed an excellent response to OVA but only a minimal response to PPD; on the other hand, the cells which had been cultured on the dish bearing PPD-pulsed adherent PECs showed only a minimal response to OVA but responded very well to PPD. Table I illustrates four such experiments, the second of which is the one

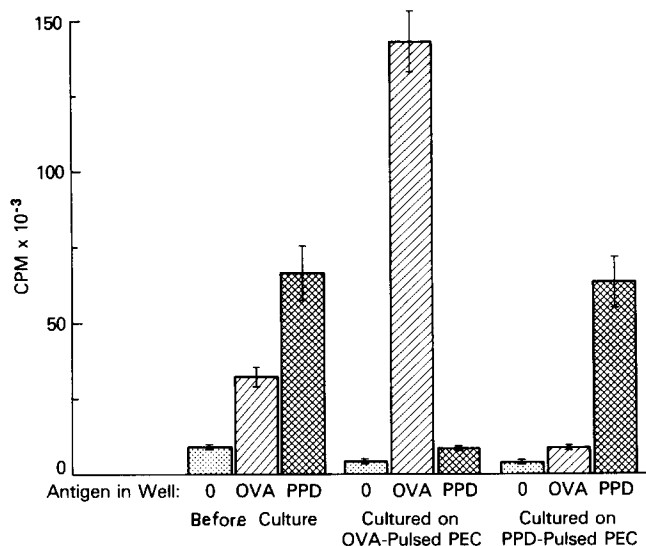


FIG. 2. Antigen-stimulated [<sup>3</sup>H]TdR uptake by cells selected for OVA or PPD. PELs from strain 2 guinea pigs immunized to OVA emulsified in CFA were either immediately cultured in microtiter wells or selected for OVA or PPD by the selection culture procedure and then tested for antigen responsiveness in microtiter wells. Responses shown are cpm  $\times 10^{-3}$  ( $\pm$ SE) in response to 0, OVA (100  $\mu$ g/ml), or PPD (100  $\mu$ g/ml).

presented in Fig. 2. These experiments involved cells from strain 2, strain 13, and  $(2 \times 13)F_1$  guinea pigs and used DNP-GL, DNP-GPA, OVA, and PPD as antigens. In each instance, the cells harvested from a dish bearing adherent PECs pulsed with a given antigen responded best to that antigen and generally displayed only minimal responses to other antigens to which the donor had been immunized.

In a series of 44 individual experiments, we found that in 40 cases cells harvested from dishes at the end of 1 wk displayed antigen responsiveness. In these 40 "successful" experiments, there were a total of 99 individual dishes bearing antigen-pulsed adherent PECs. In all but six instances, cells harvested from a dish bearing antigen-pulsed adherent PECs responded better to the antigen which had been present on the dish than to any other antigen to which the donor had been primed. Thus, it seems quite clear that a substantial degree of antigen-specific functional selection has been achieved by this procedure.

*Extended Cultures on Dishes Bearing Antigen-Pulsed Adherent PECs.* In some experiments, cells harvested from a culture dish 7 days after initial culture on antigen-pulsed adherent PECs were transferred to a new culture dish bearing freshly harvested antigen-pulsed, adherent, syngeneic PECs. The nonadherent cells were discarded at 24 and 48 h, as in the initial culture. Cells appearing in the culture supernate at 1 wk were harvested, a portion tested for antigen-stimulated DNA synthetic responses and the remainder transferred to a fresh dish bearing antigen-pulsed adherent PECs. Two such experiments are illustrated in Fig. 3. In one (3/25), high levels of specific antigen responsiveness were retained for 3 wk and measurable responses to antigen were observed for 5 wk.

In the other (4/22), excellent responses were preserved for 2 wk. In both experiments, as in the bulk of our experience with sequential selection cultures, the relative specificity of selected cells is greater after the second culture than after the first. In general, such "antigen-responsive" cultures can be propagated for 2-5 wk after which time few cells are obtained and those are not antigen responsive. As will be described below, a minority (20-30%) of the cultures become "antigen-independent" and can be retained in culture for 8-10 wks, or more.

