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David M. Spalding

William J. Koopman

John H. Eldridge

# ACCESSORY CELLS IN MURINE PEYER'S PATCH

## I. Identification and Enrichment of a Functional Dendritic Cell*

By DAVID M. SPALDING,‡ WILLIAM J. KOOPMAN, JOHN H. ELDRIDGE,§ JERRY R. MCGHEE, and RALPH M. STEINMAN‖

*From the Division of Rheumatology and Clinical Immunology, Departments of Medicine and Microbiology, and Birmingham Veteran's Administration Hospital, University of Alabama in Birmingham, Birmingham, Alabama 35294; and The Rockefeller University, New York 10021*



Peyer's patches (PP)[1] are an important source of antigen-sensitized B cells that home to mucosal tissues (1). Upon exiting PP, sensitized IgA-committed B cells migrate through the mesenteric lymph nodes (2, 3) and spleen (4), possibly encountering additional differentiation signals before arrival at distant mucosal sites. Although the induction events for eventual specific IgA synthesis occur in PP (5), the induction process has not been previously studied. In fact, PP have even been considered to be immunologically incompetent. For example, specific antibody-producing plasma cells could not be identified in PP after oral or parenteral immunization (6, 7), and early in vitro studies demonstrated that PP cells were incapable of supporting cell-mediated responses or specific antibody responses (8). An explanation forwarded for this functional inadequacy has been a deficiency of functional accessory cells (8, 9). In this regard, PP cells were capable of generating immune responses only when supplemented in vitro with either peritoneal exudate cells or 2-mercaptoethanol (8). Recently, however, we demonstrated that PP cells obtained by enzymatic rather than mechanical dissociation were fully capable of in vitro mitogenesis (10), polyclonal immunoglobulin production (10), and primary in vitro immune responses (11). Therefore, PP must possess functional accessory cells.

In this paper, we show that PP do yield accessory cells when the organ is dissociated by an enzymatic technique (10, 11). The assay we used was oxidative mitogenesis (12), the proliferation of periodate-modified T cells. In this model, small or resting T cells, obtained by a single passage over nylon wool columns, proliferate in large numbers during the second day of co-culture with suitable accessory cells (13, 14). In contrast to lectin-mediated responses, the periodate system does not require extensive manipulations to obtain accessory-dependent T cells. Using the periodate model, we have found that PP is quantitatively as good a source of accessory cells as spleen. However, the PP accessory cell is predominantly nonadherent and has required special procedures for its identification. We show here that the PP accessory cell is

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; C, complement; DC, dendritic cell; FCS, fetal calf serum; PP, Peyer's patches; SRBC, sheep red blood cells; TdR, thymidine.

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probably the dendritic cell (DC). It reacts with the 33D1 anti-DC monoclonal antibody and can be highly enriched by its ability to form aggregates with periodate-modified T cells.

## Materials and Methods

*Mice.* C3H/HeN and BALB/c mice were obtained from the Immunocompromised Mouse Core Facility, Tumor Institute, University of Alabama in Birmingham. CBA/J mice were purchased from The Jackson Laboratory, Bar Harbor, ME. Mice were generally used at 8 wk of age.

*Enzymatic Dissociation of PP and Spleen Cells.* A modification of our previously described technique (10) was used. Briefly, PP were removed from the intestines with aseptic technique. The PP were pooled and incubated at 37°C in Joklik media (Gibco Laboratories, Grand Island, NY) containing Dispase 1.5 mg/ml (grade II; Boehringer, Mannheim Biochemicals, Indianapolis, IN) and DNAase 15 μg/ml (Sigma Chemical Co., St. Louis, MO) with constant stirring. Spleens were removed and minced into small fragments with scissors before addition of enzyme. At 30-min intervals, cells were removed and fresh media added for a total of four extractions. Following each extraction, cells were washed twice in RPMI 1640 (Flow Laboratories, McLean, VA) and maintained at room temperature in RPMI 1640 containing 5% fetal calf serum (FCS) (M. A. Bioproducts, Walkersville, MD). At the completion of four extractions, the cell pools were washed twice in RPMI 1640 containing 5% FCS and resuspended in complete media (RPMI 1640, gentamycin 20 μg/ml) (Schering Corp., Kenilworth, NJ), L-glutamine 2 mM (Gibco Laboratories), 5% FCS, 2-mercaptoethanol $5 \times 10^{-5}$ M (Bio-Rad Laboratories, Richmond, CA), and indomethacin 1.0 μg/ml (Sigma Chemical Co.). Yield with this procedure was 20–25 $\times$ $10^6$ cells/animal for PP and 125–150 $\times$ $10^6$ cells/animal for spleen with viability consistently >95%, in agreement with previously reported results (10).

*Mechanical Dissociation of PP and Spleen Cells.* Spleen and PP were obtained by dissection and then gently forced through wire mesh grids. After gravity sedimentation for 10 min to remove tissue fragments, the cell suspension was washed in RPMI 1640 containing 5% FCS and resuspended in complete media. Cell yield for PP was 5–10 $\times$ $10^6$/animal with 85% viability; spleen yields were 90–120 $\times$ $10^6$/animal with 90–95% viability.

*Separation of Adherent and Nonadherent Populations.* Cells suspensions from spleen and PP were plated in 24-well (16 mm diam/well) tissue culture dishes (Costar, Data Packaging, Cambridge, MA) at appropriate concentrations (1.25 and 2.5 $\times$ $10^6$ cells/well, respectively) and incubated 1–2 h at 37°C. Nonadherent cells were removed by gentle pipetting with a pasteur pipette and placed into separate test wells.

