

ANTIGEN-INDUCED STIMULATION OF GLUCOSAMINE
INCORPORATION BY GUINEA PIG PERITONEAL
MACROPHAGES IN DELAYED HYPERSENSITIVITY*

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It is well known that the interaction between sensitized lymphocytes and specific antigen of the type occurring in delayed hypersensitivity changes the behavior of bystander macrophages (1). Under these conditions macrophages undergo a variety of light microscopic, ultrastructural, and biochemical alterations resulting in an enhanced capacity to ingest and destroy bacteria; such "activated macrophages" are thought to be the effector cells responsible for cellular immunity. Recent studies have shown that macrophages may become activated when cultured in the presence of cell-free supernatants derived from cultures of sensitized lymphocytes and antigen (2); the factor(s) responsible for macrophage activation is (are) not yet established, but appears to be similar or identical to migration inhibition factor (MIF)¹ (3). It seems probable, therefore, that macrophage activation is mediated by products of specifically sensitized lymphocytes.

A number of macrophage properties modified in the process of activation, such as motility, spreading on glass, and phagocytosis, involve functions of the cell periphery (4, 5). Moreover, using specialized electron microscopic methods for studying the cell surface, A. Dvorak et al. (6, 7) have demonstrated that macrophages whose migration from capillary tubes is inhibited by sensitized lymphocytes and specific antigen lose the layer of cell coat material normally adherent to the outer leaflet of the trilaminar unit membrane (6, 7); macrophages in such cultures aggregate to form cell clumps. For these reasons, we were stimulated to look for metabolic alterations occurring at the macrophage surface as a consequence of delayed hypersensitivity. In particular, we examined D-glucosamine incorporation since this amino sugar is an especially suitable precursor of the macromolecular polysaccharides found at the cell periphery (4, 8, 9).

We here report that incorporation of radioactive glucosamine by peritoneal

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¹ *Abbreviations used in this paper:* CFA, complete Freund's adjuvant; dpm, disintegrations per minute; HSA, human serum albumin; IFA, incomplete Freund's adjuvant; MEM, minimal essential medium; MIF, migration inhibition factor; OCB-BGG, *o*-chlorobenzoyl bovine- γ -globulin; OT, Old Tuberculin; PEC, peritoneal exudate cells; PPD, purified protein derivative.

macrophages into membrane-related macromolecular material is strikingly enhanced when these are cultured in the presence of specific antigen and lymphocytes primed to exhibit delayed hypersensitivity. This finding provides a sensitive new assay for measuring cellular hypersensitivity and, correlated with the morphologic data reported earlier (6, 7), suggests several possible mechanisms of action for lymphokines such as MIF. Portions of this work have appeared in abstract form (10).

Materials and Methods

Antigens.—Crystallized human serum albumin (HSA), lots 30–32, was obtained from Pentex Biochemical, Kankakee, Ill. Preservative-free purified protein derivative (PPD) was the generous gift of Parke, Davis & Co., Detroit, Mich. Old Tuberculin (OT) was supplied by the Massachusetts Department of Health. *o*-Chlorobenzoyl bovine- γ -globulin (OCB-BGG) was prepared by the method of Remold et al. (11). Antigens dissolved in sterile pyrogen-free saline were sterilized by passage through 0.22 μ Millipore filters (Millipore Corp., Bedford, Mass.) previously washed to remove contaminating detergent.

Immunization and Skin Tests.—350–400 g male Hartley guinea pigs (Camm Research Institute, Inc., Wayne, N.J.) were immunized in the four footpads with 0.1 ml complete Freund's adjuvant (CFA), prepared to contain 3 mg/ml killed human tubercle bacilli of strains C, DT, and PN. Other animals were sensitized with 100 μ g of OCB-BGG incorporated in either complete or incomplete Freund's adjuvant (IFA).

Skin tests were performed on occasional animals a week or more before use by the intradermal injection of 0.1 ml of saline containing 1:100 OT or 50 or 5 μ g of OCB-BGG. Reactions were read at 1, 4, and 24 hr. Prior skin testing had no detectable effect on culture results.

Cultures of Peritoneal Exudate Cells (PEC).—Exudates were induced in sensitized and nonsensitized Hartley guinea pigs by intraperitoneal injection of 25 ml of sterile Marcol 52 (Esso Chemical Co., Inc., New York). 3 or 4 days later, animals were exsanguinated and exudates were harvested by washing the peritoneal cavity with 150 ml of Hanks' balanced salt solution. Cells were collected in a separatory funnel, the aqueous phase was removed and the cells were concentrated by centrifugation at 1000 rpm for 10 min at 4°C. The cells were washed three times with sterile cold Hanks' solution, counted, and resuspended in a "complete medium" composed of Eagle's minimal essential medium (MEM; Grand Island Biological Co., Grand Island, N.Y.) supplemented with 15% fresh normal guinea pig serum and 100 units/ml penicillin and 100 μ g/ml streptomycin. An average of 2.8×10^8 cells with a mean viability of 97% were recovered from a single peritoneal exudate. Cells consisted of 10–15% neutrophils, 20–25% lymphocytes, and 60–70% oil-containing macrophages. There was no significant difference in total cell yield or differential counts between sensitized and unsensitized animals.