*Characterization of Number and Type of Cells Harvested From Dishes.* Because this culture technique may offer a procedure for in vitro selection of antigen-specific T lymphocytes, it is of importance to study both the numbers and types of cells harvested from the dishes. In a series of 10 individual experiments in which  $30 \times 10^6$  PELs, freshly purified from an immunized donor, were applied to dishes bearing adherent PECs pulsed with an antigen to which the donor had been primed, we measured the number of cells removed in the 24-h wash and the number harvested from the culture at 1 wk. In these experiments,  $27.3 \pm 0.9 \times 10^6$  (mean  $\pm$  SE) cells were removed at 24 h and  $10.0 \pm 1.9 \times 10^6$  cells obtained at 7 days. This indicated that only a fraction (less than 10%) of the initially applied PELs adhere to the culture dish at 24 h and suggests that those PELs which adhere proliferate on the dish.

TABLE I  
Specific Responsiveness of PELs Selected on Culture Dishes Bearing Adherent Antigen-Pulsed PECs

Strain	Immunogen	PELs	$^3\text{H}$ TdR incorporation in response to:			
			0	DNP-GL	PPD	
2	DNP-GL; CFA	Preculture	894	61,259	23,188	
		DNP-GL selected	882	<i>51,610</i>	2,839	
		PPD selected	1,467	2,839	<i>9,634</i>	
2	OVA; CFA	Preculture	8,660	31,750	66,239	
		OVA selected	4,545	<i>141,796</i>	8,402	
		PPD selected	3,486	7,932	<i>63,359</i>	
13	DNP-GPA; CFA	Preculture	1,974	147,421	59,035	
		DNP-GPA selected	1,175	<i>82,659</i>	2,505	
		PPD selected	1,367	7,535	<i>33,796</i>	
(2 $\times$ 13)F <sub>1</sub>	DNP-GL; DNP-GPA; CFA	Preculture	1,416	65,058	125,582	13,421
		DNP-GL selected	1,524	<i>30,060</i>	4,248	1,786
		DNP-GPA selected	916	2,523	<i>108,120</i>	1,662
		PPD selected	1,545	2,434	3,372	<i>4,512</i>

PELs were purified from peritoneal exudates induced in guinea pigs immunized with antigen emulsified in CFA. A portion of these cells were immediately cultured on U type microtiter plates in the presence or absence of antigen and uptake of  $^3\text{H}$ TdR determined 4 days later. These cells are referred to as preculture PELs. The remainder of the cells were placed on culture dishes bearing syngeneic adherent PECs which had been pulsed with antigen. Nonadherent cells were discarded at 24 and 48 h and cells were harvested at 7 days. Harvested cells were cultured in microtiter plates. In each case, the response to the antigen which had been used to pulse the macrophages used in the selection culture is in italics.

The importance of antigen-pulsing of PEC for these results is illustrated by the experiments described in Table II. In these experiments, PELs from primed donors were plated on dishes bearing adherent PECs pulsed with an antigen to which the lymphocyte donor was immune or adherent PECs which had not been pulsed with antigen. The number of cells recovered at 7 days from the dishes bearing antigen-pulsed adherent PEC were substantially greater than the number recovered from the dishes containing adherent PEC not pulsed with antigen. In one of the two experiments, essentially no cells were recovered from the nonpulsed monolayer; in the other, the number of cells which were recovered was small and these cells were not responsive to antigen (data not shown). Although the adherent PEC seem to be important in the selection procedure, it is unlikely that an appreciable number of the lymphocytes harvested at 1 wk are descendants of these adherent cells as shown by the fact that antigen-pulsed adherent PEC cultured without specific lymphocytes yield very few cells at 1 wk

