INDEPENDENT POPULATIONS OF PRIMED F_1 GUINEA PIG T LYMPHOCYTES RESPOND TO ANTIGEN-PULSED PARENTAL PERITONEAL EXUDATE CELLS

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Antigen-stimulated proliferative responses of thymus-dependent (T)[1] lymphocytes from immunized guinea pigs appear to require the presence of adherent cells which may be obtained from peritoneal exudates of nonimmune guinea pigs (1). One principal function of these peritoneal exudate cells (PECs) is the presentation and/or processing of antigen. Thus, brief exposure to antigen of PECs from nonimmune donors enables them to subsequently stimulate proliferative responses by T lymphocytes from primed guinea pigs. On the other hand, exposing macrophage-depleted immune T lymphocytes to antigens, washing these cells, and then adding PECs from nonimmune donors does not lead to a response.

The antigen-presentation function of PECs displays a clear histocompatibility restriction (2, 3). Thus, the activation of proliferative responses by T lymphocytes from primed strain 13 guinea pigs is most efficient if one employs antigen-pulsed PECs from donors that possess strain 13 *I*-region-associated (Ia) histocompatibility antigens. Similarities or differences at the closely linked *B* and *S* histocompatibility loci, the guinea pig equivalent of the *H-2K* and *H-2D* loci of mice (4, 5), do not appear important in determining whether antigen-pulsed PECs can activate primed T lymphocytes.

Successful activation of primed T lymphocytes can be obtained if the donor of the antigen-pulsed PECs and the donor of the T lymphocytes share Ia antigens encoded by only one of the two major histocompatibility complex (MHC) haplotypes present in heterozygous individuals. Thus, antigen-pulsed PECs from $(2 \times 13)F_1$ donors can activate primed T lymphocytes from either strain 2 or strain 13 donors. Similarly, T lymphocytes from primed $(2 \times 13)F_1$ donors can be activated by antigen-pulsed PECs derived either from strain 2 or strain 13 guinea pigs (2)

In order to obtain further insight into the cellular and molecular basis of the histocompatibility restriction in the interaction of antigen-pulsed PECs and primed T lymphocytes, we wished to determine whether individual "antigen-

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[1] *Abbreviations used in this paper:* BUdR, bromodeoxyuridine; CFA, complete Freund's adjuvant; CI, cellular interaction, ^{3}H-TdR, (*methyl*-^{3}H)thymidine; MHC, major histocompatibility complex; NGPS, normal guinea pig serum, OVA, ovalbumin; PECs, peritoneal exudate cells; PPD, purified protein derivative of tuberculin, T, thymus dependent

specific" T lymphocytes from primed (2 × 13)F_1 donors displayed a selective capacity to be activated by antigen-pulsed PECs derived from one or the other parental strain. Through the use of positive and negative in vitro selection techniques we show here that the T lymphocytes from primed F_1 donors capable of responding to ovalbumin (OVA)-pulsed strain 2 PECs constitute a separate population from those that may be activated by OVA-pulsed strain 13 PECs. Similarly, the population of primed F_1 T lymphocytes which respond to strain 2 PECs pulsed with purified protein derivative of tuberculin (PPD) is largely independent of the population that responds to PPD-pulsed 13 PECs.

Materials and Methods

Animals. Strain 2, strain 13, and (2 × 13)F_1 guinea pigs were obtained from the Division of Research Services, National Institutes of Health, Bethesda, Md. Strain 2 guinea pigs possess the Ia antigens 2, 4, and 5, the B region antigen 1, and are S^+ (Ia.2,4,5; B.1; S^+) while strain 13 guinea pigs are Ia.1,3,5; B.1, S^+ and (2 × 13)F_1 animals are Ia.2,4,5/Ia.1,3,5; B.1/B.1; S^+/S^+ (4,5).

Immunization. Animals were immunized with 100 μg of OVA (5 × recrystallized; Mann Research Labs, Inc., New York) emulsified in complete Freund's adjuvant (CFA; Difco Laboratories, Detroit, Mich.) or with CFA alone. A total of 0.4 ml of emulsion was injected, equally divided between the four foot pads. Immunized animals were used for study between 2 wk and 2 mo after immunization.

Preparation of PECs and Peritoneal Exudate Lymphocytes. PECs were obtained from nonimmunized strain 2, strain 13, and F_1 donors which had received intraperitoneal infusions of 25 ml of sterile mineral oil (Marcol 52; Humble Oil & Refining Co., Houston, Tex) 3 days previously. PECs were washed and incubated with either no antigen or with 100 μg/ml of OVA or of PPD (Connaught Medical Research Laboratory, Willowdale, Ontario, Canada) for 1 h at 37°C in RPMI 1640 containing L-glutamine (300 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), and fetal calf serum (10%). Mitomycin C (25–50 μg/ml) was also present in the incubation mixture. At the end of the "pulsing" period, the PECs were washed four times. They were either used immediately or incubated for 4–16 h in culture tubes at 5 × 10^6/ml and then washed three times.

Peritoneal exudate lymphocytes from immunized donors were purified from exudates induced by the infusion of 25 ml of sterile mineral oil 3 days earlier. The purification procedure involved passage over rayon wool columns as previously described (6). The cells obtained are approximately 85% lymphocytes of which less than 5% bear surface Ig or complement receptors Consequently, peritoneal exudate lymphocytes are regarded as a cell population which is principally T lymphocyte in composition and which will be referred to hereafter as T lymphocytes.

