

Plasma membrane polypeptides of resident and activated mouse peritoneal macrophages

(lactoperoxidase iodination/macrophage activation)

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ABSTRACT With the lactoperoxidase/glucose oxidase-catalyzed iodination method, we have identified at least 19 exteriorly disposed plasma membrane polypeptides on mouse peritoneal macrophages, with molecular weights ranging from 12,000 to 290,000. Resident and inflammatory macrophages could be distinguished by qualitative and quantitative differences in the display of selected polypeptides, although the overall banding patterns were similar. Some of the labeled polypeptides were identified by immunoprecipitation.

In the course of cellular differentiation, mononuclear phagocytes display a series of biochemical and functional alterations (1, 2). This maturation process is modulated by the various cellular and humoral participants in the inflammatory response and culminates with the appearance of activated macrophages endowed with enhanced secretory, endocytic, and tumoricidal capabilities. Many of the alterations recognized in activated macrophages involve functions of the plasma membrane including increased pinocytosis (3), expanded phagocytic recognition (4), changes in ectoenzyme composition (5, 6), and rapid spreading on contact with surfaces (7).

In order to correlate these functional differences with changes in the polypeptide composition of the plasma membrane, a comparative study was carried out using the lactoperoxidase/glucose oxidase procedure for surface iodination described by Hubbard and Cohn (8, 9). The banding patterns of polypeptides of the mouse macrophage plasma membrane in NaDodSO₄/polyacrylamide gel electrophoresis are highly complex. We developed an approach to overcome the difficulties usually encountered in the analysis of such patterns. The entire cell population recovered from the peritoneal cavity was iodinated prior to the adherence and selection of macrophages, with care to avoid contamination of the preparations by extraneous serum proteins. In addition, to confirm differences in banding patterns between cell populations, each preparation was labeled with a different isotope of iodine, mixed, and coelectrophoresed in the same lane of the slab gel. This technique allowed precise assignment of bands to each macrophage population. Our results indicate that at least 19 iodinated polypeptides can be recognized on the surface of resident mouse peritoneal macrophages, of macrophages elicited by endotoxin lipopolysaccharide, and of macrophages elicited by Brewer thioglycollate medium. The three macrophage populations can be distinguished by qualitative and quantitative alterations in the display of plasma membrane polypeptides, although the overall banding patterns are similar. Some of the labeled polypeptides can be identified by immunoprecipitation.

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MATERIALS AND METHODS

Mice. All experiments, except those involving immunoprecipitation, were performed with peritoneal macrophages obtained from pathogen-free Swiss mice maintained at The Rockefeller University. Some mice were injected intraperitoneally, 4 days before the experiment, with 1 ml of Brewer's thioglycollate medium (Difco) with 30 μg of 055-B5/*Escherichia coli* endotoxin lipopolysaccharide, a generous gift of David Morrison (Scripps Clinic and Research Foundation).

Iodination. The lactoperoxidase/glucose oxidase-catalyzed iodination method of Hubbard and Cohn (8, 9) was used. Peritoneal exudate cells were harvested, washed twice in phosphate-buffered saline, and resuspended at 10⁷ cells per ml. Iodination was carried out at 4°C for 25 min with 100 milliunits of lactoperoxidase (Calbiochem) and 10 milliunits of glucose oxidase (Sigma) per ml in 20 mM glucose containing 100–200 μCi (1 Ci = 3.7 × 10¹⁰ becquerels) of Na¹²⁵I (carrier-free, iodination grade; New England Nuclear) per ml and unlabeled 25 μM NaI. The iodinated cells were washed twice by centrifugation in large volumes of cold Dulbecco's medium supplemented with 10% heat-inactivated fetal calf serum and then were allowed to adhere to petri dishes for 30–60 min at 37°C under 5% CO₂ atmosphere. The cell monolayers were washed vigorously, scraped in cold phosphate-buffered saline containing 2 mM phenylmethylsulfonyl fluoride (PMSF), pelleted by centrifugation, and resuspended in a small volume of 0.1 M Tris-HCl, pH 6.8/2 mM PMSF. Aliquots were removed for protein determination by the Lowry procedure (10), radioactivity measurement after cold trichloroacetic acid precipitation onto glass fiber filters, and gel electrophoresis. Control experiments, in which lactoperoxidase was omitted from the iodination system, showed negligible incorporation of label into cells.