*Preparation of Low Density Enriched Accessory Cell Populations.* A modification of previously reported methodology (5) was used. PP populations obtained by enzymatic digestion were pelleted in 15 ml culture tubes by centrifugation at 1,750 RPM for 5 min. A discontinuous bovine serum albumin (BSA) density gradient was then made by gently resuspending the pellet in 2 ml dense ($\rho$ = 1.078) (Armour Fraction V; Reheis Chemical Co., Phoenix, AZ) and aliquoting this suspension into 5-ml cellulose nitrate tubes followed by consecutive overlays with 1 ml BSA of densities $\rho$ = 1.068, 1.063, and 1.040. Cells were centrifuged at 10,000 *g* for 20 min at 4°C in a Beckman L-50 ultracentrifuge (SW 50.1 swinging bucket rotor). Cells from each interface, and pelleted cells were aspirated separately, washed twice with RPMI 1640 containing 5% FCS, counted, and aliquoted into wells for assay. In some experiments cells floating on BSA, $\rho$ = 1.078, were separated into adherent and nonadherent populations as described above.

*Cytotoxicity Studies.* For Ia cytotoxicity studies, monoclonal anti-$Ia^k$ specificity 17 (Becton, Dickinson & Co., Sunnyvale, CA) was used. C3H/HeN ($H\text{-}2^k$) cells from enzymatically dissociated spleen and PP were adjusted to 10 $\times$ $10^6$ cells/ml in RPMI 1640 containing 1% FCS or 0.3% BSA (cytotoxicity medium). Equal volumes of cells and anti-$Ia^k$ (1 μg/3 $\times$ $10^5$ cells) in cytotoxicity medium were mixed and incubated on ice for 45 min. An equal volume of rabbit complement (C) (diluted 1:6 in cytotoxicity medium) was added, and cells were incubated at 37°C for an additional 60 min. Aliquots were removed and cytotoxicity-assessed by trypan blue exclusion. Control cells (no antibody or C), antibody-treated cells, C-treated

cells, and antibody- and C-treated cells were washed twice with RPMI 1640 containing 5% FCS and resuspended in complete medium to give equal concentrations of viable cells. Cells were aliquoted into 96-well culture dishes at appropriate concentrations and assayed for accessory activity.

Two approaches were used for cytotoxicity studies with the monoclonal anti-DC antibody, 33D1 (16): (*a*) Fresh or cultured (24 h) PP cells were suspended to $10 \times 10^6$ cells/ml and 0.5 ml was mixed with an equal volume of supernatant from 33D1 hybridoma cultures (or purified 33D1, 100 μg/ml) and rabbit C (1:5.5 dilution of rabbit serum in cytotoxicity medium). Cytotoxicity and preparation for assay was performed as in Ia experiments. (*b*) To assess the effect of 33D1 and C on adherent and nonadherent components of PP, we treated low density accessory cell-enriched populations obtained from the 1.068 and 1.063 interfaces after flotation on BSA gradients. Cells were washed three times in RPMI 1640, resuspended at $8 \times 10^6$ cells/ml in cytotoxicity medium, and 0.5 ml was mixed with an equal volume of 33D1 hybridoma supernatant and rabbit C (diluted 1:5.5 or 1:6 in cytotoxicity medium). Treated and control cell suspensions were immediately aliquoted into 96-well round-bottom culture dishes in 1:3 dilutions and incubated for 60 min at 37°C. The reaction was terminated by addition of ice-cold RPMI 1640, and cultures were gently washed with RPMI 1640 to remove nonadherent cells. The adherent cell layer was washed with RPMI 1640 containing 5% FCS and complete media were added, while the nonadherent cells were washed twice in RPMI 1640 containing 5% FCS, resuspended in complete media and aliquoted in serial dilutions into round-bottom wells for assay. In most of the above experiments, enzymatically obtained spleen cells were identically treated and assayed.

*Oxidative Mitogenesis Assays.* Spleen cells, obtained by mechanical dissociation, were washed in RPMI 1640 and resuspended in RPMI 1640 containing 10% FCS. T cells were obtained by nylon-wool column purification (17), washed in RPMI 1640, and resuspended in ice-cold phosphate-buffered saline (PBS) at $20 \times 10^6$ cells/ml. An equal volume of sodium metaperiodate 2.4 mM ($NaIO_4$; Fisher Scientific Co., Pittsburgh, PA) in ice-cold PBS was added and cells were incubated at 0°C for 10–15 min (18). Periodate-treated cells were centrifuged at 4°C, washed in minimal essential medium (MEM), and resuspended in complete media. T cells were aliquoted into either 96-well plates ($5 \times 10^5$ cells/well in 100 μl) or 24-well plates ($1 \times 10^6$ cells/well in 0.5 ml), which had been previously seeded with stimulator populations. Stimulator populations prepared as described in the preceding sections were irradiated (1,500 rad in a Picker Vangard machine; Picker Corp., Highland Heights, OH) and fractioned into culture dishes at appropriate concentrations before addition of T cells. After addition of responder T cells, cultures were incubated at 37°C in a 5% $CO_2$ atmosphere. Cultures were pulsed after 24 h incubation with 1 μCi tritiated thymidine ([$^3$H]TdR) (Amersham, Arlington Heights, IL), and 18 h later the cultures were harvested onto glass filter paper with a Mash II cell harvestor (M. A. Bio products). Although 96-well dishes could be harvested efficiently with this harvestor, cultures from the 24-well macroculture dishes had to be transferred to wells of a 96-well dish (one macroculture well transferred to three microculture wells). Filter disks were counted in 2 ml Scinti-Verse (Fisher Scientific Co.) in an LKB 1210 Ultrabeta scintillation counter (LKB Instruments, Inc., Rockville, MD.)