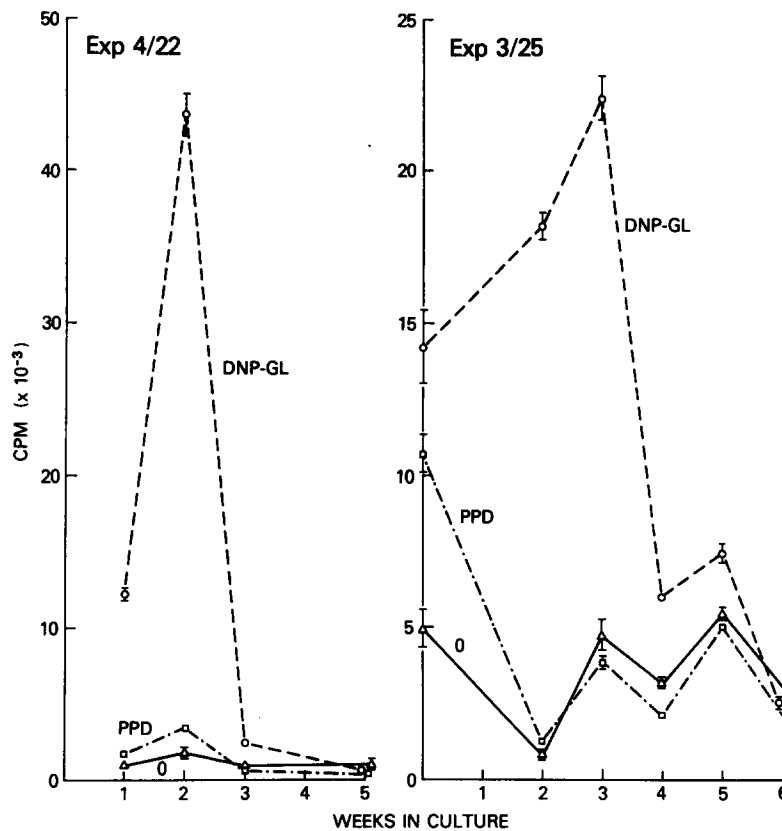


FIG. 3.  $[^3\text{H}]\text{TdR}$  uptake in microtiter wells of cells obtained from extended antigen-responsive cultures. PELs from strain 2 guinea pigs immunized to DNP-GL emulsified in CFA were subjected to sequential weekly selection cultures on "monolayers" of DNP-GL-pulsed adherent PECs from nonprimed donors. Samples of cells were taken weekly and cultured in microtiter wells with 0, DNP-GL (100  $\mu\text{g}/\text{ml}$ ), or PPD (100  $\mu\text{g}/\text{ml}$ ).  $[^3\text{H}]\text{TdR}$  uptake ( $\text{cpm} \times 10^3 \pm \text{SE}$ ) was measured 4 days later.

TABLE II  
*Cell Yields from Dishes Bearing Antigen-Pulsed or Nonpulsed Adherent PECs*

Exp.	Antigen used to pulse adherent cells	Antigens to which donor of PELs was immunized	No. of PELs placed on adherent PECs	No. of cells recovered on day 7 ( $\times 10^6$ )
1	0	DNP-GL + OVA in CFA	$16 \times 10^6$	0.8
	DNP-GL		$16 \times 10^6$	5.4
	OVA		$16 \times 10^6$	3.5
	DNP-GL		0	0.3
2	0	DNP-GL in CFA	$10 \times 10^6$	<0.1
	DNP-GL		$10 \times 10^6$	3.5
	PPD		$10 \times 10^6$	1.5

PELs from immunized guinea pigs were placed on culture dishes bearing antigen-pulsed or nonpulsed syngeneic adherent PECs. 24 h later, cells which failed to adhere were discarded. Cells which appeared in culture supernate were harvested at day 7 and counted. Viability of recovered cells was approximately 90%.