Gel Electrophoresis. Iodinated macrophages were boiled in 1% sodium dodecyl sulfate/1% 2-mercaptoethanol and electrophoresed on discontinuous pH, 5–15% polyacrylamide gradient slab gels (8, 11). The slabs were stained with 0.2% Coomassie blue, dehydrated, and exposed to x-ray films. Densitometer tracings of the gel autoradiograms were made with a Zeiss spectrophotometer (model PM6). Alternatively, the wet gel was cut into 0.9-mm slices and the radioactivity in these slices was determined in a gamma counter. In experiments involving double isotope labeling, the gamma counter was adjusted for simultaneous determination of ¹²⁵I and ¹³¹I; ¹²⁵I cpm were corrected for ¹³¹I spillover.

All gels included, as molecular weight standards, rabbit muscle myosin (heavy chain, 200,000) and muscle actin (45,000)

Abbreviation: PMSF, phenylmethylsulfonyl fluoride.

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kindly donated by M. J. Sheetz (University of Connecticut Health Center, Farmington, CT) and bovine serum albumin (66,000), ovalbumin, (43,000), and cytochrome *c* (12,700) from Sigma.

Immunoprecipitation of Radioiodinated Peptides. Iodinated cells were lysed in Nonidet P-40 in the presence of PMSF and aprotinin (Sigma). An immunoprecipitation procedure using formalin-fixed *Staphylococcus aureus* (12) was used to identify selected radioiodinated polypeptides. The following antisera were tested: rabbit anti-mouse factor B (gift from Otto Götze, Scripps Clinic and Research Foundation, La Jolla, CA) and rabbit anti-rat lysozyme that crossreacted with mouse lysozyme (gift from Elliott Osserman, Columbia College of Physicians and Surgeons, New York, NY). A rabbit antiserum to mouse C3 was prepared by injecting rabbits with serum-treated zymosan particles (13). A sheep antiserum to mouse C3 was obtained by injecting C3 purified by affinity chromatography. Both antisera were monospecific in immunoelectrophoresis against normal mouse serum and mouse serum treated with inulin. In the latter, the typical pattern of C3 conversion could be detected.

An antiserum against specificity 5 of the mouse H-2 system (D-5GP no. 56) was obtained from John G. Ray (Research Resources Branch, National Institute of Allergy and Infectious Diseases). An antiserum against Ia specificities was donated by Donald Shreffler (Washington University, St. Louis, MO). The experiments with these two antisera were performed in thioglycollate-induced macrophages from A/J mice (The Jackson Laboratory).

RESULTS

Labeling Procedure. The best labeling patterns were obtained when the entire peritoneal cell exudate was iodinated prior to the selection of macrophages by adherence to petri dishes. Attempts to label the monolayers directly always resulted in marked incorporation of ^{125}I into the protein coat bound to the dishes, making the interpretation of gel patterns rather difficult. This protein coat has been studied (14). In our case, it mostly contained components of the fetal calf serum used in the adherence step.

The ^{125}I incorporation was strictly dependent on the presence of both lactoperoxidase and glucose oxidase in the reaction mixture. A large proportion of the label could be removed by proteases (unpublished data). Most of the ^{125}I was incorporated into trichloroacetic acid-precipitable material. However, some of this material could be removed from the filters by organic solvents [chloroform/methanol, 2:1 (vol/vol); acetone/water, 9:1 (vol/vol); ethanol/ether, 3:1 (vol/vol)] without detectable alteration of the banding pattern observed in Coomassie blue-stained gels or autoradiograms, except for the disappearance of a broad band migrating with the dye front. This material was not stained by Coomassie blue and accounted for 10–30% of the acid-precipitable radioactivity. Preliminary experiments have indicated that it is not associated with sterols or phosphatides and that it does not contain free ^{125}I .

The viability of the iodinated macrophages after adherence was very high, as assessed by several criteria. More than 98% of the cells ingested sheep erythrocytes coated with IgG and complement (15) and excluded trypan blue. Pinocytotic rates measured by the uptake of horseradish peroxidase (14) were identical to those of unlabeled cells.

Distribution of the Iodinated Polypeptides. The ^{125}I -labeled plasma membrane polypeptides of resident and activated macrophages could be resolved into at least 19 well-defined bands in autoradiograms of the electrophoresis gels. Fig. 1 depicts Coomassie blue-stained gels and the corresponding autoradiograms obtained after electrophoresis of resident ma-

