THE PERITONEAL EXUDATE LYMPHOCYTE

I. Differences in Antigen Responsiveness Between Peritoneal Exudate and Lymph Node Lymphocytes from Immunized Guinea Pigs*

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Lymphocytes are now recognized to be comprised of a population of functionally heterogeneous cells. It is generally accepted that distinctions exist between lymphoid cells which are antibody-forming cell precursors and those which are involved in cell-mediated immune reactions. More recently, explicit considerations of heterogeneity among lymphocytes mediating cellular immune responses have been made. Thus, Cantor and Asofsky have demonstrated in the mouse that two distinct populations of thymus-derived lymphocytes cooperate in the graft-*versus*-host reaction (1, 2).

One important aspect of functional heterogeneity of lymphoid cells, the concentration of specific cell types in given anatomic locations, is implicit in the earliest descriptions of the cellular basis of delayed hypersensitivity. Thus, Landsteiner and Chase (3) and Chase (4) first described successful transfer of tuberculin hypersensitivity with peritoneal exudate cells. Exudate cells have also been noted to be functional in other cell-mediated immune systems including transfer of tumor immunity (5), in vitro tumor cytotoxicity (6), and the in vitro production of certain soluble mediators (7, 8). In addition, McGregor et al. (9,12) and Koster et al. (10, 11) have recently shown that lymphocytes capable of activating macrophages to kill phagocytosed bacteria are preferentially sequestered in inflammatory sites, such as the peritoneal exudate.

In the present study, we compare the relative capacity of lymphocytes purified from peritoneal exudates and from draining lymph nodes in their capacity to mediate two in vitro correlates of cellular immunity: antigen-induced lymphocyte proliferation and production of macrophage migration inhibitory factor

(MIF).[1] We find the lymphocytes isolated from the peritoneal exudate (PELS) to be much more effective than lymphocytes purified from lymph node cell suspensions in mediating these reactions. The experimental and biologic variables which might explain the striking differences observed in the in vitro reactivity of exudate lymphocytes to antigen were evaluated. The data are interpreted as indicating that the heightened antigen responsiveness of PELS is a result of the anatomic partition of functionally distinct subsets of lymphocytes.

Materials and Methods

Animals and Immunization Procedure.—Female, inbred strain 2 guinea pigs, weighing 300–400 g each (Division of Research Services of the National Institutes of Health), were immunized by footpad injection with an emulsion of antigen and complete Freund's adjuvant (CFA) containing 2 mg/ml of killed *Mycobacterium tuberculosis* H37 Ra (Difco Laboratories, Inc., Detroit, Mich.). Each animal received a total antigen dose of 100 μg for all antigens, and control animals received CFA alone. Peritoneal exudates and lymph nodes were obtained 2–4 wk postimmunization.

The following antigens were dissolved in phosphate-buffered saline (PBS, pH 7.4) for immunization, in media for in vitro assays, and sterilized by Millipore filtration (Millipore Corp., Bedford, Mass.):

(*a*) horseradish peroxidase (HRPO), a 40,000 mol wt glycoprotein (Worthington Biochemical Corp., Freehold, N. J.);

(*b*) horse spleen ferritin, 6X crystallized, cadmium removed (Pentex Biochemical, Kankakee, Ill.);

(*c*) dinitrophenyl guinea pig albumin (DNP$_{16}$-GPA) prepared by conjugation of 1-fluoro-2,4-dinitrobenzene (Eastman Organic Chemicals, Rochester, N. Y.) to crystalline guinea pig albumin (Miles Laboratories, Inc., Kankakee, Ill.) (16);

(*d*) phytohemagglutinin (PHA) (Wellcome Research Laboratories, Beckenham, England).

Media.—All washing procedures and cultures were performed in Eagle's minimal essential medium, Spinner modification, (S-MEM) (Microbiological Associates, Inc., Bethesda, Md.). Each 500 ml of S-MEM was supplemented with 5 ml each of: nonessential amino acids (100X, Grand Island Biological Co., Grand Island, N. Y.); 100 mM sodium pyruvate (Microbiological Associates; 3% glutamine, and 40% glucose; and with 2.5 ml of aqueous penicillin (20,000 units/ml). Heat-inactivated, normal strain 2 guinea pig serum (NGPS) was added to the media in the indicated concentrations.

Cell Preparation.—Animals were sacrificed by a blow to the head, exsanguinated, and cells were harvested as follows.