TABLE III  
*Characteristics of Cells Recovered from Antigen-Responsive Selection Cultures*

Exp.	Viability	Ig-bearing cells	Cells forming rosettes with IgM EAC	Cells forming rosettes with rabbit erythrocytes
	%	%	%	%
1	ND	<1	ND	ND
2	ND	<1	ND	ND
3	90	<1	ND	63
4*	80	<1	1	83
5*	80	<1	<1	90
6	>95	ND	<1	ND
7	>95	ND	<1	ND
8	94	4	ND	35

\* Cells from exp. 4 and 5 were evaluated after 4 wk of sequential selection cultures. All other cells were studied after a single selection culture.

(Table II). This point will be discussed in greater detail in the second paper of this series.<sup>2</sup>

Cells harvested from selection cultures 1 or 4 wk after removal from the donor animal were evaluated for viability, morphology, surface immunoglobulin, receptors for activated C3, the capacity to form rosettes with rabbit erythrocytes, and, in some instances, for the capacity to phagocytose latex particles (Table III). Lymphocytes bearing surface Ig and forming rosettes with IgM EAC are principally B lymphocytes (16). Formation of rosettes with rabbit erythrocytes is

<sup>2</sup> Ben-Sasson, S. Z., W. E. Paul, E. M. Shevach, and I. Green. 1975. In vitro selection and extended culture of antigen-specific T lymphocytes. II. Mechanism of selection. Manuscript submitted for publication.



a characteristic of guinea pig T lymphocytes (17). Viability of harvested cells ranged from 80 to > 90% morphologically, approximately from 50 to 90% of these cells resembled small and medium lymphocytes. In all but one case, fewer than 1% of the cells bore surface Ig and between 0 and 1% formed rosettes with EAC. The percent of cells forming rosettes with rabbit erythrocytes ranged from 35% to 90%. Thus, the great majority of lymphocytes obtained from selection cultures of PELs appear to be T lymphocytes. Variable percentages of macrophages appeared in these cultures. In general, an inverse relation existed between total cell yield and percent of macrophages.

*Antigen-Independent Cell Cultures.* When cells are harvested from an initial selection culture they retain their antigen-specific responsiveness when transferred to a dish bearing fresh antigen-pulsed, syngeneic, adherent PECs. In most cases, they exhibit such specific responsiveness for 2-5 wk and then the cell yield and antigen responsiveness diminish markedly. In a minority of cases, a different pattern is observed. Two such examples are illustrated in Fig. 4. These cultures began in a highly antigen-specific way. When assayed after the initial selection culture for [<sup>3</sup>H]TdR incorporation in microtiter wells, they responded very well to the antigen to which the adherent PEC had been pulsed (DNP-GL) and poorly or not at all to PPD. Moreover, the amount of [<sup>3</sup>H]TdR taken up by cells not exposed to antigen in the microtiter wells was very low. However, after 2 wk of sequential selection cultures in one case (4/15) and after 4 wk in the other (4/1), the recovered cells incorporated substantial [<sup>3</sup>H]TdR in the absence of antigen. After 1 or 2 additional wk of selection cultures, the [<sup>3</sup>H]TdR uptake in presence of specific antigen was no greater than in the absence of antigen. These cells thus appear to show a high degree of antigen-independent proliferation. We maintained one of these cultures for 8 wk and the other for 10 wk, at which time they became contaminated. Several other antigen-independent cultures have been maintained for at least 2 mo.

Cells harvested from two antigen-independent cultures were evaluated for various cell surface markers. As shown in Table IV, very few cells bore surface Ig or formed rosettes either with IgG EA or IgM EAC. A majority of the cells (64-68%) formed rosettes with rabbit erythrocytes. These results indicate that the cell type principally represented in the antigen-independent cultures is the T lymphocyte.