*Partial Purification of PP Accessory Cells.* The majority of accessory cells in PP are nonadherent (see Results) so a new enrichment approach was required. Toward this end, we took advantage of the previously observed characteristics of spleen DC to exhibit a low buoyant density (15, 19) and to form clusters with T cells (20). Enzymatically obtained PP cells were co-cultured with irradiated (1,500 rad) periodate-treated spleen T cells in 24-well culture dishes (generally $7.5 \times 10^6$ PP cells and $3–5 \times 10^6$ spleen T cells per well) for 16–20 h. The irradiated T cells could cluster with accessory cells but did not blast transform. As a result, most of the irradiated T cells had a high buoyant density while the DC had a low density. The large cell clusters that formed during 16–20 h of culture were completely separated from nonclustered cells on a continuous BSA density gradient ($\rho = 1.008 \rightarrow 1.030$) by centrifugation at 500 *g* for 5 min at 4°C. The cluster fraction was then incubated on ice in $Mg^{++}Ca^{++}$-free buffer for 15 min and disrupted into single cells with repeated pasteur pipetting. The cell suspension was then refloated twice on dense BSA cushions, and the low density pellicle cells were obtained by aspirating the 1.068/1.040 interface. The low density cell population was then examined for

(*a*) Ia-bearing cells using a rhodamine-labeled monoclonal anti-$Ia^k$ ($\alpha Ia^k$, hybridoma line 11-5.2 obtained from the Salk Cell Distribution Center, Salk Institute, La Jolla, CA) (21), (*b*) T cells using a fluorescein-labeled anti-Thy-1.2 reagent ($\alpha$-Thy-1.2, hybridoma line 30–42 obtained from the Monoclonal Antibody Center, Becton, Dickinson & Co., Sunnyvale, CA), (*c*) B cells using a rhodamine-labeled anti-Ig reagent ($\alpha$-Ig, rabbit anti-mouse Ig, the kind gift of Dr. John F. Kearney, Department of Microbiology, University of Alabama in Birmingham), (*d*) Fc receptor-bearing cells using IgG-coated sheep erythrocytes (SRBC) (22), and (*e*) DC using phase-contrast morphology (15, 23) and cytotoxicity with 33D1 antibody and C.

## Results

*Enzymatic Dissociation Releases a PP Cell Population With Accessory Activity.* We initially compared accessory activity of PP populations prepared by conventional (mechanical) and enzymatic dissociation using an oxidative mitogenesis assay. Mechanically dissociated PP cells exhibited negligible stimulatory activity when co-cultured with periodate-treated spleen T cells (Fig. 1A). In contrast, PP cells obtained by enzymatic dissociation from the same animals demonstrated 10-fold greater accessory activity (Fig. 1A). Unlike PP, mechanically dissociated spleen cells had potent accessory activity, and enzymatic dissociation produced less than a twofold increase (Fig. 1B). We then examined whether enzymatic treatment released a new population of cells, or merely altered the function of a cell population normally released by mechanical dissociation. PP cells were prepared with mechanical and enzymatic methods, and mechanically dissociated cells were incubated with Dispase for 2 h at 37°C. As shown in Fig. 2, Dispase treatment of conventional cell preparations did not increase stimulatory capacity, indicating that a new cell population was being released by enzymatic dissociation. Moreover, enzyme treatment did not alter the stimulatory activity of mechanically obtained spleen cells (data not shown). Spleen and PP cell populations prepared by enzymatic dissociation from three murine strains (BALB/c,

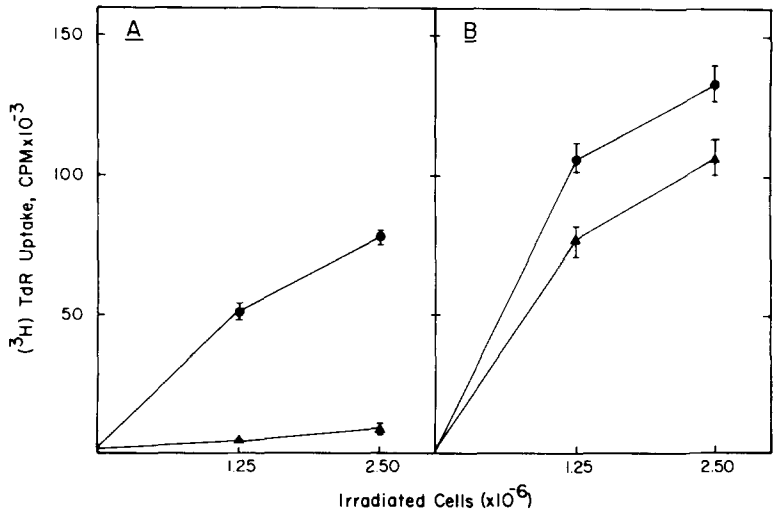


FIG. 1. Enhancement of accessory activity in enzymatically dissociated PP. (A) Whole BALB/c PP: cells obtained by mechanical dissociation (▲) versus enzymatic dissociation (●) as stimulators in oxidative mitogenesis. (B) Whole BALB/c spleen: mechanical (▲) vs. enzymatic dissociation (●). Irradiated stimulator populations cultured alone did not incorporate [$^3$H]TdR. Background incorporation of [$^3$H]TdR in this and subsequent experiments was always less than 2,000 cpm. Mean and range of duplicate microcultures are presented.

