PLASMA MEMBRANE LOCALIZATION AND METABOLISM OF ALKALINE PHOSPHODIESTERASE I IN MOUSE PERITONEAL MACROPHAGES*

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We have previously reported on the metabolism of the macrophage plasma membrane enzyme 5'-nucleotidase (1, 2). However, although this enzyme is easily detected in resident peritoneal macrophages, it is absent in thioglycollate-stimulated cells. In an effort to learn more about the physiology of the plasma membrane of inflammatory cells we therefore chose to examine the enzyme alkaline phosphodiesterase I (EC 3.1.4.1). This enzyme has been previously reported to be a component of the plasma membrane of rat and mouse liver cells (3, 4, 5) and rabbit alveolar macrophages (6). In this work we present evidence that it is also associated with the plasma membrane of mouse peritoneal macrophages. We also report on the rates of synthesis and degradation of the enzyme in resident and inflammatory populations.

Materials and Methods

Macrophages. Cells were collected from female NCS mice weighing 25–30 g, and were cultivated as previously described (7) in Dulbecco's modified eagle's medium supplemented with 20% heat inactivated fetal calf serum (DMEM-20% FCS).[1] Serum-free cultures were maintained in DMEM supplemented with 0.2% lactalbumin hydrolyzate. Inflammatory macrophages were obtained by injecting endotoxin or thioglycollate medium intraperitoneally (8). Medium and lactalbumin hydrolyzate were purchased from Grand Island Biological Co., Grand Island, N. Y. Serum was purchased from Flow Laboratories, Inc., Rockville, Md. Endotoxin bacto-lipopolysaccharide (E. Coli 0111:B4) and thioglycollate medium (Brewer thioglycollate medium, dehydrated) were purchased from Difco Laboratories, Detroit, Mich.

Assay for Alkaline Phosphodiesterase. Alkaline phosphodiesterase I activity was measured by the method of Beaufay et al. (3) using 1.5 mM *p*-nitrophenyl thymidine-5'-monophosphate (Calbiochem, San Diego, Calif.) in 0.1 M glycine-NaOH buffer (pH 9.6) containing 2 mM zinc acetate. Cells to be assayed are rinsed twice in phosphate-buffered saline, and then scraped into a small volume of freshly prepared 0.05% Triton X-100. The usual lysate vol for cells in a 35 mm culture dish is 0.1 ml. Lysates can be stored frozen and thawed once before being assayed without a change in enzyme activity. Routinely, 50 μl of cell lysate is mixed with 0.5 ml substrate. Both the substrate and the reaction mixtures are protected from light with aluminum foil. The mixture is shaken vigorously and then incubated for 30 min in a 37°C water bath. The reaction is stopped with 1.0 ml of 0.1 N NaOH and the absorbance of the reaction mixture is read at 400 nm. Enzyme blanks are prepared by substituting 0.05% Triton for the cell lysate. The absorbance of these samples is subtracted from that of the lysate samples. Results are reported

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[1] *Abbreviations used in this paper:* DASA, diazonium salt of sulfanilic acid; DMEM, Dulbecco's modified eagle's medium; FCS, fetal calf serum.