Positive Selection Procedure. Two techniques were used to obtain positive selection of antigen-responsive T lymphocytes. In one positive selection technique (method a), 1 × 10^6 antigen-pulsed PECs and 5 × 10^6 T lymphocytes from primed donors were cultured together in 1.5 ml of RPMI 1640, supplemented as described above, in 15-ml culture tubes for 1 wk. The cells harvested at the end of that time were tested for responsiveness to various stimulants

In a more elaborate positive selection technique (method b), which has been described in detail (7), 30 × 10^6 antigen-pulsed PECs which had been cultured overnight were washed and allowed to adhere to plastic culture dishes. Nonadherent cells were discarded and 10–20 × 10^6 T lymphocytes from immunized donors were added and incubated for 24 h. Those T lymphocytes which failed to bind to the "monolayer" of antigen-pulsed adherent cells were discarded and the remaining cells were cultured for 6 additional days. These cells were then harvested and their capacity to respond to various stimulants was tested

Negative Selection Procedure T lymphocytes (6 × 10^6) from immunized donors were mixed with 2 × 10^6 antigen-pulsed PECs which had been preincubated for 4 or 16 h. The cells were cultured for 2 days in medium consisting of RPMI 1640, supplemented as already noted. Bromodeoxyuridine (BUdR; ICN Nutritional Biochemicals Div., International Chemical & Nuclear Corp., Cleveland, Ohio) was added to a final concentration of 3 × 10^{-6} M and the cells incubated for an additional 24 h. The cultures were illuminated by placing them directly over three Westinghouse Cool-Ray fluorescent tubes (Westinghouse Electric Corp., Pittsburgh, Pa.) for 90 min. The cells

were then washed, the cell yield determined, and the cells cultured with a variety of stimulants The use of this procedure for guinea pig T cells has been fully described by Janeway and Paul (8).

Stimulation of (methyl-^{3}H)Thymidine (^{3}H-TdR) Incorporation. T lymphocytes (2×10^5) obtained from positive or negative selection cultures or directly purified from immunized donors were cultured with antigen-pulsed PECs (1×10^5) in a volume of 0.2 ml in round bottom microtiter plates (Cook Laboratory Products Div., Dynatech Laboratories, Inc., Arlington, Va.). After 48 h, 1 μCi of ^{3}H-TdR (6 7 Ci/mol; New England Nuclear, Boston, Mass.) was added; the cells were cultured for an additional 16 h and incorporation of ^{3}H-TdR measured using a semiautomated harvester

Alloantisera. Strain 2 anti-strain 13 (anti-Ia.1,3) and strain 13 anti-strain 2 (anti-Ia.2,4) sera were prepared as previously described (9). Sera were heat inactivated before use.

Results

Positive Selection. We have previously shown that positive selection of antigen-specific T lymphocytes can be achieved by culturing T lymphocytes from primed donors on antigen-pulsed monolayers of PECs and discarding cells that fail to adhere to the monolayer (7). After 1 wk of such culture, the residual T cells show augmented responses to the antigen that was present on the PECs and are markedly depleted in responsiveness to other antigens to which the T-lymphocyte donor had been primed. Indeed, simply co-culturing antigen-pulsed PECs and T lymphocytes from primed donors leads to a substantial degree of antigen-specific selection (10).

In the current studies, we carried out antigen-specific positive selection by culturing T lymphocytes from $(2 \times 13)F_1$ donors, which had been primed to OVA in CFA, with strain 2 or strain 13 PECs which had been pulsed with OVA. We then determined the responsiveness of the harvested cells to OVA- or PPD-pulsed strain 2 or strain 13 PECs. Table I illustrates the results of such an experiment. F_1 T lymphocytes freshly harvested from an immunized donor were tested for their responsiveness to OVA-pulsed 2, OVA-pulsed 13, PPD-pulsed 2, and PPD-pulsed 13 PECs. Each of these antigen-pulsed PECs stimulated substantial incorporation of ^{3}H-TdR. Another portion of the F_1 T lymphocytes was cultured for 1 wk with OVA-pulsed 2 PECs and then tested for its responses to OVA-pulsed 2 or 13 PECs and to PPD-pulsed 2 or 13 PECs. These T lymphocytes, which had been positively selected by OVA-pulsed 2 PECs, gave marked proliferative responses to OVA-pulsed 2 PECs and a very meager response to PPD-pulsed 2 PECs, indicating that a striking antigen-specific selection had been achieved. More importantly, the selected cells responded very poorly to OVA-pulsed strain 13 PECs, indicating that selected F_1 T cells clearly discriminated between OVA-pulsed 2 and OVA-pulsed 13 PECs. F_1 T lymphocytes which had been selected by initial culture with OVA-pulsed 13 PECs responded well to OVA-pulsed 13 PECs and poorly to both PPD-pulsed 13 PECs and OVA-pulsed 2 PECs. Thus, PEC-specific selection can be achieved by preculturing with either OVA-pulsed 2 or OVA-pulsed 13 PECs.