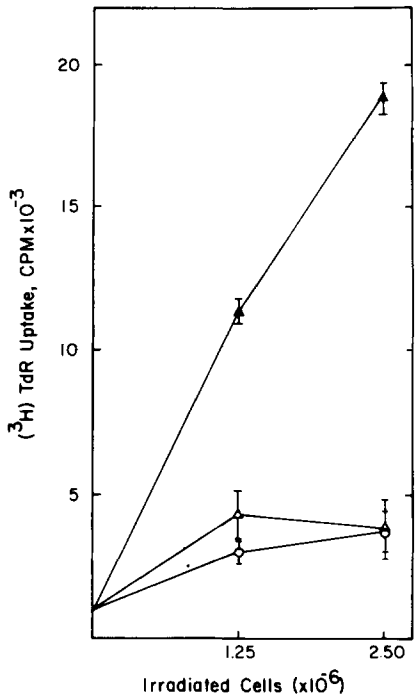


FIG. 2. Enhanced PP accessory activity is not an artifact induced by enzymatic treatment. Mechanically prepared cell populations without (○) or with (△) Dispase exposure for 2 h show minimal accessory activity when compared to enzymatically dissociated population (▲). Mean and range of duplicate microcultures are presented.

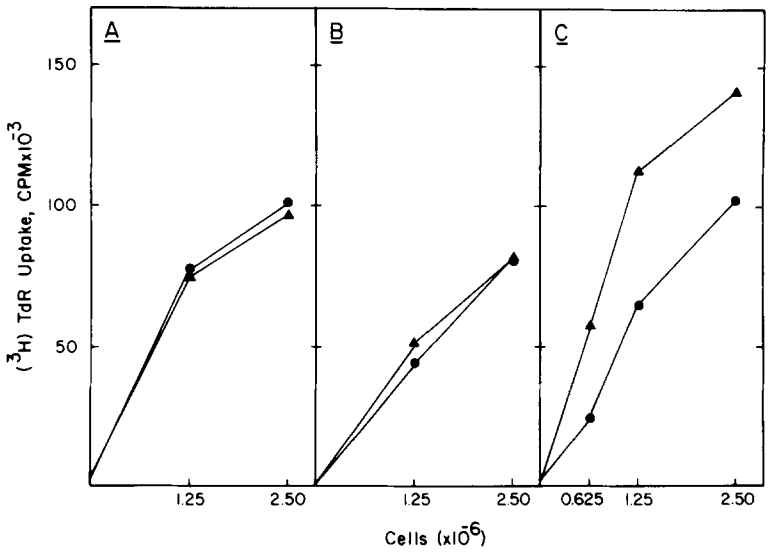


FIG. 3. Comparison of spleen and PP cells from different murine strains. Irradiated stimulator populations assayed in oxidative mitogenesis. (A) CBA/J: PP (○); Spleen (△) (B) C3H/HeN: PP (○); Spleen (△) (C) BALB/c: PP (○); Spleen (△). Mean and range of duplicate microcultures are presented.

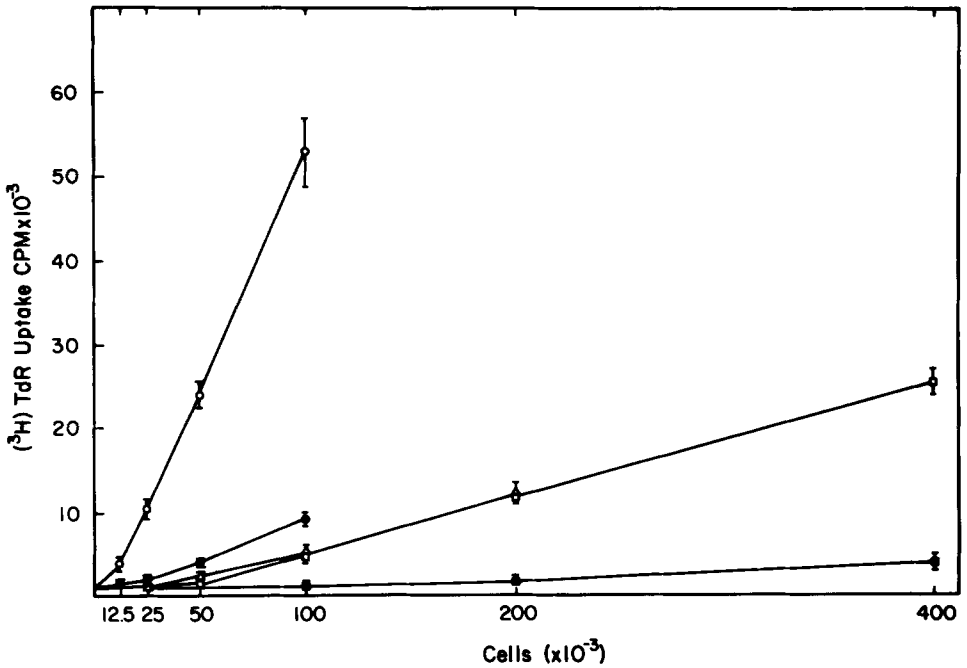


FIG. 4. Enrichment of PP accessory activity using discontinuous BSA gradient. Irradiated stimulators were populations obtained from: whole PP (□); pelleted cells ($\rho > 1.078$) (□); floating cells at 1.078 interface (△)); floating cells at 1.068 interface (○); floating cells at 1.063 interface (○). Mean and standard deviation of triplicate microcultures are presented.