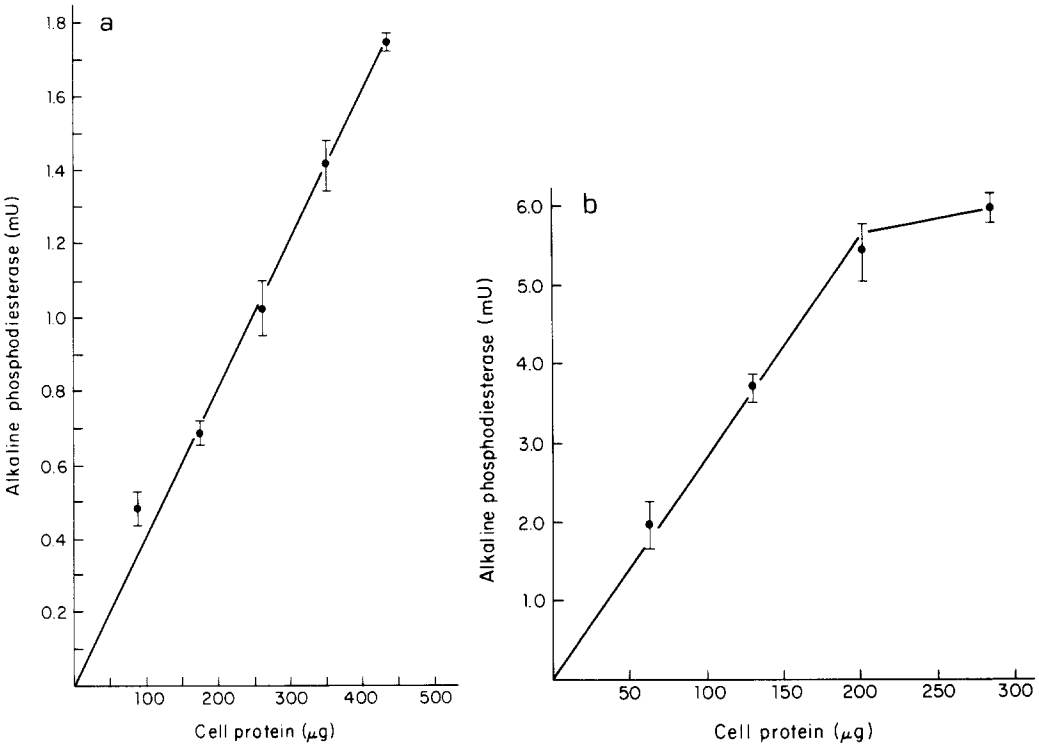

FIG. 1. Relationship between quantity of cell lysate assayed and alkaline phosphodiesterase activity measured. (a) resident peritoneal macrophages. (b) thioglycollate-stimulated cells. Lysates were incubated with substrate for 30 min at 37°C, the reaction was stopped and the absorbance at 400 nm was measured.

in units of activity per milligram cell protein. 1 U of activity is that quantity of enzyme which hydrolyses 1 μmol of substrate per minute at 37°C.

The enzyme activity measured is linearly related to the amount of cell lysate assayed, up to about 45 μg cell protein for resident macrophages, and about 20 μg cell protein for thioglycollate-stimulated cells (Fig. 1 a and b). The amount of substrate hydrolyzed is also linearly related to the incubation period, between 15 and 60 min (Fig. 2). As Beaufay and his collaborators noted for the hepatic enzyme, the macrophage activity is also dependent upon the presence of zinc ions, and little or no activity is seen when lysates are assayed in the absence of the metal, or in the presence of 1 mM EDTA.

Estimation of Rates of Enzyme Synthesis and Degradation. We followed the analysis of Berlin and Schimke as previously applied by us to the study of the metabolism of 5'-nucleotidase (1). Briefly, the rate of enzyme decay, when enzyme synthesis was inhibited by cycloheximide, was used to calculate the fractional rate of enzyme degradation. This rate, and the equilibrium value for enzyme activity, allowed us to calculate the rate at which the enzyme is synthesized.

Preparation and Assay of the Diazonium Salt of Sulfanilic Acid (DASA). The DASA was prepared by the method of DePierre and Karnovsky (89), as previously described (2). It was, however, more convenient to assay the reagent by allowing it to form a colored product with resorcinol as suggested by Koltun (10). The assay is performed by combining 1.0 ml of an appropriate dilution of the DASA solution with 9.0 ml of 0.01 M resorcinol in 0.2 M acetate buffer (pH 4.5). The mixture is kept at room temperature for 30 min, and the absorbance is read at 385 nm. The concentration of DASA is calculated with a molar extinction coefficient for the product of 21,500.

Cell Lines. Cultures of the macrophage-like cell lines J774, WEHI-3, and PU5-1.8 were

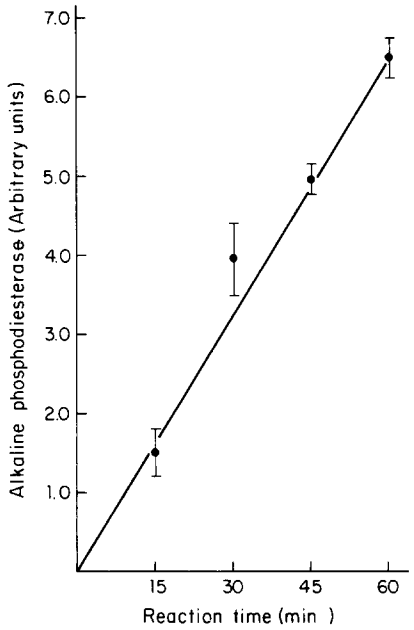

FIG. 2. Time course of alkaline phosphodiesterase assay. The reactions were carried out by adding 100 μg cell protein to prewarmed substrate and incubating the mixture for up to 60 min more.

generously supplied to us by Dr. Peter Ralph, Sloan-Kettering Cancer Center, Rye, N. Y.; and were maintained in DMEM-10% FCS. These lines are more fully described elsewhere (11).