Table II gives results for four individual experiments, including the experiment presented in Table I. In order to compare the results of these experiments, we introduced the parameter "relative responsiveness" which is the net counts per minute (Δ counts per minute) of ^{3}H-TdR incorporated in response to the OVA-pulsed PECs which had been used for selection divided by Δ counts per minute incorporated in response to OVA-pulsed PECs derived from the other parent. The value for relative responsiveness ranged from 2 4 to 105 for T lymphocytes selected by culture with OVA-pulsed 2 PECs and from 2.5 to 7.7 for lymphocytes selected by culture with OVA-pulsed 13 PECs. These results strongly suggest that the population of F_1 T lymphocytes capable of responding to OVA-pulsed strain 2 PECs is largely independent of the population that responds to OVA-pulsed strain 13 PECs

Negative Selection An alternative approach to the analysis of the specificity of T-

TABLE I

Positive Selection of F_1 T Lymphocytes by OVA-Pulsed Parental PECs

T-lymphocyte population	^{3}H-TdR uptake (cpm × 10^{-3})				Relative responsiveness, Δ cpm (OVA-pulsed PECs used for selection)/Δ cpm (OVA-pulsed PECs not used for selection)
	PECs	Antigen used for pulsing			
		None	OVA	PPD	
Freshly harvested from OVA-CFA-primed F_1 donors	2	2.5	44 5	33.5	
	13	3.9	52 3	32.7	
Cultured for 1 wk with OVA-pulsed 2 PECs	2	0.8	85.3	2.4	105
	13	1.0	1.8	0.9	
Cultured for 1 wk with OVA-pulsed 13 PECs	2	0.9	23.4	1.6	3.6
	13	2.7	83.3	1.1	

T lymphocytes from (2 × 13)F_1 donors primed with OVA in CFA were purified from peritoneal exudates One portion was tested for responsiveness by culturing in microtiter plates with strain 2 or strain 13 PECs which had been pulsed with nothing, OVA (100 μg/ml), or PPD (100 μg/ml). Uptake of ^{3}H-TdR was measured by the addition of 1 μCi of ^{3}H-TdR 48 h later and determination of incorporated radioactivity 16 h thereafter. Other portions of the T lymphocytes were co-cultured with OVA-pulsed 2 or OVA-pulsed 13 PECs for 1 wk and their responsiveness measured by subsequent culture in microtiter wells with pulsed 2 or 13 PECs. Underlined values indicate cultures in which positive selection had occurred. Relative responsiveness to OVA-pulsed PECs is counts per minute incorporated in response to OVA-pulsed PECs of the type used for selection minus counts per minute incorporated in response to nonpulsed PECs of the type used for selection divided by counts per minute incorporated in response to OVA-pulsed PECs of the type not used for selection minus counts per minute incorporated in response to nonpulsed PECs of the type not used for selection.

TABLE II

Positive Selection of F_1 T-lymphocytes by OVA-Pulsed Parental PECs, Summary Data

Exp	Method	PELs selected with OVA-pulsed 2 PECs			PELs selected with OVA-pulsed 13 PECs		
		Response to OVA-pulsed PECs (Δ cpm × 10^{-3})		Relative Response (Δ cpm_2/Δ cpm_{13})	Response to OVA-pulsed PECs (Δ cpm × 10^{-3})		Relative Response (Δ cpm_{13}/Δ cpm_2)
		2	13		2	13	
1	a	84 5	0 8	105	22 5	81 2	3 6
2	a	41 0	17 4	2 4	7 6	56 6	7 4
3	b	16 5	4 8	3 4	4 5	11 1	2 5
4	b	18 2	4 5	4 0	0 3	2 3	7 7

F_1 T lymphocytes were selected with OVA-pulsed 2 or OVA-pulsed 13 PECs and their responsiveness measured as described in the legend to Table I Method a is a positive selection procedure in which primed and antigen-pulsed PECs are co-cultivated in tubes and the cells present 1 wk later are tested for responsiveness Method b is a positive selection procedure in which primed T lymphocytes are "plated" on monolayers of adherent antigen-pulsed PECs Cells that fail to adhere are discarded 24 h later and cells appearing in the culture fluid at 1 wk tested for antigen responsiveness

lymphocyte activation by antigen-pulsed PECs is to selectively eliminate the population of F_1 cells which proliferate upon stimulation with antigen-pulsed PECs derived from one parent and then to test responsiveness of residual cells to antigen-pulsed PECs from both parents and from F_1 donors. To accomplish this, BUdR, a thymidine analogue, was added during the time responding cells were synthesizing DNA. The cultures were then exposed to light which activates BUdR and leads to cross-linking of chromatin strands, preventing any further proliferation by such cells (11). The procedure employed is outlined in Fig. 1.