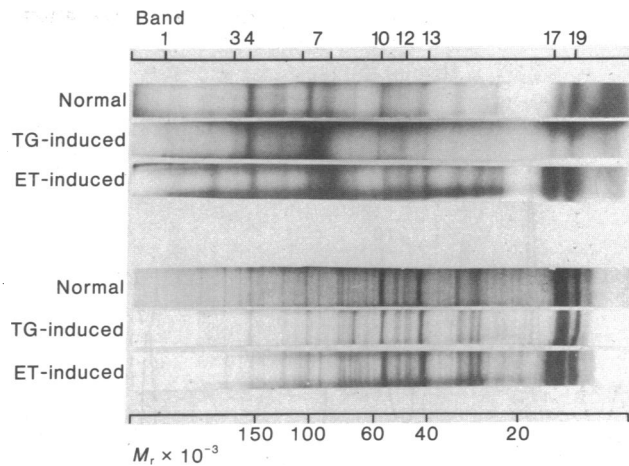


FIG. 1. Comparison of Coomassie blue-staining patterns (Lower) and autoradiograms (Upper) of NaDodSO₄/polyacrylamide gel electrophoretic analysis of radioiodinated resident, thioglycollate-induced (TG-induced), and endotoxin-induced (ET-induced) mouse peritoneal macrophages. Numbers are assigned to the major radioiodinated bands.

crophages and of macrophages induced by endotoxin and by thioglycollate. The bands revealed by the protein stain did not always coincide with the major radioactive bands. For example, a major protein band comigrating with rabbit muscle actin was not iodinated.

For identification purposes, numbers were assigned to the major radioactive bands, according to their increasing mobility in the gels. Table 1 lists some of these bands and the apparent molecular weights determined in a large number of experiments performed with thioglycollate-induced macrophages. The molecular weights ranged from 292,000 to 12,000.

Difference Between Macrophage Populations. The band distribution patterns of iodinated polypeptides of the three macrophage populations examined were basically similar, as expected from their common origins. However, specific qualitative and quantitative differences, characteristic for each macrophage population, could be identified. These alterations could be visualized better when densitometer scans of autoradiograms were superimposed (Fig. 2). The most evident differences were seen in bands, 7, 12, 17, and 19. Band 7 was broad and occupied an area of the gel with molecular weights ranging from 85,000 to 103,000. It was very intense in activated macrophages. The central area was not present in resident cells. Band 12 (M_r , 48,000) was weak in resident cells, more intense

Table 1. Apparent molecular weights of major iodinated plasma membrane polypeptides of thioglycollate-induced macrophages

Band	Apparent $M_r \times 10^{-3}$ *
1	291.8 ± 14.9 (7)
3	187.4 ± 4.4 (8)
4	166.5 ± 2.5 (12)
7 (a, top)	103.1 ± 1.1 (8)
(c, bottom)	84.7 ± 0.7 (7)
10	57.8 ± 0.8 (7)
12	48.2 ± 1.0 (7)
13	40.3 ± 0.9 (7)
17	13.7 ± 0.3 (7)
19	11.8 ± 0.3 (7)

Mouse peritoneal macrophages were obtained 4 days after intraperitoneal injection of thioglycollate, and iodinated.

* Determined in relation to standards described in *Materials and Methods*. All the polypeptides listed are glycoproteins (15) and may have migrated anomalously. Results are shown as mean ± SEM, with number of determinations in parentheses.

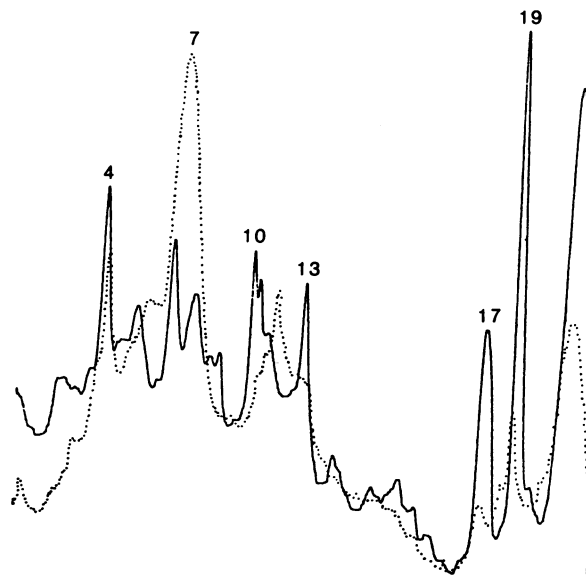


FIG. 2. Superimposed densitometer scans of autoradiograms of electrophoresis gels of mouse peritoneal macrophages labeled with ^{125}I . —, Resident macrophages, ·····, thioglycollate-induced macrophages. Numbers indicate band number.

in endotoxin-induced cells, and very intense in thioglycollate-induced macrophages. On the other hand, bands 17 and 19 were much more intense in endotoxin-induced cells than in the other two populations. They had apparent molecular weights of 14,000 and 12,000, respectively.