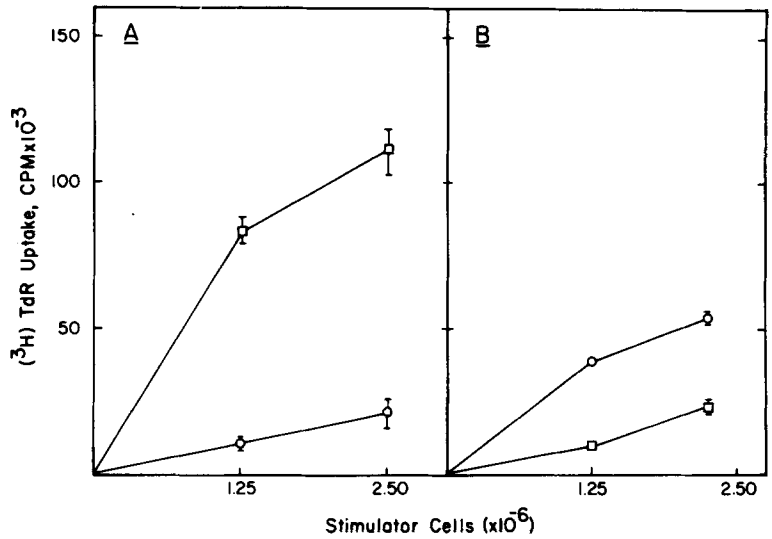


FIG. 5. Adherence characteristics of accessory activity from enzymatically dissociated BALB/c spleen and PP. (A) In spleen, 80–85% of the accessory activity resides in the adherent fraction (□) while ~15–20% resides in the nonadherent fraction (○) (B) PP shows a complete reversal with 70–75% of the accessory activity in the nonadherent fraction (○) and 25–30% remains in the adherent fraction (□). Cell numbers reported for adherent cells represent the number of cells plated from which adherent cells were obtained. Mean and range of duplicate macrocultures are presented.

C3H/HeN, and CBA/J) exhibited similar accessory activity (Fig. 3).

*Physical Characteristics of the PP Accessory Population.* In other murine lymphoid organs, accessory cells have two notable physical properties, low buoyant density and adherence to glass or plastic surfaces. As anticipated, the majority of accessory cell activity in PP resided in a very low density population. The greatest enrichment (eightfold) was found in the cell population with density <1.063 (Fig. 4), which accounted for ≦5% of the total population. Greater than 90% of the total PP accessory activity was recovered in cell populations with a density <1.078 (<1.063 ~ 60%; 1.063 → 1.068 ~ ~10%; 1.068 → 1.078 ~20%).

Adherence properties were next examined with the unexpected finding that most activity resided in the nonadherent fraction (Fig. 5A). In contrast, the accessory activity of identically prepared spleen populations was predominantly in the adherent fraction (Fig. 5B). In three experiments with unfractionated PP, 75–90% of the accessory activity was in the nonadherent fractions. The low density populations (from dense BSA columns) yielded similar results. The adherent PP cells were studied further. 70% were $Ia^+$. Most cells had the appearance of macrophages and would rosette and ingest opsonized erythrocytes. Adherent DC were apparent but represented <10% of the population. Additional experiments with mechanically and enzymatically digested spleen showed that Dispase treatment did not alter Fc receptor function (data not shown). A more complete characterization of PP adherent cells will appear later (manuscript in preparation) but the weak accessory activity of adherent PP cannot be ascribed to a lack of $Ia^+$ macrophages.

*Surface Markers of the PP Accessory Cell Population.* Treatment with anti-$Ia^k$ and C reduced the accessory activity of C3H/HeN ($H\text{-}2^k$) PP cells >90% (Table I). PP cells cultured overnight (to exclude enzyme-induced artifact) were also sensitive to anti-Ia and C (Table I).

PP accessory cells were also eliminated with the anti-DC monoclonal antibody 33D1 and rabbit C (Table II). Accessory activity was reduced 75% in freshly prepared PP cells and >90% in both adherent and nonadherent low density populations (Table II). Finally, PP and spleen cells that had been cultured overnight exhibited a 60–75% reduction in accessory activity. In several other experiments (not shown) the effect of 33D1 on spleen and PP functions was similar. We conclude that 33D1 interacts with the adherent and nonadherent accessory cell in PP.

*Partial Purification of DC from PP.* To identify DC as the 33D1 positive component of PP, we took advantage of the fact that clusters of lymphoblasts and DC routinely form during DC-mediated responses (20, and unpublished observations). Prominent clusters were in fact observed during the first day of culture of PP cells and nylon wool-passed periodate-treated T cells. Few aggregates were seen in 1-d cultures of PP alone, periodate-treated T cells alone, or PP and unmodified T cells. To purify DC from the clusters by flotation on dense albumin columns, we had to reduce contamination of low density lymphoblasts (26). We found that clusters would still form when irradiated periodate-treated T cells, which did not blast transform, were used. The clusters were isolated, and 65–75% of the cells were Thy-1 positive. We then dissociated the clusters, retrieved the low density population, and tested for surface Ig, Thy-1.2, Ia, 33D1, Fc receptors, and function. Table III shows the cell yields in a typical experiment.