We initially carried out an experiment to validate the use of this procedure for

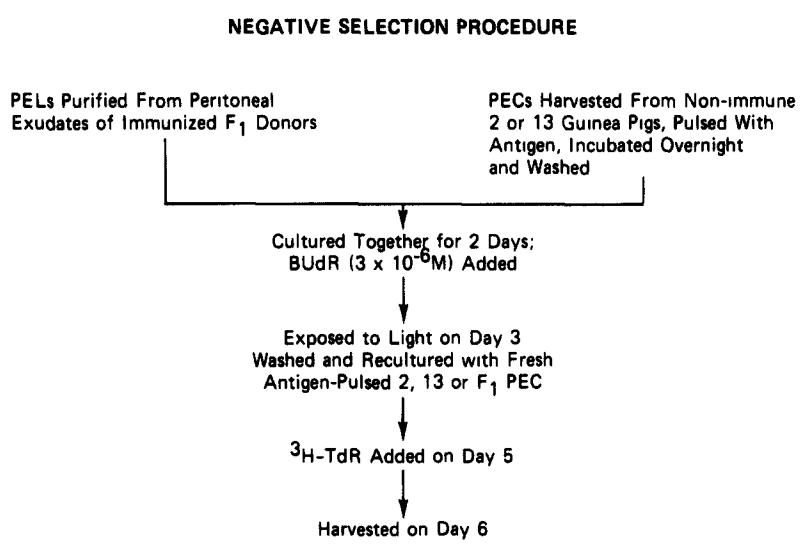

FIG. 1 An outline of the BUdR and light negative selection procedure is presented. The abbreviation PELs signifies peritoneal exudate lymphocytes, which is principally a T-lymphocyte population.

TABLE III
Specificity of Negative Selection Procedure

First culture	Second culture	^{3}H-TdR uptake (cpm × 10^{-3})		Residual response, (Δ cpm [BUdR + light])/(Δ cpm [light])
		BUdR and light	Light only	
2 PEC-PPD + 13 PEC-PPD	2 PEC-0	1 5	22.8	
	2 PEC-PPD	6 1	80.2	8 0
	2 PEC-OVA	130 2	155 1	97 2
	13 PEC-0	1.1	17.6	
	13 PEC-PPD	15.8	68 1	29 1
	13 PEC-OVA	44.8	64 6	93 0

T lymphocytes from (2 × 13)F_1 donors primed to OVA in CFA were cultured (first culture) with both PPD-pulsed 2 PECs and PPD-pulsed 13 PECs. At 48 h, half of the cultures received BUdR (3×10^{-6} M) and 24 h later, all the cultures were illuminated. The cells were washed and recultured (second culture) with strain 2 or strain 13 PECs which had been pulsed with nothing (0), OVA, or PPD ^{3}H-TdR uptake was determined at day 3 of the second culture. Underlined values indicate negative selection

lymphocyte stimulation using antigen-pulsed PECs (Table III). T lymphocytes from (2 × 13)F_1 donors which had been primed to OVA in CFA were cultured with a mixture of PPD-pulsed strain 2 and PPD-pulsed strain 13 PECs. 2 days later, BUdR was added to some cultures; after an additional 24 h, all cultures were exposed to light. The cells were washed and recultured with PPD-pulsed strain 2 or 13 PECs or with OVA-pulsed strain 2 or 13 PECs. Responses to OVA-pulsed strain 2 or 13 PECs were quite substantial and were similar in cultures that had been treated with BUdR and light or with light only. On the other hand, responses of T lymphocytes which had been exposed to BUdR and light to PPD-pulsed PECs from either strain 2 or strain 13 donors were much lower than were the responses of T lymphocytes which had been exposed to light only. The net response to

TABLE IV

Negative Selection of OVA and PPD Immune F_1 T Lymphocytes by Antigen-Pulsed Parental PECs

First culture		Second culture		
		^{3}H-TdR uptake (cpm × 10^{-3})		
Origin of PECs and antigen used for pulsing	Antigen used for pulsing (100 μg/ml)	2 PECs	13 PECs	F_1 PECs
PPD-pulsed 2 PECs	0	1.1	3.7	1.8
	PPD	4 1	59.7	52.2
	OVA	125.5	52.6	89 3
PPD-pulsed 13 PECs	0	5.2	1.9	7 8
	PPD	91.0	8 7	79.4
	OVA	141 9	54.4	101.6
OVA-pulsed 2 PECs	0	1.0	4.9	3.4
	PPD	86 8	55 9	90.1
	OVA	1.9	25.4	19.1
OVA-pulsed 13 PECs	0	26.8	2.4	22.3
	PPD	95 3	67 7	102.7
	OVA	102.7	4.5	65 9

T lymphocytes from OVA-CFA-primed F_1 donors were cultured with PPD- or OVA-pulsed strain 2 or strain 13 PECs for 2 days, at which time BUdR (3×10^{-6} M) was added. After an additional 24 h, the cultures were exposed to light for 90 min, washed, and recultured with 2, 13, or F_1 PECs which had been pulsed with nothing, OVA, or PPD. Incorporation of ^{3}H-TdR was measured 3 days later Underlined values indicate instances of specific negative selection

PPD-pulsed 2 PECs of T lymphocytes exposed to BUdR and light was 8% of the response of cells exposed to light only. Responsiveness of BUdR- and light-treated T lymphocytes to PPD-pulsed 13 PECs was 29% of the response of T lymphocytes treated with light only.

It should be noted that both the positive and negative selection procedures involve co-cultivation of immune T lymphocytes and antigen-pulsed PECs. In the negative selection procedure, the unstimulated cells retain responsiveness over the 3-day selection period. In the positive selection procedure, it appears that responsiveness of unstimulated cells is lost during the 7-day selection period.