(*a*) *Lymph node cells (LNC):* Axillary, epitrochlear, posterior cervical, and popliteal nodes were collected in cold, sterile S-MEM. Perinodal fat was removed and cells were teased free with forceps and needle. Large cell clumps were removed by passage through a double

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[1] *Abbreviations used in this paper:* CFA, complete Freund's adjuvant; DNP-GPA, dinitrophenyl guinea pig albumin; HRPO, horseradish peroxidase; LNC, lymph node cells; MIF, macrophage migration inhibitory factor; NGPS, normal guinea pig serum; PBS, 0.01 M phosphate-buffered saline (pH 7.4); PEC, peritoneal exudate cells; PELS, peritoneal exudate lymphocytes; PHA, phytohemagglutinin; S-MEM, Eagle's minimal essential medium (Spinner modification).

thickness of cotton gauze and the cells were washed three times in S-MEM containing 1% NGPS. Differential cell counts showed 95% small and medium-sized lymphocytes, 1-2% monocytes and macrophages, 1-2% intact reticular cells, and 2% blast cells, plasma cells, and neutrophiles. Viability as judged by a dye exclusion test using trypan blue was 60-70%.

(b) *Peritoneal exudate cells (PEC):* Animals were injected intraperitoneally with 25 ml of sterile mineral oil (Marcol 52, Humble Oil & Refining Co., Houston, Tex.) to induce peritoneal exudates. 3 days later, under sterile conditions, the peritoneal cavity was exposed via a midline incision, and lavaged with three 50-ml vol of S-MEM containing heparin (5 units/ml). Cells were washed three to four times in heparin-free S-MEM containing 1% NGPS until free of residual oil. The average differential count revealed 60-70% macrophages and monocytes, 15-20% lymphocytes, and 15-20% neutrophiles. More than 90% of PEC excluded trypan blue.

(c)*Peritoneal exudate lymphocytes (PELS):* Lymphocytes were separated from the whole exudate cell population by a modification of the column technique of Lamvik (13). Viscose rayon (Johnson & Johnson, New Brunswick, N.J.), a pure, filamented cellulose (14), was used instead of natural cotton because of structural uniformity and to avoid the potential toxic contamination with endotoxin that may be seen with natural products.

Before packing of the column, the rayon wool was washed with copious amounts of distilled water, firmly packed in 50-ml glass syringes to a final wet volume of 40 ml, and autoclaved. Immediately before use the columns were washed with 150 ml of sterile, warm S-MEM, then 250×10^6 cells suspended in 5 ml of NGPS were added to the column and the cells were allowed to percolate into the rayon wool. The columns were incubated at 37°C for 30 min, nonadherent cells were eluted at 2 ml/min with a 50 ml volume of warm S-MEM containing 10% guinea pig serum, and the eluted cells were washed once in serum-free medium.

To determine the number of residual phagocytic cells, cells suspended in S-MEM containing 10% NGPS were slowly rotated at room temperature for 20 min in the presence of 0.1% polystyrene latex beads (1.1 μ diameter, 1.05 g/ml, Dow Chemical Co., Midland, Mich.) and cell differential counts were performed on Wright's-stained smears (15). Any mononuclear cell that contained either large lipid vacuoles or phagocytosed beads was considered a macrophage or monocyte while those small, basophilic, mononuclear cells with scanty cytoplasm that did not exhibit phagocytosis were considered to be lymphocytes. Using these criteria, the columns yielded 80% small and medium-sized lymphocytes, 10% macrophages and monocytes, and 10% neutrophiles. Greater than 98% of PELS excluded trypan blue. The average yield of lymphocytes by this procedure was 15% of the total exudate cells placed over the columns (60-80% recovery of lymphocytes initially present in exudate population).

In Vitro Assays.—Lymphocyte proliferation was determined by incorporation of tritiated thymidine into newly formed DNA. 1.5 ml of cell suspension (3×10^6 cells) in S-MEM containing 10% NGPS, with or without appropriate concentrations of antigen, was added to round-bottom, 16×150 mm glass screw-top tubes (Bellco Glass, Inc., Vineland, N. J.) in triplicate and incubated upright at 37°C for 72 hr in an atmosphere of 5% CO_2 and 95% air. 4 hr before harvesting, 3.0 μCi of tritiated thymidine (TdR-^{3}H, 6.7 Ci/mM; New England Nuclear Corp., Boston, Mass.) was added to each culture tube. The cultures were harvested by washing the cell button twice in PBS, twice in 5% trichloroacetic acid, and twice in absolute methanol. The cell buttons were dried, digested by boiling with 0.5 ml 0.2 N sodium hydroxide, neutralized with 0.2 ml 3% acetic acid, and transferred to counting vials with 10 ml of a scintillation cocktail consisting of 4% Liquifluor (New England Nuclear Corp.) and 10% Bio-Solv (BBS-3; Beckman Instruments, Inc., Fullerton, Calif.) in toluene. Radioactivity was counted in a Packard scintillation counter and results were expressed as the average stimulation (S/C), where

$$S/C = \frac{\text{mean cpm TdR-}^3\text{H incorporated in stimulated cultures}}{\text{mean cpm TdR-}^3\text{H incorporated in unstimulated cultures}}.$$