Monolayer cultures were prepared in 35 \times 10 mm plastic Petri dishes (Falcon Plastics, Los Angeles, Calif.). Each contained 5×10^6 viable cells in a final volume of 3.0 ml of complete medium. Appropriate amounts of OCB-BGG, PPD, or saline were added to duplicate cultures. Cells were incubated for 1–7 days in an atmosphere composed of 5% CO₂-95% air at 37°C. Beginning at 24 hr of incubation, cultures were supplemented daily with 0.1 ml of a solution containing MEM, dextrose, essential and nonessential amino acids, and NaHCO₃ (12).

Incorporation of Radioactive Precursors.—After 1, 2, 3, or 7 days of cultivation, each culture received radioactive precursors as follows: 0.25 or 0.5 μ Ci of D-glucosamine-¹⁴C or L-leucine-¹⁴C, both uniformly labeled; 5 μ Ci of thymidine-methyl-³H, L-leucine-4,5-³H, L-fucose-³H, choline-methyl-³H, or D-glucosamine-6-³H; or 0.25 μ Ci sodium sulfate-³⁵S (all obtained from New England Nuclear Corp., Boston, Mass.). Frequently double isotope ex-

periments were performed on the same Petri dish using one ^{14}C -labeled precursor (e.g., glucosamine) together with another labeled with ^3H (e.g., leucine or thymidine). After a pulse period of 1–24 hr, the medium containing nonadherent cells was removed, and the adherent monolayer was washed four times with cold Hanks' solution supplemented with a large excess of appropriate unlabeled precursors (8.9 mg/ml D-glucosamine, 20 $\mu\text{g}/\text{ml}$ thymidine, 20 $\mu\text{g}/\text{ml}$ L-leucine). Culture plates were then frozen and thawed. Cold 10% trichloroacetic acid (TCA) was added, and the monolayers were scraped and transferred quantitatively to test tubes. After a minimum incubation of 1 hr in ice these were centrifuged at 1500 rpm for 15 min at 4°C, and the pellets were dissolved in Hyamine hydroxide 10X (Packard Instrument Co., Inc., Downers Grove, Ill.) at 37°C. Solubilized pellets, and in some instances aliquots of TCA-soluble supernates, were transferred to Bray's solution; and radioactivity was measured in a Packard TriCarb liquid scintillation spectrometer. Activities were converted to disintegrations per minute (dpm) by reference to an external standard.

Preliminary studies indicated that both normal and sensitized PEC incorporated radioactive glucosamine into acid-insoluble material with linear kinetics for at least 6 hr, and hence 6-hr pulse periods were employed in this study unless otherwise indicated. Absolute amounts of glucosamine incorporated by sensitized PEC in the absence of antigen or by normal PEC in the presence or absence of antigen increased slightly from 1 to 3 days of culture but were reduced below initial values at 7 days.

Expression of Data and Statistical Analysis.—Incorporation of radioactive precursors into TCA-precipitable macromolecular material was expressed in the form of stimulatory ratios. These are defined as the mean dpm of duplicate peritoneal exudate cultures containing antigen divided by the mean dpm of duplicate cultures of similar cells incubated for the same interval without antigen. Results from 4 to 12 animals were averaged at each dose level and time interval. Statistical differences between experimental and control groups were assessed using the Mann-Whitney test (13) and by calculation of the standard errors of the means.

Measurement of Cell Counts and Adherent Protein.—Cell counts were performed after scraping attached cells into 1 ml of normal saline; viability was determined by trypan blue exclusion before scraping. Lowry protein determinations (14) were carried out on cell monolayers dissolved in 0.5 N NaOH after four washes in Hanks' solution.

Radioautography.—Cultures for radioautography were pulsed with 10–30 μCi glucosamine- ^3H or thymidine- ^3H for 6–48 hr. Cultures were then washed four times in Hanks' solution supplemented with excess unlabeled glucosamine or thymidine, fixed, scraped into BEEM capsules, and embedded in Epon as previously described (15). 1- μ sections were coated with NTB-2 emulsion and exposed for 1–8 wk, developed, and stained with Giemsa reagent (15).

Subcellular Fractionation.— 15×10^6 PEC were cultured in 60 \times 15 mm plastic Petri dishes (Falcon) in 10 ml of complete medium. After 3 days each culture was pulsed for 6 hr with 5 μCi glucosamine- ^{14}C and was then washed four times with Hanks' solution containing excess unlabeled glucosamine and once in phosphate-buffered saline. Cells were then scraped off the Petri dishes into 10 ml of 0.01 M Tris-HCl buffer, pH 7.0, and homogenized in an ice-cooled, tight-fitting, Dounce homogenizer. Approximately 25 strokes were required for adequate cell breakage as monitored by phase-contrast microscopy. Homogenates were subjected to differential centrifugation in the cold to obtain nuclear (10 min, 2000 g), mitochondrial (10 min, 9000 g), and microsomal (1 hr, 100,000 g) pellets and a soluble supernate. Pellets were resuspended in water. Aliquots of all fractions were analyzed for protein (14); TCA precipitates of other aliquots were analyzed for radioactivity.