Having demonstrated the effectiveness of the negative selection procedure, we carried out such a selection on $(2 \times 13)F_1$ T lymphocytes from donors primed with OVA in CFA using OVA-pulsed 2 PECs, OVA-pulsed 13 PECs, PPD-pulsed 2 PECs, and PPD-pulsed 13 PECs (Table IV). In each instance, the response of these cells to the antigen-pulsed PECs used for negative selection was very low. For example, if $(2 \times 13)F_1$ T lymphocytes were precultured with PPD-pulsed PECs from strain 2 donors, the net incorporation of ^{3}H-TdR upon restimulation with PPD-pulsed 2 PECs was only 3.1×10^3 cpm. The same cells responded well to OVA-pulsed 2, 13, or F_1 PECs (net uptake 48.9×10^3 cpm or more) indicating that antigen-specific negative selection had been achieved. Furthermore, the selected cells gave excellent responses to PPD-pulsed 13 or F_1 PECs (50.4×10^3 cpm and 56.0×10^3 cpm, respectively) indicating that a negative selection specific for the strain of origin of the antigen-pulsed PEC had also been achieved. As indicated in Table IV, cells negatively selected with any given antigen-pulsed PEC responded well to antigen-pulsed PECs other than those used for the negative selection.

TABLE V

Negative Selection of OVA and PPD Responsive F_1 T Lymphocytes in the Same Culture

First culture	Antigen used for pulsing	Second Culture (^{3}H-TdR uptake, cpm × 10^{-3})			
		Exp 1		Exp. 2	
		2 PECs	13 PECs	2 PECs	13 PECs
PPD-pulsed 2 PECs + OVA-pulsed 13 PECs	0	0.25	0 22	0.56	0.38
	OVA	56.1	3.2	33.0	3 7
	PPD	1.6	15 3	17.7	35.3
OVA-pulsed 2 PECs + PPD-pulsed 13 PECs	0	0 35	0.36	0 46	0 55
	OVA	8.5	20.1	9.0	17 8
	PPD	12 1	3 6	47.3	9.3

T lymphocytes from (2 × 13)F_1 donors which had been primed to OVA in CFA were cultured with PPD-pulsed 2 PECs and OVA-pulsed 13 PECs or with OVA-pulsed 2 PECs and PPD-pulsed 13 PECs (first culture). BUdR was added after 48 h and the cells were illuminated 24 h later. The cells harvested from these cultures were then tested for their responsiveness to 2 or 13 PECs which had been pulsed with nothing, OVA, or PPD. Underlined values indicate cultures demonstrating negative selection

These experiments extend the positive selection experiments in two important ways. Firstly, they show that selective responsiveness to antigen-pulsed parental PECs does not depend on two independent populations of T cells, one that is specific for antigen and the other for MHC gene products. If that were the case, we would anticipate that negative selection with PPD-pulsed 2 PECs should either eliminate the response to PPD-pulsed 13 PECs, by destroying the antigen-specific cell, or should diminish the response to OVA-pulsed 2 PECs, by eliminating the T-cell specific for the strain 2 MHC gene product.

Secondly, these experiments strongly suggest that selective responsiveness is not due to the development of some type of suppressive phenomenon initiated by exposure to the MHC structures expressed on the antigen-pulsed PEC which fails to stimulate proliferative responses. If such suppression were operative, then cells negatively selected by PPD-pulsed 2 PECs should fail to respond both to PPD-pulsed (2 × 13)F_1 PECs and to PPD-pulsed 2 PECs. However, such negatively selected cells respond to PPD-pulsed (2 × 13)F_1 PECs as well as they respond to PPD-pulsed 13 PECs.

The question of suppressive factors artifactually influencing these results was also addressed by attempting a "double"-negative selection (Table V). T lymphocytes from (2 × 13)F_1 donors primed with OVA in CFA were cultured with PPD-pulsed 2 PECs and OVA-pulsed 13 PECs or with OVA-pulsed 2 PECs and PPD-pulsed 13 PECs. After addition of BUdR and subsequent illumination, the cells were recultured with OVA-pulsed 2, OVA-pulsed 13, PPD-pulsed 2, and PPD-pulsed 13 PECs and the uptake of ^{3}H-TdR measured. T lymphocytes harvested from cultures negatively selected with PPD-pulsed 2 PECs and OVA-pulsed 13 PECs responded well to PPD-pulsed 13 PECs and to OVA-pulsed 2 PECs but poorly to PPD-pulsed 2 PECs and to OVA-pulsed 13 PECs. Similarly, F_1 PELs negatively selected with OVA-pulsed 2 PECs and PPD-pulsed 13 PECs responded well to OVA-pulsed 13 PECs and to PPD-pulsed 2 PECs and poorly to OVA-pulsed 2 PECs and to PPD-pulsed 13 PECs. This experiment makes it very unlikely that the failure of negatively selected cells to respond can be due to the development of suppressor cells specific for either strain 2 or strain 13 alloantigens. If such cells were present, then we would expect that neither antigen-pulsed 2 or 13 PECs should stimulate responses in double-negative selection cultures.