Double-Labeling Experiments. To confirm the above observations, resident macrophages and macrophages induced by endotoxin or by thioglycollate were labeled with different isotopes of iodine, mixed, and electrophoresed in the same lane of the gel. The gels were sliced and assayed for radioactivity. In the experiment shown in Fig. 3, resident macrophages were labeled with ^{131}I and thioglycollate-induced macrophages were labeled with ^{125}I . The profiles confirmed the differences ob-

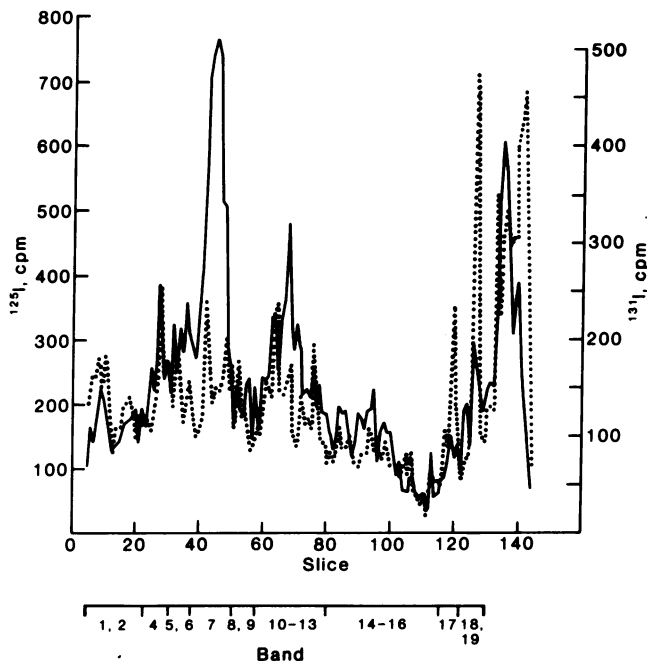


FIG. 3. Distribution of radioactivity among plasma membrane polypeptides from ^{125}I -labeled resident and ^{131}I -labeled thioglycollate-induced macrophages. The two cell types were mixed and co-electrophoresed; radioactivity in gel slices was determined by double isotope counting. See Table 2 for analysis of data.

Table 2. Relative distribution of radioactivity upon electrophoresis of plasma membrane polypeptides from resident and thioglycollate-induced macrophages

Band	% of total radioactivity	
	Resident ^{131}I	Thioglycollate-induced (^{125}I)
Excluded fraction	6.9	4.4
1, 2	3.7	7.0
3	1.8	1.5
4	8.0	6.1
5, 6	5.2	6.3
7	10.5	18.5
8, 9	4.1	4.2
10-13	15.7	17.4
14-16	11.6	12.3
17	1.1	0.8
18, 19	11.2	7.7
Dye front	20.2	13.7

These data are from the experiment described in Fig. 3.

served in single label experiments. A detailed analysis of the data obtained in this experiment is shown in Table 2. It confirms the marked increase in label in band 7. Also evident is the higher intensity of the high molecular weight bands 1 and 2.

Double-labeling experiments were also performed with endotoxin-induced macrophages. The results substantiated those obtained in the initial experiments. Fig. 4 summarizes the major differences observed among the three macrophage populations.

Immunochemical Characterization of Radiolabeled Peptides. Some of the components of the radioiodinated peptides could be identified by immunoprecipitation (Table 3). All experiments were performed with thioglycollate-induced macrophages from A/J mice. Factor B of the properdin pathway of complement comigrated with band 7. The antiserum to specificity 5 of H-2K brought down materials that migrated as bands 12 and 18. The antiserum to Ia precipitated a radioactive band migrating as band 16. No precipitate could be obtained with two different antisera to C3 and an antiserum directed to rat lysozyme. No attempts were made to assess in each band the proportion of radioactivity that carried the antigenic specificity.

DISCUSSION

The lactoperoxidase/glucose oxidase-catalyzed iodination has been shown to label exteriorly disposed plasma membrane polypeptides of various cell types (16). In the present study, the iodination conditions were optimized for adequate labeling of plasma membrane proteins. Iodination of the whole peritoneal cell exudate prior to the adherence of macrophages to petri dishes ensured the selection of viable cells and avoided the iodination of serum-derived proteins that usually coat the dish. Artfactual modification of the plasma membrane proteins was prevented by the addition of protease inhibitors to all reaction mixtures, by carrying out the procedure at 4°C , and by boiling the labeled cells in 1% NaDodSO₄ immediately after iodination.