Reagents. Trypsin and chymotrypsin, were purchased from Worthington Biochemical Corp., Freehold, N. J. Pronase was purchased from Calbiochem. Cycloheximide, Triton X-100 (octyl phenoxy polyethoxyethanol, Rohm and Haas Co., Philadelphia, Pa.) and papain were purchased from Sigma Chemical Co., St. Louis, Mo. Papain was kept refrigerated and was activated with dithiothreitol immediately before use. Other chemicals used were reagent grade.

Results

Alkaline Phosphodiesterase Activity in Resident or Inflammatory Macrophages. As Table I shows, alkaline phosphodiesterase activity is readily detected in both resident and inflammatory macrophages which have been purified of contaminating lymphocytes by overnight cultivation. Nonadherent peritoneal cells have low levels of activity, but the characteristics of this activity were not explored. This enzyme is also expressed in several long-term cell lines which have various characteristics of macrophages, but it is also present in L-929 mouse cells, which is a fibroblast line.

Among macrophages, thioglycollate-stimulated macrophages have considerably more activity than resident cells. Endotoxin stimulation has no effect on the specific activity of the enzyme.

Effect of Macrophage Cultivation on Alkaline Phosphodiesterase Activity. Cultivation of macrophages in serum-supplemented medium has very little effect on the enzyme activities of either resident or inflammatory macrophages. However, cultivation in serum-free conditions decreases the alkaline phosphodiesterase activity 40–50% (Table II).

TABLE I
*Alkaline Phosphodiesterase Activity of Cultured Mouse Cells**

Cell type	Alkaline phosphodiesterase activity
	mU/mg
Resident macrophages	1.43 ± 0.18‡
Endotoxin-stimulated macrophages	1.36 ± 0.15
Thioglycollate-stimulated macrophages	3.91 ± 0.70
Nonadherent peritoneal cells	0.21 ± 0.09
L929	1.24
J-774D1	0.78
WEH1-3	0.42
PU5-1.8	0.81

* Peritoneal macrophages were cultivated overnight in DMEM-20% FCS. Nonadherent peritoneal cells were recovered from a mixture of unstimulated peritoneal cells, after the cells were allowed to adhere to plastic, in DMEM-20% FCS, for 60 min. Cell lines were maintained in DMEM-10% FCS.

‡ Mean ± SD 1 U of enzyme activity hydrolyzes 1 μmol of substrate per minute at 37°C.

TABLE II
*Effect of Cultivation on Alkaline Phosphodiesterase Activity**

	4	24	48	72
			h	
Resident cells	1.63	1.43 ± 0.18	1.36 ± 0.47	1.09
Endotoxin-stimulated cells	1.16	1.36 ± 0.15	0.94	1.18
Thioglycollate-stimulated cells	3.60	3.91 ± 0.70	4.88 ± 0.38	4.23 ± 0.90
Thioglycollate-stimulated cells (cultivated in lactalbumin hydrolyzate)	–	1.99 (50.1%)‡	2.62 (53.4%)	1.66 (39.2%)

* Macrophages were cultivated for the periods indicated in DMEM-20% FCS, or in DMEM-0.2% lactalbumin hydrolyzate. All enzyme activities are given as milliunits per milligram cell protein.

‡ Percentage of corresponding cultures maintained in DMEM-20% FCS.

Plasma Membrane Location of Alkaline Phosphodiesterase. The ability of DASA to inactivate alkaline phosphodiesterase in intact cells was used as a measure of the extent to which the enzyme was associated with the outer surface of the plasma membrane (2). As Fig. 3 shows, when macrophages are exposed to increasing concentrations of DASA, progressively larger fractions of the total alkaline phosphodiesterase activity are inhibited, until a plateau of about 19% is reached at a concentration of DASA of 5×10^{-4} M. Thus, for thioglycollate-stimulated cells, about 80% of the total enzyme activity is externally disposed on the plasma membrane. As shown in Table III, the same fraction of enzyme is exposed in resident or endotoxin-stimulated cells. We have also found that at least 70% of the total activity in L-cells is inactivated by DASA (not shown).