Role of Ia Antigens in Negative Selection by Antigen-Pulsed PECs. Rosenthal and

Shevach (2) and Shevach (3) have previously reported that sharing of Ia antigens between donors of antigen-pulsed PECs and donors of primed T lymphocytes was an important factor in the collaboration of these cells. Similarity at the *B* locus, which is the guinea pig equivalent of the mouse *H-2D* and/or *K* loci, was not required for such interaction nor in the face of differences in Ia antigens, would similarity at the *B* locus allow for effective collaboration. Consequently, it seemed most likely that the specific selection of subpopulations of primed F_1 T lymphocytes by antigen-pulsed parental PECs depended upon the Ia antigens of the PECs and/or those of the T lymphocytes. We are currently performing formal studies to establish this by examining the potential of PECs from backcross and outbred guinea pigs with known Ia antigens to activate T lymphocytes from primed F_1 donors which have been selected by culture with antigen-pulsed parental PECs.

An alternative approach to establishing a role for Ia antigens in this selection procedure is based on the finding that anti-Ia antibodies block antigen-specific proliferative responses. In particular, the activation of T lymphocytes from primed (2 × 13)F_1 donors by antigen-pulsed PECs from strain 2 donors can be inhibited by antisera with principal specificity for Ia antigens of strain 2 guinea pigs (2). Similarly, the activation of F_1 lymphocytes by antigen-pulsed strain 13 PECs is inhibited by antisera specific for strain 13 Ia antigens. We reasoned that if Ia antigens were critical in the antigen-specific selection of primed F_1 T lymphocytes, the addition of anti-Ia sera during the negative selection culture should prevent ablation of antigen-responsive T lymphocytes. Furthermore, if negative selection of primed F_1 T lymphocytes by antigen-pulsed F_1 PECs were carried out in the presence of anti-2 sera, we would expect that those F_1 T lymphocytes capable of responding to antigen-pulsed 13 PECs should be eliminated while those responsive to antigen-pulsed 2 PECs should be spared. Anti-13 sera should have the opposite effect.

In order to test these possibilities, T lymphocytes from CFA-primed F_1 donors were negatively selected by culture with PPD-pulsed F_1 PECs in the presence of normal guinea pig serum (NGPS), in the presence of anti-2 serum (anti-Ia.2,4), or in the presence of anti-13 serum (anti-Ia.1,3) (Table VI). In one of the experiments presented in this table, negative selection of F_1 T lymphocytes with antigen-pulsed parental PECs was also carried out in order to give an indication of responsiveness to the alternative antigen-pulsed PEC in the subsequent culture. Thus, in experiment 1, F_1 T lymphocytes negatively selected with PPD-pulsed 2 PECs subsequently exhibited a net incorporation of 0.5 × 10^3 cpm in response to PPD-pulsed 2 PECs and a net incorporation of 7.5 × 10^3 cpm in response to PPD-pulsed 13 PECs. When negatively selected with PPD-pulsed 13 PECs, the subsequent response of these cells to PPD-pulsed 2 PECs was 22 × 10^3 cpm and that to PPD-pulsed 13 PECs was 0.2 × 10^3 cpm. Negative selection with PPD-pulsed F_1 PECs in the presence of NGPS diminished responsiveness to both 2 and 13 PECs pulsed with PPD, although not to the extent achieved by negative selection with PPD-pulsed PECs from the appropriate parent. However, negative selection of primed F_1 T lymphocytes with PPD-pulsed F_1 PECs in the presence of anti-2 serum yielded a cell population which responded to PPD-pulsed 2 PECs almost as well as did F_1 T lymphocytes which had been negatively selected with PPD-pulsed 13 PECs. These negatively selected T lymphocytes responded to PPD-pulsed 13 PECs no better than did F_1 T lymphocytes negatively selected with F_1 PECs in the presence of NGPS. Consistent results were obtained when negative selection of F_1 T lymphocytes by PPD-pulsed F_1 PECs was carried out in the presence of anti-13 serum.

These results show that negative selection can be specifically inhibited by anti-Ia sera. In turn, this indicates that the capacity of T lymphocytes from primed F_1 donors to distinguish between parental PECs pulsed with the same antigen is based, at least in part, on the Ia antigens of the PECs and/or the T lymphocytes.

TABLE VI
Anti-Ia Sera Inhibit Negative Selection

First culture		Second culture			
		^{3}H-TdR uptake in response to PPD-pulsed PEC (Δ cpm × 10^{-3})			
		Exp. 1		Exp. 2	
PPD-pulsed PEC	Serum	2	13	2	13
2 (Ia 2, 4, 5)	NGPS	0.5	7 5		
13 (Ia.1, 3, 5)	NGPS	22 0	0 2		
(2 × 13)F_1	NGPS	6.6	3.7	4.8	1.2
(2 × 13)F_1	Anti-2(anti-Ia.2,4)	17 0	4 8	19 3	1.5
(2 × 13)F_1	Anti-13(anti-Ia.1,3)	8.5	7.2	4.4	3.4

T lymphocytes from primed (2 × 13)F_1 guinea pigs were cultured (first culture) with PPD-pulsed 2, 13, or F_1 PECs in the presence of normal guinea pig serum (NGPS), anti-2 serum, or anti-13 serum at a final concentration of 5%. BUdR was added at 48 h and the cultures were illuminated 24 h later. The cells were harvested and recultured (second culture) in the presence of strain 2 or strain 13 PECs which had been pulsed with nothing or PPD. ^{3}H-TdR uptake was measured 3 days later and is reported as ^{3}H-TdR uptake in the presence of PPD-pulsed PECs minus ^{3}H-TdR uptake in the presence of unpulsed PECs (Δ cpm) Underlined values indicate cultures in which negative selection had occurred