Several observations (this paper and unpublished data) indicate that the label was restricted to the plasma membrane. The viability of the macrophages was high, the number of labeled species was decreased compared to Coomassie blue patterns, all labeled polypeptides bound concanavalin A, and a large proportion of the label could be removed by protease treatment of the cells.

The NaDodSO₄/polyacrylamide gel electrophoresis profiles of macrophage plasma membrane proteins was complex. At

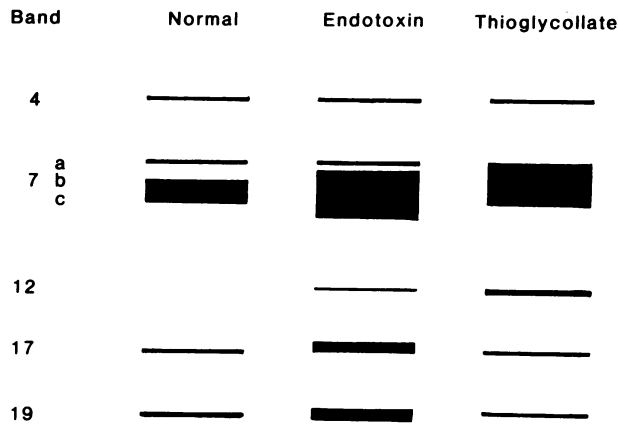


FIG. 4. Schematic representation of the differences between selected bands in NaDodSO₄/polyacrylamide gel electrophoresis of iodinated plasma membrane polypeptides from resident, endotoxin-induced, and thioglycollate-induced macrophages.

least 19 polypeptides could be clearly identified. The marked similarity of the band distribution of resident, endotoxin-induced, and thioglycollate-induced macrophages confirms at the molecular level the close relationship among these cell populations. On the other hand, the unique modifications of the labeling patterns characteristic for each of these groups of macrophages can be included in the catalogue of functional, morphological, and biochemical changes that characterize the activated state. In particular it will be of interest to assign functions to the enriched peptides of band 7, which range from 85,000 to 103,000 daltons.

The different labeling patterns of inflammatory macrophages may reflect phenotypic changes that accompany cell differentiation and may be directly associated with the enhanced functional capabilities of activated macrophages. Obviously, other phenomena could explain the observed modifications. Topographical rearrangement of the plasma membrane polypeptides may alter their accessibility to the iodinating enzymes. Or neutral proteases, secreted by the activated macrophage or present in the inflammatory environment, may affect the banding patterns. However, it should be stressed that a number of the modified peptides are resistant to digestion by proteases *in vitro* (unpublished data).

The labeled polypeptides appeared to be tightly associated with the plasma membrane because they resisted extensive washing and in several cases remained associated with the cell after prolonged time in tissue culture (15). However, further

Table 3. Immunochemical identity of radioiodinated plasma membrane polypeptides from thioglycollate-induced macrophages

Antiserum to	Apparent $M_r \times 10^{-3}$	Associated with band no.*
Factor B (properdin pathway of complement)	91	7
H-2K, specificity 5	46	12
	12	18
Ia (ATH anti-ATL)	27.6	16
Mouse C3		Not detected
Rat lysozyme		Not detected

The radioiodinated macrophages from A/J mice were solubilized with Nonidet P-40 and exposed to the antiserum. Formalin-fixed *S. aureus* was added, recovered, washed, and boiled in NaDodSO₄; the supernatant was electrophoresed.

* Determined by comparison with parallel lanes containing lysates of radioiodinated thioglycollate-induced macrophages.

investigation is necessary to determine if all labeled polypeptides are integral membrane proteins.

Some of the labeled proteins could be identified immunochemically. Both H-2K and Ia specificities could be precipitated from the iodinated material by antisera and migrated with apparent molecular weights close to those reported for lymphocytes (17, 18). These studies were restricted to thioglycollate-induced macrophages. Also, the procedure did not indicate the degree to which these antigens were distributed among macrophages. Actually, it is possible that the expression of antigens is restricted to a small macrophage subpopulation. Attempts to identify secretory proteins among the labeled polypeptides were successful only with factor B of the properdin pathway of complement. Lysozyme and C3 could not be detected. The molecular weight of the membrane-associated factor B was 91,000, closer to that determined for the native molecule found in mouse serum and secreted by mouse macrophages (19). The cleaved form of factor B (Bb) was not detected. Bb is known to induce rapid macrophage spreading by an enzymatic mechanism (20).

In conclusion, the present paper defines the distribution of plasma membrane polypeptides of resident mouse peritoneal macrophages and of inflammatory macrophages induced by endotoxin lipopolysaccharide and by thioglycollate medium and defines specific alterations of the banding patterns characteristic for each of these macrophage populations.

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