MIF was assayed by a modification of the capillary migration technique (17). Peritoneal exudate cells, 60×10^6/ml, suspended in S-MEM containing 15% NGPS, were drawn up in 50 μl capillary tubes (Scientific Products Div., Evanston, Ill.), one end sealed by flaming, and then spun into a pellet at 130 g for 10 min. The capillary tubes were cut at the cell-fluid interface and fixed to the bottom of Sykes-Moore chambers (Bellco Glass Inc.) with sterile silicone grease (Dow Corning Corporation, Midland, Mich.). The chambers were filled with S-MEM containing 15% NGPS and appropriate antigen concentrations, and incubated at 37°C for 24 hr. The areas of migration were projected 20 times enlarged on a Nikon Profile Projector (Nippon Kogaku K. K., Japan), traced, and measured with a planimeter. The areas of migration of four capillaries (two duplicate chambers) were averaged, and the results were expressed as migration inhibition, where

$$\text{migration inhibition } (\%) = \left(1 - \frac{\text{mean area of migration with antigen}}{\text{mean area of migration with media}}\right) \times 100.$$

Skin Tests and Antibody Determinations.—Skin reactivity was assessed, after intradermal injection into the flank of 20 μg of antigen in a volume of 0.1 ml, and the reactions were read at 4, 6, and 24 hr. Erythema and induration of greater than 10 mm diameter at 24 hr was considered to be positive. Immunized animals manifested specific delayed hypersensitivity to each of the three antigens tested: HRPO, ferritin, and DNP-GPA. No false positive reactivity was seen in any nonimmunized animals.

Guinea pigs immunized to DNP-GPA and ferritin develop precipitating antibodies that are detectable by a simple ring test (18). Guinea pigs immunized to HRPO by our protocol were not found to have precipitating antibodies by this technique or by direct hemagglutination of HRPO-coated sheep red cells (19). However, using a more sensitive Farr assay (20), employing ^{125}I-labeled HRPO, a small amount of antibody was detected (less than 1 μg of antigen bound/ml of undiluted serum). This binding material has been further characterized and appears to be a nonprecipitating immunoglobulin, studies of which are to be presented in a subsequent publication. High-titer anti-HRPO antibody prepared by hyperimmunization of NZW rabbits was used as a positive control for all HRPO antibody determinations.

RESULTS

Antigen-Stimulated Production of MIF by PELS: Use of a Transfer MIF Technique.—In order to compare specific lymphocyte populations directly and in a quantitative fashion, a transfer MIF assay was employed (21). Peritoneal exudate lymphocytes were mixed in varying concentrations with peritoneal exudate cells from nonimmunized guinea pigs (nonimmune PEC) and antigen-mediated inhibition of migration was determined. This transfer procedure eliminated the need for separate manipulation of lymphocyte supernatants.

3 million PELS obtained from immunized animals (5%) were mixed with 60×10^6 nonimmune PEC and inhibition of migration with increasing concentrations of HRPO was determined (Fig. 1). MIF production by PELS was stimulated by 0.01 μg/ml HRPO, and inhibition increased to a maximum of 61% with 1 μg/ml HRPO. By comparison, whole, unseparated, immune PEC (direct MIF), which contain 15–20% total lymphocytes, display a somewhat greater inhibition at every antigen concentration. Nonimmune PELS or PEC did not produce significant inhibition (<4%) at concentrations of HRPO up to 1 μg/ml. At higher concentrations, nonspecific inhibition of nonimmune mac-

rophages becomes significant (10–15%). For this reason, 1 μg/ml HRPO was used for all subsequent MIF assays.

Antigen-Stimulated Production of MIF by PELS and LNC.—In order to compare the relative ability of the two lymphocyte populations to elaborate MIF, PELS and lymph node cells from the same animals were mixed with nonimmune PEC in varying concentrations and assayed for MIF production using 1 μg/ml of HRPO (Fig. 2). Using this system, significant migration inhibition was found to be at a level of 15% or greater. PELS produced significant MIF at a lympho-
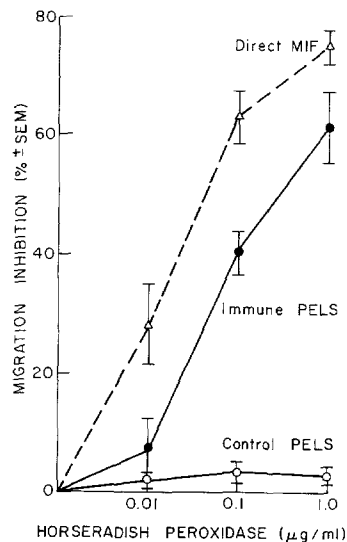

HORSERADISH PEROXIDASE (μg/ml)

Fig. 1. MIF production by PELS; use of a transfer assay. 3×10^6 (5%) PELS were mixed with 60×10^6 nonimmune PEC and tested for MIF production induced by the indicated concentrations of HRPO. *Control PELS* refers to lymphocytes from a CFA-immune animal. *Direct MIF* refers to the assay with whole, unseparated, immune peritoneal exudate cells (containing 15–20% lymphocytes). The values are expressed as per cent migration inhibition and represent the arithmetic mean ± SE of the mean of four experiments.