Although antigen-independent cultures incorporate large amounts of [<sup>3</sup>H]TdR without antigen stimulation, they appear to require the presence of a cell (or soluble component) found in the PEC population both for maintenance in tissue culture dishes and for proliferation in microtiter wells. The results of experiments demonstrating both the need for adherent PECs and the lack of requirement for antigen in the maintenance of antigen-independent cultures on dishes are shown in Table V. In exp. 1, a culture of strain 2 PELs which had been originally selected for responsiveness to DNP-GL but which had entered an antigen-independent phase was harvested and the cells obtained were divided into two portions. One portion was plated on a dish bearing DNP-GL-pulsed adherent PECs and the other was plated on a dish bearing nonpulsed adherent PECs. 1 wk later, the cells were harvested and placed in microtiter wells to assess their baseline [<sup>3</sup>H]TdR incorporation and their responsiveness to DNP-GL and

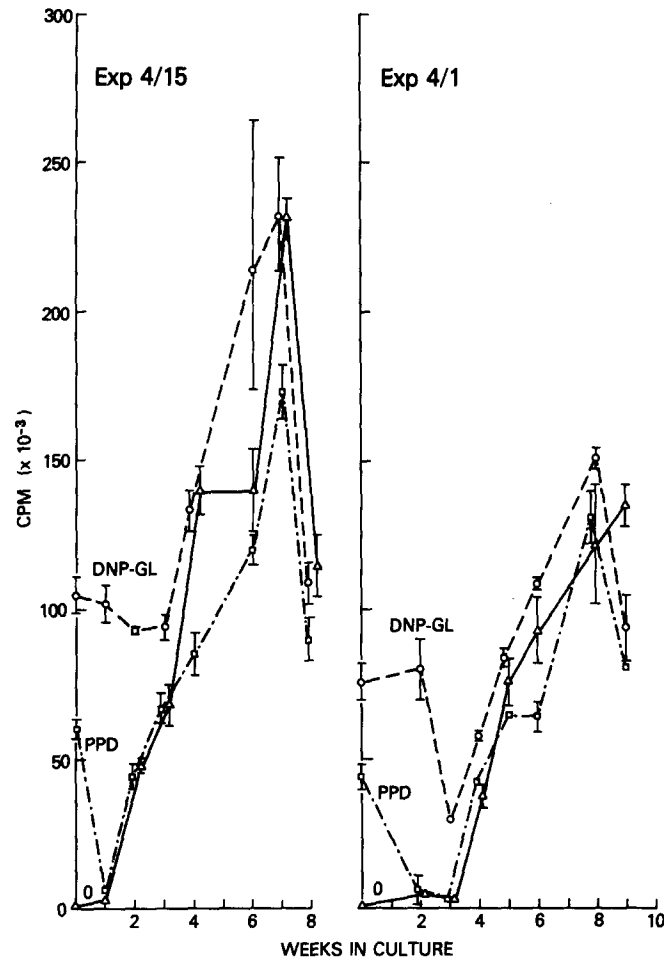


FIG. 4. [ $^3\text{H}$ ]TdR uptake in microtiter wells of cells obtained from antigen-independent cultures. PELs from strain 2 guinea pigs immunized to DNP-GL emulsified in CFA were subjected to weekly selection cultures on monolayers of DNP-GL-pulsed adherent PECs from nonprimed donors. Samples of cells were taken weekly and cultured in microtiter wells with 0, DNP-GL (100  $\mu\text{g}/\text{ml}$ ), or PPD (100  $\mu\text{g}/\text{ml}$ ). [ $^3\text{H}$ ]TdR uptake (cpm  $\pm$  SE) was measured 4 days later.