RESULTS

Antigen-Induced Stimulation of Glucosamine Incorporation by Sensitized PEC.—Incorporation of glucosamine- ^{14}C into acid-precipitable material by peritoneal exudate cells from sensitized guinea pigs was greatly stimulated

by specific antigen (Fig. 1). Plastic adherent PEC from animals sensitized 3-8 wk previously with CFA so as to develop delayed hypersensitivity incorporated 1.5 to 17 times more glucosamine in the presence of PPD than similar cells cultured without antigen. Antigen-enhanced incorporation was not apparent after 1 day of culture, but thereafter became highly significant

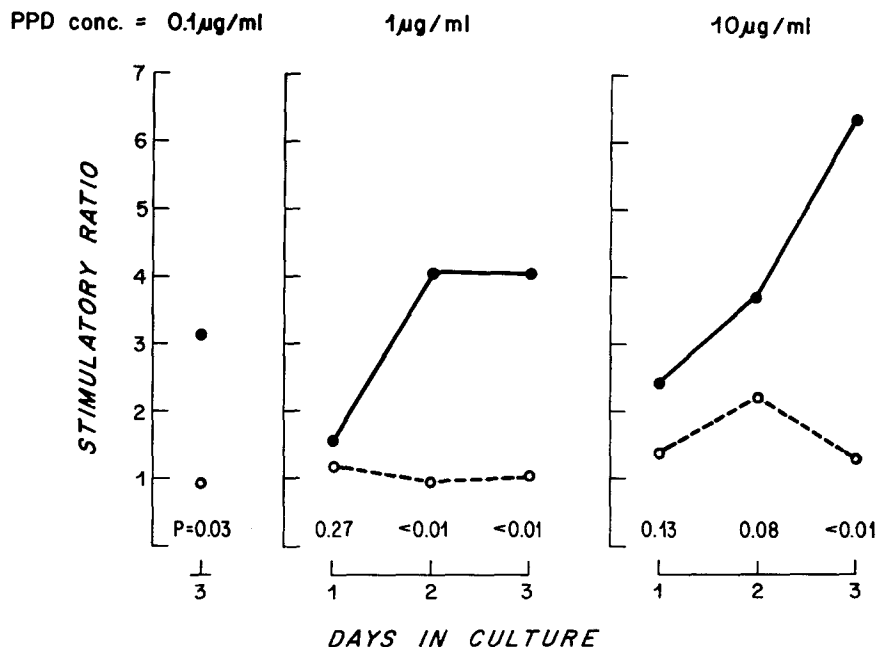


FIG. 1. Antigen-induced stimulation of ^{14}C -labeled or ^3H -labeled glucosamine incorporation into TCA-precipitable material by guinea pig peritoneal exudate cells from previously sensitized (●—●) or unsensitized (○----○) animals. Dishes were cultured for 1-3 days and were pulsed with isotope for the final 6 hr before harvest. Each point represents the mean stimulatory ratio determined from replicate cultures of cells from 5-12 guinea pigs over an antigen concentration range of 0.1-10 $\mu\text{g}/\text{ml}$ PPD. Probability (P) values for statistical differences between the response of PEC from sensitized and unsensitized animals to antigen were calculated with the Mann-Whitney test.

(Fig. 1). Maximal stimulation was observed in 3-day cultures, and was no longer significant at 7 days. Significant stimulation was achieved with as little as 0.1 μg PPD/ml, and mean stimulatory ratios were somewhat greater with antigen concentrations up to 25 $\mu\text{g}/\text{ml}$ (Fig. 2). No significant stimulation was detected with 0.01 $\mu\text{g}/\text{ml}$ PPD. Approximately a twofold increase was observed in the cell-associated, acid-soluble glucosamine pool in adherent cells from sensitized animals cultured in the presence of antigen.