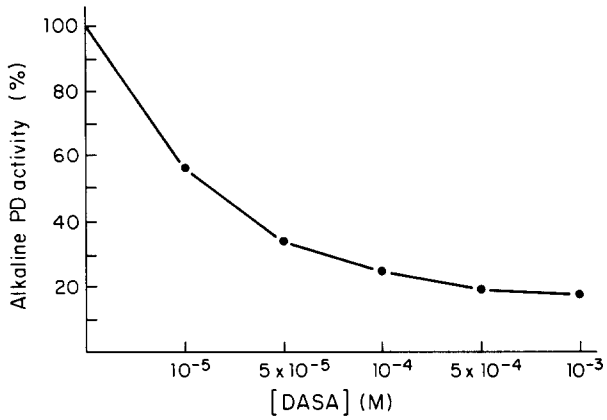

FIG. 3. Inactivation of alkaline phosphodiesterase in intact thioglycollate-stimulated macrophages by DASA. Cells were rinsed twice in a balanced salt solution free of phenol red, and then exposed to DASA for 20 min at 37°C, rinsed, lysed, and assayed for enzyme activity.

TABLE III
*Effect of DASA on Macrophage Alkaline Phosphodiesterase**

Cell type	DASA treatment‡	Alkaline phosphodiesterase activity	
		mU/mg	%
Resident	+	0.34	24.2
	−	1.39	
Endotoxin-stimulated	+	0.32	21.9
	−	1.46	
Thioglycollate-stimulated	+	0.94	19.6
	−	4.79	

* Macrophages were cultivated for 48 h in DMEM-20% FCS before treatment with DASA.
‡ Cells were exposed to 5×10^{-4} M DASA for 20 min at 37°C.

Sensitivity of Alkaline Phosphodiesterase Activity to Proteases. The bulk of the enzyme activity of intact thioglycollate-stimulated cells is sensitive to papain treatment (Table IV). About half of the total activity is lost at 100 μg/ml of enzyme, and about 23% remains after treatment with 250 μg/ml. Higher enzyme concentrations were toxic to the cells. Trypsin chymotrypsin, or pronase were less effective in inactivating the enzyme (Table V), although some effect was seen with each. Less than 3% of papain-treated cells were stained by trypan blue, and such cultures recovered their full enzyme activity after a 24 h incubation in serum-supplemented medium. This recovery was sensitive to cycloheximide.

Surprisingly, considerably less of the enzyme activity of endotoxin-stimulated cells was sensitive to papain. Papain (250 μg/ml) reduced the activity in these cells only about 25%. Because thioglycollate-stimulated cells secrete a battery of neutral proteases (12) which might work co-operatively with papain, or which might be activated by the added protease, we examined the effect of papain on endotoxin-stimulated cells which had been preincubated for 24 h in

TABLE IV
*Effect of Papain Treatment of Intact Cells on Alkaline Phosphodiesterase Activity**

Treatment	Alkaline phosphodiesterase activity	
	mU/mg	%
None	4.47	(100)
Papain, 10 μg/ml	3.35	85.3
25 μg/ml	3.36	83.1
50 μg/ml	3.11	69.6
100 μg/ml	3.12	58.9
250 μg/ml	1.05	23.2

* Thioglycollate-stimulated macrophages cultivated for 48 h in DMEM-20% FCS were exposed to papain at the indicated concentrations in PBS for 30 min at 37°C. The reaction was stopped by washing three times with PBS, and cell lysates were prepared and assayed.

TABLE V
*Effect of Protease Treatment on Alkaline Phosphodiesterase Activity of Thioglycollate-Stimulated Macrophages**

Protease	Alkaline phosphodiesterase activity	
	mU/mg	%
None	4.54	(100)
Trypsin, 250 μg/ml	3.24	71.4
Chymotrypsin, 250 μg/ml	3.94	86.5
Pronase, 250 μg/ml	2.45	53.9
Papain, 250 μg/ml	0.91	20.0

* Macrophages were cultivated for 72 h in DMEM-20% FCS, rinsed three times with phosphate-buffered saline, and then exposed to the enzyme solutions in phosphate-buffered saline for 15 min at 37°C.

thioglycollate-cell conditioned medium. Endotoxin-cell conditioned medium was used as a control. Papain removed 29.6% of the enzyme activity of cells treated with medium which had been conditioned by endotoxin-stimulated cells, and 39.4% of the enzyme activity of cells treated with thioglycollate-cell conditioned medium. Additionally, incubation in thioglycollate-cell conditioned medium did not effect the basal levels of enzyme activity in these cells.