cyte concentration of 0.1% (60×10^3 lymphocytes + 60×10^6 nonimmune PEC) with near-maximal inhibition at a lymphocyte concentration of 5%. MIF production by LNC was significantly less than PELS, with significant inhibition being found only at an LNC concentration of 5%. At the 5% transferred cell level, PELS produce 61.5% migration inhibition, compared to only 26.9% inhibition by LNC ($P < 0.01$). With added lymphocyte concentrations of 10% or more, the nonadherent lymphocytes layer on top of the macrophages in the capillary tube, and some are lost into the media. Because direct lymphocyte-macrophage contact is important in this type of transfer assay (21), migration inhibition with 10% or more added lymphocytes was not significantly

greater than at 5%. Nevertheless, it is clear that at every level of added lymphocytes, PELS produce more MIF than LNC.

Effect of Column Purification on HRPO-Induced MIF Production by Lymph Node Lymphocytes.—It was necessary to column purify PELS to remove nonlymphoid cells; however, it was possible that column purification nonspecifically enhanced the reactivity of the exudate lymphocytes. To study this possibility, lymph node cells were column purified in an identical fashion to PELS, and then tested for antigen-mediated MIF production in the transfer MIF system.
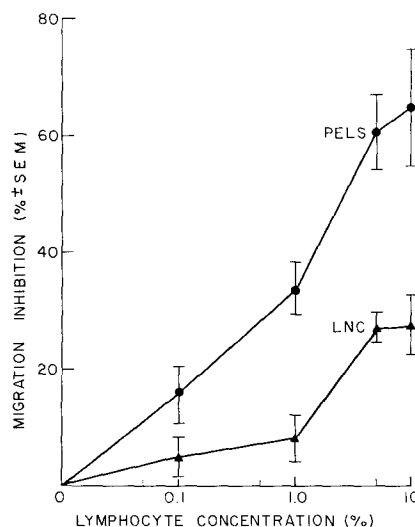

Fig. 2.   Comparison of MIF production by PELS and LNC induced by HRPO. Lymphocytes, varying in number from 60×10^3 (0.1%) to 6×10^6 (10%) were mixed with nonimmune PEC (60×10^6) and tested for MIF production with HRPO (1 μg/ml). The results are expressed as per cent migration inhibition and represent the arithmetic mean $\pm$ SE of the mean for five experiments.

Lymphocytes purified in this fashion were 99% viable and free of macrophages, monocytes, and neutrophiles, as tested by phagocytosis of polystyrene latex beads.

Unpurified lymph node cells produced 28.2% inhibition, while after column purification the inhibition was 22.8% (Table I). There is no significant difference between these two numbers. PELS from the same animals, tested simultaneously, produced significantly greater inhibition (68.8%). Thus, column purification of cell populations did not appear to introduce a significant variable into this system, and cannot of itself account for the difference between LNC and PELS.

Comparison of MIF Production by PELS and LNC Induced by Other Soluble

Protein Antigens.—In order to determine if the marked differences between PELS and LNC were unique to HRPO or could be shown with other antigens, MIF production by these two cell populations was compared using ferritin and dinitrophenyl guinea pig albumin (DNP-GPA). PELS and LNC were obtained

TABLE I

Effect of Column Purification on MIF Production by Lymph Node Lymphocytes

Cell population	Mean migration‡ inhibition (%)	P value§
1. LNC	28.1 ± 4.2	1 vs. 2, n.s.
2. Column-purified lymph node lymphocytes	22.8 ± 3.1	
3. PELS	68.8 ± 2.5	1 or 2 vs. 3, < 0.01

* MIF production assayed by transfer technique; 5% immune lymphocytes mixed with nonimmune PEC; see text for details.

‡ Value represents the arithmetic mean ± SE of the mean of three experiments.

§ Statistical comparisons for all experiments in this communication were performed using Student's *t* test (two-tailed).

TABLE II

Comparison of PELS and LNC: MIF Production Induced by Ferritin, DNP-GPA, and HRPO

Tested antigen (dose)	Cell popula- tion*	Mean migration inhibition (%)‡			
		Immune lymphocytes	P§	Control lymphocytes	P§
DNP-GPA (50 µg/ml	PELS	61.5 ± 2.7	<0.01	8.5 ± 2.3	n.s. (3)
	LNC	30.1 ± 3.2		7.2 ± 3.0	
Ferritin (10 µg/ ml)	PELS	53.3 ± 6.2	<0.01	5.2 ± 1.8	n.s. (3)
	LNC	22.3 ± 9.4		3.8 ± 2.1	
HRPO (1 µg/ml)	PELS	61.0 ± 6.1	<0.01	3.1 ± 2.0	n.s. (5)
	LNC	26.9 ± 2.5		3.7 ± 2.9	

* 5% lymphocytes for transfer MIF assay.