As depicted in Table IV, ~70–75% of the low density cluster-derived cells were Ia-

TABLE I

*Abrogation of Accessory Cell Activity in C3H/HeN PP Cell Preparations with Anti-I-$a^k$ and C**

| Cell preparation | Number of cells added | Accessory cell activity | |
|---|---|---|---|
| | | C only | Anti-I-$a^k$ + C |
| | × $10^5$ | *cpm* | |
| Noncultured PP cells | 4.0 | 48,331 ± 219 | 6,709 ± 937 |
| | 2.0 | 28,578 ± 1,450 | 5,516 ± 74 |
| | 1.0 | 15,039 ± 1,257 | — |
| | 0.5 | 9,303 ± 1,254 | 4,222 ± 268 |
| | None | 3,962 ± 884 | |
| Cultured (24 h) PP cells | 4.0 | 62,972 ± 3,540 | 27,795 ± 1,425 |
| | 2.0 | 55,840 ± 640 | 23,925 ± 1,185 |
| | 1.0 | 31,560 ± 710 | 11,230 ± 460 |
| | 0.5 | 13,940 ± 1,470 | 5,240 ± 810 |
| Noncultured spleen cells | 4.0 | — | — |
| | 2.0 | 56,574 ± 5,836 | 3,618 ± 764 |
| | 1.0 | 27,048 ± 495 | 2,281 ± 538 |
| | 0.5 | 10,561 ± 35 | 2,388 ± 413 |
| | 0.25 | 5,580 ± 528 | 1,440 ± 54 |
| | None | 1,300 ± 321 | |
| Cultured (24 h) spleen cells | 4.0 | 60,292 ± 1,190 | 6,695 ± 75 |
| | 2.0 | 33,860 ± 1,610 | 2,630 ± 260 |
| | 1.0 | 18,275 ± 2,645 | — |
| | 0.5 | 5,705 ± 985 | — |

* $5 \times 10^5$ nylon wool nonadherent, periodate-treated spleen cells were cultured with graded numbers of irradiated stimulator cells treated with C or Ia plus C. Control values for no treatment or antibody alone, were similar to values for the C control. All data represent cpm of [$^3$H]TdR incorporated during a 16-h pulse (mean and standard deviation) of triplicate microcultures.

positive, Thy-1-negative, and Ig-negative cells. 55–60% of cells were killed by 33D1 supernatant and rabbit C. Additional experiments revealed that <5% of the cells were able to rosette IgG-coated SRBC, indicating a lack of Fc receptors. Phase contrast microscopy revealed typical DC (Fig. 6). Finally, we demonstrated that the DC-enriched population was 60–80-fold enriched in accessory cell activity relative to cultured or fresh PP suspensions (Table V).

## Discussion

Previously, it has been difficult to identify significant accessory cell activity in murine PP. We had noted that PP suspensions, obtained with Dispase, were capable of in vitro mitogenesis, polyclonal Ig production, and antigen-specific immune responses (10, 11). One would, therefore, assume that appropriate accessory cells had been released, and in this paper we find that this is indeed the case. Enzymatically dissociated PP from several mouse strains have accessory activity that is quantitatively similar to a standard source, mouse spleen (Figs. 1–3). The assay system used to detect accessory cells was the proliferation of periodate-modified T cells. Previous studies have demonstrated the usefulness of this system as a simple, quantitative, and polyclonal assay of accessory cell-dependent, T cell mitogenesis (13, 14, 18). Our

TABLE II

*Abrogation of Accessory Cell Activity in C3H/HeN PP Cell Preparations with 33D1 and C**

| Cell preparation | Number of cells tested | Accessory cell activity | |
|---|---|---|---|
| | | C only | 33D1 + C |
| | × $10^5$ | *cpm* | |
| Unfractionated PP cells | 5.0 | 31,402 ± 3,481 | 9,061 ± 90 |
| | 2.5 | 14,258 ± 1,254 | 3,562 ± 376 |
| | 1.25 | 6,200 ± 247 | 2,136 ± 144 |
| | 0.625 | 2,657 ± 377 | 1,175 ± 170 |
| | None | 1,128 ± 75 | |
| Flotation-enriched | | | |
| PP adherent cells | 8.0‡ | 26,147 ± 4,277 | 2,388 ± 19 |
| | 2.65 | 9,064 ± 562 | 1,573 ± 106 |
| | 0.8 | 3,274 ± 89 | 1,350 ± 138 |
| PP nonadherent cells | 1.0 | 12,195 ± 891 | 2,020 ± 91 |
| | 0.50 | 5,698 ± 226 | 1,291 ± 81 |
| | 0.25 | 2,776 ± 261 | 1,014 ± 88 |
| | 0.125 | 1,457 ± 34 | 694 ± 43 |
| | None | 1,398 ± 59 | |
| Cultured PP cells | 1.0 | 40,456 ± 2,051 | 12,814 ± 567 |
| | 0.5 | 20,635 ± 551 | 7,140 ± 781 |
| | 0.25 | 6,918 ± 114 | 2,188 ± 140 |
| | 0.125 | 2,708 ± 682 | 611 ± 83 |
| Cultured spleen cells | 4.0 | 61,811 ± 3,598 | 27,488 ± 460 |
| | 2.0 | 50,917 ± 4,221 | 10,572 ± 46 |
| | 1.0 | 29,834 ± 565 | 3,715 ± 182 |
| | 0.5 | 13,955 ± 291 | 1,026 ± 62 |
| | None | 468 ± 60 | |

* 5 × $10^5$ nylon wool nonadherent, periodate-treated spleen T cells were cultured for 42 h with graded numbers of irradiated stimulator cells that had been treated wih complement or 33D1 and C. Control values for no treatment and antibody alone were similar to the C control. The data represent cpm of [$^3$H]TdR incorporated during a 16-h pulse (mean and standard deviation) of triplicate microcultures.