The antigen-induced stimulation of glucosamine incorporation observed

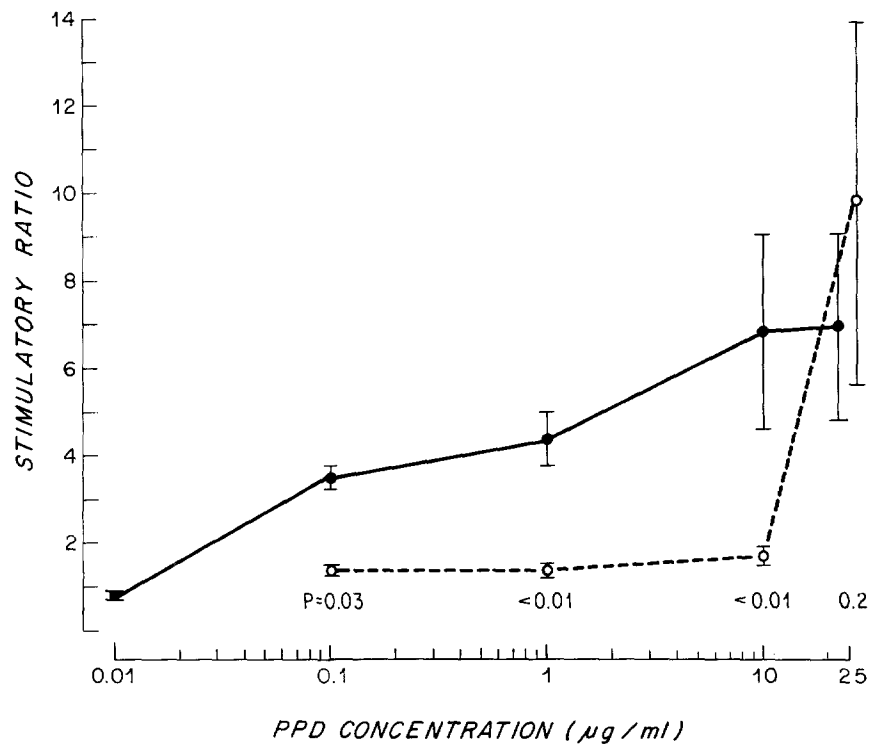


FIG. 2. ^{14}C or ^3H -labeled glucosamine incorporation by sensitized (●—●) and unsensitized (○---○) peritoneal exudate cells after 3 days of culture as a function of PPD concentration. Culture conditions, *P* values, and isotope pulse as in Fig. 1. Standard errors are indicated for each point, affording a second statistical method of evaluating variation between stimulatory ratios of cells from sensitized and control animals.

in sensitized exudates over the range of 0.1–25 μg PPD/ml could not be attributed to an increase in the number of cells adherent to the culture dish. Thus, counts of sensitized peritoneal exudate cells adherent to Petri dishes after 3 days of culture in the presence or absence of 1 $\mu\text{g}/\text{ml}$ PPD were $4.85 \pm 0.26 \times 10^6$ and $4.85 \pm 0.13 \times 10^6$, respectively; corresponding counts of unsensitized cell cultures were $4.55 \pm 0.33 \times 10^6$ and $4.35 \pm 0.24 \times 10^6$. Total protein adherent to Petri dishes was slightly greater in cultures of sensitized cells to which antigen (10 μg PPD/ml) was added; however, the increment, although statistically significant, was small (approximately 17%) and cannot explain the observed differences in glucosamine incorporation.

By contrast, PEC from unsensitized animals failed to show antigen-enhanced incorporation of glucosamine over a PPD concentration range of 0.1–10 $\mu\text{g}/\text{ml}$. At the high dose of 25 $\mu\text{g}/\text{ml}$, normal cells sometimes exhibited increased glucosamine incorporation; but the effect was irregular, as reflected in the large standard error shown in Fig. 2.

A similarly striking antigen-induced increase in glucosamine incorporation was observed in PEC obtained from animals sensitized with OCB-BGG in CFA (Table I). Significant stimulation was obtained with antigen concentrations of 33 $\mu\text{g/ml}$ or 7 $\mu\text{g/ml}$ (not shown), and was again maximal on days 2 or 3. Similar concentrations of OCB-BGG failed to stimulate glucosamine incorporation by normal PEC.

Importance of Nonadherent Cells (Lymphocytes) to Glucosamine Incorporation.—By analogy with other instances of immunologically mediated macrophage activation, it seemed likely that antigen-induced glucosamine incorporation would require the presence of sensitized lymphocytes. To test this hypothesis, we removed cells that did not adhere to plastic, mostly lymphocytes, early in culture, before their exposure to antigen. Freshly obtained PEC from sensitized animals were placed in Petri dishes in complete medium lacking antigen.

TABLE I
Effect of 33 $\mu\text{g/ml}$ OCB-BGG on Glucosamine- ^{14}C Incorporation by Plastic Adherent Peritoneal Exudate Cells Obtained from Unsensitized Guinea Pigs and from Guinea Pigs Sensitized with 100 μg OCB-BGG in Either CFA or IFA 5 wk Previously

| Sensitization (OCB-BGG) | Stimulatory ratios after culture for 2 or 3 days* | |
|--------------------------|---|------------|
| | 2 | 3 |
| 100 μg in CFA | 3.1 (0.03) | 2.0 (0.05) |
| 100 μg in IFA | 2.1 (0.2) | 1.1 (0.4) |
| 0 | 0.8 | 1.0 |

* Probability (P) values for statistical differences between the stimulatory ratios of PEC from sensitized and unsensitized animals to antigen are included in parentheses. Each point represents mean stimulatory ratio determined from replicate cultures of cells from four animals.

After 45 min of culture, the medium containing nonadherent cells was poured off and replaced by an equivalent amount of fresh, cell-free medium containing 0, 1, or 10 $\mu\text{g/ml}$ PPD. Nonadherent cells consisted of approximately 60% small lymphocytes and 25–30% macrophages; by contrast, 90% of adherent cells were macrophages, 1–2% were lymphocytes, and the remainder consisted of granulocytes and erythrocytes.