‡ Value represents the arithmetic mean ± SE of the mean of the number of experiments in parentheses.

§ Inhibition produced by PELS compared to inhibition produced by LNC.

from animals immunized to either ferritin or DNP-GPA and tested for MIF production induced by specific antigen in a transfer MIF assay with 5% added lymphocytes (Table II). Control refers to animals immunized with CFA alone. With DNP-GPA, PELS from an immune animal produced 61.5% migration

inhibition, while LNC from the same animal produced only 30.1% migration inhibition.

The response to ferritin demonstrated a similar superiority of PELS over LNC with 53.3% *versus* 22.3% migration inhibition, respectively. HRPO-induced MIF production is included for comparison with the other antigens.

Comparative Lymphocyte Proliferation of PELS and LNC Stimulated by HRPO.—PELS and LNC from the same animal were cultured with increasing
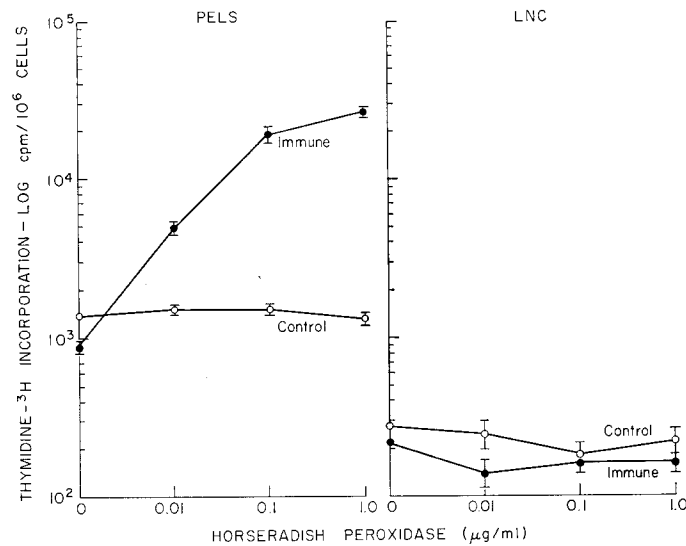

FIG. 3. In vitro lymphocyte proliferation induced by HRPO. Lymphocytes (3 $\times$ 10⁶ cells) were cultured with the indicated concentrations of HRPO and tested for proliferation by the incorporation of tritiated thymidine. Results are expressed as the total counts per minute of incorporated thymidine-³H corrected for 10⁶ cells, and are plotted as the log of the geometric mean $\pm$ SE of the mean. These values summarize the results of eight experiments. *Control* refers to lymphocytes from CFA-immune animals.

concentrations of HRPO and tested for in vitro proliferation by the incorporation of tritiated thymidine into DNA. Over the dose range of HRPO employed in vitro, there was no significant proliferative response by LNC from guinea pigs immunized to horseradish peroxidase (Fig. 3).

In contrast, the response of PELS to antigen was quite significant. Proliferation was detectable at an HRPO concentration of 0.01 ug/ml and was near maximal at 1 μg/ml of HRPO. With 1 μg/ml of HRPO, there was a 31-fold increase in exudate lymphocyte DNA synthesis as compared to unstimulated immune PELS. This response was immunologically specific; PELS from a CFA-immune animal (control) were not stimulated by HRPO. Also demon-

strated in Fig. 3 is the interesting observation that even in the absence of added antigen, PELS have a higher proliferative rate than equivalent numbers of lymph node lymphocytes.

Possible Critical Determinants in the In Vitro Antigen-Mediated Proliferative

TABLE III

Lymphocyte Proliferation Induced by HRPO and PHA: Comparison of PELS and LNC

Immunity	Cell type	Mean stimulation index (S/C)*	
		HRPO‡	PHA
HRPO	PELS	28.1 ± 6.6	261 ± 55
	LNC	1.2 ± 0.3	375 ± 106

* Values represent arithmetic mean ± SE of the mean of five experiments.
‡ HRPO, 1 μg/ml; PHA, 1 μg/ml.

TABLE IV

Comparison of PELS and LNC: Effect of Column Purification and Reconstitution with Nonimmune Peritoneal Exudate Cells on Lymphocyte Proliferation Induced by Horseradish Peroxidase

Cell population	Mean cpm* TdR-³H		Mean stimulation index (S/C)
	Un-stimulated	Stimulated	
LNC	855	1,504	1.76
LNC + PEC	1513	3,646	2.41 (6)
Column purified lymph node lymphocytes (LNL)	229	347	1.52
LNL + PEC	1610	4,186	2.60 (6)
PELS	2690	54,630	20.3
PELS + PEC	2370	49,350	20.8 (3)
PEC alone‡	950	850	0.89 (3)

* Values represent the geometric mean of the number of experiments indicated in parentheses.
‡ PEC = 0.3 × 10⁶ cells/culture tube.