TABLE IV  
Characteristics of Cells Recovered from "Antigen-Independent" Cultures

Duration of culture	Antigen used for selection	Viability	Ig-bearing cells	Cells forming rosettes with:		
				EAC	EA	Rabbit erythrocytes
<i>wk</i>		%	%	%	%	%
5	PPD	85	<1	1	1.5	64
7	DNP-GL	65	<1	0.5	0	68

TABLE V  
*Requirements for Maintenance of Antigen-Independent Cultures*

Exp.	Immunogen	PELs selected for	PECs on dish	PECs on dish pulsed with:	Antigen in well	[ <sup>3</sup> H]TdR uptake
1	DNP-GL; CFA	DNP-GL	Yes	0	0	<i>cpm ± SE</i> 108,780 ± 6,090
					DNP-GL	100,780 ± 14,212
					PPD	112,360 ± 4,212
			Yes	DNP-GL	0	121,060 ± 17,951
					DNP-GL	104,203 ± 7,188
					PPD	80,198 ± 2,188
2	DNP-GL; CFA	PPD	No	—	0	1,248 ± 110
					DNP-GL	1,773 ± 241
					PPD	1,019 ± 59
			Yes	PPD	0	22,189 ± 4,738
					DNP-GL	29,335 ± 4,607
					PPD	12,050 ± 1,248

Cells from antigen-independent cultures were transferred to culture dishes bearing (or not bearing) adherent PECs from nonimmune syngeneic donors. The adherent PECs had either been pulsed or not been pulsed with antigen as indicated. After 1 wk,  $1 \times 10^5$  cultured cells were placed in microtiter U wells together with  $1 \times 10^5$  fresh PECs and antigen, as indicated.

to PPD. As can be seen, cells harvested from antigen-pulsed and nonpulsed "monolayers" had comparable high baseline levels of [<sup>3</sup>H]TdR incorporation and were unresponsive to antigen added to the microtiter wells. Exp. 2 indicates that maintenance of antigen-independent cells is dependent on the presence of adherent PECs on the dish. In this case, cells were harvested from an antigen-independent culture which had been initially selected for responsiveness to PPD. These cells were plated either on a dish which lacked adherent PECs or a dish which bore PPD-pulsed PECs. Because of the importance of adherent PECs in the initial attachment of lymphocytes to culture dishes, we did not discard nonadherent cells at 24 h from either dish in this experiment. When cells were harvested at 7 days and placed in microtiter wells, those which had been cultured without adherent PECs had very low baseline [<sup>3</sup>H]TdR incorporation and failed to respond to antigen while the cells which had been cultured with PPD-pulsed adherent PEC had a substantial baseline incorporation.

Finally, we examined the requirement for the addition of fresh PECs to the microtiter wells to obtain antigen-independent [<sup>3</sup>H]TdR uptake. Strain 2 cells which had been selected for responsiveness to DNP-GL and which had entered an antigen-independent phase were added to microtiter wells with or without fresh syngeneic PEC (Table VI). Those wells which had not received fresh PECs incorporated little [<sup>3</sup>H]TdR (<1,000 cpm) whereas those to which fresh syngeneic PECs were added incorporated in excess of 100,000 cpm of [<sup>3</sup>H]TdR.

TABLE VI  
*Requirement for Added PECs for [<sup>3</sup>H]TdR Incorporation by  
 Antigen-Independent Cell Cultures*

Exp.	PEC in well	[ <sup>3</sup> H]TdR uptake
1	0	760 ± 117
	1 × 10 <sup>5</sup>	121,697 ± 20,004
2	0	103 ± 32
	1 × 10 <sup>5</sup>	138,966 ± 14,245

Lymphocytes were harvested from antigen-independent cultures; 1 × 10<sup>5</sup> syngeneic adherent PECs. The latter had not been pulsed with antigen and no antigen was added to the wells. The amount of [<sup>3</sup>H]TdR incorporated was measured 4 days later.