Dishes thus freed of the bulk of lymphocytes were cultured for 2 or 3 days and processed as usual for radioactive counting. It was found, in experiments repeated on from four to nine animals, that adherent PEC, cultured in the absence of nonadherent cells, were not stimulated to incorporate glucosamine at an enhanced rate. Thus at 2 days of culture the mean stimulatory ratio in the presence of 1 $\mu\text{g/ml}$ PPD was 4.1 in the presence of adherent cells and 1.2 in the absence of adherent cells; corresponding values after 3 days of culture were 4.0 and 0.9, respectively. At both 2 and 3 days the differences in stimulatory ratios between cultures with and without nonadherent cells were highly significant ($P < 0.01$). Similar results were obtained with 10 $\mu\text{g/ml}$ PPD.

Glucosamine Incorporation by PEC from Animals Primed for Antibody Formation but not Delayed Hypersensitivity.—In order to determine whether the presence of delayed hypersensitivity was essential for antigen-induced stimulation of glucosamine incorporation, we prepared peritoneal exudates from guinea pigs sensitized 5 wk previously with OCB-BGG in IFA. Animals immunized to soluble protein antigens in this fashion develop a transient form of lymphocyte-mediated skin test reactivity characterized by the presence of numerous basophilic leukocytes (cutaneous basophil hypersensitivity [6]). However, this cellular hypersensitivity largely disappears by 2 or 3 wk after immunization, and skin test of the animals used in these experiments 4 wk after immunization elicited strong Arthus reactivity, maximal at 1–3 hr, with little or no visible residual reaction at 24 hr. Peritoneal exudate cells from four such animals were not induced to incorporate glucosamine at an enhanced rate after 1–3 days of culture by 7 or 33 $\mu\text{g/ml}$ OCB-BGG (Table I), concentrations that regularly stimulated incorporation in animals primed with the same quantity of antigen in CFA. Glucosamine incorporation was stimulated when OCB-BGG was used at a level of 100 $\mu\text{g/ml}$, but in four successive experiments this high concentration of antigen stimulated exudate cells from unsensitized animals as well.

Effect of Antigen on Incorporation of Other Radioactive Precursors.—Incorporation of leucine- ^3H or ^{14}C was not detectably increased in sensitized cultures in the presence of 0.1–10 $\mu\text{g/ml}$ PPD and nonadherent cells at any test interval. Similar results were obtained in cultures grown in medium prepared with leucine-free MEM. Fucose- ^3H , choline- ^3H , and sodium sulfate- ^{35}S were all tested in similar experiments; no increase in stimulatory ratios was found in the presence of antigen and sensitized lymphocytes.

By contrast, incorporation of thymidine- ^3H into macromolecular material was increased significantly in cultures containing sensitized lymphocytes and specific antigen (Fig. 3). This stimulation could not be attributed to increased cell numbers since repeated cell counts and total protein determinations on cell monolayers were little changed in such cultures (see above). The stimulatory ratios obtained were regularly lower than those measured with glucosamine (compare Figs. 2 and 3), and at 3 days of culture were significant only at a single PPD concentration (1 $\mu\text{g/ml}$). It will be shown (see next section) that thymidine was incorporated by an entirely different cell population than glucosamine.

Radioautography of Macrophage Monolayers Cultured with Glucosamine- ^3H and Thymidine- ^3H .—In order to determine the cellular and intracellular localization of incorporated radioactive precursors, radioautographs were prepared of sensitized and normal PEC cultured in the presence or absence of 1 or 10 $\mu\text{g/ml}$ PPD. When glucosamine- ^3H was included in 3-day cultures for a 6-hr pulse, nearly all glass-adherent cells (approximately 90% macrophages) were labeled, including cultures of nonsensitized cells and of sensitized cells grown

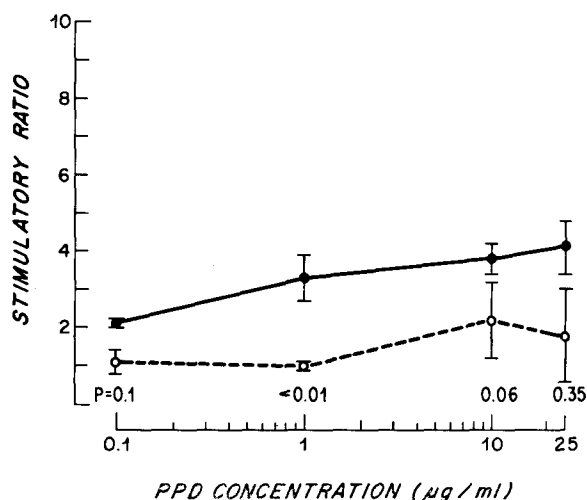


FIG. 3. Incorporation of ^3H -labeled thymidine by sensitized (●—●) and unsensitized (○----○) PEC after 3 days of culture as a function of PPD concentration. Culture conditions, isotope pulse, P values, and standard errors are the same as in Fig. 2.

in the absence of antigen. Grains were localized over the cytoplasm of macrophages and were frequently concentrated in the perinuclear zone corresponding to the Golgi region. Moreover, grains were strikingly localized to the general region of the cell surface. Lymphocytes were less intensely labeled but exhibited a similar distribution of grains. Rare granulocytes and erythrocytes in culture were not labeled above background levels. With longer pulse periods (e.g., 16 or 48 hr) labeling was more intense, and a greater proportion of grains was associated with the cell surface (Fig. 4).