Rate of Enzyme Inactivation. As we did previously for 5′-nucleotidase (1), we used cycloheximide to estimate the endogeneous rate of enzyme inactivation in the absence of any enzyme synthesis. Fig. 4 displays the data obtained for each of the three macrophage types studied. In each case, the enzyme decay was an exponential function of time, consistent with the assumption of first-order kinetics. Cycloheximide itself does not appear to affect this decay, as calculations made on the rate of enzyme recovery of endotoxin-stimulated cells, after their removal from cycloheximide, gave $t_{1/2}$ = 9.4 h (see Fig. 4, insert), as compared with a value of 8.2 h in the presence of cycloheximide (Table VI).

The rate of inactivation of enzyme in unstimulated cells is about three times slower than it is in thioglycollate-stimulated cells, and about two times slower than it is in endotoxin-stimulated cells. Endotoxin-stimulated cells have about

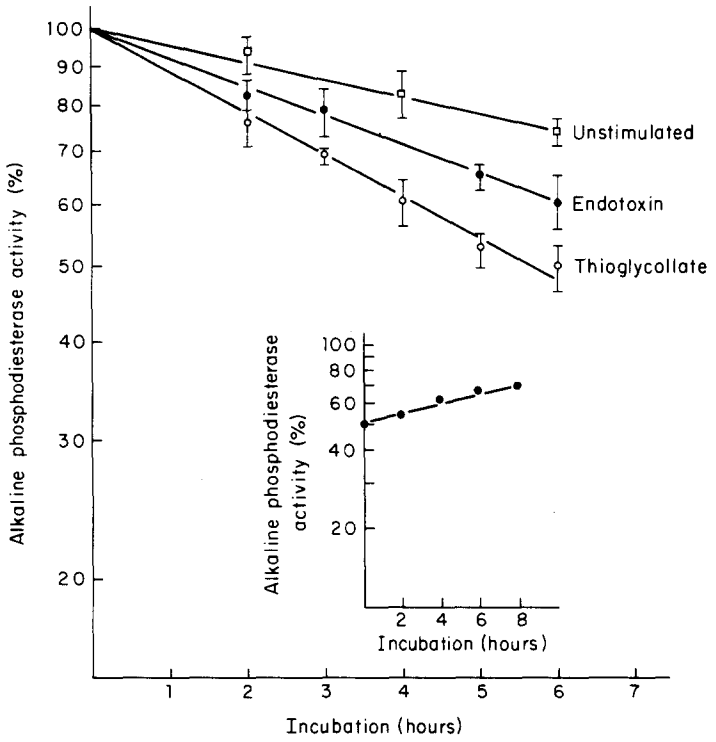

FIG. 4. Decay of alkaline phosphodiesterase activity in cells treated with cycloheximide (10 μg/ml). Curves were fit by the method of least squares. (Inset) Recovery of enzyme activity after reincubation of cells in medium free of cycloheximide.

TABLE VI

*Rates of Synthesis and Degradation of Alkaline Phosphodiesterase in Resident and Inflammatory Macrophages**

Cell type	Alkaline phospho-diesterase activity	$t_{1/2}$	Kd	Ks
	mU/mg	*h*	h^{-1}	*U/h*
Resident	1.32	14.1	.049	.065
Endotoxin-stimulated	1.38	8.2	.085	.117
Thioglycollate-stimulated	3.88	5.7	.122	.473

* Cells were maintained in DMEM-20% FCS for 48 h. Cycloheximide (10 μg/ml) was added and the rate constant for degradation (Kd) was calculated from the observed half-life of the enzyme. The synthetic rate (Ks) was calculated from Kd and the equilibrium enzyme activity.

the same enzyme activity as resident cells because they have also increased their synthetic rate two-fold. Thioglycollate-stimulated cells have a synthetic rate about seven times greater than that of resident cells, and this accounts for their increased enzyme levels.

Effect of Thioglycollate-Cell Conditioned Medium on the Rate of Degradation of Enzyme in Endotoxin-Stimulated Cells. Because alkaline phosphodiesterase *in situ* is liable to proteolytic inactivation, we examined the possibility that

the rate of enzyme degradation in thioglycollate-stimulated cells might be artifactually increased by extracellular enzyme inactivation. We therefore compared the rate of enzyme degradation in endotoxin-stimulated cells incubated in their own, or thioglycollate-cell conditioned medium. Such treatment had no effect on the intrinsic rate of enzyme degradation. In cells treated with thioglycollate-cell conditioned medium, the $t_{1/2}$ of the alkaline phosphodiesterase activity was 10.6 h, as compared with 9.5 h for control cells.