Response of Lymph Node Cells.—(a) It was possible that the lack of response by LNC to antigen resulted from a suppressive or toxic effect of residual immunizing antigen (HRPO) present in the lymph node. To test this possibility, lymphocytes from an HRPO-immune guinea pig were tested with the nonspecific mitogen, phytohemagglutinin, and with HRPO (Table III). PHA-induced DNA synthesis was not significantly different for PELS and LNC. The average stimulation index with LNC was 375 compared to 261 with PELS.

These data suggest that, given the proper stimulus, LNC were capable of proliferation in vitro, and their lack of response was specific for the immunizing antigen, HRPO.

(b) We were not able to demonstrate significant enhancement of MIF activity by column purification of LNC. However, previous work has shown that two variables, purification over glass bead columns and readdition of macrophages, may enhance the antigen-mediated proliferative response of guinea pig LNC (22). PELS are a column-purified population of lymphocytes containing 10% monocytes and macrophages. It was possible, therefore, that the heightened proliferative response of PELS represented augmentation by these two variables rather than the inherent reactivity of the lymphocytes themselves. To evaluate this, whole LNC were column purified in a manner

TABLE V

*Influence of an Induced Peritoneal Exudate on the In Vitro Response of LNC to HRPO**

| Presence of peritoneal exudate in guinea pig | Lymph node lymphocytes assayed for‡ | |
| | MIF | Lymphocyte proliferation |
	Mean Migration§ inhibition (%)	(S/C)§
Yes	24.8 ± 4.1	2.41 ± 0.42
No	26.0 ± 3.2	3.00 ± 0.51

* HRPO, 1 μg/ml.
‡ Lymph node cells column purified for both in vitro assays and reconstituted with 10% nonimmune PEC for lymphocyte proliferation assay.
§ Values represent the arithmetic mean ± SE of the mean of three experiments.

identical to PELS and their proliferative response to HRPO was tested with, and without, the addition of 10% PEC from nonimmunized animals (Table IV). Column purification of LNC by itself did not increase the HRPO-induced proliferation. Addition of PEC to both LNC and column-purified LNC resulted in a small but significant ($P < 0.05$) increase in antigen-mediated DNA synthesis. In contrast, addition of PEC did not have any significant effect on the proliferative response of PELS. These experiments show that there exists, in the draining lymph nodes, a population of cells capable of responding to this antigen (HRPO). However, the marked difference in in vitro proliferation between PELS and LNC could not be accounted for solely by the presence of macrophages or the column purification procedure.

(c) To determine if the presence of an oil-induced peritoneal exudate decreased the response of LNC by depleting the lymph nodes of reactive cells, the reactivity of LNC from guinea pigs with and without oil-induced exudates

were compared (Table V). MIF activity was measured with 5% immune lymph node cells in the transfer MIF assay. There was no significant difference in MIF production between LNC from immunized guinea pigs which had not received oil and guinea pigs that had peritoneal oil for 3 days. Similarly, the presence of a peritoneal exudate did not alter the proliferative response of LNC to antigen.

Other technical factors important in the in vitro proliferative response to antigen were also examined. LNC were tested for proliferation after 1, 3, 5, and 7 days in culture with HRPO. There was no increase in stimulation at any time tested, either before or after the standard 3 day culture time. Concentrations of LNC in culture of 1×10^6, 2×10^6, 5×10^6, 10×10^6, 15×10^6 cells/ml were tested for proliferation with HRPO with no significant increase

TABLE VI

Comparison of PELS and LNC: Lymphocyte Proliferation Induced by Ferritin and DNP-GPA

Tested antigen (dose)	Cell type*	Mean cpm TdR-³H Incorporated‡		Mean stimulation index	
		Unstim-ulated	Stimulated	(S/C)	P
Ferritin (10 µg/ml)	PELS	543	123,306	227.1	<0.001
	LNC	589	1,499	2.6	
DNP-GPA (100 µg/ml)	PELS	612	29,560	48.3	<0.01
	LNC	522	6,639	12.7	

* 3×10^6 cells/culture tube; see text for details.

‡ Values represent the geometric mean of three experiments with each antigen.

in the stimulation index. Finally, increased stimulation of LNC could not be demonstrated after different immunizing doses of HRPO (1, 10, 100, 1000 µg/ guinea pig), or by increasing the concentration of HRPO in culture (10, 100 500, 1000 µg/ml).

Comparison of Lymphocyte Proliferation of PELS and LNC Induced by Ferritin and DNP-GPA.—Since the draining lymph nodes in guinea pigs immunized to some soluble protein antigens are capable of an in vitro proliferative response (23), the fact that draining nodes from HRPO-immune animals responded poorly suggested a possible peculiarity of HRPO as an antigen. For this reason, the proliferative response of PELS and LNC from animals immunized to two other soluble protein antigens, ferritin and DNP-GPA, was examined (Table VI).