‡ Represents number of cells plated to obtain adherent cells.

experiments indicate that the PP accessory cell is similar to the DC identified in other tissues and species (13, 14, 24, 25). Identification of PP DC, which occurred in very small numbers, proved to be difficult and required three types of experiments.

We first looked at the physical properties of the PP accessory cell. Greater than eightfold enrichment of function was obtained by flotation on dense albumin columns (Fig. 4). Low bouyant density has been a constant feature of DC in previous work (13–16, 19, 24). However, unlike previous studies in mouse and in humans, the PP accessory cell was predominantly in the nonadherent fraction. We suspect that nonadherence is an intrinsic property of PP accessory cells, rather than the direct consequence of the enzymatic dissociation procedure. Specifically, exposure of spleen cells to this procedure did not interfere with the well-known capacity of spleen accessory cells to adhere firmly to glass or plastic (Fig. 5). Enzymes might be required to dissociate those regions of the PP that surround areas rich in accessory cells; alternatively, PP accessory cells might themselves be anchored differently than their spleen counterparts and require enzymes for their release.

TABLE III

*Steps in the Enrichment of PP DC*

| Procedure | Number of cells |
|---|---|
| (*a*) Mix PP and irradiated, periodate-treated T cells | |
| Total PP cell population* | $150 \times 10^6$ |
| $NaIO_4$ splenic T cells‡ | $60 \times 10^6$ |
| (*b*) Isolate the cell clusters formed after 16-20 h of culture | |
| Clustered cells§ | $33 \times 10^6$ |
| (*c*) Dissociate clusters‖ and refloat on dense albumin | |
| First flotation ($\rho = 1.072$) | $6.5 \times 10^6$ |
| Second flotation ($\rho = 1.068$) | $1.9 \times 10^6$ |

* Enzymatically obtained PP cells (generally $7.5 \times 10^6$) and periodate-treated spleen T cells (generally $3 \times 10^6$) were cultured in complete media for 16-20 h.

‡ Periodate-treated spleen T cells were irradiated (1,500 rad) before addition of PP cells.

§ After co-culture, large cell clusters were separated from nonclustered cells on continuous BSA density gradients. Clusters were dissociated and number of viable cells counted.

‖ Dissociated cluster cells were floated on dense BSA. The floating cells from the 1.072 interface were refloated in BSA ($\rho = 1.068$) and the floating cells from this pellicle were characterized. 1.8% of the starting population of PP cells were recovered in the final low density fraction in this experiment (recovery was 1.07 ± 0.68% in 12 experiments).

TABLE IV

*Surface Markers of Enriched PP Accessory Cells**

| Immunofluorescence experiments | Surface markers‡ | | | |
|---|---|---|---|---|
| | $I\text{-}A^k$ | $I\text{-}A^k$, sIg | $I\text{-}A^k$, Thy-1.2 | Thy-1.2 |
| 1 | 78.0 | 0 | 0 | 17.7 |
| 2 | 78.0 | 3 | <1 | 18.3 |
| 3 | 67.0 | <1 | 2 | 16.7 |

| Cytotoxicity experiments§ | Cell death with antibody ± C | | |
|---|---|---|---|
| | $I\text{-}A^k$ | 33D1 | Thy-1.2 |
| 1 | 69 | 70 | 26 |
| 2 | 80 | 64 | — |

* Cells were obtained from the second flotation of dissociated clusters (see Table III).

‡ Represents percent positive of cells counted (generally ≧200 cells counted for each marker).

§ Cytotoxicity performed as in Materials and Methods with 10-μl aliquots of cells at $8 \times 10^6$ cells/ml. Two separate counts of 100 cells each were averaged. Values given represent percent of cells specifically killed by antibody ± C. Antibody controls, C controls, and no treatment controls had 11 and 10% cell death in each experiment, respectively.

In the second set of experiments, we studied the surface markers of the PP accessory cell. It was $Ia^+$ (Table I), Ig- and Thy-1-negative (Table IV), and was killed by the monoclonal anti-DC antibody, 33D1, and C (Table II). This monoclonal antibody has been shown to react specifically with DC in mouse spleen and node, and does not appear to react with any other leukocyte (16). 33D1 eliminated accessory function from adherent PP low density cells, which were predominantly $Ia^+$ macrophages, and also depleted most of the accessory function in nonadherent populations, which were predominantly $Ia^+$ lymphocytes. It is known that Ia is not a cell-specific marker and it would appear that the principal if not only $Ia^+$ cell that stimulates oxidative

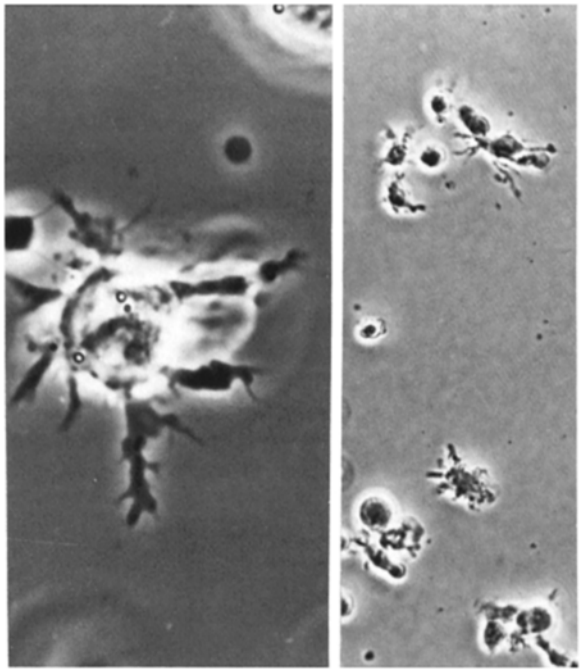

FIG. 6. Phase-contrast micrographs of irregularly shaped DC isolated from PP.

mitogenesis is the DC. We also obtained data (not shown) demonstrating that the PP accessory cell was Fc receptor negative, since it did not bind to surfaces coated with immune complexes. Unfortunately, we have not yet established if our technique removes all Fc-bearing leukocytes, although macrophages were efficiently retrieved.