The radioautographic distribution of incorporated thymidine- ^3H was quite different from that observed with glucosamine. In nonsensitized cultures or in cultures of sensitized cells without antigen, only 1% of the adherent cells were labeled, including rare mature macrophages and medium-sized mononuclear cells and grains were sharply localized to cell nuclei. In the presence of 10 $\mu\text{g}/\text{ml}$ PPD, 2–5% of the cells were labeled in 3-day cultures from sensitized animals. Labeled cells in these cultures were primarily lymphocytes, only rare macrophages being labeled.

Subcellular Distribution of Incorporated Glucosamine- ^{14}C .—The subcellular distribution of TCA-precipitable radioactivity was evaluated in sensitized PEC cultured for 3 days in the presence or absence of 10 $\mu\text{g}/\text{ml}$ PPD and pulsed for 6 hr with glucosamine- ^{14}C . After being rinsed, cells were scraped from dishes, homogenized, and subjected to differential centrifugation. The results of a typical experiment are presented in Table II. In the absence of antigen the highest specific activity was found in the membrane-rich microsomal fraction. Moreover, the microsomal fraction showed the largest antigen-

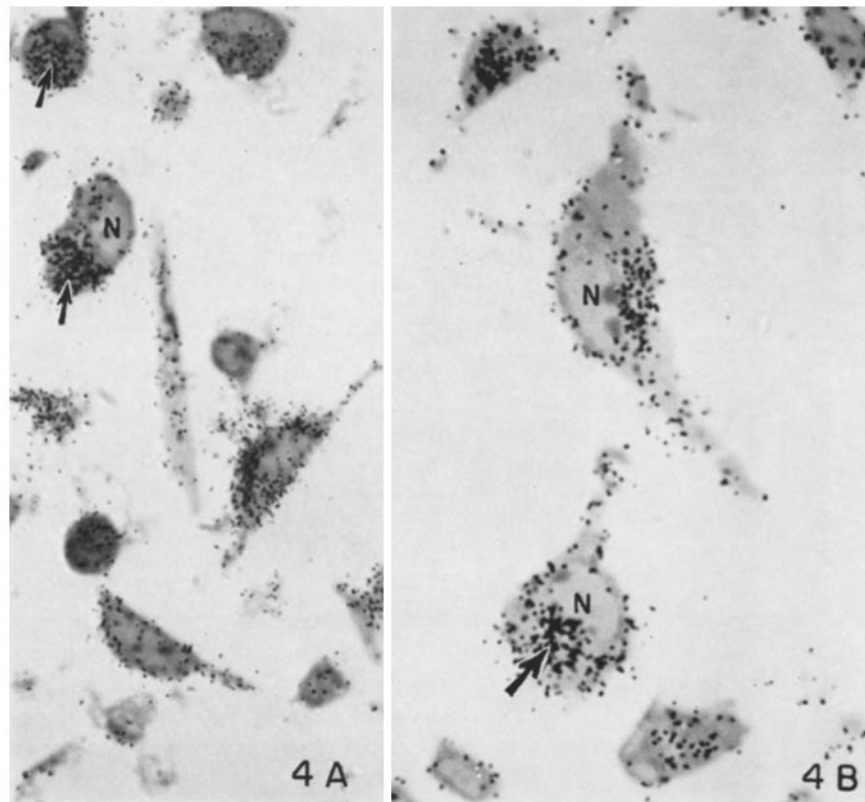


FIG. 4. Radioautographs of PEC from unsensitized animals pulsed with $30 \mu\text{Ci}$ glucosamine- ^3H for 48 hr. Note that nearly all cells, mostly macrophages, are labeled and that grains are concentrated in the vicinity of the perinuclear zone (presumed Golgi region, arrows) and on the peripheral cell membrane, particularly obvious on elongate cell processes. Nuclei (N) are unlabeled. The camera was focused in all instances on the plane of the emulsion; hence the underlying cell detail is somewhat blurred in these high magnification photomicrographs. A, $\times 1000$; B, $\times 1200$.

induced increment in glucosamine incorporation, having a specific activity 2.5 times that of the microsomal fraction of a parallel culture lacking antigen; the same ratio calculated for the starting homogenate was only 1.5. These data indicate that glucosamine is preferentially incorporated into the microsomal fraction of normal macrophages and that the increment in glucosamine incorporation observed when sensitized PEC are cultured in the presence of specific antigen is localized principally to the microsomal fraction.