Discussion

Alkaline phosphodiesterase I hydrolyzes polyribonucleotides or oligodeoxyribonucleotides which have a free 3′-OH group, sequentially liberating 5′-nucleoside monophosphates (13). It is most conveniently assayed with the artificial substrate *p*-nitrophenyl thymidine-5′-phosphate which was developed by Razzell and Khorana (14). This enzyme has been recognized as a component of the plasma membrane of liver cells of rats (3, 5) or mice (4), and of rabbit alveolar macrophages (6). In mouse liver cells, the enzyme has been placed on the outer face of the plasma membrane (15).

We have found the enzyme in resident or inflammatory peritoneal macrophages, in some mouse macrophage cell-lines, and in mouse L-cells. The specific activity of the enzyme is from two to fourfold higher in thioglycollate-stimulated macrophages than it is in any of the other macrophage varieties examined. The enzyme shows little change in activity when cells are cultured in serum-supplemented medium, but there is a striking decrease in activity, to about 40% of normal levels, when cells are maintained in serum-free medium. This is quite similar to the behavior of 5′-nucleotidase (1) and, as in that case, may be due to an enhanced rate of enzyme degradation during conditions of serum deprivation.

The ability of a nonpenetrating reagent, the DASA, to inactivate about 80% of the macrophage enzyme activity indicates that the bulk of this enzyme is arranged in the plasma membrane, with its active site facing outward. As with 5′-nucleotidase, there is a second pool of enzyme which is presumably intracellular, and which may represent vesicles of internalized plasma membrane or newly synthesized enzyme in plasma membrane precursors.

The ability of several proteases to attack the enzyme in intact cells would support our locating it on the outer face of the plasma membrane. As we would expect, the papain-sensitive pool in thioglycollate-stimulated cells is the same size as the pool of enzyme which is sensitive to DASA. It is not clear why the enzyme in resident or endotoxin-stimulated cells is less sensitive to protease attack. Our experiments with medium conditioned by thioglycollate-cells would appear to be against a major role for secreted proteases, or enzyme inhibitors, in this process. It is possible that the enzyme in thioglycollate-stimulated cells is situated differently in the plasma membrane, that in some way its environment makes it more vulnerable to protease attack, or that it is in fact a different protein from the one present in resident cells.

Quantitatively, the metabolism of the enzyme is quite different among the three macrophage varieties studied. In resident cells, the enzyme is inactivated with a half-time of 14.1 h. This half-time is decreased to 8.2 h in endotoxin

cells, and to 5.7 h in thioglycollate-stimulated macrophages. We have previously reported that endotoxin-stimulated cells pinocytise at a rate about 1.5 times that of resident macrophages, and that thioglycollate-stimulated cells pinocytise at a rate about 3.4 times the resident rate (8). Thus, our data on the rates of alkaline phosphodiesterase inactivation is consistent with our earlier hypothesis that the pinocytic rate is a major, though not the sole, determinant of the rate at which plasma membrane is degraded (1).

We have also found differences in the rates of enzyme synthesis among the three cell types. With inflammatory stimulation, the rate of enzyme synthesis increases from two to sevenfold. This, too, is consistent with previous work showing that the pattern of protein synthesis is distinct in resident and inflammatory macrophages (16), and supports a concept of macrophage activation as a process of cell differentiation leading to the generation of qualitatively distinct cell types (17).

Summary

Alkaline phosphodiesterase I activity is demonstrable in lysates of mouse resident peritoneal macrophages (1.43 mU/mg), endotoxin-stimulated macrophages (1.36 mU/mg), and thioglycollate-stimulated macrophages (3.91 mU/mg), as well as in the lysates of several mouse cell lines. The enzyme showed little variation in culture, although serum deprivation caused a 50% decrease in enzyme activity. In each of the three macrophage types about 80% of the enzyme is inactivated by the diazonium salt of sulfanilic acid, indicating that this enzyme is a component of the plasma membrane. In thioglycollate-stimulated cells about the same fraction of enzyme can be inactivated with papain, corroborating this assignment. The enzyme is inactivated with a half-time of 14.1 h in resident cells, but this is decreased to 8.2 h in endotoxin cells, and to 5.7 h in thioglycollate cells. These results are consistent with the hypothesis that the endogenous pinocytic rate is a major determinant of plasma membrane turnover. In addition, the different synthetic rates measured in resident and inflammatory cells support the concept that macrophage activation is a differentiative process leading to a qualitatively new cell type.

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