Discussion

The results presented here indicate that in primed (2 × 13)F_1 guinea pigs, the group of T lymphocytes responsive to OVA or to PPD can each be subdivided into two largely independent subpopulations, one that can be activated by antigen-pulsed strain 2 PECs and the second that can be activated by antigen-pulsed strain 13 PECs. The critical observation supporting this contention is that it is possible to obtain cell populations which respond to antigen-pulsed strain 2 PECs but which fail to respond to strain 13 PECs pulsed with the same antigen. Similarly, F_1 T cells uniquely responsive to antigen-pulsed strain 13 PECs can be obtained. These results complement the finding that in vitro priming of F_1 guinea pig T lymphocytes with antigen-pulsed parental PECs generates a population of cells which respond to antigen-pulsed PECs of the type used in priming but not to antigen-pulsed PECs from the alternative parental strain (12). In many respects our results are comparable to previous demonstrations that mouse F_1 T lymphocytes which are capable of killing virus-infected or trinitrophenylated target cells from one parent are distinct from those that can kill virus-infected (13) or trinitrophenylated cells (14) from the alternative parental strain. In the cytotoxicity experiments, in contrast to the ones described here, the MHC gene products important in the distinction of parental cells were the H-2K and/or D antigens rather than the Ia antigens.

The results that we have obtained depended upon both positive and negative selection techniques, which gave comparable results. In reviewing the results of the positive selection experiments, it might be argued that the unique responsiveness to OVA-pulsed 2 PECs of F_1 T cells positively selected by culture with OVA-pulsed 2 PECs was due to a selection of two independent populations of F_1 cells, one specific for OVA and the other for strain 2 Ia antigens and that the observed proliferative response required the action of both cell populations. This explanation is incompatible with the results of the negative selection experiments If two cell populations were required, one responsive to OVA and the other to strain 2 Ia antigens, then elimination of either would abolish responsiveness to OVA-pulsed 2 PECs. However, if it were the OVA-responsive population which was

eliminated, then the remaining cells should fail to respond to OVA-pulsed 13 PECs; if the cell population capable of responding to strain 2 Ia antigens were eliminated then the population capable of responding to strain 2 Ia antigens were eliminated then the remaining cells should fail to respond to PPD-pulsed 2 PECs. Since the negatively selected cells gave excellent responses to OVA-pulsed 13 PECs and to PPD-pulsed 2 PECs but failed to respond to OVA-pulsed 2 PECs, it must be concluded that the capacity of F_1 T lymphocytes to distinguish between antigen-pulsed 2 and 13 PECs is not based on the concerted action of an Ia-specific cell and an antigen-specific cell.

Before a discussion of the implications of the existence of independent populations of F_1 T cells which distinguish antigen-pulsed PECs from parental strains, it should be pointed out that our results cannot easily be explained by the action of an alloantigen-specific suppressor cell. The major pieces of evidence which argue against this are: (*a*) negatively selected F_1 T lymphocytes which fail to respond to antigen-pulsed 13 PECs respond to $(2 \times 13)F_1$ PECs pulsed with antigen as well as they respond to antigen-pulsed 2 PECs; (*b*) negative selection can be achieved simultaneously to OVA-pulsed PECs of one parent and PPD-pulsed PECs from the other parent. In the first case, alloantigen-responsive suppressor cells, if generated during the selection culture, should be activated by the antigens expressed on the F_1 PECs and thus antigen-pulsed F_1 cells should fail to stimulate a response. In the second instance, alloantigen-specific suppressors directed at both strain 2 and strain 13 Ia antigens should be generated during selection. The cells obtained should fail to respond to antigen-pulsed strain 2 or 13 PECs. Of course, it is possible that the putative suppressor is specific for both antigen and Ia antigen and only suppresses responses of T lymphocytes with similar specificity. If that were true, it would require the existence of F_1 cells capable of discriminating antigen-pulsed PECs from one parent from PECs from the alternative parent pulsed with the same antigen. Thus, even a suppressor mechanism would require cells of the specificity that we have already postulated to exist on the basis of these selection experiments. In any case, a suppressor mechanism seems most unlikely in the negative selection system since this procedure involves elimination of proliferating cells and might very well delete the suppressing as well as the responding cells.

The histocompatibility restriction in the interaction of primed T lymphocytes with antigen-pulsed PECs or with B lymphocytes has previously been interpreted in terms of self-recognition by cellular interaction (CI) structures borne by the interacting cell types (15,16). This hypothesis held that activation of primed T lymphocytes by antigen-pulsed PECs requires that the T lymphocyte recognize antigen by virtue of its specific antigen-binding receptor and that PEC and T lymphocyte each express CI structures, encoded within the *I* region of the MHC, which are capable of mutual recognition. Our demonstration that the F_1 T lymphocytes which respond to antigen-pulsed PECs from each parent are largely separate cell populations places important constraints on such CI theories. Thus, one could postulate that only one of the two CI loci were expressed in any cell (allelic exclusion). For example, in $(2 \times 13)F_1$ T cells, some cells would express the strain 2 CI structure and lack the strain 13 structure, whereas the remaining cells would express the opposite phenotype. In the selection experiments, we would be selecting for (or against) those antigen-responsive T lymphocytes which expressed one of the two allelically excluded CI genes. If the CI structures were not allelically excluded, one could still explain the existence of independent sets of F_1 cells which recognize antigen-pulsed macrophages from each parent by postulating a stable association between antigen receptor and CI structure on the T-lymphocyte membrane (15) and by requiring that, on any individual T lymphocyte, the allelic forms of CI structures were associated with distinct antigen receptors. Thus, $(2 \times 13)F_1$ cells which recognize OVA-pulsed 2 PECs would do so because they possessed a receptor for OVA closely associated with the strain 2 CI structure. The same cells might also express strain 13 CI structures but these would be associated with receptors specific for antigens other than OVA. This would make it