In other experiments macrophage monolayers cultured with antigen and labeled with glucosamine- ^{14}C for 6 hr were rinsed four times with Hanks' solution containing unlabeled glucosamine and digested with trypsin, $500 \mu\text{g}/$

TABLE II
*Subcellular Distribution of Glucosamine-¹⁴C Incorporated into TCA-Precipitable Material by Adherent Peritoneal Exudate Cells from Sensitized Animals Cultured without Antigen or in the Presence of 10 µg/ml PPD**

| | Starting homogenate | Subcellular fractions | | | |
|--|---------------------|-----------------------|---------------|------------|-------------|
| | | Nuclear | Mitochondrial | Microsomal | Supernatant |
| -PPD | | | | | |
| dpm | 66,006 | 19,735 | 9,647 | 18,829 | 17,795 |
| % dpm | — | 45 | 14.6 | 28.5 | 26.9 |
| Protein (mg) | 2.44 | 0.33 | 0.27 | 0.30 | 1.55 |
| % Protein | — | 13.6 | 11 | 12 | 63.3 |
| Specific activity | 26,037 | 59,443 | 35,756 | 63,935 | 11,502 |
| +PPD | | | | | |
| dpm | 118,261 | 30,766 | 19,681 | 42,073 | 25,741 |
| % dpm | — | 26 | 16.6 | 35.6 | 21.8 |
| Protein (mg) | 3.07 | 0.50 | 0.28 | 0.26 | 2.03 |
| % Protein | — | 16.4 | 9.1 | 8.6 | 66 |
| Specific activity | 38,799 | 61,043 | 70,289 | 159,973 | 12,711 |
| Ratio, specific activities (+PPD/-PPD) | 1.49 | 1.03 | 1.96 | 2.50 | 1.11 |

* The methods of cell homogenization and preparation of subcellular fractions by differential centrifugation are described in Materials and Methods.

ml, for 30 min at room temperature. This treatment released cells quantitatively from the culture dish with a viability in excess of 99% as judged by trypan blue (but see Kraemer [4] for possible reservations regarding interpretation of results obtained with the dye exclusion method). After centrifugation, 30-40% of incorporated ¹⁴C label was found in the supernatant, suggesting that a significant fraction of counts was localized at the cell periphery where it could be solubilized without cell disruption. The labeled material released by trypsinization and centrifugation is currently being characterized. Preliminary studies by Dr. R. C. Hughes, National Institute for Medical Research, Mill Hill, London, reveal that this material contains both labeled glucosamine and galactosamine. However, neutral sugars were unlabeled, indicating that there was no significant conversion into the glycolytic pathway.

DISCUSSION

We have shown that incorporation of glucosamine into macromolecular material is a general property of guinea pig macrophages and is increased up to 17-fold in the presence of specific antigen and sensitized lymphocytes. Good evidence exists that this antigen-induced stimulation of glucosamine incorporation represents an *in vitro* correlate of delayed hypersensitivity. Thus cultures of sensitized peritoneal exudate cells from which the lymphocyte-rich, nonadherent cell population had been decanted were not stimulated

to incorporate increased amounts of glucosamine. Furthermore, in cultures from animals sensitized so as to develop Arthus reactivity without delayed hypersensitivity, antigen failed to stimulate glucosamine incorporation. In our hands this assay has afforded an exceedingly reliable and sensitive measure of delayed hypersensitivity. Significant antigen-induced stimulation was obtained in 35 of 36 consecutive sensitized animals studied and was never observed in unsensitized animals unless very high concentrations of antigen (e.g., 25 $\mu\text{g}/\text{ml}$ PPD; 100 $\mu\text{g}/\text{ml}$ OCB-BGG) were employed. Moreover, threefold stimulation was achieved with as little as 0.1 $\mu\text{g}/\text{ml}$ PPD in specifically sensitized guinea pigs. The minimum antigen dose required to enhance glucosamine incorporation, therefore, is at least as low as that required for inhibition of macrophage migration in comparably immunized animals (17, 18).

The antigen-stimulated glucosamine incorporation described here cannot be attributed simply to a general, nonselective enhancement of macrophage synthetic activity. Incorporation of radioactive leucine, choline, inorganic sulfate, and fucose was not affected under conditions in which glucosamine uptake was strikingly elevated. Although thymidine incorporation was enhanced, and followed kinetics roughly similar to those observed with glucosamine, radioautographic studies indicated that this precursor was incorporated by only a small fraction (1–5%) of adherent cells, many of which had the light-microscopic morphology of lymphocytes. By contrast, glucosamine was incorporated by nearly all (>90%) adherent cells and preferentially by macrophages. Lymphocyte activation with enhanced DNA synthesis regularly occurs when sensitized lymphocytes are cultured with antigen (19, 20) and is distinct from the macrophage glucosamine response.

The delayed time course of antigen-stimulated glucosamine incorporation by macrophages is of interest. In contrast to the standard migration-inhibition assay (17), significant stimulation occurred at 48 and 72 hr of culture but was not apparent earlier. These kinetics could be explained if time were required for the synthesis and release of a lymphokine(s) or if the response of target macrophages to lymphokine(s) was not immediately evident. Present evidence, based on experiments using culture supernatants containing preformed lymphokines, favors the latter possibility. Thus Nathan et al. (2) found that enhanced hexose monophosphate shunt activity, macrophage adherence to plastic, and phagocytic capacity developed only after a lag phase of 3 days. In preliminary experiments we have found that cell-free supernatants containing MIF, and very likely other lymphokines, enhance glucosamine incorporation as measured at 3 days.