### Discussion

The experiments presented in this paper demonstrate that a substantial degree of specific in vitro T-cell selection can be achieved by culturing immune PELs on dishes to which antigen-pulsed PECs from nonimmune donors had been allowed to adhere. Superficially, this system resembles the depletion and relative enrichment which has been achieved for T lymphocytes cytotoxic for alloantigen-bearing fibroblasts and tumor cells (5, 6). However, the technique employed here allows the in vitro selection of cells responding to relatively simple and well characterized protein, hapten-protein, and hapten-polypeptide antigens. Indeed, one of the antigens with which we have had most success in this procedure is DNP-GL, an antigen, responsiveness to which is controlled by a histocompatibility linked immune response (*Ir*) gene (12). Thus, if these cells can be demonstrated to be enriched for T cells specific for such antigens and can be propagated in vitro for extended periods, they may be excellent sources for the molecular analysis of specific T-lymphocyte receptors and *Ir*-gene products. Although we will consider the mechanisms by which cells are selected in these cultures in more detail in a subsequent paper,<sup>2</sup> certain aspects of the selection culture procedure bear comment. Our technique involves the plating of PELs from immune guinea pigs on culture dishes bearing adherent PECs which have been previously pulsed with an antigen to which the donors were immunized. This approach was suggested both by the difficulty encountered in depleting specific T lymphocytes by means of conventional immunoadsorbents and by the detailed studies of several workers demonstrating the great importance of adherent cells (presumably macrophages) in the activation of antigen-specific lymphocyte responses (7-9). A series of studies by Rosenthal and his colleagues (for review see 18) indicate that macrophage-associated antigen is particularly efficient for the activation of specific guinea pig T lymphocytes. Moreover, Lipsky and Rosenthal have demonstrated that guinea pig lymphocytes will bind to guinea pig macrophages which are adhering to glass or plastic surfaces (19). Initially (at 2 h), this phenomenon is antigen independent (19) but by 24 h, the number of lymphocytes from immunized donors adhering to macrophages pulsed

with antigen is greatly in excess of the number adhering to nonpulsed macrophages or to macrophages pulsed with an antigen to which the lymphocyte donor was not immune (10). With this in mind, we allowed purified PELs to incubate on dishes bearing antigen-pulsed adherent PECs for 24 h before discarding nonadherent cells. We reasoned that this should add a substantial degree of selection to our procedure. As demonstrated in the second paper of this series,<sup>2</sup> discarding nonadherent cells at 24 and 48 h does improve the degree of selection, but substantial selective effects are seen even if the nonadherent cells are not discarded at 24 and 48 h.

Because of the potential importance of cultures selected for antigen-specific T lymphocytes, we will review the evidence that the cells are specifically selected and that they are predominantly T lymphocytes and then discuss the problem of whether they are enriched in specific cells as well as selected for specific responsiveness. In each experiment presented in the Results and in 93 of 99 individual cases, cells harvested from a dish bearing adherent PECs pulsed with a given antigen responded better to that antigen than to other antigens to which the donors of the PELs had been primed. In many cases, the harvested cells were virtually unresponsive to the alternate antigen(s). In particular, in sequential cultures on dishes bearing antigen-pulsed adherent PECs, cells obtained after a second selection culture were almost always unresponsive to alternate antigens. Thus, in a functional sense, a high degree of selection has clearly been obtained.

The cell population which is initially plated, PELs has been previously demonstrated to be highly enriched in T lymphocytes (20) and to be very reactive to antigen (13), both in terms of antigen-stimulated uptake of [<sup>3</sup>H]-TdR and production of migration inhibition factor. Finally, analysis of the recovered cell population indicates that there are few cells bearing surface Ig or receptors for activated C3, suggesting that few B lymphocytes are present. In most cases, very few of the cells form rosettes with sheep erythrocytes coated with IgG antibody, indicating that relatively few of the cells recovered are monocytes or macrophages. In some instances, however, substantial numbers of macrophages do appear in the selected cell population. A majority of, but by no means all, the cells harvested from selection cultures form rosettes with rabbit erythrocytes. Guinea pig cells forming such rosettes have been previously shown to be T lymphocytes (17), although it is not clear that all T lymphocytes form rosettes with rabbit erythrocytes. Thus, it seems certain that a majority of the recovered cells are T lymphocytes; in some cases, most of the remainder may also be T lymphocytes but this determination is mainly based upon their lack of surface Ig, C3 receptors, or receptors for IgG. Interestingly, some plasma cells are also observed and, in preliminary experiments, DNP-specific plaque-forming cells have been found in cultures harvested from DNP-GL-bearing monolayers.