possible to select F_1 T-lymphocyte populations which distinguish antigen-pulsed PECs of one parental strain from antigen-pulsed PECs derived from the other parent. If neither of these alternatives is correct, it is not clear how a CI based system could lead to independent sets of F_1 T cells of the type described here.

As an alternative to self recognition CI theories, it can be envisioned that T lymphocytes recognize both antigen and MHC gene products either through two receptors, one specific for antigen and the other for Ia molecules, or through a single receptor which recognizes both antigen and Ia molecules. In this case, one would expect that in the course of initial in vivo priming, antigen would have become associated with cells equivalent to the PECs used in these experiments. In an F_1 animal, these "physiologically" antigen-pulsed cells would then stimulate two broad T-cell classes, one which could recognize antigen and strain 2 Ia molecules (separately or together) and one which could recognize the same antigen and strain 13 Ia molecules. Indeed, according to this view, there is no particular reason why such recognition should be limited to Ia antigens possessed by the individual that has been immunized. The MHC restriction normally observed would result from the fact that priming was done under conditions in which antigen was presented on syngeneic cells. If priming could be achieved with antigen-pulsed allogeneic cells, then a subsequent restriction to responsiveness to antigen-pulsed cells of that allogeneic MHC type would be anticipated. Indeed, studies on the T-lymphocyte proliferative responses to trinitrophenylated PECs in guinea pigs,[2] on the in vitro antibody response to terpolymer of glutamic acid, alanine, and tyrosine in mice (17), and on the transfer of delayed hypersensitivity to fowl gamma globulin in mice (18) are all consistent with this concept. Finally, one would anticipate that no MHC restriction in primary responses should exist, as the restriction would be a function of the initial priming regime. Studies of B lymphocyte-T lymphocyte collaboration suggest that there is no restriction in primary antibody responses, provided that allogeneic effects can be eliminated (19, 20).

A final possibility is that T lymphocytes recognize only conventional antigens and are not directly specific for Ia molecules. However, Ia molecules, or the closely genetically linked immune response (*Ir*) gene products, might function by selecting which antigenic determinants on a complex molecule were presented to the T-lymphocyte population. Thus, the distinction made by F_1 T lymphocytes between OVA-pulsed 2 PECs and OVA-pulsed 13 PECs might be a function of the presentation of distinct determinants on OVA as a result of the action of strain 2 and strain 13 *Ir* gene products. This concept has been referred to as specific determinant selection (21) and assumes that differences between different PECs as antigen-presenting cells is directly attributable to *Ir* gene function in the presenting cell and not to distinctive antigenic determinants of Ia molecules.

A definitive choice between these alternative classes of explanations for restrictions in cellular interaction is not yet possible. It seems likely that establishing the basis of such restriction will require precise chemical analysis of the T-lymphocyte receptor for antigen and of the nature of the events that occur when PECs are pulsed with antigen.

Summary

Thymus-dependent (T) lymphocytes from (2 × 13)F_1 hybrid guinea pigs immunized to ovalbumin (OVA) in complete Freund's adjuvant can be stimulated to proliferate in vitro by antigen-pulsed peritoneal exudate cells (PECs) derived from either strain 2 or strain 13 donors. In this communication, we show that the population of primed F_1 T lymphocytes which can be activated by antigen-pulsed strain 2 PECs is largely independent of the population of cells that can be activated by antigen-pulsed strain 13 PECs. This was demonstrated by both positive and negative selection procedures. In the former, T lymphocytes from OVA-primed (2 × 13)F_1 donors were enriched by initial culture with

[2] Thomas, D W , and E. M. Shevach. Manuscript submitted for publication

OVA-pulsed strain 2 or strain 13 PECs for 1 wk. Cells selected by culture with OVA-pulsed strain 2 PECs responded well to OVA-pulsed strain 2 PECs and poorly to OVA-pulsed strain 13 PECs. If positive selection had been carried out with OVA-pulsed strain 13 PECs, the selected F_1 T cells responded well to OVA-pulsed 13 PECs and poorly to OVA-pulsed 2 PECs. Negative selection was achieved by short term culture with antigen-pulsed PECs and by eliminating proliferating cells by treatment with bromodeoxyuridine and light. This procedure demonstrated that the population of primed F_1 T lymphocytes which are responsive to OVA or to purified protein derivative of tuberculin can be divided into subpopulations uniquely responsive to antigen on either strain 2 or strain 13 PECs. Evidence was presented to indicate that this selective responsiveness was not the result of the action of alloantigen-specific suppressor cells. The results are considered in terms of current concepts of the genetic and molecular regulation of the interaction of PECs and T lymphocytes.

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