Our findings indicate that a large fraction of the glucosamine incorporated by adherent macrophages in the presence or absence of specific antigen and sensitized lymphocytes is localized to the cell surface. Cell fractionation studies reveal that nearly 30% of radioactivity incorporated during a 6-hr pulse is found in the membrane-rich microsomal fraction; in cultures containing sensi-

tized lymphocytes and antigen, this fraction increases in both absolute and relative terms, at the expense of other cell compartments. Radioautographic data supplement these results by localizing grains to the vicinity of the cell membrane and to the perinuclear zone that corresponds to the membrane-rich Golgi region.

The precise anatomic localization and biologic significance of this glucosamine containing cell surface material are not yet established. It is tempting to postulate that at least some of the surface membrane-associated macromolecular material labeled with glucosamine in this study corresponds to the extramembranous cell coat that we and others, using special electron-microscopic stains with a predilection for mucopolysaccharides (6, 7, 21), have shown to surround macrophages. The resolution afforded by electron microscopic radioautography will be required to settle this point. If, in fact, the two are homologous, an interesting paradox arises whose resolution may contribute significantly to an understanding of the mechanism of action of the lymphokine(s) responsible for inhibition of macrophage migration and enhanced glucosamine incorporation. Although PEC are normally endowed with a prominent cell coat, this extramembranous glycocalyx is not observed when macrophages are cultured in the presence of sensitized lymphocytes and specific antigen (6, 7). This finding is not peculiar to tissue culture since a similar absence of cell coat material has been noted during the rejection of ascites tumor cells in previously sensitized guinea pigs (22). Both *in vitro* and *in vivo* lack of demonstrable cell coat is particularly prominent among aggregated macrophages, which suggests that this coat may have a role in keeping cells apart (6, 7, 23). On the other hand, the metabolic studies reported here indicate that macrophages cultured in the presence of specific antigen and sensitized lymphocytes are stimulated to incorporate greatly increased amounts of glucosamine into their membrane fractions. Presumably this material represents glycoprotein and/or glycolipid (4, 8), and it is not yet known whether it is chemically identical with or different from that normally synthesized by unstimulated macrophages.

Considering these morphologic and biochemical findings together, we propose that MIF or another lymphokine(s) produced in delayed hypersensitivity exerts an important influence on the macrophage surface. Possibly the cell coat synthesized under these conditions is chemically abnormal so that it is readily lost from the cell membrane or has lost its affinity for the electron microscopic stains employed for its recognition. According to this view, lymphokine would induce a qualitative change in cell coat synthesis. Alternatively, lymphokine(s) might act by removing chemically normal cell coat material. This could be accomplished by a direct mechanism (the lymphokine itself could be an enzyme capable of digesting the cell coat) or by an indirect mechanism (e.g., the lymphokine might release macrophage lysosomal enzymes that digest the cell coat). In this instance the enhanced glucosamine incorporation

observed in delayed hypersensitivity could represent a compensatory attempt to restore the normal cell coat material that has been lost. Experiments to distinguish among these and other possible explanations of our data are now in progress.

SUMMARY

The interaction between sensitized lymphocytes and specific antigen occurring in delayed hypersensitivity causes bystander macrophages to undergo a variety of light-microscopic, ultrastructural, and biochemical changes, which are reflected in alterations in cell movement and intercellular contacts. Since such alterations involve functions of the cell periphery, we postulated that metabolic changes in this polysaccharide-rich zone would accompany the expression of delayed hypersensitivity.

We here demonstrate that the incorporation of radioactive glucosamine by peritoneal macrophages into TCA-precipitable, membrane-associated material is regularly enhanced when these are cultured in the presence of specific antigen and nonadherent cells (lymphocytes) primed for delayed hypersensitivity. Lymphocytes from unsensitized animals, or from animals immunized so as to form antibody but not delayed hypersensitivity, do not stimulate such incorporation. Antigen-induced glucosamine incorporation is maximal at 2 or 3 days of culture and is not observed earlier; it may be elicited with as little as 0.1 $\mu\text{g}/\text{ml}$ PPD, and affords an exceedingly reproducible and sensitive index of delayed hypersensitivity.

Radioautographic studies indicate that nearly all plastic adherent cells (90% macrophages) incorporate glucosamine and that grains are concentrated in the regions of the perinuclear zone and cell membrane. Subcellular fractionation indicates that nearly 30% of counts and the highest specific activity are associated with the membrane-rich microsomal fraction; the microsomal distribution of counts increases in both absolute and relative terms when macrophages are cultured in the presence of specific antigen and sensitized lymphocytes.

Taken together, these data indicate that a sizable fraction of incorporated glucosamine is localized to the vicinity of the cell periphery but lack sufficient resolution to determine whether this material is associated with the cell membrane itself or with the extramembranous cell coat. This last possibility is of particular interest since we have previously shown that macrophage cell coat material is lost or altered as a consequence of an interaction between sensitized lymphocytes and specific antigen.

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