The problem of whether enrichment of specific T lymphocytes, as opposed to functional selection, has been achieved is more difficult to answer on the basis of the information now available. Clearly, responses to unrelated antigens are markedly diminished, which would be most consistent with a loss of the cells responsible for such responses. Since our data suggests that adherent PECs provide relatively few cells to the population harvested at day 7,<sup>2</sup> it would be logical to conclude that enrichment has occurred. On the other hand, the

absolute amount of [ $^3\text{H}$ ]TdR incorporated by selected cultures in response to the antigen for which they were selected is often no higher and indeed may be lower than the absolute response to the cells assessed for [ $^3\text{H}$ ]TdR responses before the selection culture (i.e., immediately after purification from the cell donor). Although this would suggest that enrichment has not occurred, such a conclusion depends upon the existence of a linear relation between the number of specific cells and the amount of [ $^3\text{H}$ ]TdR incorporated. If other, nonspecific, cells play a critical role in the proliferative response, an absence of such cells might serve to diminish this response. Indeed, we will show in a subsequent paper<sup>2</sup> that adding PELs from nonprimed donors to cells obtained from selection cultures often increases the response of the latter cells to the antigen for which they were selected. A final determination of whether enrichment has actually occurred will require an assay which allows the enumeration of specific cells.

In addition to our ability to achieve an initial selection with this procedure, we have been able to sequentially transfer the cell cultures and retain responsiveness for 5 wk. Some cultures transferred in this way enter an antigen-independent phase during which they can proliferate and be maintained in the absence of antigen although they continue to require the presence of adherent PECs. Cells obtained from both extended "antigen-responsive" and antigen-independent cultures are principally T lymphocytes. It would be tempting to conclude that the antigen-independent cells are derived from the cells which were responsive to antigen in the initial selection cultures. However, we have no evidence that these antigen-independent cells do not derive from other cells present in the selection cultures.

Another example of extended culture of specifically responsive T-cell populations has recently been reported by MacDonald et al. (21). They demonstrated that mouse cells mediating mixed lymphocyte responses and specific T-lymphocyte-dependent cytotoxicity could be preserved in cultures for long periods (up to 64 days). Although they did not report as to whether a specific selection had been achieved, Howard and Wilson (22), using a rat mixed lymphocyte response system, obtained a clear selection and apparent enrichment of specific T lymphocytes. Whether the procedures described in our paper or those employed by authors studying alloantigen-responsive T cells will provide highly enriched lines of antigen-specific T lymphocytes remains to be established. The potential importance of such cell populations in the study of mechanisms and control of T-cell activation and of the recognition structures of T cells is clear.

### Summary

Specific selection of antigen-responsive guinea pig peritoneal exudate lymphocytes (PELs) was achieved by a selection culture procedure. This procedure involved the addition of PELs from immune donors to monolayers of antigen-pulsed adherent peritoneal exudate cells from nonprimed syngeneic donors. PELs which failed to adhere were discarded at 24 and 48 h; after 1 wk of culture, lymphocytes were obtained which were highly responsive to the antigen for which they were selected but which demonstrated little or no response to other antigens to which the original donor of the lymphocyte was immune. These selected cells were largely T lymphocytes and could be maintained in culture for 2-5 wk in an

antigen-responsive state and, in 20–30% of cases, for 8–10 wk in an antigen-independent state.

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