The proliferative response, stimulated by both ferritin and DNP-GPA in culture, was significantly greater with PELS than that produced by an equiva-

lent number of LNC. The difference was most striking with ferritin where the mean stimulation of LNC is 2.6 compared to a response of 227.1 with PELS. The average stimulation of PELS to a 100 µg/ml dose of DNP-GPA was 48.3, while that of LNC was 12.7. For both antigens, no significant stimulation of lymphocytes from nonimmune guinea pigs was noted.

Absence of Syngery between PELS and LNC in the In Vitro Proliferative Response to HRPO.—Synergy between different lymphocyte populations has been demonstrated with one cell-mediated immune system, the graft-*versus*-

TABLE VII

Absence of Synergy between LNC and PELS in the Proliferative Response Induced by HRPO

Cell type (immunity)*	cpm-TdR-³H incorporated‡	
	Un-stimulated	Stimulated§
Exp. I		
1. LNC (HRPO)	172	164
2. PEL (CFA)	2670	1970
3. LNC (HRPO) + PEL (CFA)	2110	2960 (2134)‖
Exp. II		
4. LNC (CFA)	165	127
5. PEL (HRPO)	1870	11,850
6. PEL (HRPO) + LNC (CFA)	4150	13,150 (11,977)
Exp. III		
7. PEL (HRPO)	1420	14,510
8. LNC (HRPO)	180	236
9. LNC (HRPO) + PELS (HRPO)	3280	13,450 (14,746)

* Immunity refers to immune status of animal from which cells were derived.

‡ Geometric means of triplicate cultures.

§ Stimulated = HRPO (1 µg/ml). For each experiment, individual populations were 1.5 × 10⁶ cells/culture tube, and mixed cell populations were 3 × 10⁶ cells per tube.

‖ The numbers in parentheses are the expected responses calculated by adding the counts per minute incorporated by each individual population.

host response in mice (1, 2). It was therefore possible that the difference in reactivity between PELS and LNC was because of the presence of a synergistic lymphocyte population in the PELS that was absent in LNC. To investigate this, PELS and LNC were mixed in equal numbers and tested for in vitro proliferation with HRPO (Table VII).

In order to decrease the number of residual macrophages present in the PELS, column purification was performed using fine glass beads (22) instead of rayon wool. The resultant PEL population was 95% small lymphocytes and less than 2% macrophages and the average viability was greater than 98%.

In the first experiment, the influence of PELS from a control guinea pig (CFA immune) on LNC from an HRPO-immune animal was tested (Exp. I). Individually (1.5 × 10⁶ cells), neither population responded to HRPO (lines 1 and 2, Table VII). The mixture of equal numbers of each population (3 × 10⁶ cells total, line 3) did not result in a significant increase in proliferation (2960 cpm observed *versus* 2134 cpm calculated).

To determine if the presence of LNC would inhibit the proliferation of PELS, control LNC were mixed with PELS from an HRPO-immune guinea pig (Exp. II, Table VII). Decreasing the number of macrophages by purification of PELS with glass bead columns both increases the unstimulated DNA synthesis and decreases antigen-stimulated DNA-synthesis (line 5), compared to purification with rayon wool. Addition of control LNC (line 6) did not result in a significant change in the DNA-synthetic response of PELS (13, 150 cpm observed *versus* 11,977 cpm calculated).

Finally, to determine if synergy required antigen-specific lymphocytes of both populations, PELS and LNC from guinea pigs immunized to HRPO were mixed (Exp. III, Table VII). Again, there was no significant difference in proliferation between this cell mixture (line 9) and the proliferation of immune PELS by themselves.

In other experiments, no significant synergy between PELS and LNC was found using either varying concentrations of PELS (50, 10, 5, 1, 0.1%) or with another antigen (tuberculin-purified protein derivative).

These experiments indicate that the relative lack of responsiveness of LNC to antigen was not the result of a deficiency of a necessary cooperating cell that was present in the PELS.

DISCUSSION

We have demonstrated that a population of lymphocytes present in oil-induced inflammatory exudates has enriched cellular immune activity as contrasted to lymph node cells. The data suggest that lymphocytes sequestered into inflammatory sites represent a population selected on the basis of some inherent physiological property. Thus the heightened reactivity of exudate lymphocytes was not because of the presence or absence of macrophages nor peculiarities of the antigen used. Conversely, the cell mixing experiments, as well as the assay of PHA-mediated DNA synthesis, indicated that the lesser response of lymph node cells to antigen was not the result of an inhibitory cell population or the lack of an accessory cell required for the expression of in vitro correlates of cellular immunity. Furthermore, column purification of lymph node cells did not factitiously increase DNA synthesis or production of macrophage inhibitory factor.