We needed a third set of experiments to identify the PP DC directly. In the rat, where DC are typically nonadherent, Klinkert et al. (13) were able to enrich DC by preparing a radioresistant, low density fraction. This procedure has not been successful in mice or humans, since the viability of DC 1 d after irradiation has been low, and since significant numbers of other cell types exhibited a low buoyant density after irradiation (unpublished observations). We, therefore, took advantage of another property of the DC, its ability to aggregate with blast-transformed lymphocytes to produce large cell clusters (20). Such T cells have a low buoyant density (26) comparable to DC. However, we were able to generate large cell aggregates during the first day of culture of PP cells and irradiated, periodate-modified T cells. These aggregates were isolated and shown to contain primarily T cells. When the dissociated cells were separated on dense albumin columns the low density population was predominantly (~60%) DC with typical morphology (Fig. 6) and surface markers (Table IV). Thus periodate-modified T cells in the absence of blast transformation interact with DC and can be used as a vehicle for isolating this trace cell type.

Most important, these enriched DC were some 60–80-fold more active than unfractionated PP in triggering oxidative mitogenesis (Table V). We think this indicates 60–80-fold enrichment in DC numbers. However, it is possible that the interaction of DC and contaminating T cells in the purified fraction is responsible for enriched accessory function; e.g., by generating interleukins. Austyn et al. (25) have

TABLE V

*Enrichment of Accessory Cell Activity With Cluster-derived Low Density Cells* *

| Cell preparation | Number of cells added ($\times 10^3$) | Accessory cell activity |
|---|---|---|
| Experiment 1 | | |
| Cultured PP cells | 200 | 21,243 ± 1,726 |
| | 100 | 6,459 ± 1,120 |
| | 50 | 2,350 ± 319 |
| | 25 | 1,775 ± 194 |
| Cluster-derived low density cells | 10 | 67,185 ± 3,968 |
| | 5 | 37,667 ± 2,088 |
| | 2.5 | 16,913 ± 4,080 |
| | 1.25 | 8,727 ± 2,176 |
| T cells only | | 1,789 ± 213 |
| Experiment 2 | | |
| Noncultured PP cells | 400 | 21,531 ± 2,206 |
| | 200 | 9,589 ± 2,087 |
| | 100 | 2,457 ± 203 |
| | 25 | 1,169 ± 156 |
| Cluster-derived low density cells | 10 | 36,289 ± 1,633 |
| | 5 | 15,171 ± 476 |
| | 2.5 | 5,472 ± 590 |
| | 1.25 | 2,911 ± 193 |
| T cells only | | 741 ± 182 |

* Cells obtained from the second flotation of dissociated clusters, or from the starting population, were irradiated and cultured with $5 \times 10^5$ nylon wool nonadherent periodate-treated spleen T cells. The data represent cpm of [$^3$H]TdR incorporated during a 16-h pulse. Mean and standard deviation of triplicate cultures are presented.

recently studied the function of DC in inducing responsiveness to, and release of, interleukin during oxidative mitogenesis. They were able to show that interleukins do not synergize with intact DC over a wide dose range.

The identification of DC in PP will now allow us to further compare PP DC and macrophages, as well as PP and spleen DC, in other assay systems. PP exhibit unique immunologic properties distinct from spleen and other peripheral lymphoid organs: specialization for IgA isotype induction (1); generation of lymphocytes that exit PP without terminal differentiation and that subsequently populate distant mucosal sites (2, 3); and immune response properties distinct from other lymphoid tissues (27). It is possible that some of the differences between PP and other lymphocyte organs relate to distinctive features of their accessory cells. This possibility is being pursued in our current experiments.

## Summary

Previous studies have suggested that in vitro and in vivo immune responses are defective in Peyer's patch (PP) as a result of a deficiency in accessory cell number or

function. However, we report here that enzymatic dissociation of PP does release a cell population with accessory activity in oxidative mitogenesis, i.e., the proliferation of periodate-modified T cells. The accessory activity present in PP is quantitatively similar to that of spleen. Accessory function is mediated by a cell type(s) that has the following characteristics: low buoyant density, lack of adherence to plastic or glass surfaces, lack of Fc receptors, and presence of surface Ia and the 33D1 dendritic cell (DC)-specific determinants.

This PP accessory cell was markedly enriched by a novel technique. PP cells formed large aggregates when cultured for 16 h with irradiated, periodate-treated spleen cells. From the clusters we obtained a low density cell population that was 60% Ia positive, 33D1 positive, non-T and non-B, Fc receptor-negative, and dendritic in morphology. The DC-enriched populations were 60–80-fold enriched in accessory function relative to unfractionated PP. We can now compare PP accessory cells with accessory cells from other organs, and try to determine how PP dendritic cells contribute to the unique functions of this lymphoid organ.

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