The data presented in this communication demonstrating the superiority of PELS over LNC in inhibition of macrophage migration, in addition to the work of several other laboratories showing increased killing of tumor cells (6), and

enhanced macrophage viricidal (32) and bactericidal capacity by peritoneal exudate cells, emphasize that exudate lymphocytes not only recognize antigen, but also exhibit effector cell function. Using an in vitro model of antigen-mediated macrophage bactericidal activity (31), Simon and Sheagren have used a similar column-purification procedure, and demonstrated that peritoneal exudate lymphocytes are markedly superior to lymph node cells in activation of macrophage bactericidal capacity.[2]

Recent findings of McGregor et al. (9) and Koster et al. (10) would support a conclusion that anatomic partition of lymphocytes mediating cellular immunity occurs. Their studies in the rat demonstrated that in response to new antigenic challenge, lymphocytes formed in the draining lymph nodes, entered the circulation via the thoracic duct, and accumulated in peritoneal exudates. More importantly their observations indicated that the less rapidly dividing or "long-lived" lymphocytes were excluded from the inflammatory site. That is, those cells with specificity directed towards recently encountered antigens were selected over those lymphocytes that were present before the test immunization. This selectivity may account in part for the heightened antigen reactivity of peritoneal exudate lymphocytes.

Moreover, the findings that the exudate contains certain specific lymphocytes while other lymphocytes are excluded and our observation that PELS do not adhere to fiber or glass beads suggest that these cells may possess distinct surface membrane characteristics. Indeed, on ultrastructural analysis, surface membrane modifications characterized by regions of microvilli were found in 50% or more exudate lymphocyte profiles while less than 10% of lymph node cells or thymocytes exhibited such features (26). The relationship of these membrane structures to surface adherence and egress from small capillaries is currently under investigation.

Since antibody-forming cells and complement receptor–bearing lymphocytes are depleted by column adherence techniques (24, 25), the ability of such columns to fractionate complex lymphoid populations and separate antibody-forming cells and their precursors from cellular-immune reactive lymphocytes was evaluated. We observed that in the guinea pig a single passage of lymph node cells over glass bead columns depleted 80-90% of antibody-forming cells and cells with immunoglobulin associated with their surface membranes, while antigen and mitogen-induced lymphocyte proliferation and antigen-mediated MIF production were maintained in the nonadherent lymphocytes. Furthermore, PELS obtained by identical column purification techniques contain less than 2% immunoglobulin-bearing cells and less than 0.002% antibody-forming cells (26).

The observation by Raff in the mouse that thymus-derived lymphocytes do

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[2] Simon, H. B., and J. S. Sheagren. Cellular immunity in vitro. II. Lymphocyte-macrophage interactions. Manuscript in preparation.

not contain detectable surface immunoglobulin (27), and by Rabellino and
Grey (28) and Kincade et al. (29) in the chicken that those lymphocytes
possessing surface immunoglobulin are a bone marrow–derived population,
suggests by analogy, in the guinea pig, that PELS and column-purified LNC
may be considered to be thymus-derived lymphocyte populations. Since the
response of lymphoid cells to PHA is another marker for thymus dependency
(30), support for such an interpretation is found in the observation that PELS
and LNC respond similarly to PHA (Table III) (26). Furthermore, we have
demonstrated that a specific rabbit anti–guinea pig thymocyte antiserum
developed by Drs. E. Shevach and I. Green kills >90% of PELS and column-
purified lymphocytes derived from draining lymph nodes but only 60% of
unfractionated lymph node cells.[3] The differences observed in antigen reactivity
between PELS and LNC are therefore not the result of a relative deficiency of
thymus-derived lymphocytes in the lymph nodes.

In summary, we have shown that peritoneal exudates contain a distinct
subset of antigen-reactive lymphocytes that mediate cellular immune effector
functions. The data presented support a concept of heterogeneity of thymus-
derived lymphocytes. Whether this functional and anatomic heterogeneity
occurs as the result of the existence of separate cell lines or as the result of
differentiation of a single cell line is not known. Characterization of the immu-
nobiologic properties of inflammatory lymphocytes may provide insight into
the events underlying cellular immunocompetence.

SUMMARY

Peritoneal exudate lymphocytes from guinea pigs immunized with horse
radish peroxidase, dinitrophenyl guinea pig albumin, or ferritin in complete
Freund's adjuvant have been shown to be significantly more reactive than
other lymphocytes in two in vitro assays of cellular immune function: produc-
tion of macrophage migration inhibitory factor and antigen-induced lympho-
cyte proliferation. The enhanced reactivity of peritoneal exudate lymphocytes
cannot be accounted for by artifacts introduced by column purification or by
the presence of nonlymphoid accessory cells. These observations suggest that
the peritoneal exudate lymphocyte pool is a highly enriched source of cellular
immune effector cells with specificity directed towards those antigenic determi-
nants to which an animal has been recently exposed.

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